



# Dynamics of the cerebral microvasculature during the course of memory consolidation in the rat : physiological and altered conditions induced by hypertension and hypergravity

Alice Pulga

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**Alice PULGA**

**Dynamics of the cerebral microvasculature during the course of  
memory consolidation in the rat:**

**physiological and altered conditions induced by hypertension and hypergravity**

Sous la direction de : Jean-Luc MOREL

Soutenue le 20 décembre 2016

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# **Titre : Dynamique des microvaisseaux cérébraux pendant la consolidation de la mémoire chez le rat en condition physiologique et en situation d'hypertension artérielle ou d'hypergravité**

## **Résumé :**

Les réseaux vasculaires cérébraux adaptent leur activité à la demande métabolique des neurones environnants, mais leur contribution fonctionnelle à la consolidation de la mémoire, processus par lequel les traces mnésiques se stabilisent dans le temps, reste inconnue. A l'aide d'un test de mémoire olfactif associatif couplé à des approches biochimiques et d'imagerie cérébrale chez le rat, nous avons étudié la dynamique des changements vasculaires au cours de la consolidation mnésique qui nécessite une interaction transitoire entre l'hippocampe et les régions corticales constituant les sites dépositaires des souvenirs. Nous montrons que la formation d'une mémoire durable est associée, dès l'encodage, à un signal hypoxique qui déclenche une angiogenèse transitoire dans des régions corticales spécifiques impliquées plus tard dans le stockage des souvenirs. Manipuler cette angiogenèse corticale précoce (ACP) par blocage ou stimulation spécifique de la voie de signalisation de l'angiopoïétine-2 perturbe, ou améliore, le rappel des informations anciennement acquises. Stimuler l'ACP chez un modèle de rats hypertendus présentant des déficits d'activation de la voie de l'angiopoïétine-2 et de formation de la mémoire pallie le déficit mnésique observé, confirmant l'importance fonctionnelle de l'ACP comme un prérequis à la formation des souvenirs. L'hypergravité, connue pour altérer les fonctions vasculaires, n'a pas modifié l'organisation de la mémoire. Nos résultats identifient l'ACP comme un processus neurobiologique crucial sous-tendant la formation et la stabilisation des souvenirs. Ils révèlent l'importance de la plasticité vasculaire dans la modulation des fonctions cognitives et suggèrent que les changements structurels précoces du réseau vasculaire cérébral constituent un mécanisme permissif pour la régulation de la plasticité neuronale au sein des réseaux corticaux impliqués dans la formation progressive et le stockage des souvenirs.

**Mots-clés :** Angiogenèse - Consolidation mnésique - Hypergravité - Hypertension artérielle – Plasticité cérébrale - Rat - Rappel - Réseau vasculaire cérébral

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# **Title: Dynamics of the cerebral microvasculature during the course of memory consolidation in the rat: physiological and altered conditions induced by hypertension and hypergravity**

## **Abstract:**

While the cerebral microvasculature is known to adapt its activity according to the metabolic demand of surrounding neurons, the functional contribution of vascular networks to memory consolidation, the process by which memory traces acquire stability over time, remains elusive. By using an associative olfactory memory task in rats coupled to biochemical and imaging techniques, we investigated the dynamics of vascular changes during memory consolidation which requires a transitory interaction between the hippocampus and distributed cortical regions that ultimately support storage of enduring memories. We found that remote memory formation was associated, upon encoding, with a hypoxic signal that triggered transitory angiogenesis in specific cortical regions which support memory storage and retrieval only weeks later. Manipulating early cortical angiogenesis (ECA) by selectively blocking or stimulating the angiopoietin-2 signalling pathway impaired or improved remote memory retrieval, respectively. Enhancing ECA in spontaneously hypertensive rats, which exhibit reduced angiopoietin-2 expression when cognitively challenged and are unable to properly stabilize and/or retrieve remotely acquired information, was efficient in rescuing the observed memory deficit, thus confirming the functional importance of ECA as a prerequisite for the formation of remote memories. Hypergravity, known to impair vascular functions, failed to alter the organization of recent and remote memory. Altogether, our findings identify ECA as a crucial neurobiological process underlying the formation and stabilization of remote memory. They highlight the importance of vascular plasticity in modulating cognitive functions and suggest that the early structural changes within vascular networks constitute a permissive mechanism for the regulation of neuronal plasticity within cortical networks which support the formation and storage of enduring memories.

**Keywords:** Angiogenesis – Arterial hypertension - Cerebral plasticity – Cerebral vascular network - Hypergravity - Memory consolidation - Rat - Retrieval

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*“Le véritable voyage de découverte  
ne consiste pas à chercher de nouveaux paysages,  
mais à avoir de nouveaux yeux. ”*

*À la recherche du temps perdu, Marcel Proust*

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Anyway thank you all to take part to this adventure!

## List of Abbreviations:

- 5-HT: serotonin
- 20-HETE: 20-hydroxyeicosatetraenoic acid
- A: antero
- AA: arachidonic acid
- ACh: acetylcholine
- aCSF: artificial cerebrospinal fluid
- ACA: anterior cerebral artery
- ACC: anterior cingulate cortex
- AD: Alzheimer's Disease
- AICA: anterior inferior cerebellar artery
- Ang-1: Angiopoietin 1
- Ang-2: Angiopoietin 2
- azACA: azygos of the anterior cerebral artery
- BA: basilar artery
- BBB: blood brain barrier
- BK:  $\text{Ca}^{2+}$  dependent  $\text{K}^{+}$  channels
- CA: Cornu Ammonis
- Cam: calmodulin
- CaMKII: calmodulin-dependent protein kinase II (CaMKII)
- Cav: voltage-operated channel
- CBF: cerebral blood flow
- CICR: Calcium-Induced Calcium-Release
- cGMP: guanosine 3',5'-cyclic monophosphate
- COX: cyclooxygenase
- CSF: cerebrospinal fluid
- dHPC: dorsal hippocampus
- HPC: hippocampus
- ECs: endothelial cells
- ECM: extracellular matrix
- EDHF: endothelium-derived hyperpolarizing factor
- EETs: epoxyeicosatrienoic acids
- eNOS: endothelial nitric oxide synthase
- Ent: entorhinal cortex
- EXP: experimental
- FP: food preference
- FGF: fibroblast growth factor
- GABA:  $\gamma$ -Aminobutyric acid
- GAP: growth-associated protein
- GC: guanylate cyclase
- GD: Gyrus dentatus
- GFP: green fluorescent protein
- Glu: glutamate
- HIF-1 $\alpha$ : hypoxia-inducible factor 1 $\alpha$
- HRE: hypoxia response elements
- HPC; hippocampus
- IC: internal carotid
- IEGs: immediate early genes
- iGluR: ionotropic glutamate receptor
- IKCa: intermediate conductance  $\text{Ca}^{2+}$  activated K channels
- KATP: ATP-sensitive potassium channel
- IL: infralimbic cortex
- iNOS: inducible nitric oxide synthase
- I.P.: intraperitoneal
- IP<sub>3</sub>: inositol 1,4,5-trisphosphate

- IP<sub>3</sub>R: inositol 1,4,5-trisphosphate receptor
- JAM: junction adhesion molecules
- l-NNA: as l-nitro-arginine
- LTM: long-term memory
- LTP: long term potentiation
- MAP: mitogen-activated protein
- MCA: middle cerebral artery
- mGluR: metabotropic glutamate receptor
- MTT: multiple trace model of memory consolidation
- MPPs: metalloproteases
- MLC: myosin light-chain
- mPFC: medial prefrontal cortex
- MTL: medial temporal lobe
- NA: noradrenaline
- nNOS: neuronal nitric oxide synthase
- NOS: nitric oxide synthase
- NO: nitric oxide
- NOTCH:
- NRPs: neuropilins
- OAM: olfactory associative memory
- ODDD: Hif- $\alpha$  O<sub>2</sub>-dependent degradation domain
- OFC: orbitofrontal cortex
- P: posterior
- P<sub>A</sub>: arterial pressure
- PAI-1: plasminogen activator inhibitor-1
- PBS: phosphate buffer solution
- PC: Parietal cortex
- PCA: posterior cerebral artery
- PcomA: posterior communicating artery
- PDGFB: platelet-derived growth factor-B
- PFA: paraformaldehyde
- PG: prostaglandin
- PHD: prolyl hydroxylase enzymes
- PKA: protein kinase A
- PL: prelimbic cortex
- PLC: phospholipase C
- PLGF: placental growth factor
- PMCA: plasma membrane Ca<sup>2+</sup> ATPase
- PO<sub>2</sub>: partial oxygen pressure
- REM: rapid eyes movements
- RM: remote memory
- RS: retrosplenial cortex
- RyR: ryanodine receptor
- SC: sarcoplasmic reticulum
- SCA: superior cerebellar artery
- SERCA: Sarco-Endoplasmic Reticulum Ca-ATPase
- SKCa: small conductance Ca<sup>2+</sup> activated K channels
- SM: standard model of memory consolidation
- SMC: smooth muscle cell
- STFP: social transmission of food preference
- SHRs: Spontaneously Hypertensive Rats
- SHRSPs: Stroke-Prone Spontaneously Hypertensive Rats
- SDs: Sprague Dawleys Rats
- SMC: smooth muscle cells
- STM: short-term memory
- Sub: subiculum
- SWS: slow wave sleep

- Tie-2: angiopoietins receptor
- TIMPs: tissue inhibitors of metalloproteinases
- TRP: transient receptor potential channels
- TXA2: thromboxane A2
- VA: vertebral arteries
- VDCC: voltage-dependent calcium channels
- VEGF: vascular endothelial growth factor
- VEGFR: vascular endothelial growth factor receptor
- VHL: Von Hippel–Lindau protein

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## GENERAL INTRODUCTION

## **Chapter 1: Memory and vascular contribution: the aim**

## **Chapter 1: Memory and vascular contribution: the aim of the thesis**

What is the aim of our memory? To remember, of course!

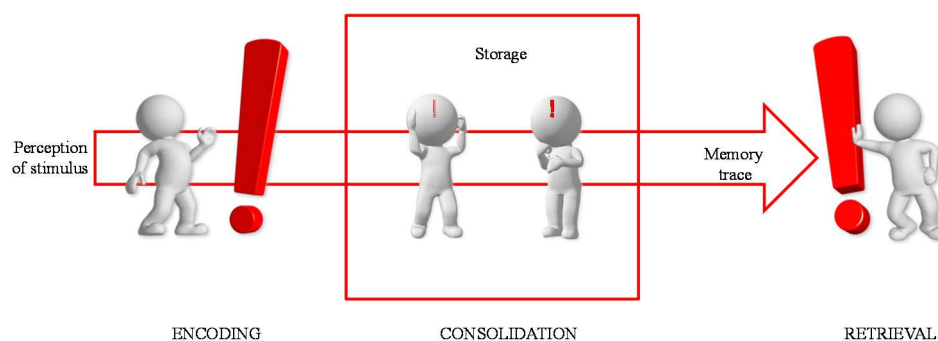
Nevertheless, more deeply the memory helps us to think and to shape our knowledge.

Why is our culture so enriched by systems that help us to remember, to take trace of the events? Humanity grew up creating systems for transmitting the information acquired during their life, starting from paintings, books to the most modern databases. These are forms of “external memories”, but what about “our internal memory”? Why is it so irritating not remember? It is so annoying when you do not remember the code of your credit card! And it is even worse when you don’t remember what you did for a certain period of time because you feel your personality compromised. Memory, as a matter of facts, is fundamental for building up your individual identity and in the construction of social entity. We are our memories; without our memories we modify our identity, at least from a psychological point of view (Maurizio Ferraris). But memory is not always infallible. How many times has it happened to discuss with a childhood friend and you realize that the events that he reminds are different from yours? But even so, he was with you when that episode was happening.

The study of human memory is dating back at Ancient Greece philosophers e.g. when Aristoteles defined it as “the scribe of the soul”. From Plato’s point of view, all humans were born free of any knowledge and the human mind/soul was a blank slate on which memory makes impressions in wax, Aristoteles refines this idea adding the concept of “associations”: two ideas are associated when the retrieval of one of them let us remember the second one and this process is possible thanks to an organized network of associations; this theory of memory held sway for many centuries (Plato, Teeteto; Aristoteles, De memoria et reminiscencia).

Nowadays we consider memory the intellectual attitude that let us store and retrieve information belonging to the past. In other words, memory is the physiological process by which environmental stimuli are encoded, stored and reused in our daily living activities. Thus, our brain is capable of create and store long-lasting memories that we can recall and relive, so memory is not just related to the past but also to the present and the future.

The memory process (Fig. 1) can be summarized in different fundamental steps (Josselyn et al., 2015):  
Encoding: the acquisition and the registration of new information, during a period of learning;  
Retention and storage: the period during which the information, previously acquired, is assimilate and organized in order to be stocked and conserved;  
Retrieval: the return of the memory/recall of this information.



**Figure 1:** Memory process.

Memories are formed from spatial-temporal patterns of synchronized firing in specific and dynamic neuronal assembly; firings constitute a transient activate network that underlie to cellular

modifications, in order to store these representations. These activity-driven cellular modifications produce a synaptic remodelling and an increase of strength of synapses such that the specific distribution of activity can later form a readout pattern during recall (Veyrac et al., 2014).

In new memories formation process, information recently acquired is gradually transformed from an initial labile state, in which they are vulnerable to interferences, into enduring and stable state resistant to disruption. These post-experience processes of memory stabilization are called memory consolidation (Müller and Pilzecker, 1900). But how, when and where these processes concerning memory create an engrams or a memory trace remain a fundamental question.

Therefore, the purpose of my thesis is to shed light on a particular phase of memory process that is the memory consolidation. However, when we speak about memory and memory consolidation we are often focusing on neuronal changes and networks, which of course are the principal players in this process but they cannot carry out this process alone. Like an orchestra, different instruments (neurons) form a complex musical symphony but they need musical notations (energy and oxygen) to produce music.

As a matter of facts, the brain is one of the most highly perfused organs of the body, having a very low ability to store energy. For that reason, it is highly dependent on cerebral blood flow. The cerebrovascular network adapts its activity in order to provide energy and nutrients according to the metabolic need of neuronal networks (Cipolla, 2009). Consequently, there is a close interaction between the cerebral vasculature and neuronal networks during cognitive functions. However, the functional contribution of vascular networks during memory consolidation remains unknown. Thus, the first aim of my thesis is to investigate the dynamics of vascular changes during the course of memory consolidation.

Moreover, in recent years, several studies described the existence of a potential link between the disruption of the vascular system and the onset or the acceleration of cognitive pathologies (Carnevale et al., 2012; Faraco and Iadecola, 2013). For instance, hypertension has been suggested as one of the most relevant risk factors able to induce and/or increase cognitive impairments associated with vascular dementia or Alzheimer's disease, although the underlying mechanism remains to be explored. Therefore, the second aim of my thesis is to investigate to what extent dysfunctions of vascular networks impair memory performance using an animal model of hypertension.

Finally, why the CNES founded this research? The exploration projects as "mission to Mars" required a long exposure to space (closed to 2 years) with a long delay to communicate with the Earth (20min), then the possible impairment of cognitive functions as memory should have crucial consequences for the crew and the mission success. Since cardiovascular deconditioning is known to be one the first effect measured due to the modification of gravity during spaceflights, we address the question if it is possible that the gravity alteration, through the modification of cerebrovascular physiology, can affect the memory and especially the memory consolidation.

In the first part of the introduction (Chapter 1) we introduce the memory process, in particular the process concerning a memory called "ancient memory". We start from what we know from patients that lost their memory and from literature concerning the modification of memory function and their neuro-anatomical correlates. Then we explore the concept of memory consolidation, thus the system leading the storage of our memories, considering the modifications at synaptic and systemic levels and the different models explaining the link between neuro-anatomical structures.

In the second part (Chapter 2), we point out the contribution of vascular network in supporting memory process. We investigate the link between vascular network and neurons, how they communicate and how they interact. In particular, we focus on how neuron can induce vascular

changes to support memory process through the mechanism of hypoxia that in turn stimulates the modification of vascular architecture via angiogenic mechanism.

Then, in the last part (Chapter 3 and 4), we reveal how pathologic or non-physiologic conditions could affect memory process. In particular, we take advantage of two particular models; the first model is a hypertensive model: we selected the Spontaneously Hypertensive Rats (SHRs) that develop a spontaneous hypertension since 2 months, focusing on the vascular components that can affect memory during a long period of time. The second model, the rats exposed to hypergravity, is used as a tool to control the insurgence of vascular alteration and the duration of this perturbation.

Since the transdisciplinary topic of the thesis, it will be difficult to explore in details all the mechanisms implicated in the processes considered in this manuscript. In any case, we tried to summarize them and add some references of the reviews treating these topics.



## **Chapter 2: The memory process**

## **Chapter 2: The memory process**

### **2.1. Historical overview: milestones in memory study**

While learning is the process whereby we acquire new information (an adaptive change in behaviour caused by experience), memory includes any changes in activity and connectivity of neuronal system, triggered by exterior stimuli or brain state that persist over the time (Chaudhuri and Fiete, 2016). Memory is an adaptive process by which experience-based information is encoded, stored and finally retrieved (Lesburgueres et al., 2011). Memory is like glue, without it our life is made by disconnected fragments without meaning.

But where the memory is stored and how it is conserved in our brains? In the study of neuroscience, we have always looked for a link between cognitive functions and structures tasked with that function.

However, if today it seems logical that the brain functions reside in the brain, in the past the concept of the dualism, that is the separation between cognitions and logical knowledge, was a common thought, with the former housed either in heart or in the soul, and the latter, residing in the brain. William Shakespeare in *The Merchant of Venice* (Act 3, Scene 2) writes “*Tell me where is fancy bred. Or in the heart or in the head?*”. In the XVIII century, a physician named Franz Joseph Gall attempted to abolish mind-brain dualism providing insights that are important for modern neuroscience; based on anatomical studies, he argued for the first time that brain is the organ of the mind and all mental functions arise from the brain; moreover he noticed that the cerebral cortex is not homogeneous and different mental functions are localized in different regions of the cortex; Gall thought to localized mental faculties by examining the surface of the skulls of humans and, not surprisingly, he misidentified the functions of most parts of the cortex; nevertheless he produced these major conceptual contributions that has opened the door to the study of cognitive process localized in the brain. After more than one century, this insight was reviewed thanks to the study of the correlation between cognitive impairment and cerebral lesions. Studying the language and the aphasia process Broca and Wernicke understood that a complex mental function is not localized to a single brain region but involves interconnections between several regions. This intuition drives in the XX<sup>th</sup> century a plethora of studies concerning the localization of cognitive functions, and among these also memory was investigated (Milner et al., 1998).

Nowadays we know that memory is not stored in one single structure, but it spreads in different brain areas, in other words, it is a multi-system process. However, the fundamental question about how and where the memories are formed and stored in our brains has not yet been answered. A new interesting insight that started to scratch the surface of this intriguingly field was given by Richard Semon at the beginning of XX<sup>th</sup> century that was the first to propose the “engram theory” in memory process. His idea was that the information, which is encoded and transformed in memory, could be retrieved even if we find just an element resembling to a component of the original stimulus thanks to a memory trace called “engram”. He defined the engram as “...the enduring though primarily latent modifications in the irritable substance produced by a stimulus...”, to be distinguished from the “ecphory” the process which “...awakens the mnemonic trace or engram out of its latent state into one of manifested activity...” (Semon and Simon, 1921); currently, these two concepts are called respectively memory trace and retrieval. Nowadays we know that the formation and the storage of a memory lay on physical and biochemical modifications in brain structures induced by learning. These modifications that last over the time are summarized with the name of engram or memory trace (Josselyn et al., 2015). In other words, engram refers to enduring physical and chemical changes produced by learning, which are concretized in the formation of newly formed memory associations.

Moreover, Semon amazingly pointed out the re-integrative aspect of the retrieval: only a part of the total stimulation needs to be present in order to recall the retrieval of the memory (Tonegawa et al., 2015a).

The theory of engram was supported by Karl Lashley (Tonegawa et al., 2015a) that empirically tried to localize the spatial memory trace in rodent brain structures through cortex lesions of different size. The rats were let to perform a maze with food rewards. He showed that little cortical lesions did not affect memory spatial performance; but when the lesions were more extended memory was affected; he concluded that the severity of memory impairment was directly proportional to the size of the lesion and that memory was not localized in any specific region of the brain. He formulated the “law of mass action” (Lashley, 1929; Milner et al., 1998), according to which the extent of memory deficit was correlated with the size of the cortical area removed, not with its specific location. Two weaknesses biased this study: first the lesions were addressed just to the cortex and second rats are able to develop other strategy to perform the maze. Despite Lashley’s conclusions were partially incorrect, encouraged by these findings, several studies were produced in order to localize and characterize the engram both on animals and in humans. Among these Donald Hebb in 1949 tried to explain Lashley’s results suggesting that learning induces the reinforcement of synaptic connection and facilitates the formation of neuronal assemblies, a population of neurons that shows coordinated firing activity; these assemblies can be distributed over large areas of cortex (Josselyn et al., 2015). This concept is the base of modern theory of memory engram formation.

Today, we often use the terminology of engram pathways to indicate that the memory trace of an event is not necessarily located to a single anatomical region but it is distributed in several areas directly or indirectly connected in a scheme specific to that given information (Tonegawa et al., 2015a).

Another important step in the localization of memory structures in the brain was achieved by neurosurgeons Wilder Penfield and Theodore Rasmussen that showed, for the first time, that episodic memory is localized in specific areas of the brain.

For a pre-surgery procedure, they stimulate a part of the medial temporal cortex of a patient, inducing a vivid recall of episodic memory: “Yes, Doctor, yes, Doctor! Now I hear people laughing - my friends in South Africa. Yes, they are my two cousins, Bessie and Ann Wheliaw.” (Penfield, 1950). This study revealed the involvement of this structure in retrieval of episodic memory.

A particular acceleration in the memory study was given by patient HM and other patients that make possible the correlation of different kind of amnesia with brain lesions; we will discuss the interesting case of this patient in the chapter “The landmark study of Henry Gustav Molaison (Feb. 26, 1926 – Dec. 2, 2008)” in order to understand the important insights that allowed us to define the different kinds of memory and the temporal process underlying memory formation.

Nowadays thanks to imaging technology, behavioural paradigms coupled with invasive or non-invasive techniques, immediate early genes (IEGs, that underlie synaptic plasticity and modification of neurons required for memories) or optogenetics studies we can identify and manipulate the neurons implicated in memory process. Moreover, we know that the engram associated to a souvenir is not necessarily located in one single region, but it can be scattered in a broad neuronal network, and it was probably for this reason that Lashley faced so many issues to find out the engram localization.

All these studies reveal that each memory recruits a specific subpopulation of neurons activated during the encoding and re-activated during retrieval (Veyrac et al., 2014). The supporting evidences regarding this concept can be summarized in three types of study: the observational studies, which demonstrate a correlation between neuronal assemblies and correlated to the behavioural expression of that specific memory; the loss-of function studies, which demonstrate that inhibiting the neuronal

assemblies results in the impairment of the memory expression; the gain-of function studies that show how the behavioural expression of the memory can be triggered through the activation of neuronal population (Tonegawa et al., 2015a).

Regarding observational studies, examining IEGs like c-fos or zif268, several teams demonstrated that the engram associated to a memory is not necessarily located in one single region, but it can be localized in a wide neuronal network (Frankland et al., 2004; Lesburgueres et al., 2011; Maviel et al., 2004). Moreover, they confirmed that a selected population of neurons, active during acquisition of a memory, are preferentially reactivated during the retrieval of the same memory. It is the case of fear memory: Reijmers and colleagues showed using transgenic mice (TetTag mouse model) that neurons activated during pavlovian fear conditioning encoding are re-activated during the memory retrieval in the amygdala (Reijmers et al., 2007). Similar studies demonstrated the same phenomenon during contextual fear conditioning test in hippocampus (Deng et al., 2013; Tayler et al., 2013) and cortex (Tayler et al., 2013; Zelikowsky et al., 2014).

In humans, other studies showed that the activity of single neurons in hippocampus and entorhinal cortex when the subjects view for the first time a cinematic episode are the same that are reactivated during verbal reports of memories of these specific episodes at the time of free recall (Gelbard-Sagiv et al., 2008).

In accordance, loss-of function studies proved that the reversible or irreversible inhibition of neuronal population either in amygdala (Han et al., 2009) or in hippocampus, in particular in hippocampal areas such as gyrus dentatus (GD), CA3 (Denny et al., 2014) and CA1 (Tanaka et al., 2014), can affect the retrieval of memory during fear conditioning task. Similar results were obtained regarding the essential role of retrosplenial cortex neurons in spatial navigation memory during Morris Water Maze task (Czajkowski et al., 2014). Since disrupting this neuronal population the memory is impaired, these studies revealed that these areas contain an indispensable part of engram complex.

Finally, the gain-of-function studies provide the most direct evidence for the existence of memory engram cells, since they demonstrate that selected neuronal assemblies activated by learning can elicit memory recall once they are reactivated. In others words, some studies showed that the reactivation of the same neurons in hippocampus (Liu et al., 2012) or in amygdala (Kim et al., 2014) that aroused during encoding of fear conditioning task is sufficient to trigger the behavioural expression of the memory, in this case freezing behaviour, normally induced by the original stimulus. Interestingly, the artificial activation of neurons can make the memory fleet and able to incorporate new information to form a new memory.

The capacity of the memory to be modified during reactivation can be responsible of the formation of false memories. As a matter of fact, during fear conditioning test, Ramirez and colleagues showed that the reactivation in another context of the same neurons initially activated in a first context induces freezing behaviour in absence of foot-shock, being able to demonstrate that the recall of this false memory was context-specific (Ramirez et al., 2013).

In view of above, the engram notion of Semon can be actualized; in fact when a person has a new experience a plethora of specific stimuli constituting the experience activates a specific subpopulation of neurons, in order to strengthen the connections between the neurons which in turn create a neuronal assembly that takes in charge the storage of the memory.

Successively, when the person is re-exposed to at least one part of the original experience, the neuronal network depositary of the memory trace is reactivated in order to synchronize the coherent retrieval of the original experience. Thus, during consolidation, the engram can exist in a “dormant state” between two active states, the encoding and the retrieval (Josselyn et al., 2015). The engram is

not yet a memory: the memory trace is different from the memory per se, since the first one is the neuronal substrate eliciting the expression of the second one.

Therefore, the formation of a memory trace is a complex phenomenon involving the reinforcement of the connections between different populations of neurons activated during learning. This activation of synchronized firing neurons leads to experience-induced epigenetic changes, increase of synapsis strength and neuronal excitability. In other words, the engram is constituted by long-lasting physical and biochemical modifications induced by encoding at cerebral level, but even if it starts to be formed during the learning, it is not static. The consolidation process can alter the physical or chemical organization of the engram, undergoing to strength or quality alterations (Josselyn et al., 2015).

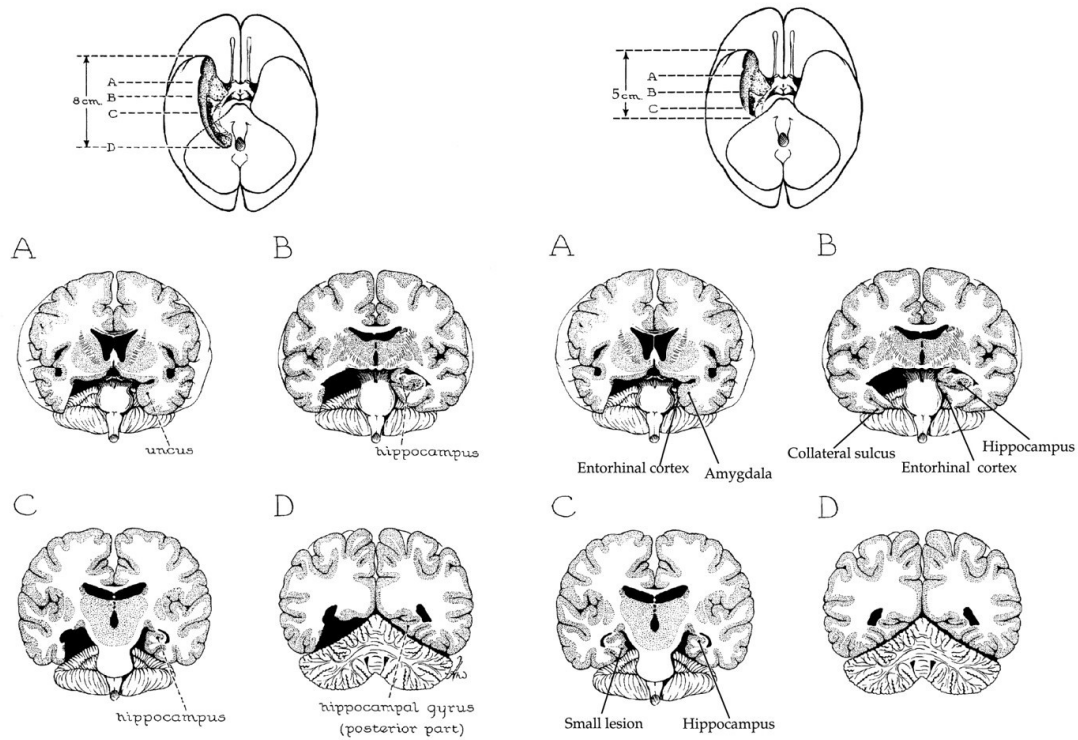
As a matter of facts, the memory trace can change its nature and its location, from molecular cellular level (synaptic remodelling, epigenetic modifications and variation of neuronal excitability) to systemic level (different structures implicated in memory consolidation process). In others words, memory trace is formed by both synaptic dynamics, that alter the efficacy of synaptic transmission (functional plasticity) and changes in the structure and number of synaptic connections (structural plasticity) (Korte and Schmitz, 2016). Moreover, the storage of the information is highly dependent from the neuronal networks.

In the next part of introduction we will consider the synaptic and systemic modification of memory trace, starting from a general overview of what concerns declarative memory consolidation and exploring the brain areas related to memory consolidation, since the objective of my thesis is to investigate part of this complex phenomenon.

## **2.2. The landmark study of Henry Gustav Molaison (Feb. 26, 1926 – Dec. 2, 2008)**

Regardless of the stimulant work of Penfield, the scientific community was not convinced that the temporal lobe was the critical area in memory process until the study of the patient Henry Gustav Molaison (known for many years as patient H.M. till his death in 2008); he was the first and most famous case, studied by Brenda Milner and William Scoville, revealing the presence of memory deficits induced by a bilateral ablation of a portion of medial temporal lobe (MTL) (Scoville and Milner, 1957) (Fig. 2).

H.M. suffered of a progressive and pharmacologically uncontrolled form of epilepsy caused by a bike crash happened when he was 9 or 10 years old, (Corkin et al., 1984). When became adult, H.M was not able to have neither a normal job nor a normal life and for this reason, at the age of 27, in 1953, he underwent to a surgical ablation of MTL in order to control the epileptic attacks (Squire, 2009). During the surgery hippocampal formation (parahippocampal-entorhinal cortex and anterior hippocampus), amygdala and parts of associative multimodal areas of temporal lobe were removed (Corkin et al., 1997).



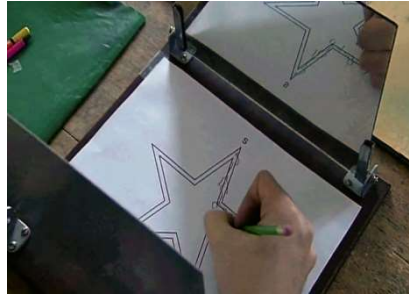
**Figure 2:** On the left, schemes show the estimation of surgical ablation of medial temporal lobe (MTL) in H.M. (Scoville and Milner, 1957), with the right hemisphere intact in order to show the structures removed. On the right, the rectified version of the original diagram indicating the extent of the ablation based on the MRI studies (Corkin et al., 1997).

After the surgery, the epileptic attacks were effectively controlled, but unfortunately for him and hopefully for us, a strong memory deficit was detected.

This memory deficit (amnesia) was peculiar, since the short-term memory, from seconds to minutes, was normal. Moreover, he possessed a long-term memory of the events happened years before the surgery. He remembered his name, his previous job and events of his childhood, even if he had a certain degree of retrograde amnesia of the events happened during few years (10 years) preceding the surgery (*partial retrograde amnesia*).

H.M. possessed a normal mastery of language, comprehending a reach vocabulary, and his IQ was unchanged (normal-high); he was able to carry on normal conversations (i.e. had some capacity for working memory) but he would forget what the conversation was about immediately (*anterograde amnesia*); practically, he was incapable to transfer new memory traces from short-term memory to long-term memory (See also the paragraph “human amnesia”).

If he was asked to remember a number like 8414317, he was able to repeat it during few minutes, showing a good short-term memory, but once briefly distracted, he forgot it. He was unable to remember information regarding people, places or objects more than few minutes, being unable to store new memories for longer. For example, he was not able to remember Milner even if he met her at least once for month and for several years. Moreover, he presented some issues in spatial orientation. He required more than one year to acquire an orientation in the thereabouts of the new home where he went to live. But while at the beginning Milner thought that all memories of H.M. were compromised, she was astonished regarding his ability to acquire new skills of motor activity that was comparable with normal subject. For example, he was able to learn how to draw a star looking the reflex of his hand in a mirror, but he did not remember to have drawn it before (Fig. 3).



**Figure 3:** Star-tracing experiment performed by H.M. to assess his ability to learn new skills involving the formation of motor memories Photo credit: © WGBH Educational Foundation.

Moreover, he was able to retain repetitive or familiar information (he remembered to have memory problems) or emotionally relevant for him (J.F. Kennedy's murder, 1963), since the repetition or the emotions are linked to other brain's areas (i.e. amygdala) without requiring of MTL.

It was clear that memory deficit was not total and it was dependent from the kind of memory considered; H.M possessed an unconscious memory.

Practically, the memory capacity of H.M., but also in others patients presenting a lesion in MTL, regards: first, habits, perceptive or motor skills comporting the execution of reflexes but not reflection/meditation; secondly these skills do not require awareness or complex cognitive processes like comparison or evaluation. These patients answer to stimuli or suggestions but when doctors ask why the execution of a work is so improved after training they do not remember to have done it before.

The study of Molaison's amnesia and the work of Brenda Milner provide important insights into memory knowledge:

- i. The lesions of MTL do not affect neither the intellectual aspects of daily life nor the perceptions and cognitions of global aspects.
- ii. There is dissociation from anatomical and systemic point of view between the short-term memory (STM) and the long-term memory (LTM), since the STM of H.M was intact; it means that the hippocampal formation plays a critical role in converting the experience from STM to LTM in order to store them in a permanent way.
- iii. In any case, since he was able to remember events happening long time before his surgery, the MTL is not the site of the permanent storage of information, but he plays an important role in how these memories are organized and stored in other different regions of the brain.
- iv. H.M. did not suffer from a total amnesia; this means that there are different kinds of long-term memory subtended by distinct brain areas. The MTL lesion's is related to memories concerning events, called declarative memory.

### 2.3. Systems of memory

The landmark case of H.M. underlined the necessity to create taxonomy systems and definitions of the different kinds of long-term memory but nowadays it is still under debate. Despite this there is a common consensus on the principal systems of memory and the cerebral regions underling these processes.

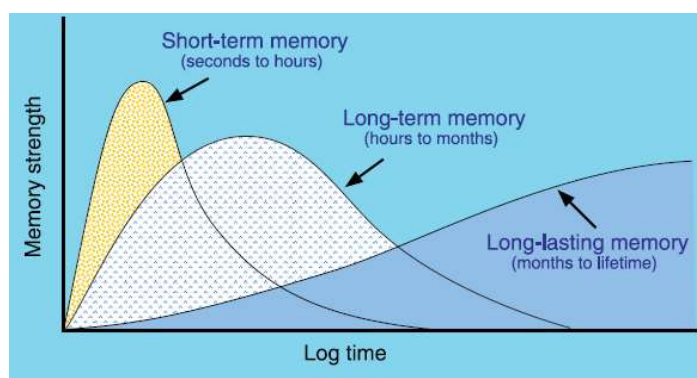
As mentioned before memory is an adaptive and dynamic process: thank to memory we can learn from experience, rapidly generalize the information acquired and retrieve the information required (Chaudhuri and Fiete, 2016) to better predict and prepare a reaction that can be used to improve the future choices that we have to make (Schultz et al., 1997).

But how the brain is organized in order to recall this information? And which are the brains areas involved in the storage and recall of memory trace, even after a long period of time?

It is not easy for our brain to process continuously the huge amount of inputs from different sensory organs and at the same time store the memories that can last for days, years or even for all our life.

But while the learning is the process by which we acquire the knowledge of the world around us, the memory is the process by which our knowledge are codified, conserved and used; it is a complex phenomenon but traditionally it is possible to distinguish three kinds of memory, depending on the duration of the storage, in other words the persistence of the memory during the time (Fig. 4): Short Term Memory (STM), that last for minutes or hour; Long Term Memory (LTM), that last for hour or days; Remote Memory (RM) also named long-lasting memory, that last for days, months, years and all our life (McGaugh, 2000).

The STM is related to transitory modifications in neuronal communication while the LTM/RM leads to stable modifications of neuronal structure.



**Figure 4:** Time-dependent memory consolidation process showing the STM, LTM and RM time courses (McGaugh, 2000).

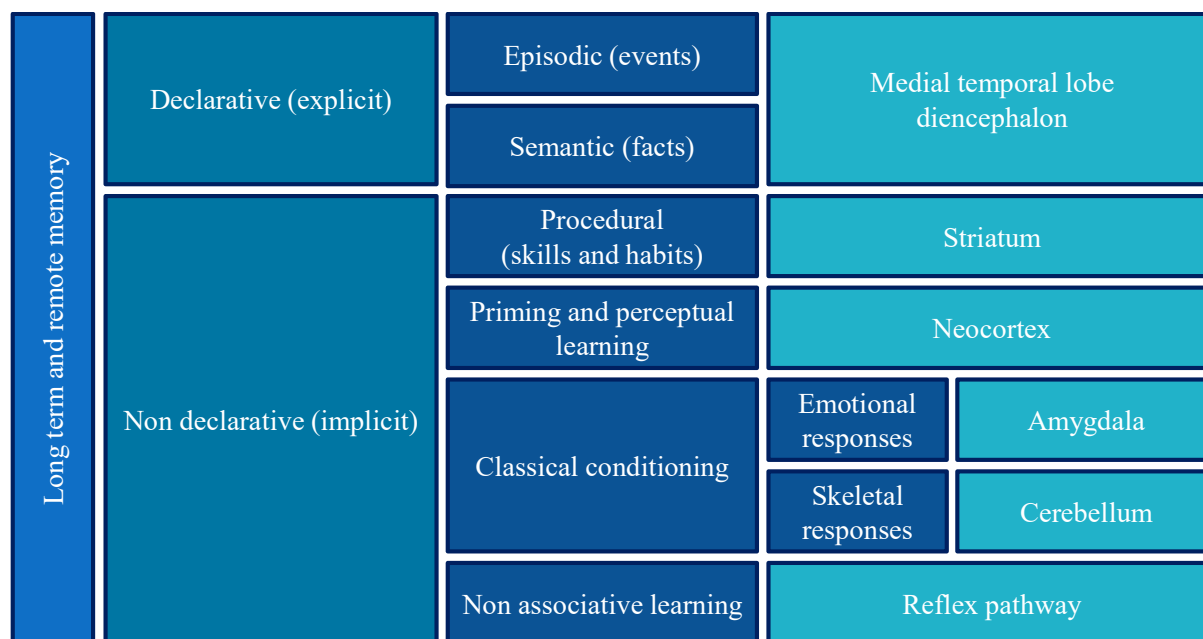
STM and LTM/RM differ considering the time-dependent memory decay but in reality the system is more complex since they differ also from the capacity, meaning that there is a limit in the number of the items stored (Cowan, 2008). Another distinction between these forms of memory is the changes in molecular or cellular structures: while STM regards sustained changes in activity, LTM refers to changes in the presence of connections and the strengths of the corresponding synapses between neurons (Chaudhuri and Fiete, 2016).

Even if the definitions are under debate it is well known that while the STM is a fleeting form of memory, the LTM and RM are stable and long-lasting and the process rendering the acquisition stable over time is called memory consolidation.

The most famous classification of long term and remote memory systems is proposed by Larry Squire in 2004 (Levy et al., 2004; Squire, 2004) (Fig. 5); the main distinguishing criterion is based on the level of consciousness with which we use our memories: the ability to retrieve the facts and events consciously (explicitly) or unconsciously (implicitly) defines the difference between declarative and non-declarative memory. In the next part of the paragraph we are going to explore in particular the



explicit memory since it is necessary to better understand the methods that will be used in this thesis, but for more information concerning the implicit memory you can read (Kandel, 2012; Squire, 2004).



**Figure 5:** Long-term memory mammalian taxonomy and mostly related structure involved. Modified from (Squire, 2004).

**The explicit or declarative memory** is the concept of memory that everybody uses, speaking in everyday language. It concerns the facts and events (Tulving, 1972 ), details regarding people or things and their meaning; the memory is recalled with deliberate and conscious efforts.

Declarative memory supports the encoding of the relationship between multiple events and items; in other words, it concerns the capacity to associate different aspects all together; it is a ductile memory in which the information stored is flexible and can guide the performance of a task even in different contexts and situations. It is able to detect and encode multiples information of a single event happened in a particular time and place (different from implicit memory).

Impairment of this memory is detected in patients with MTL and midline diencephalon lesions.

Declarative memory can be divided in semantic memory and episodic memory. The *semantic memory* (memory of facts) concerns the real knowledge of facts and evidences regarding the world, independent from personal experiences (i.e. Shakespeare wrote “Romeo and Juliet”) and it includes the general knowledge that we possess.

The *episodic memory* (also called autobiographic memory or memory of events) regards the retrieval of personal experience (i.e. playing drama club's performance of “Romeo and Juliet” at the high-school) and is described as the capacity of re-experience an event in the context in which it is originally occurred (Tulving, 1985). As affirmed by the name episodic, this memory is able to encode both the factual aspect and the spatial and temporal contexts. It is the same memory that let us to perceive the own identity and integrate the past events with future perspectives.

In summary, the declarative memory gives us a representation, a model of the surrounding world but it could be either a true or a false model.

In contrast, the **implicit or non-declarative memory** is neither true nor false and it relates to the modalities of execution of an action and it is recalled unconsciously; it is more connected with the

performance of the execution of motor skills or perceptive reflexes rather than a recollection. It is connected to the original conditions in which the information was acquired (i.e. learn how to ride a bike), but it helps us to extract the common elements from a series of separate event (i.e. we are able to ride a bike everywhere). Moreover, the formation of this memory correlates with changes in specialized performance systems.

From a biological point of view, each memory system is defined by a network of structures working together on the same type of information and participate to the storage of the same (Sporns et al., 2000). Traditionally, the experimental studies pretend to investigate the memory traces as a compartmentalized physiological substrate; even if this simplified approach is necessary to unravel the complexity of the memory pathway, we have to deal with the evidence that all these traces and structures interact each other in active manner, and that all the events occurred create a dynamic relationship of synergism, competition or independence (Kim and Baxter, 2001).

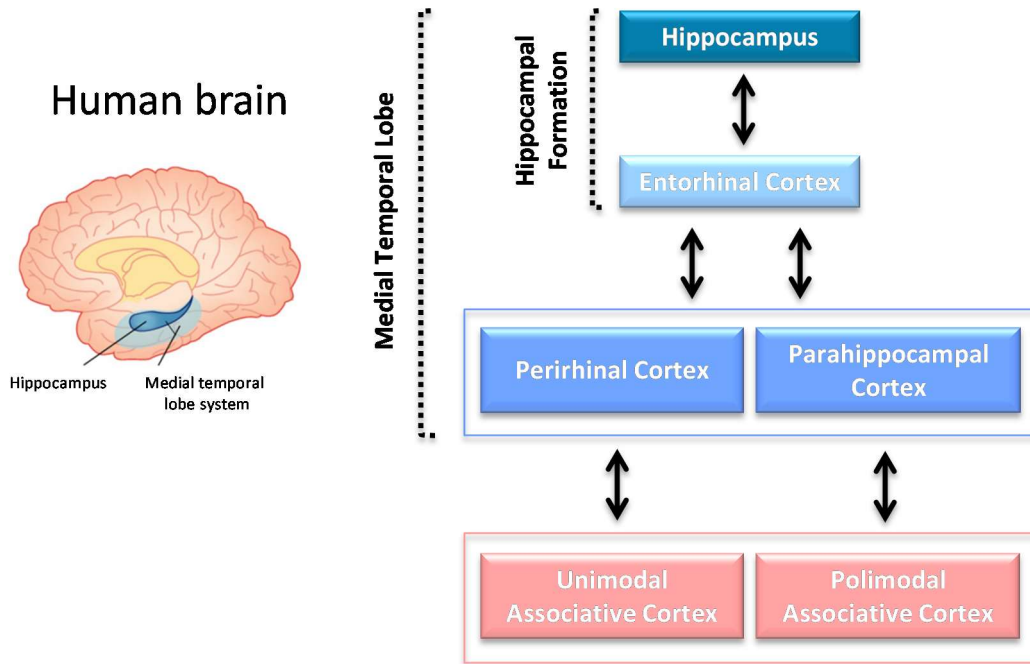
What is known is that MTL, as mentioned before, is a crucial actor in the formation of declarative memory.

Coming back to H.M. now we can better understand why he suffered from a persistent anterograde amnesia specifically restricted to declarative memory; in fact he could not transfer new semantic and episodic memories because the lesion of LTM, left the procedural memory intact, suggesting that the declarative memory is different from non-declarative memory since is not reliant on the hippocampus. Moreover, these lesions only impaired recent memories, indicating that hippocampus is the anatomical locus where newly acquired memories reside, whereas older memories are stored permanently elsewhere (Santini et al., 2014).

### **2.3.1. The role of Medial Temporal Lobe (MLT) and memory pathway**

MTL is composed by several cerebral areas, in particular it includes the hippocampal formation consisting in the hippocampus (HPC) and parahippocampal cortex that includes entorhinal (Ent), perirhinal and postrhinal cortices, as well as presubiculum and parasubiculum (Amaral and Witter, 1989). The perirhinal and parahippocampal cortices receive different unimodal and polimodal afferents from associative areas of frontal temporal and parietal lobes. These cortices are reciprocally connected with Ent which represents the principal entry of cortical afferents directed to HPC (Fig. 6). The neuroanatomic organization of HPC is similar among the species and this phylogenetic stability suggests the crucial role of central nervous system.

In the following part we are going to explore briefly the cytoarchitecture of hippocampal formation.



**Figure 6:** Medial temporal lobe (MLT) system and hippocampal circuitry in the human brain (Bizon and Gallagher, 2005).

### 2.3.2. Hippocampal formation: cytoarchitecture and neuronal pathways

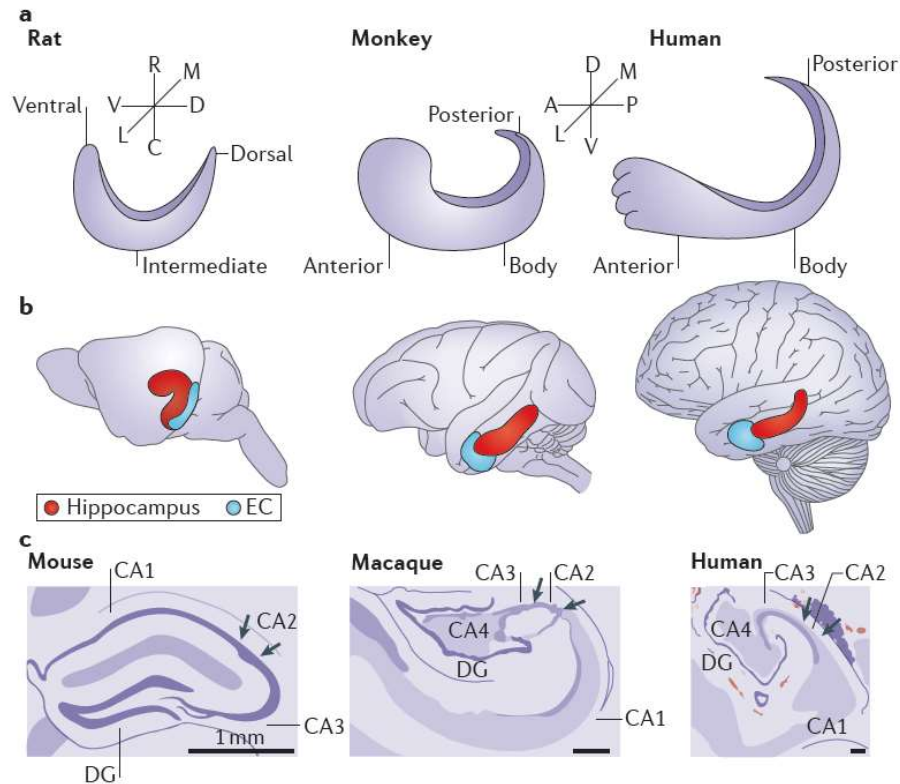
Hippocampus, as mentioned before, is one of the phylogenetically oldest parts of the brain. Its name is derived from Greek words *hippos* (horse) and *kampos* (sea), due to its similarity with the seahorse. In the Homer poem, the god of the sea Poseidon was used to ride the hippocampus (Fig. 7), an animal with the head of a horse and the body of a dolphin (Okten, 2016).



**Figure 7:** “Thetis Riding Hippokampos”, J Paul Getty Museum in Malibu, California, USA. (Okten, 2016).

As the mythology suggests, the hippocampus is a long and curved structure included in the grey matter of the MTL.

The hippocampus can be morphologically divided in three parts (named in relation with the position with respect to corpus callosum): precommissural, supracommissural and retrocommissural hippocampus; the first two are little vestigial structures, in contrary, the retrocommissural hippocampus is more developed and in this thesis when we speak about HPC, we refer to this last part. It is located along a dorsal to ventral axis in rodents, corresponding to a posterior to anterior axis in human (Strange et al., 2014) (Fig. 8).



**Figure 8:** Comparison between hippocampal anatomy of rats, monkeys and humans; a) orientation of HPC long axis among these species; b) Localization of HPC (red) and entorhinal cortex (EC; blue) in brains of rats, monkeys and humans; interestingly, 90-degree rotation is required for the rat hippocampus to have the same orientation as that of primates; c) Drawings of Nissl cross-sections of mouse, rhesus and human hippocampi. A, anterior; C, caudal; D, dorsal; DG, dentate gyrus; L, lateral; M, medial; P, posterior; R, rostral; V, ventral. From (Strange et al., 2014).

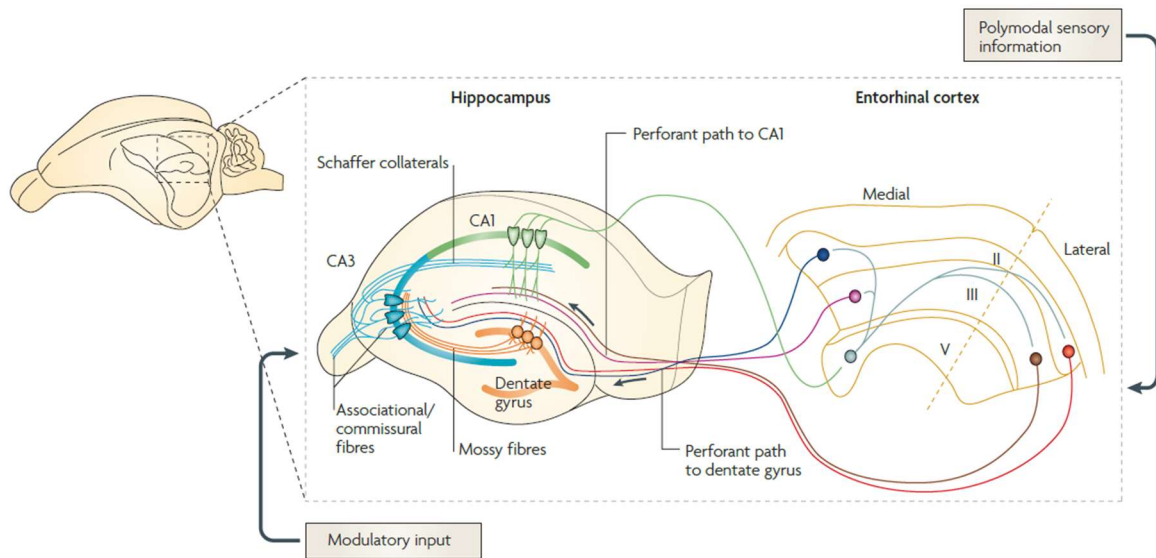
HPC is composed by three longitudinally organized structures: *Girus Dentatus* (GD), *Cornu Ammonis* (CA) and *Subiculum* (Sub). HPC resembles two C, composed by distinct cellular layer, and symmetrically included one into the other, respectively represented by GD and Sub together with CA.

The GD is composed by three different layers: molecular, granular and polymorphic layers (from external to internal part); the granular layer is composed by neuronal granular bodies whose dendritic spines penetrate in the molecular layer branching out and taking contacts with afferents pathways (e.g. Ent, commissural afferents and ascending axons from polymorph layer); the axons of the granular cells penetrate into the CA constituting the Mossy fibres.

In turn CA, called in this way since the resemblance of the outer surface to a ram's horn, can be divided in 4 different zones: CA1, CA2, CA3 and CA4. At dorsal level of HPC (dHPC), the CA1 constitute the distal region while the CA2, CA3, CA4 form the proximal part of the dHPC. Other authors consider the CA2, since its reduced size, a distal part of CA.

Hippocampal formation can be compared to cortical areas regarding its pyramidal shaped projections and small interneurons but it possesses two important characteristics: in primis, its capacity to transmit unidirectional information. As a matter of facts, Ramòn y Cajal in 1893 noticed a peculiar characteristic of hippocampal formation. While cortices are linked by bidirectional connections, HPC does not, owning a unidirectional projection. Secondly, it owns a tridimensional organization of distributed associative connections. These characteristics allow the HPC to quickly receive and integrate highly processed multimodal sensory information from different cortical areas, mix, and

compared together. In other words, the role of HPC in coordinates cortical input is due to its cytoarchitectonic organization forming a unidirectional tri-synaptic circuit with a precise topography (Fig. 9; Ent-GD, 1<sup>st</sup> synapsis; GD-CA3 2<sup>nd</sup> synapsis; CA3-CA1 3<sup>th</sup> synapsis), communicating prevalently with glutamatergic signalling (Amaral and Witter, 1989).



**Figure 9:** Schema of trisynaptic circuit of HPC and its connections with Ent. The principal input of hippocampal formation is the perforant path conveying the polymodal sensory information from layer II of Ent to GD. The axons of perforant path make excitatory synaptic with dendrites of granular cells, which, in turn, send projections (via mossy fibres) to proximal apical dendrites of CA3 pyramidal cells; CA3 pyramidal cells project to ipsilateral CA1 pyramidal cells and to contralateral CA3 (Schaffer collaterals) and CA1 pyramidal cells (commissural connections). In addition to the trisynaptic circuit, there is a wide CA3 interconnection associative network between ipsilateral CA3. CA3 and CA1 pyramidal cells receive direct input respectively from layer II and III of Ent. (Neves et al., 2008).

Cortical inputs reach HPC through the neurons of superficial layer of Ent, whose axons project to GD. This is the principal income pathway of cortical inputs and it is called perforant path. The GD does not project backwards, forming a non-reciprocate network. In fact, the principal cells of GD, granular cells, give rise to axons (Mossy fibres) ending on CA3 pyramidal cells. Likewise, these cells do not project reciprocally back but they create the input source directed to CA1 (Schaffer collaterals). CA1 projects unidirectionally to Sub, which represent its most important excitatory input. Once arrived to the level of CA1 the pathway starts to be more complex since CA1 projects to Ent and the Sub sends its output mainly to Ent but also to the presubiculum and parasubiculum areas. These connections close the loop circuit: the superficial layer of Ent provides the most prominent input to HPC while the deep layers of Ent receives the broadest output of HPC.

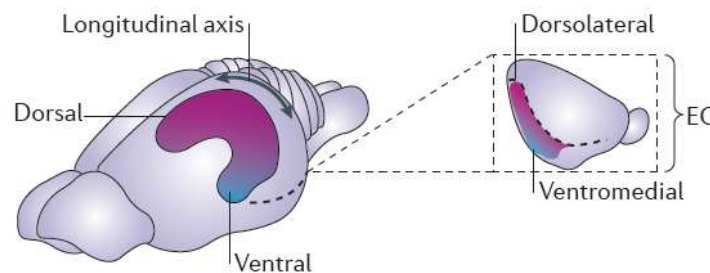
The hippocampal formation may appear as simple in organization and for a long time it was described as a structure with a unique laminar organization. Nowadays, we know that the pathway is more complex due to the interconnections within the HPC; e.g. CA3 cells send numerous axon collateral projections to other CA3 pyramidal cells. The existence of this recurring circuit (recurrent collateral system) makes a CA3 strongly interconnected HPC area, suitable for the rapid acquisition of information (Rolls and Kesner, 2006), in contrast to CA1 where pyramidal cells are only loosely interconnected. In addition, there are direct Ent projections (layer III) to CA3 and CA1. CA1 moreover projected backwards to the entorhinal cortex via subiculum, as previously mentioned. To this complex internal architectural organization, we have also to take into consideration different relationships between HPC and other surrounding structures, in particular the cortical ones such as prefrontal cortex and anterior cingulate cortex (Godsil et al., 2013).

The majority of hippocampal afferences come from Ent (performant path), which receives several inputs from different neocortical areas. In particular, the projections arriving to the II or III layers (superficial layers) are coming from olfactory bulb and perirhinal cortex. The deeper layers of Ent receive afferences from insular cortex, medial prefrontal cortex, anterior cingulate cortex and retrosplenial cortex (Amaral and Witter, 1989; Godsil et al., 2013). More recently, it was highlighted the existence of monosynaptic connections between anterior cingulate cortex and CA3 and CA1 (Rajasethupathy et al., 2015).

Regarding HPC efferences, CA1 pyramidal cells project to Sub (Amaral et al., 1991), that constitute the principal exit pathway of HPC which innervate the majority of extrahippocampal structures (e.g retrosplenial, orbitofrontal cortex, medial prefrontal areas and Ent). CA1 project directly to perirhinal, retrosplenial and prelimbic cortex (Burette et al., 1997). Moreover, the connections are even more complex since the dorsal part of HPC (dHPC) and the ventral part (vHPC) take on different roles.

In the next part, I am going to refer to rodent hippocampus considering that dorso-ventral organization in rodent brain corresponds to poster-anterior axis in human, as previously mentioned. Of course there are differences between rodent and human hippocampus in term of size (monkey HPC is tenfold bigger than rat one and in turn human HPC is 100 fold bigger than non-human primate, reflecting the evolution and the complexity of information elaborated) and in term of architecture (CA1 of rats appears a compact layer, the human one is thicker and heterogeneous the commissural interconnections of GD are more prominent in rat than in human; the subdivisions of human Ent are than rats ones, reflecting a higher development and in turn a stronger interconnections between associational area of neocortex), but the connectivity pattern is similar (for more details see (Andersen, 2007)). The distinction between dHPC and vHPC was based on anatomical studies which underlined segregated connections along the dorsoventral hippocampal axis (Moser et al., 1993; Moser and Moser, 1998), and deficit-lesions studies which shown that dHPC (but not vHPC) lesions affect cognitive functions and in particular the spatial memory (Moser et al., 1993) and vHPC (but not dHPC) lesions alter stress and emotional behaviour (Henke, 1990). This view was criticized since the distinction between what is dHPC and vHPC was not clear but fortunately nowadays we are able to study the link between molecular and functional domains of HPC (Thompson et al., 2008).

Regarding the intra-hippocampal connectivity, there is a dorsolateral-to-ventromedial gradient of Ent projections to dorso-ventral hippocampus (Strange et al., 2014) (Fig. 10).



**Figure 10:** Extrinsic connectivity gradients: topographical arrangement of Ent-HPC reciprocal connections in rodents. The dorsolateral part of Ent (EC; magenta) is preferentially connected to the dorsal HPC. Ventral and medial part of the EC (purple to blue) are connected to increasingly more ventral levels of the hippocampus (Strange et al., 2014).

Ent can be rostrocaudally divided in caudolateral, intermediate rostromedial zones. Caudolateral area receives visuospatial information, via perirhinal and postrhinal cortex transferring them to dHPC; the



intermediate zone collects a widespread input projecting to the intermediate part of HPC; rostromedial part receives primary olfactory, gustative, and visceral inputs, in turn sent to vHPC.

Regarding the neuronal connectivity of dHPC, dorsal CA1 and dorsal Sub send the most prominent projections to retrosplenial and anterior cingulate cortex, areas involved in cognitive processing of visuospatial and memory information (Frankland et al., 2004) and spatial navigation, while dorsal Sub is connected with mammillary body and anterior thalamic nuclei, implicated in the navigation/direction system enabling rodents to properly orient and execute behaviours in a learned environment (Jeffery, 2007; Muller et al., 1996; Taube, 2007; Taube et al., 1990).

Regarding the neuronal connectivity of vHPC in particular, there are two strong connections: the first involves ventral CA1, ventral Sub and posterior amygdala pathways controlling neuroendocrine activity and the second implicates ventral CA1 and central amygdala mediating the contribution of vHPC in fear learning (Clark and Bernstein, 2009) as in the case of taste aversion learning (Fanselow and Dong, 2010).

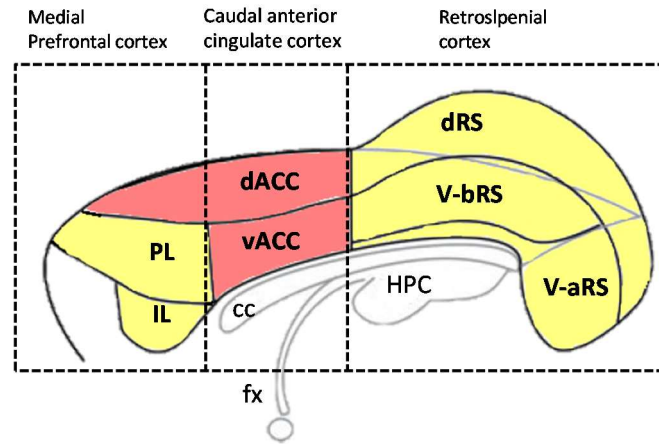
In conclusion evidences suggest that the gradient along hippocampal longitudinal axis (from dorsal to ventral areas of rat HPC which find its homologous in human HPC from posterior to anterior zones) reflects genetic expression and anatomical connectivity as well as physiological and behavioural functions (Sigurdsson and Duvarci, 2015)

### **2.3.3. Hippocampal-Anterior Cingulate Cortex pathway**

Although HPC has a preponderant role in memory, we have to remember that it interacts with other cortical players that are activated depending on the type of memory formed and the time-course of memory process.

In this part of the chapter we are going to focus our attention on a particular cortical area, the anterior cingulate cortex (ACC), which is activated during olfactory associative memory, a type of memory that we explore during this thesis.

The rat cingulate cortex is a part of the limbic system situated in the medial part of cerebral cortex and in a simplified view it can be divided anterior-posteriorly in 3 main zones: the medial prefrontal cortex, caudal anterior cingulate cortex and retrosplenial cortex. Medial prefrontal cortex includes the infralimbic (IL) and prelimbic (PL) cortices and one third of the dorsal ACC, while caudal anterior cingulate region is composed by the two thirds of dorsal ACC and ventral ACC and finally the retrosplenial (RS) cortex is divided in retrosplenial granular and dysgranular regions (Fig. 11).



**Figure 11:** Sub regions of the cingulate cortices. ACC (in red) is composed by dorsal and ventral parts which seem implicated in different memories. Abbreviations: dACC, dorsal anterior cingulate cortex; vACC, ventral anterior cingulate cortex; cc, corpus callosum; fx, fornix; HPC, hippocampus; IL, infralimbic cortex; PL, prelimbic cortex; dRS, dorsal (or dysgranular) retrosplenial cortex; V-aRS retrosplenial ventral (or granular) cortex A; V-bRS retrosplenial ventral (granular) cortex B; Modified from (Insel and Takehara-Nishiuchi, 2013; Jones and Witter, 2007).

The connections between cingulate cortex and hippocampal formation, but in general all the areas of cingulate cortex give rise to a wide connections to the parahippocampal region.

In particular IL and PL project extensively to perirhinal and EC, while the remaining areas of cingulate cortex send their projections to postrhinal cortex, EC, presubiculum and parasubiculum (for more details: (Jones and Witter, 2007)).

But what it is more interesting for our purpose is the existence of a bidirectional monosynaptic projection from HPC (CA1 and CA3) and ACC in mice (Rajasethupathy et al., 2015), founded using retrograde and anterograde tracers. Moreover, to assess the functional importance of this connection, they manipulate it with optogenetic techniques, evoking contextual memory retrieval. Furthermore, recording hippocampal cellular-resolution neuronal activity during stimulation of the ACC projections in mice performing virtual navigation, they show that learning drives the rise of a spare population of CA2 and CA3 neurons, which induce wide-population synchronous activity events with same neurons preferentially recruited by the ACC-HPC projection during memory retrieval. This implemented mechanism in HPC, via ACC bidirectional pathway, could act on memory stabilization. In the following part, we are going also to explore the implication of the ACC-CA connection and the emerging role of ACC in memory consolidation.



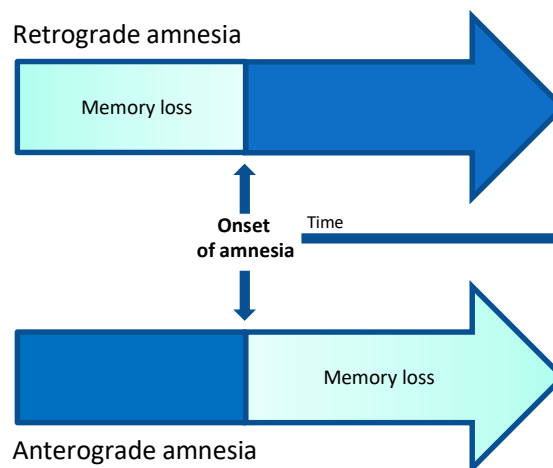
## 2.4. Lessons from amnesias: the role of Medial Temporal Lobe (MLT)

Amnesia is the inability to memorize or recall information stored in memory without the presence of others significant cognitive impairments (Markowitsch and Staniloiu, 2012). Amnesia can appear following narrow brain damage of the areas through which the information pass before being consolidated in a stable manner or following widespread cortical damage.

The retrograde (backward) amnesia (Fig. 12) is the inability to consciously reactivate information previously stored inducing memory loss of the events that occurred before the brain damage; generally the subjects permanently forgot the event occurred immediately before the accident due to an interruption to the consolidation process, caused by lesions of temporary lobe.

Anterograde amnesia (forward) is the inability to acquire, store or retrieve new information consciously after the memory accident, inducing memory loss of events that occurred after brain damage; in other words, new declarative memories cannot be consolidated and it is usually induced by hippocampal damage, since people affected can not transfer informations from STM to LTM.

The two movies “the Bourne identity” and “memento” can show the effect of respectively retrograde and anterograde amnesia. The case of H.M. shows clearly how the lesion of the bilateral ablation of hippocampal formation and amygdala was able to induce a loss of conscious new long-lasting memory formation, preserving semantic and procedural memory. The oldest episodic memory (autobiographical events) were still present but the episodic memory of few years preceding the surgery was impaired since he was not able to transfer information from STM to LTM/RM. However he was able to acquire new information if the processing of them was at procedural, priming or conditioning level.



**Figure 12:** Schema representing of retrograde and anterograde amnesia.

### 2.4.1. Anterograde amnesia and the inability to transfer memories

In case of MTL damage, as in the case of H.M., the patients suffer for anterograde amnesia, being unable to form and stabilize new declarative memories during the time. This inability to consolidate memories is independent by the sensory input the information is presented (Levy et al., 2003; Squire et al., 2001) since the MTL is a fundamental site of converging sensory cortical inputs (Lavenex and Amaral, 2000).

The severity of anterograde amnesia is depending to the size of MTL lesions (Zola-Morgan et al., 1986) as confirmed by the study of patient E.P.

E.P. presents a more extensive MTL lesion compared to H.M. (Corkin et al., 1997; Stefanacci et al., 2000). Regarding the case history, E.P. become amnesic when he was 70 years old after an episode of herpes simplex encephalitis in November 1992. His initial reduced loss of memory became severe and persistent after the clinical recovery period. Magnetic resonance studies revealed that E.P. had an extensive bilateral damage of MTL, including amygdala, HPC, Ent, perirhinal cortex and rostral parahippocampal cortex; moreover, the volume of lateral temporal lobe and left parietal lobe was reduced.

Following cognitive tests revealed his interactive and cooperative attitude but he showed repeated amazement about the invention of portable computer. During daily routine he was independent and autonomous, choosing his clothes and bathing or shaving, even if sometime his wife had to remind it to do. He woke up having the breakfast but, if he felt asleep after that, he wasn't able to remember that he has woke up, having his breakfast again. He remembered about his childhood, his teenager life and his travel during the 2<sup>nd</sup> World War, but during a session of 1 hour he repeated the same history as many as 10 times. He was attentive alert but disoriented by places and time periods.

As H.M., E.P. showed normal intellectual functions, and the immediate and the non-declarative memories were intact. In contrast, he showed a profound anterograde amnesia supported by impairment in several verbal and non-verbal tests of recall and recognition. He also had a severe retrograde amnesia affecting the events and the facts, autobiographical memory and personal semantic memory, even though he was able to recall memories acquired in his early life. If we compare the studies conducted by Corkin (Corkin et al., 1997) and by Stefanacci, it is clear that the extension of MTL lesion was bigger in E.P. (in particular concerning the perirhinal cortex, the parahippocampal cortex and HPC) supporting a more severe anterograde amnesia (verbal and non-verbal retrieval tasks).

Vice versa, in the case of the patient R.B., where the ischemic-induced lesion was circumscribed to bilateral CA1 field of HPC, the anterograde amnesia was moderated but sustained at least during the 5 years until his death (he also show a slight retrograde amnesia). In any case, this case report was sufficient to prove that hippocampal dysfunction affects memory consolidation and retrieval (Zola-Morgan et al., 1986).

#### **2.4.2. Retrograde amnesia and the stabilization of the memories**

The MTL lesions are equally accompanied by retrograde amnesia, as mentioned before, the inability to recall memories encoded before the brain damage.

As anterograde amnesia, the severity of the retrograde amnesia is proportional to the extension of the lesion (Squire and Zola, 1996). Nevertheless, in the majority of cases, included the ones previously mentioned in the paragraph of anterograde amnesia, retrograde amnesia is *temporally graduated*: as a matter of facts, the more recent information are less accessible compared to the ones acquired formerly, which remain mostly preserved. In fact, the retrograde amnesia of H.M. and E.P. covered a period of 11 and 15 years, respectively, time beyond which the memories were intact (Corkin, 2002; Squire, 2009). The temporal window is even more significant concerning the patients with more spread lesions, including Ent, perirhinal and parahippocampal cortex, affecting the memories during decades. In contrast, patients with less extended lesions, e.g. R.B., retrograde memories are just only marginally impaired (Zola-Morgan et al., 1986), nevertheless, this capacity to retrieve is diminished if lesions affect neocortical areas (Squire et al., 2015), meaning that others areas exterior to MTL can

contribute to retrograde amnesia. Anyway, in some studies presenting a flat gradient of retrograde amnesia (Rosenbaum et al., 2005), the patients presented the expected hippocampal lesion assumed to be responsible to the memory deficit, but they showed also a concomitant lesion to the posterior temporal cortex. Thus, the retrograde amnesia without temporal gradient appears when, in addition to hippocampal damage, others neocortical areas are lesioned (Kirwan et al., 2008), since the cortex is recognized to be responsible of the storage of long term memories (Frankland and Bontempi, 2005).

Moreover, other theories suggest that the severity degree of amnesia is not only linked with the kind of lesion (size and place), but it depends to the kind of declarative memory that should be retrieved. Thus, independently from the age of the memory, the episodic memory will rely on MTL, in particular HPC, while the semantic memory is dependent to HPC just during a limited period after acquisition becoming independent during the time (Nadel and Moscovitch, 1997) .

If this hypothesis is true, hippocampal lesions are able to impair the number of retrieved contextual details (time and place), resulting in the retrieval of essential semantic information. It is the case for the patients K.C. (Rosenbaum et al., 2005), G.T. (Bayley et al., 2005) and A.D. (Gilboa et al., 2006) which present, following hippocampal lesion, a deficit in autobiographic memory resulting in an impersonal knowledge of the past; however, it is possible that non graduated episodic amnesia is associated to significant modifications (volume or structural alterations) of frontal lobes, areas implicated in the autobiographic events (Bayley et al., 2005; Bayley et al., 2003).

#### **2.4.3. Animal models of amnesia**

It is out of question the fact that animals are always been useful in the study of physiological and pathological conditions and the memory field is not an exception. The rodents and even more the non-human primates help us to better understand the implication of MTL and cortex in declarative memory. The goal is to understand whether and how memory are formed and conserved over the time.

Even if the absence of language made more difficult the parallelism in characterizing the declarative memory, different studies were conducted to reproduce the effect of human amnesia in animal models in order to dissect the effects of specific lesions in different regions of HPC or more extensively of MTL (McDonald et al., 2004) and this is also possible thanks to the preservation of MTL among the species; the advantage of animal models is not just the possibility to *localize* the lesion but also to control the *size* of the lesion.

Studying non-human primates' models it was possible to investigate what was observed in human regarding the correlation between the degree of lesion of MTL and the severity of the memory deficit (Zola-Morgan et al., 1994). As for human, the lesion of HPC was sufficient to produce a memory deficit. In addition, it was possible to show in these animals that additional lesions in Ent or parahippocampal cortex induce higher memory impairment; moreover it was possible, in turn, to increase the impairment with the extension of the lesion in perirhinal cortex.

Furthermore, the difficulty of retrospective studies in sufficient homogeneous groups of patients suffering of retrograde amnesia is the limit that boosts the use of animal models. Furthermore, they let us not only to target and reproduce the zone and the extension of the lesion but they give us the possibility to correlate the memory performance to the *age* of the memories acquired and the kind of *information* acquired. Therefore, a plethora of animal studies, confirmed the temporally-graduated retrograde amnesia following hippocampal lesion or inactivation, in different cognitive tasks (Squire et al., 2015). This was demonstrated, for example, in a study conducted by Zola-Morgan and Squire (Zola-Morgan and Squire, 1990) where monkeys firstly performed novel object discrimination task at five different time point during 16 weeks preceding the hippocampal lesion. After hippocampal

damage, the monkeys were able to remember better the task acquired 12 weeks before the lesion compared to the one acquired just before the lesion. These results show that the HPC is required for the memory storage, but only for a limited time window after the information acquisition. During the time its role decreases leading the cortex taking in charge of the phenomenon. Winocur (Winocur, 1990) demonstrated the same concept using rats performing the Social Transmission of Food Preference task (STFP): rats acquired the food preference before hippocampal lesion performed at different time points after encoding (Day 0, 2, 5, 10) displaying a temporally-graded retrograde amnesia (normal memory performance at day 10), even if shorter, compared to monkey in Zola-Morgan's study. The same effect was reproduced using other behavioural paradigms like in context fear conditioning with rats undergoing to hippocampal lesion at different interval after the conditioning (Anagnostaras et al., 1999; Kim and Fanselow, 1992).

These studies prompted that the observed temporal gradients of retrograde amnesia are produced by inactivation of the same cerebral regions in humans, suggesting the reliability of animal models.

Despite this, as observed also in human studies, the time-limited contribution of HPC is not always detectable. As a matter of facts some studies failed to show a temporal gradient in fear conditioning task (Lehmann et al., 2007; Sparks et al., 2011; Sutherland et al., 2008) and in Morris Water Maze paradigm (Bolhuis et al., 1994; Broadbent et al., 2006; Clark et al., 2005; Martin et al., 2005; Sutherland et al., 2001; Teixeira et al., 2006). Similarly, Squire and collaborators were not able to show hippocampal disengagement in the same task, although modulating different parameters like the conditioning protocol, the time windows between conditioning and hippocampal lesion, the lesion size and the lesion protocol (Broadbent and Clark, 2013). Thus in some case it seems that memory trace remain dependent to HPC for more long-lasting delay, or in a permanent manner. Other theories suggest that HPC will be always required for the aspects related to the realization of the task, without any link with the memory consolidation. In this case, during the time, the memory trace will be available in others regions beyond the HPC, but independently from the delay, HPC is necessary in the realization of the task (Squire and Bayley, 2007; Sutherland et al., 2001). Moreover, to make the analysis more puzzling, a recent study highlights an interesting standpoint showing in the same rat the existence of a temporal gradient of retrograde amnesia is associated to contextual memory formed during context fear conditioning but not to a spatial memory formed during Morris Water Maze (Winocur et al., 2013): rats underwent to contextual fear conditioning and were trained on Morris Water Maze 1 or 28 days before hippocampal lesion. When tested after surgery, lesioned rats showed retrograde amnesia for spatial memory both at day 1 and at day 28 (Morris Water Maze) independently from the delay, while they displayed temporally graded retrograde amnesia for the contextual fear response. This phenomenon was detected in some patient with hippocampal lesions strengthening the idea, that retrograde amnesia depends to the nature of the tasks and the type of memory encoded. But why the temporal gradient of retrograde amnesia is generated in one task and not in the other is still under debate.

Even if in these different studies it is possible to detect significant difference between the presented stimuli or the type of memory associated (e.g. spatial vs non spatial), each paradigm show some parallelism regarding human declarative memory. As a matter of fact, the behavioural tasks force the animal to express in behavioural manner the memory of the association between different stimuli (contextual, spatial, temporal aspects of the encoded information), and reuse them flexibly (Eichenbaum, 2000, 2004). Even if the role of MTL in the declarative memory is evident and it can explain both retrograde and anterograde amnesia, it is still under debate the correlation between the type of declarative memory (episodic and semantic) and the presence or absence of temporal gradient of retrograde amnesia.

The formation of declarative memory requires the MTL integrity, even if the MTL lesions do not affect the immediate memory. This suggests that the information is initially supported by the

neocortex. Despite this, if the delay of retention is longer, the information is not more available, suggesting that the cortex it is not sufficient to transform the memory trace in a permanent memory.

Indeed, the remote memory, in order to become stabilized, requires a gradual maturation during the time following the encoding; this process of maturation and consequently stabilization of the memory trace is called consolidation (Dudai, 2004). According with the studies concerning the temporal graded retrograde amnesia, the retrieval of declarative memory is less sensitive to MTL lesion over the time suggesting that the neocortex can sustain the process without MTL. This induced the postulation of the existence of interaction between MTL and neocortex promoting the stabilization of memory trace at cortical level. Despite this, the cortical mechanisms concerning this process are not well established.

#### **2.4.4. Cortical lesions and amnesia: The role of cingulate cortex in memory consolidation**

The first study suggesting an implication of cingulate cortex in remote memory was performed in 1999 by Bontempi and colleagues who investigate the reorganization of the brain circuitry underlying remote memory (Bontempi et al., 1999).

It was clear that HPC has a limited role in memory storage, since retrograde amnesia was temporally graduated in case of hippocampal lesion, without affecting the oldest memories; moreover the cortex plays a role in memory consolidation underlying the storage of remote memories.

But invasive studies, as lesions, cannot describe the effective contribution of HPC at the moment of memory retrieval. They therefore performed a non-invasive functional brain imaging with ( $^{14}\text{C}$ ) 2-deoxyglucose during the retrieval of recent and remote memories of mice performing spatial discrimination task. Mice were trained and tested in Radial Arm Maze after 5 or 25 days later and the uptake level of ( $^{14}\text{C}$ ) 2-deoxyglucose was correlated with the regional metabolic activity during the retrieval. A relative increase in metabolic activity was found in frontal (ACC included) and temporal cortices in mice tested for the remote retrieval.

Upon the time after learning, there is a shift of the roles played by the different brains area: in particular the memory performance is strongly related to metabolic activity in HPC just after learning. As memory consolidation proceed over the time, the functional hippocampal contribution decreases and simultaneously the cortices involved in this task, in particular frontal, ACC and temporal cortices, mediate the retrieval of information previously acquired.

The consolidation process is accompanied by a hippocampal-cortical dialogue that allows the stabilization of cortical representations.

Summarizing the concept, the HPC is a required to encode the new information and to maintain the memory until the cortical mastery; cortices participate to the organized triggering, to the accessibility and to the usage of spatial information acquired.

Other experiments confirm the involvement of ACC, together with other cortical regions, when animals are exposed to associations learned in the past. The paradigms, showing this differential activation of ACC are different: mazes using a reward (Gusev and Gubin, 2010), aversive tasks like fear conditioning (Frankland et al., 2004; Restivo et al., 2009) or social transmissions food preference tasks (Lesburgueres et al., 2011; Ross and Eichenbaum, 2006; Smith et al., 2007). These correlative studies clearly shown the ACC involvement during consolidation and retrieval of remote memories, but the invasive studies demonstrated the necessity of ACC for remote memory expression. As a matter of facts, affecting the functionality of cingulate cortex can cause an impairment of remote memory expression using a battery of different task.

In particular, we would like to mention some of them starting from the studies performed by (Frankland et al., 2004).

Firstly, Frankland and colleagues showed a time-structure-dependent variation of the expression of the IEGs in mice performing the contextual fear conditioning, a task that associate an aversive event that take place in a certain context. Secondly, they use CaMK-II<sup>+/-</sup> mutant mice, which have specific deficits in remote memory (Frankland et al., 2001), as a tool to show the decrease of early gene activation, and the consequent decrease of neuronal activity in ACC. They therefore demonstrated the involvement of ACC in processing remote contextual fear and the integrative role of ACC in the storage of memory and in the dialogue with others regions.

In the same year, another invasive study confirmed these results: in fact Bontempi's group (Maviel et al., 2004) demonstrated a progressive activation in zif and c-fos expression and in synaptogenesis during the time of consolidation in ACC ad PC, in mice undergoing to Radial Arm Maze and tested at day 1 or 30. They suggested that remote memory storage in neocortical regions is accompanied by the increase of cortical-cortical connections and laminar reorganization, simultaneously complemented by a disengagement of HPC and posterior cingulate cortex. Moreover the pharmacological inactivation with lidocaine of HPC or ACC was able to affect respectively recent or remote memory, confirming the progressive establishment of cortical memory during consolidation.

It is not possible to conclude this paragraph without citing the elegant study of Rajasethupathy in 2015 (Rajasethupathy et al., 2015) who proved for the first time the presence of CA1/3-ACC direct pathway; moreover, using optogenetics approach, he showed that the manipulation of this projection was able to suppress contextual memory retrieval in mice performing context fear conditioning.

Other studies show this pattern of activity between HPC and cortical areas. (for a review see (Insel and Takehara-Nishiuchi, 2013))

## 2.5. Memorizing Memories: The role of consolidation

The explicit memory is composed by distinct but correlated processes (previously mentioned in the paragraph "Memory and vascular contribution: the aim of the thesis"): the encoding, the consolidation, the storage and the retrieval.

The **encoding** is the process by which we focus our attention on new information and we analyse them as soon as we get in touch. The nature and the degree of the codified information determine the quality of the retrieval, since, in order to obtain a persistent memory trace and a correct retrieval, it is necessary that the new information is accurately codified. To perform this process we need to focus our *attention* to the information and associate it in a logical and systematic way in accordance with the already present memories, in order to have a coherent and matched integration. Moreover, the strength of memory storage of this information depends on the *motivation* of acquisition.

The **storage** involves the mechanisms and the sites by which memories are maintained during the time and the amazing characteristic of the remote memory storage is that it seems unlimited, in contrary to STM/Recent memory that is limited. The "condicio sine qua non" for the storage to happen is the **consolidation**, which refers to the processes that transform the information just acquired, but still fleeting, in stable and long-lasting memories. It requires gene expression and the synthesis of new proteins in order to give rise to structural modifications enabling the stable memory trace conservation during the time.

Finally, the **retrieval** includes the processes that recall and reuse the information contained in memory trace. This recall involves different kinds of information acquired in several moments and stored in different cortical areas. The retrieval is similar to the perception and as this last is susceptible to distortions, as perception is prone to illusions. The retrieval of information is more efficient if the context is similar to the acquisition one and in particular for explicit memory, is dependent from the recent memory (Kandel, 2012).

In this thesis, we focus the attention to the consolidation process and the progressive post-acquisition stabilization mechanisms. Consolidation is usually referred to declarative memory (Dudai et al., 2015), even if it was proposed to exist in non-declarative memory. For this reason, hereinafter, consolidation will be referred to the declarative memory..

The first concept of consolidation was given during I century B.C. by Marcus F. Quintilianus who underlined the fact that the night increases the memory strength and remarked the possibility that the power of restitution can be supported by a process of maturation (*Institutio Oratoria* 11.2.43): “... curious fact ... that the interval of a single night will greatly increase the strength of the memory ... the power of recollection ... undergoes a process of ripening and maturing during the time which intervenes” (Dudai et al., 2015).

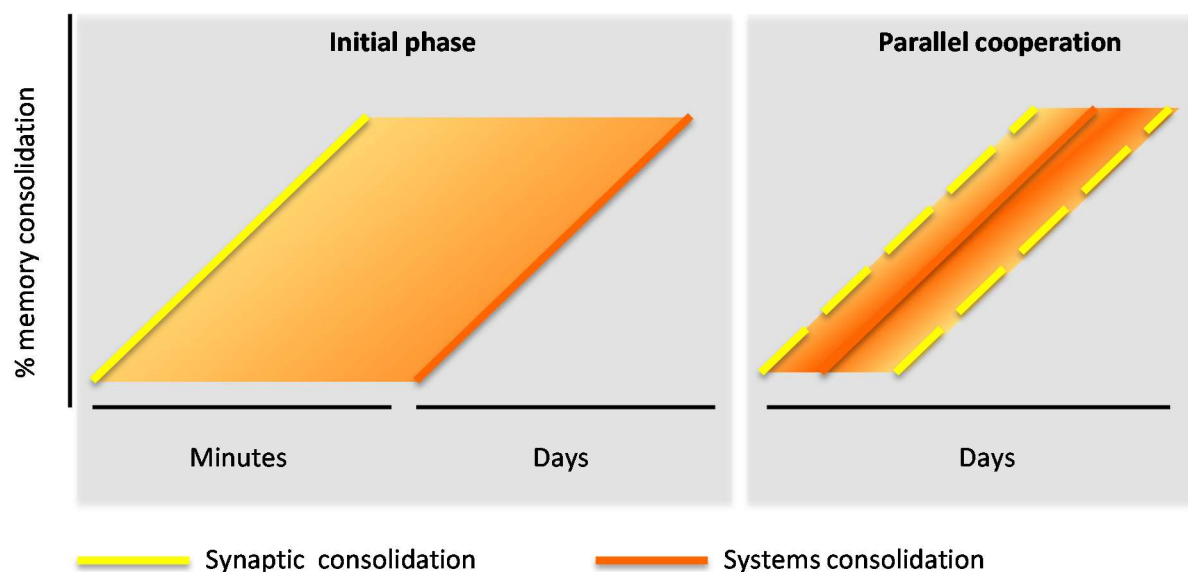
Another milestone in the concept of consolidation was described in 1881 by the French psychologist Théodule Armand Ribot, who assumed that memory is gradually transformed and reorganized during the time. As a matter of facts, he correlated the degree of memory loss induced by brain damage to the age of memory; in other words, he described the existence of a temporal gradient affecting retrograde amnesia: in particular, there was a greater loss of recent memories compared to older memories. This effect, known as Ribot’s Law, implies the existence of a process of dynamic and progressive reorganization of memory circuitry subtending memory process in order to stabilize the memory trace. Today we call this process memory consolidation.

The term “consolidation” is attributed to Müller & Pilzecker (1900), who firstly studied the acquisition and the retrieval of syllable pairs in human subjects (Dudai, 2004; McGaugh, 2000). These studies showed that the achievement of a correct retrieval of syllable pairs required a period of time, and the presentation of “distractors” like new materials during the initial period after encoding, thus before the consolidations of syllable pairs, impair its correct recall (Santini et al., 2014). Accordingly, the information recently encoded has to be stabilized and transformed in a stable long-lasting mental representation in order to not be quickly forget elicited by distractors (Lechner et al., 1999). In other words, there is a time windows during which is possible to interfere with newly learned information and its consolidation; consequently, memory trace cannot be reorganized and stabilized, inducing the incorrect recall or a fast forgetting. The consolidation is the post-experience processes of memory stabilization (Frankland and Bontempi, 2005), that allows the formation of a stable long term memory, in other words its passage from a fleeting form to a stable one.

This progressive reorganization of neuronal circuits involved in long term memory formation could takes days and years but attention is drawn to the fact that starts immediately after learning.

The memory consolidation is a complex phenomenon that includes all the plethora of mechanisms that starts after encoding during the wake state and during the sleep, and regards the molecular, synaptic and cellular aspects that are able, in turn, to influence functional and behavioural characteristics; the temporal window of this process may ranging from seconds to days or to years. This can give the idea of the dynamic mechanism that consolidation is.

The traditional view distinguishes two types of consolidation, based on the time windows and the localisation of modifications induced by memory encoding: the cellular and synaptic level and the brain system level namely the synaptic and systems consolidation (Fig. 13).



**Figure 13:** Schema illustrating the time course of synaptic and systems memory consolidation; modified from Benjamin Bessieres PhD thesis.

The synaptic consolidation is a fast process, occurring within the first minutes or hours after encoding, inducing the stabilization of synaptic changes induced by learning. These changes can include the formation of new synaptic connections or the strengthening of existent synapsis (Dudai, 2012; Squire et al., 2015). All the elements disturbing the synaptic consolidation induce the perturbation of memory process. Vice versa, the same distractors applied after this period do not affect memory formation. The time course of synaptic memory depends from the signalling pathways considered, from the structures implicated in memory process and, of course, by the nature of the memory task, but in the common vision the end of this process match with the beginning of systems consolidation.

On the other hand, systems consolidation is a slow phenomenon lasting weeks in rodents and years in human subjects, even if, also in this case, the kinetics depends on the kind of memory used (declarative or non-declarative) and the nature of the memory task. Systems consolidation is characterized by a reorganization of brain circuitry and systems supporting the formation of long term memories during the time (Frankland and Bontempi, 2005; Squire and Alvarez, 1995), but in the modern theory (Dudai, 2012; Dudai et al., 2015) it involves recurrent waves of synaptic consolidation in order to reprocess or integrate new experiences in coherent way. In other words, synaptic and systems consolidations do not act as consecutive events but they synergistically cooperate in parallel (Dudai, 2012; Dudai et al., 2015; Takeuchi et al., 2014).

In fact, as previously described in the section regarding the “Human amnesia”, the engram can be encoded by different regions including HPC and neocortex. During the time, the memory trace becomes less dependent from HPC and better supported by the cortex (McClelland et al., 1995). This systemic reorganization, able to stabilize the engram at cortical level, is induced and sustained by parallel waves of synaptic modifications. Therefore, the synaptic consolidation as traditionally thought can be assimilated to the concept of “initial consolidation” (Takeuchi et al., 2014), according to which the cellular and synaptic changes are necessary to the initial stabilization of memory in HPC. In this sense, we can affirm that synaptic consolidation is a fast process that last for minutes or hours after



encoding and it has the goal to stabilize the synaptic changes started during encoding. Thus, it induces the formation of new synaptic connections or the restauration of existent synapses; synaptic consolidation triggers systems consolidation which is a slow process lasting weeks in rodents and years in humans but it drives to the reorganization of cerebral circuits. In fact, the memory trace can be codified in more than one region (HPC, cortices).

Moreover, we have to keep in mind that even if memory consolidation seems to start and end once, the appearance of a “reminder cue” makes an apparently consolidated memory information labile again, when an “amnesic agents” is presented (Misanin et al., 1968). The reorganization of the consolidation-like process induced by the reactivation of the memory trace is called reconsolidation.

### **2.5.1. Systems consolidation**

Systems consolidation refers to the post-encoding time-dependent processes assuring the transformation of encoded information in long-term memory representations spread over brain circuit. As a matter of facts, it is clear that if we speak about different anatomical regions in which memory is located over the the time we are focusing not on cellular or molecular mechanisms between the neurons and their synapsis (synaptic consolidation), but rather on connection pathways between different regions with different dynamics.

Systems consolidation commonly refers to declarative memory, but it may exist in non-declarative memory as well (Dudai, 2012). The systems consolidation of the declarative memory is the process by which the HPC orchestrates and coordinates in coherent manner the progressive reorganization of memory trace at cortical level, in order to enable the storage and the recall of long-term memory even without the involvement of HPC itself (Alvarez and Squire, 1994). In other words the memory trace initially reliant on HPC becomes progressively independent to it, leading the cortex to take in charge of the process. The progressive reorganization of memory trace does not refers to a simple transfer of the engram between different areas, since the trace is indeed codified both at hippocampal and cortical level during the encoding phase but it is then actively consolidated via repetitive, organized, temporal-limited interactions between cortex and HPC, producing a reinforcement (via creation of new connection or potentiation of pre-existing ones) of synaptic connection; this coordinate and progressive process of cortical synaptic remodelling is responsible for the formation and the storage of long-lasting and stable cortical memory. It is important to notice the dynamic nature of this process that can be modulated from the complexity of the souvenirs and the similarity of previous memories. It seems, indeed, that consolidation kinetic depends from the dissonance between the novel information brought to encoding and the knowledge already present in our memory (Dudai et al., 2015).

It is important to point out that consolidation is not just a status, but it is a dynamic representation, able to integrate the information in a functional way.

But when does it start or end? How the engram is transformed?

To answer the first question we have to consider the different actors involved in this process while regarding the second question we can analyse the theories of systems consolidation

Regarding the timeline of consolidation, despite more than one century of studies, it is difficult to define specific changes in brain that can represent a signature of consolidation: we can just call consolidation the time window in which memory trace is susceptible to amnesic agents.

As discussed in the previous paragraph, the traditional point of view on memory consolidation considers that the fixation of a memory has a beginning and an end but since the process regarding the

memorization of information can be relatively easily targeted, it does not reflect the dynamic nature of behaviour. Thus, the concept of fixation of memory trace was reviewed, since the presentation of a remainder cue (normally the conditioned stimulus) is able to re-open and reactivate the memory; interestingly, the perturbation of this time window with amnesic agents can impair the retrieval of the memory (Misanin et al., 1968). This process of reactivation and the following reopening of a consolidation-like time windows that can challenge the memory maturation is called reconsolidation.

In this thesis we are not going to study the reconsolidation aspect, even if it will be relevant to explore in order to extend our results to a more complex system of stimuli and memories (Dudai, 2012), but in the following part we can dissect the kinetic of system memory consolidation.

#### **2.5.1.1. Neocortex: slow-learner?**

Marr (Marr, 1970, 1971) was the first that hypothesizes the existence of a dialogue between HPC and cortex after the encoding and that HPC was responsible to rapidly store the memory trace and organize the transfer and the progressive reorganization in the cortex, which in turn store it permanently. Thus, two important questions rise from this model:

Why, then, do we need the HPC if the memory depends on induced changes in cortical connectivity?

Moreover, why the cortex takes such a long time to integrate the encoded information? And consequently, why HPC is able to quickly take in charge the organization of the initial memory trace?

According to the computational model of systems consolidation proposed by McClelland and colleagues or O'Reilly and Norman (McClelland et al., 1995; O'Reilly and Norman, 2002), HPC is particularly suitable to rapidly encode the information. Moreover, as previously mentioned, HPC and in particular CA3 neurons are already strictly connected. In any case, there is no reason why the cortical plasticity can be slower than hippocampal ones,

This different kinetic of the cortex reflects the complexity of the cortical network.

To firstly answer to this question we have to consider that, at the moment of acquisition, the different characteristics of the event are individually and independently treated within a wide cortical network. Moreover, cortex possesses several partially overlapped processing pathways and these pathways can be treated in different cortical areas (Miller, 1999). McClelland and colleagues assumed that each information gives rise to small adaptive adjustments to the level of neuronal connections, which are small in magnitude and consequently having subtle effects. Over the time, these continuous repetitions accumulate changes, creating a relationship between inputs and outputs, providing the basis of an acquired memory.

In our life, the presence of the stimulus is limited in time and the derived representations last just for few seconds at conscious level. The HPC, which receives sets of data from different neocortical associative regions and sends back projections to these areas, seems to orchestrate the progressive remodelling of connections between different neocortical regions activated by the encoding and containing the different components of original experience.

During the hippocampal-cortical dialogue, the cortical modifications can benefit from persistence of representations provided by HPC, in order to assure progressively the formation of complex associations between the elements of the event either treated by different neocortical areas or encoded in different times by the same cortical area (Manns and Eichenbaum, 2006).

According to McClelland, this meticulous organization provides different advantages: firstly, it allows a finest structuration of the characteristics of the new information acquired; secondly, it avoids the redundant assimilation of the same characteristic and its energetic cost; thirdly, enable an efficacious integration of the new event with the linked memories already present, without forgetting or alterations of these, and eventually with other stored memories. Of course this fine regulation requires time and can explain why this process is slow and why the hippocampus is necessary for cortex (McClelland et al., 1995). Moreover, the model proposed is even more complicated as suggested by others studies (McClelland, 2013; Tse et al., 2007; van Kesteren et al., 2012) supporting the concept that the processing kinetics of the new memories depends on the nature of the memories already integrated at cortical level. If the nature of new memories is similar to the older ones already consolidated, creating an associated cortical network, the integration of the new memory at cortical level results to be faster, without creating interferences to other memories.

To summarize, this organization of memories allow us to answer the question previously mentioned (Why then do we need the HPC? Why the cortex takes such a long time to integrate the encoded information?). The hippocampus is the medium that allows the initial storage of memories, enabling a coherent integration without interference with the memories already present at cortical level. The incorporation of the new memory mediated by the cortex requires time to be stuck and incorporated into existing cortical memories, avoiding the loss of old memories that can be affected by fast changes destabilizing the structured knowledge built up from the previous experiences.

As the index of the book guides us to find the good chapter, in the same way hippocampus drives the cortex in memory processing. But the hippocampal role is not just to link distributed cortical network but is also to reactivate cortical assemblies, activated during encoding (Wiltgen et al., 2004).

#### **2.5.1.2. Index and chapters: hippocampal index and cortical tagging**

The process of memory consolidation requires a sustained time-limited communication between hippocampus and cortex; hippocampus has a role in organizing the consolidation for a limited period of time, to sustain the establishment of a stable long term memory at cortical level; after that, neocortical regions independently mediate retrieval of remote memories (Alvarez and Squire, 1994; Lesburgueres et al., 2011; Marr, 1971; Maviel et al., 2004; Squire and Alvarez, 1995; Wiltgen et al., 2004). This hippocampal-cortical dialogue allows the recurrent reactivations between these structures, leaving to a reinforcement of neocortical synapses previously recruited during encoding in order to enable the storage of long-lasting memories. But how the HPC is able to induce synaptic changes at cortical neuronal level, during the early phase of consolidation process? How HPC is able to address the cortical neurons involved in a specific memory trace?

Since the cortical synaptic changes seem to appear slower than the hippocampal ones, it was assumed that a mechanism of temporal tagging of cortical neurons during encoding is required to identify the cortical assembly involved in the specific memory and allow the consequent reactivation mediated by hippocampus. In other words, the acquisition induces simultaneously early synaptic modifications both at hippocampal and cortical level. These modifications create the substrate for the following coordinate reactivations of the structures implicated in the consolidation of the memory, in order to assure a coherent retrieval of the initial information encoded.

## Hippocampal index:

An event is composed by an ensemble of different characteristics (i.e. temporal, spatial,...) perceived by different sensory systems. Thus, the encoding of an event has an associative nature, and the circuit mediating this encoding requires the ability of fast integration of association of these characteristics. The different wide and scattered cortices activated by the encoding are not an efficient system to support both a fast and complex storage of the different characteristics composing the event and maintain the associations between the different cortical structures implicated in the event (Morris, 2006). Differently, HPC is a particularly suitable region to associate the different areas since possesses several reciprocal connections with different cortical areas (Amaral and Witter, 1989). This characteristic lead to important implications in the process of the consolidation of memories since it is the anatomical converging site that provides to the information received an elevated level of integration and abstraction (Lavenex and Amaral, 2000). Thus, these characteristics make the HPC an ideal structure not only for encoding the different informations forming the event but also to associate the different elements of the information.

Due to these anatomic and functional characteristics of HPC, Teyler et DiScenna (Teyler and DiScenna, 1986) proposed that during the encoding, the acquisition of the different sensorial elements constituting an event activates specific cortical areas, and this pattern of activity is also encoded by HPC, like a topographic snapshot of the firing activity which contain the cortical addresses of the event that is being processed. The hippocampal index does not contain just the topographic characteristic of the pattern of activity but also the temporal co-occurrence of the patterns induced by acquisition (Teyler and Rudy, 2007). This theory is known as the theory of hippocampal index.

Thus, the time limited role of HPC in memory consolidation does not reduce its importance: the associative ability of HPC and the storage of hippocampal index allow the restitution of a unified and coherent representation of the event during the retrieval, using the cortical addresses activated at the moment of the encoding. In other words, during the cortical consolidation the information coming from different cortical areas is rapidly and temporally linked through the hippocampus via several plasticity mechanisms.

Moreover, the neocortical areas recruited at the moment of encoding are subsequently activated by HPC during the periods of inactivity and sleep. Thus, the HPC participate to the reinforcement of cortex-cortical connection, inducing the stabilization of the long-term memory, by reactivating the cortical patterns concerning the memory trace. But how the HPC is disengaged at a certain moment? The mechanism of hippocampal deactivation is probably mediated by cortical direct (Rajasethupathy et al., 2015) or indirect efferences, even if the phenomenon is still not totally understood.

### **2.5.1.3. Reciprocity of hippocampal index: cortical tagging**

As previously discussed, the retrieval of a declarative memory induces the reactivation of hippocampal and/or cortical assembly activated during encoding (Tonegawa et al., 2015a; Tonegawa et al., 2015b). This hypothesis was confirmed by the creation and the use of transgenic models, enabling to temporally but clearly target a specific neuronal population of neurons activated during the encoding of behavioural task and subsequently check their reactivation at the moment of retrieval (Reijmers et al., 2007). With fluorescence in situ hybridization was possible to target neurons activated by encoding after a short time window, since mRNAs for Arc or others IEGs rapidly decay. While it was difficult to follow the activity of individual neurons weeks after learning, Tayler and colleagues (Tayler et al., 2013) addressed this issue using a long-lasting, stable but inducible form of GFP protein (H2B-GFP, green fluorescent protein); in this model the GFP expression is regulated by c-fos

promotor and tTA/tetO system and allow them to label neurons activated by encoding in a permanent way during the fear conditioning. In this way they were able to demonstrate that the retrieval of a recently (2 days) formed context memory is able to reactivate hippocampal, amygdala and cortical neurons tagged during encoding. Differently, after 2 weeks from learning, the same degree of activation is maintained in cortex but a decrease of neuronal activation was reported in hippocampus and amygdala. These results shown that the same cortical neurons activated by the encoding were also reactivated during retrieval of recent and long-term memory; in addition, they confirmed the hypothesis of hippocampal disengagement during long-term memory consolidation process of a fear context memory.

In support to these results, a previous PhD student of the team, Edith Lesburgères in 2011 (Lesburgueres et al., 2011) showed that during the acquisition of social transmission of food preference (STFP) task, the recruitment of the neurons of orbitofrontal cortex (OFC) is fundamental for the stabilization of a long-term memory. In fact, the pharmacological inactivation of this region during encoding was able to prevent the progressive organization of OFC neurons (decrease in dendritic spines density), blocking the retrieval of an associative olfactory memory after 30 days from encoding. The “tagging” of the OFC during the encoding is the prerequisite for the beginning of hippocampal-cortical dialogue and consequently the consolidation of this memory.

Moreover, it has been proved that the pharmacological blockade of synaptic transmission of OFC neurons during encoding does not affect the expression of recent memory (7 days after encoding) but rather impaired the consolidation and thus the retrieval of the remote memory (30 days later). Thereby, the OFC was not enough “mature” to sustain alone the consolidation of a remote memory at the moment of encoding. Thus, the dialogue between HPC and OFC is fundamental in this phase. During the time this dialogue decrease and cortical connections are strong enough to support alone the engram, independently to hippocampus.

Confirming Tayer’s findings, Lesburgères and colleagues underlined the necessity of the early OFC tagging in order to allow their subsequent reactivation triggered by HPC, to assure the strengthening of cortical connections and thus to enable the stabilization of a remote memory.

Other studies confirmed these results, allowing the generalization of the concept with others kind of memory (i.e. fear conditioning memory (Sierra-Mercado et al., 2015)).

Despite this, the complexity of the memory process and the study of the amnesic patients or animal models produce a plethora of heterogeneous results regarding the role of HPC during memory consolidation, enhancing the rise of different hypothetical model of memory consolidation.

#### **2.5.1.4. Memory reactivation during sleep**

As previously described, the reactivation of cortical and hippocampal neuronal networks previously activated during encoding is one of the most important feature of the consolidation process.

Even if it does not represent an aspect investigated in this thesis, we discuss briefly the importance of the sleep process in memory consolidation.

The coordinate reactivations of hippocampal and cortical neuronal population activated during encoding, called “reply “, occurs during an “off-line phase” which corresponds to rest or sleep periods during which there is not the neuronal activation induced by external stimuli. This mechanism allows the integration of memories without the intrusion of new information (Sejnowski, 1995).

This phenomenon was highlighted by electrophysiological records, which showed a selective reactivation during sleeping of the same coordinate patterns, firing during encoding in hippocampal neurons. This replay process was in particular recorded during “slow wave sleep” (SWS) and less frequently during the paradoxal sleep (rapid eye movement sleep; REM) (Walker and Stickgold, 2004). This “recapitulation” mechanism was recorded also at cortical level (Hoffman and McNaughton, 2002).

Moreover, it was demonstrated the presence of coordinate reactivations of hippocampal and cortical activities: during the SWS the oscillatory high frequency activity of HPC (Sharpe waves ripples) (Hasselmo, 1999) increased the activity of the cortical regions engaging plasticity mechanisms (Chrobak and Buzsaki, 1996; Siapas and Wilson, 1998), in turn correlated to cortical slow waves spindles (Molle et al., 2006; Siapas and Wilson, 1998; Sirota et al., 2003).

Furthermore, Gais and collaborators, using MRI technique, showed that post-learning sleep enhances hippocampal responses during the recall recent memories (48 h after learning), accompanied by an increase of connectivity between HPC and medial prefrontal cortex (mPFC). Six months after learning, memories activated the mPFC more strongly when they were encoded before sleep. These data show us that sleep participates to the progressive increase of neocortical contribution for memory retrieval; moreover, the sleeping phase contributes to progressive reorganization of neuronal network involved in the consolidation and retrieval of declarative memory (Gais et al., 2007). This replay can reflect the hippocampal-cortical dialogue and promote the stabilization of the new information acquired.

In addition, even if exists a double sense communication between HPC and cortex during sleep (Axmacher et al., 2010; Ji and Wilson, 2007; Sirota et al., 2003), it seems that hippocampal activity can initially act on neuronal activity (Wagner et al., 2010; Wierzynski et al., 2009).

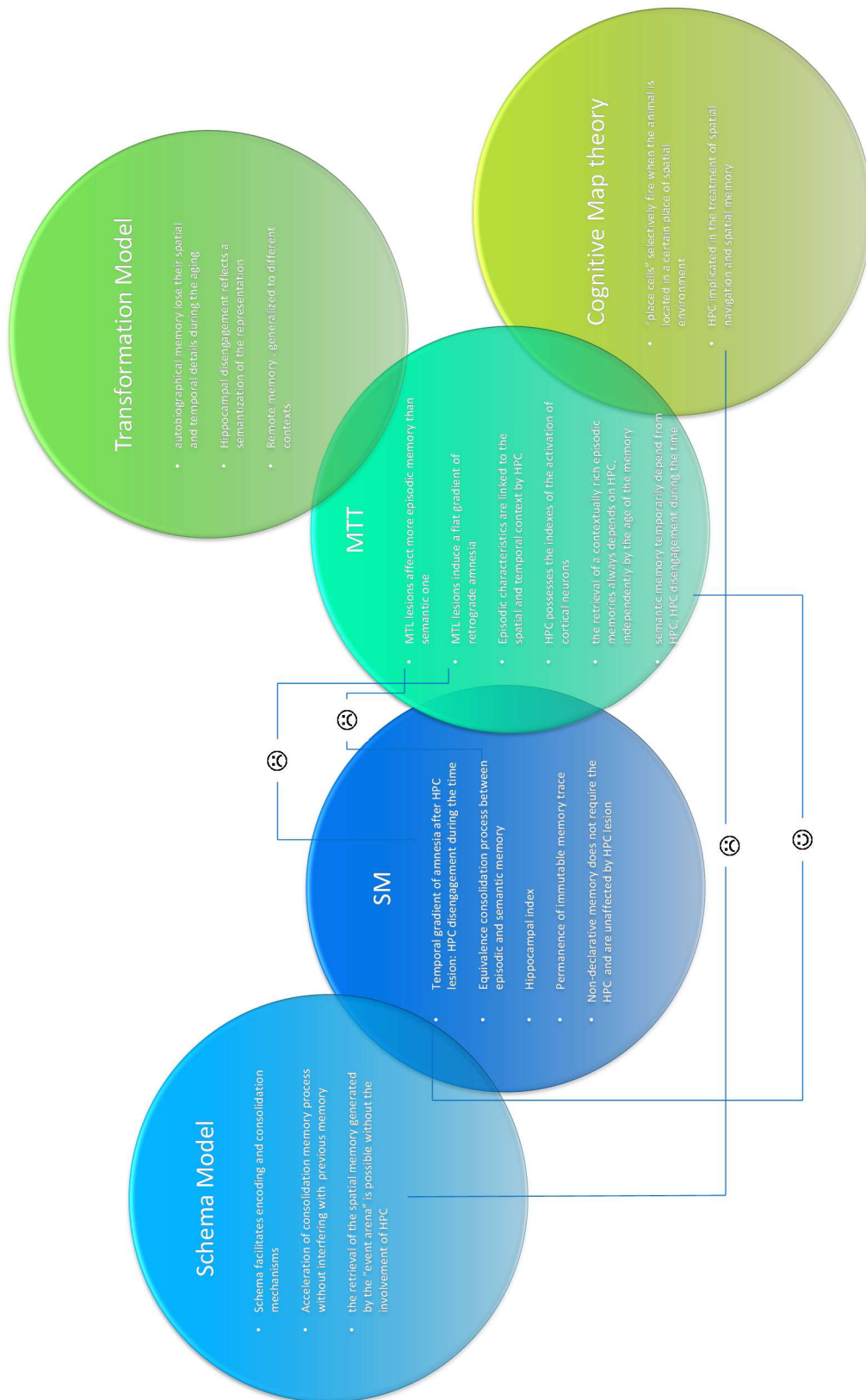
The SWS phase, although accompanied by important neuronal activation (Sutherland et al., 2001), is characterized by a decrease in IEGs’ expression involved in plasticity mechanism and a reduced induction of LTP (Ribeiro and Nicolelis, 2004), differently to REM phase implicated in synaptic plasticity changes and regulation of transcriptional activity (Ribeiro et al., 1999).

Thus, it was proposed that memory reactivation during SWS allows the redistribution and reorganization of systems consolidation, while the REM phase is responsible to the remodelling and reinforcement of reactivated synapsis, participating to synaptic consolidation (Siegel, 2001). We have to mention that there is a sequence of SWS and REM periods, thus this sequence contribute to the complementary reorganization of synaptic connections.

The memory reactivation, however, are not only restricted to the period of sleep but they can also exist during post-acquisition periods in the awake animal (Karlsson and Frank, 2009) and humans (Peigneux et al., 2006). The meaning of these reactivations seems to be related to modifications and the update of memory traces face to new elements that we can find in the environment (Dudai, 2006).

#### **2.5.1.5. Models of memory consolidation**

The complexity of memory consolidation process leads, during the time, to the formulation of different theories that try to analyse the involvement of different brain areas taking into consideration their contribution during the time and the different types of memory involved; in the following part we underline the similar and different aspects between them (summarized in Fig. 14).



**Figure 14:** Different theories of systems consolidation: in blue, theories showing a temporally graduated retrograde amnesia, indicating a transitory participation of HPC in memory consolidation. In green, theories showing a flat gradient of retrograde amnesia induced by a permanent functional implication of HPC in memory retrieval, independently from the age of the memory. Happy emoticons show the common features, sad emoticons indicates different point of view.

### **i. Standard Model of Memory Consolidation (SM)**

The standard model of memory consolidation (SM) is based on the existence of the temporal gradient of retrograde amnesia following hippocampal damage.

In this model, the HPC is considered an “anatomical index” capable to integrate the information coming from different cortices that in turn supports the different aspects of the experience. During encoding the HPC fuse all the elements provided, creating a coherent memory trace. During consolidation, repeated and coordinates reactivations of HPC-cortices paths induce a regional/systemic reorganization that leads to a progressive increase of the strength and stability of cortical-cortical connections, the holders of original experience. This reorganization leads to the transformation of the memory trace in stable long-lasting and coherent memory. Throughout the consolidation, as time went on, after days and weeks, the HPC gradually decrease its role leaving the cortex able to sustain independently the permanent memories and their retrieval (Frankland and Bontempi, 2005; Squire and Alvarez, 1995).

Thus the HPC is the site of temporary storage of memory trace, while the cortex is the site of permanent storage. Moreover, since the hippocampal temporal involvement, the retrieval of memory is dependent from HPC as long as the cortex is able to sustain alone the storage of the remote memory.

But what does it happen exactly during encoding and consolidation? (Fig. 15)

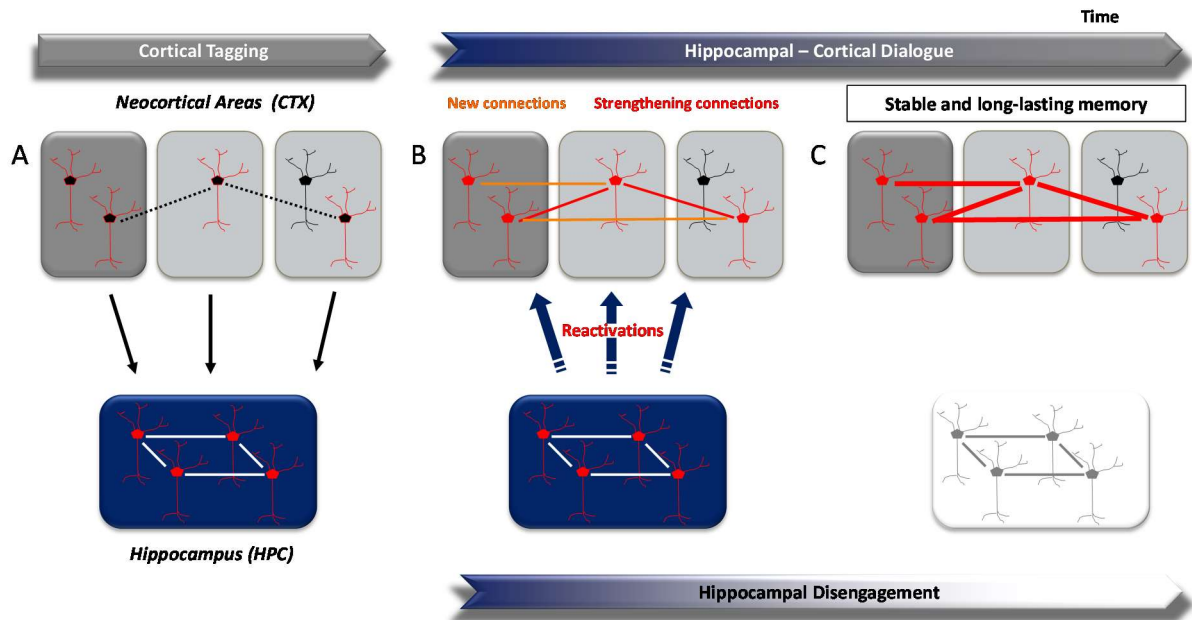
During encoding:

The information is treated and codified at cortical level; the temporal and topographical patterns of neuronal assembly tagged at this moment are addressed to HPC (see previous paragraph “Index and chapters: hippocampal index and cortical tagging”), which, as result of its associative ability and rapidity, encode cortical firings addresses in the form of hippocampal index in order to enable a coherent and unified representation of the event treated.

During hippocampal-cortical dialogue:

During the first days, the hippocampal index mediate the repeated activation of spread cortical neurons mediated by HPC (during active phase or during sleep); this synchronized reactivation of this wide population of cortical neurons lead to the creation and/or the reinforcement of cortical-cortical connections in order to allow the formation of a coherent memory trace. Once the memory trace is consolidated (i.e. gradually transformed from an initial labile state into enduring stable memories, and consequently retrieves by the cortex), the role of HPC as a consolidation-organizing device ends and the neocortical regions independently mediate retrieval of remote memories (Dudai, 2004; Maviel et al., 2004; McGaugh, 2000; Squire et al., 2015).





**Figure 15:** Standard model of systems memory consolidation: A) the initial information is initially encoded at primary and associative neocortical level (CTX, black neurons with red contour line) through the tagging process. Hippocampus (HPC), creating the “hippocampal index” (red HPC neurons), trigger a rapid integration of the different aspects of the information coming from the different neocortical areas, in order to create a coherent memory trace. B) Successive cortical-hippocampal reactivations induce both the progressive reinforcement of pre-existing connection (red connection lines) and the creation of new connections (orange connection lines), stabilizing the cortical information. C) The modifications of these connections induced by hippocampal-cortical system are fast and transitory, but during the time the cortical modifications become stable and long-lasting (thick red connection lines). Adapted from (Frankland and Bontempi, 2005; Lesburgueres et al., 2011).

Several invasive approaches conducted on animal models confirm this hypothesis (previously reported in the former paragraphs, in particular in the section Animal models of amnesia; for a review (Winocur et al., 2010)); moreover neuroimaging studies performed on patients showed that the retrieval of declarative memory is correlated, during the time, to a progressive reductions of hippocampal activity associated to an increase of cortical activity (Smith et al., 2010; Smith and Squire, 2009; Takashima et al., 2006; Takashima et al., 2009). Confirming these results, studies performed by Bontempi’s team on animal models identified the neuroanatomical cartography of the metabolic (using ( $^{14}\text{C}$ ) 2-deoxyglucose) (Bontempi et al., 1999) or neuronal activity (using IEGs, i.e. c-fos and zif) (Frankland et al., 2004; Lesburgueres et al., 2011; Maviel et al., 2004) during memory consolidation, as explained more in details in the section “Cortical lesions and amnesia: The role of cingulate cortex in memory consolidation”. Others teams confirmed this cerebral dynamic using different behavioural paradigms and different techniques ((Ross and Eichenbaum, 2006; Takehara-Nishiuchi and McNaughton, 2008; Takehara et al., 2003; Thomas et al., 2002; Wheeler et al., 2013), showing that after encoding there is a shift on the roles played by different areas: just after encoding, the memory performance, thus the relative neuronal activation, is strongly linked to HPC; but as time went on and the consolidation gradually proceed, the functional contribution of HPC decreases and cortical areas are able to mediate the retrieval alone. In others words, HPC is more active during the retrieval of a recent memory compared to remote one and the cortex (especially medial prefrontal and anterior cingulate cortices) are more active during the retrieval of a remote memory.

Moreover, the stabilization of the remote memory trace is accompanied by an increase of dendritic spines density at level of ACC during the consolidation of context memory induced by fear conditioning (Restivo et al., 2009) or at OFC level during the consolidation of olfactory associative memory during STFP, together with an increase of synaptophysin, a marker of synaptogenesis

(Lesburgueres et al., 2011). In the same way, an increase of the expression of the presynaptic protein GAP-43 (growth-associated protein, used as a marker of synaptogenesis) in ACC is measured 30 days after the encoding of spatial memory task, compared to Day1 (Maviel et al., 2004).

This presynaptic and postsynaptic structural remodelling at cortical level reflects the gradual increase of synaptic activity and of cortical-cortical connections of the neuronal network implicated in the memory consolidation and the retrieval of remote memory, becoming a morphologic correlate of the memory stabilization during the time.

These correlative approaches are valued and confirmed by invasive strategies, which confirm the importance of neocortical regions at the moment of remote memory retrieval.

For instance, the pharmacological inhibition of ACC, achieved by lidocaine injection before testing the memory in Radial Arm Maze, specifically perturbs the expression of remote spatial memory, without affecting the recent one (Maviel et al., 2004). Moreover, the HPC inhibition induces an impairment of the retrieval of the recent spatial memory, without effect on the retrieval of remote memory. In the same year, Frankland (Frankland et al., 2004) showed the same effect of lidocaine on memory retrieval using the contextual fear conditioning paradigm, confirming the progressive establishment of cortical memory during consolidation.

Moreover, in another study using a Morris Water Maze task it has been shown that c-fos expression was elevated after expression of a remote but not recent memory (increasing of ACC role as a function of time), that the lidocaine-induced inactivation of ACC impairs remote memory retrieval, sparing the recent memory (Teixeira et al., 2006).

In the same way, Kim and collaborators in 1995 tested the memory response (quantified as blink of nictitating membrane) in eye-blink conditioning paradigm in rabbits, hippocampectomized 1 day or 30 days after learning. Rabbits receiving surgery 1 day after learning showed impairment in conditioned responses, while no effects were found on rabbits operated 1 month after encoding (Kim et al., 1995), confirming the time-limited participation of HPC in memory processing, being necessary for the retrieval of recent memories but not remote ones. Using the same protocol Takehara and colleagues (Takehara et al., 2003) extended the study showing that the lesion of medial prefrontal cortex strongly impacts remote memory, while the recent memory was just marginally affected. In any case this area seems to mediate recently acquired memory.

All together, these data show the progressive importance of cortical regions for the expression of the remote memory, as suggested by studies performed on human suffering from semantic dementia or cortical damages, characterized by specific loss of remote memory (Levy et al., 2004; Moscovitch and Nadel, 1998).

## **ii. The schema theory**

Everybody can notice that it is easier learn a task and remember it if the task is related with something that we already know. This idea was developed by an English psychologist, Frederic Bartlett (Bartlett, 1932 ) which proposed the concept of mental “schema” referring to a mental model as a framework of knowledge (Tse et al., 2007; Tse et al., 2011), and in this network of knowledge the new information can be easily integrated. This schema is made by cognitive structures that facilitate encoding and consolidation mechanisms (Ghosh and Gilboa, 2014). In the beginning this concept was not considered by the neuroscientists, but hopefully was recently reconsidered by Tse and collaborators.

Before exploring this study we have to open a little parenthesis on laboratory animal models. As mentioned before, the progressive corticalization of a declarative memory takes several weeks in rodents and years in humans, in order to avoid the wrong integration of memory trace in memory “library” (see paragraph “Neocortex: slow-learner?”). Despite this, the limit of laboratory animal models is the impoverished environment in which they live all their life, compared to rodents in natural environment. This depleted condition makes them relatively naïve to the encoding. In nature the animals have to integrate the new encoded information in a wide network of memories. By the way these models allow us to understand a relatively simplification of pathways studied. Tse and colleagues tried to study a memory network more developed, even if still simplified. In any case, they demonstrated that the temporal gradient of retrograde amnesia induced by hippocampal lesion is not a rigid time window but it can change dependently to the knowledge acquired.

They showed that in rats new information can be rapidly incorporated at cortical level if it is similar to cortical representation (schema) of a complex spatial environment already established. In this task was used an arena (event arena) in which rats were trained to learn an association between a flavour and a place in the arena (i.e. banana flavour is in location A, bacon flavour is in location B), forming a complex spatial environment. The rats exposed to a flavour before entering in the arena have to reach the right place associated to this flavour in order to obtain the food; the time spent by the animal to reach the place is recorded as index of memory performance. This paired associated learning task requires a transitory initial involvement of HPC and a corticalization of long-term memory. It was shown that rats take 6 weeks to learn six flavour-place associations (a flavour is presented once during a session; 3 session/week). After this learning phase, rats were able to learn 2 new flavour-place association in one train session (being able to retrieval them for at least two weeks) showing that cortical schema induced by the first six association can facilitate the incorporation of new association.

Furthermore, even more interesting, they showed that a bilateral lesion of HPC executed 48 hours after encoding does not affect the memory retrieval showing a faster corticalization of the memory. By the way; the same lesion performed 3 hours after encoding, affects the memory retrieval of these 2 new flavors, showing a hippocampal-dependent mechanism (Tse et al., 2007).

In another set of experiments (Tse et al., 2011), the same groups complementarily proved that the presentation of the two new flavour-place associations in a familiar environment induce a fast learning associated to the expression of IEGs (Zif268, Arc) in different cortical areas such as the medial prefrontal cortex. Differently, if these 2 associations are presented in a different environment does not neither induce a rapid learning nor the expression of IEGs. These results showed that the exposition to new associations, that are similar to a previously formed schema, induces important cortical changes, which are not evoked in case of either re-exposition to the information already known or to the presentation of new pairs in absence of the schema of knowledge.

These studies revealed that the cortex is not necessary a “slow learner” in presence of a mental schema, since it is able to quickly integrate new information. The acceleration of consolidation memory process can be ascribed to a cortical schema previously established, allowing the fast integration of similar and compatible information, without creating interferences with the memories (McClelland, 2013; Wang and Morris, 2010).

Moreover, unexpectedly, the results obtained by Tse showed that the retrieval of the spatial memory generated by the “event arena” is possible without the involvement of HPC. Rats trained in the event arena and exposed to the new association, were lesioned after 24 h, without showing any memory deficit for the original association and the new ones. This means that the spatial memory trace is already stored outside the HPC. This finding is in contrast with multiple trace model and the theory of the transformation (described below) which considered the HPC the site of storage of spatial memories (spatial maps). According to the schema theory, the high level of the familiar environment in which

the memory was formed can allow the formation of the memory trace, (enriched in spatial and contextual characteristic), to become independent from HPC. Winocur (Winocur et al., 2010) showed that rat habituated to a high rich environment (“the village”), with different accesses to water and food in different compartments of the arena, do not show spatial memory deficit after hippocampal lesion, confirming that the familiarity for the spatial environment allows to maintain some aspects of spatial memory without hippocampal involvement but with a cortical cognitive map of spatial environment (Moscovitch et al., 2005; Rosenbaum et al., 2000; Rosenbaum et al., 2001). The standard model, and the associated schema model, is supported by different studies with invasive (lesion/inactivation) or non-invasive (imaging) approaches in rodents’ and primates’ models in spatial and non-spatial tests (Dudai, 2004; Squire et al., 2015; Wang and Morris, 2010). Despite this, these theories present some limits and they do not take into account the phenomenon of flat gradient retrograde amnesia observed in other studies (previously mentioned in the paragraph “Retrograde amnesia and the stabilization of the memories” and examined in the following part). As a matter of fact, the corticalization of all the forms of declarative memory is under debate, and new theory of memory consolidation have been proposed, as exposed in the following part.

### iii. **The cognitive maps theory**

In 1971, electrophysiological recording of HPC in rat exploring a new spatial environment showed that some neurons in CA1 are able to record spatial information regarding the position of the animal in the environment (O’Keefe and Dostrovsky, 1971). These neurons called “place cells” selectively fire when the animal is located in a certain place of spatial environment. O’Keefe and Nadel (O’Keefe, 1978) suggested that the HPC is able to create a “cognitive map” (spatial cartography) of external environment that the animal can use in a flexible way in order to localize its position in the space, confirmed also by data showing the involvement of the hippocampus in learning and spatial memory in man (Ekstrom and Bookheimer, 2007; Maguire et al., 1997).

The ensemble of neuronal place cells recruited is specific of the environment that the animal is exploring and this specificity takes time to be created, suggesting the triggering of modifications of neuronal network recruited by the spatial exploration and the consolidation of a spatial memory. Contrarily to the topographic organization that characterized primary sensory cortex or motor cortex, the HPC present an aleatory organization of the place cells (i.e. neighbouring place cells do not necessarily represent adjacent regions of the spatial environment). Thus, the same spatial environment can recruit different neuronal population in different animals/humans and the same animal/human can represent different environment with different place cells (Dombeck et al., 2010). These studies shed light on the contribution of HPC in the treatment of declarative information, showing its essential role in the treatment of spatial navigation and spatial memory. Even if the creation of cognitive maps is a priority for hippocampal network, studies performed on patients and lesioned animal models suggests that HPC can integrate associative information of spatial and non-spatial nature (Squire, 2004).

### iv. **The multiple trace theory**

This theory was proposed in 1997 by Nadel and Moscovitch (Nadel and Moscovitch, 1997) and it is derived from the observations that MTL lesions affect more episodic memory than semantic one in human subjects. Moreover, MTL lesions induce a flat gradient of retrograde amnesia leading to the preservation of episodic memories acquired more the ten years before the lesion. Despite the flat gradient of retrograde amnesia was associated with extensive damage to extra-hippocampal regions, which can consequently affect other sites of permanent storage, Nadel and Moscovitch observed that

the dimension of the gradient depends not only on lesion size but also on the type of memory probed (Frankland and Bontempi, 2005). In particular, they noticed that if the whole hippocampal formation was damaged the retrograde amnesia for episodic memories was extensive.

Consequently, it was proposed this new theory, the multiple trace theory (MTT), which confirmed that the initial phase of declarative memory formation requires the mandatory implication of HPC, that the memory trace is encoded in a spread hippocampal-cortical networks and that the characteristics of the information encoded are distributed in different cortical networks. Furthermore and interestingly, this theory affirmed that these characteristics are linked to the spatial and temporal context by HPC and it possesses the different indexes of cortical neuronal populations allowing the creation and retrieval of coherent and unified representation of initial event encoded. Thus, the retrieval of the contextually rich episodic memories, characterized by a precise spatial and temporal context, always depends on hippocampus, independently by the age of the memory (Eldridge et al., 2000; Moscovitch et al., 2005).

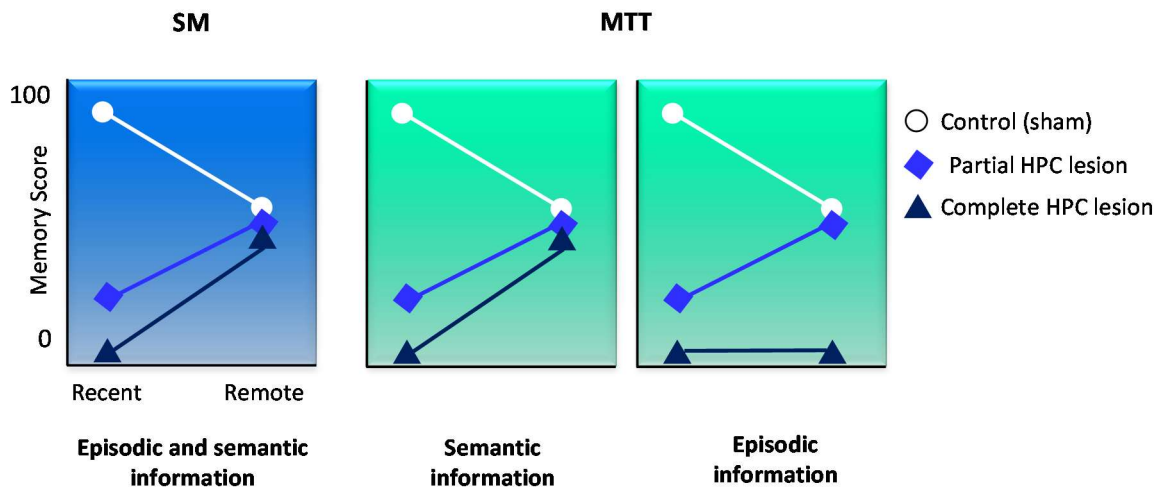
Thus, according to this theory, each time that the episodic memory is retrieved it is re-encoded; while the HPC encodes all the information consciously perceived, the reactivation of memory trace creates a new memory trace that will share common characteristics with the initial trace. In other words, the memory reactivation of the episodic information generates different multiple traces in the HPC which are linked to cortical networks. The ensemble of these multiple traces are widely distributed, making the retrieval of older (remote) episodic memory less sensitive to hippocampal damage, compared to recent memories. In this way is possible to explain why partial MTL lesions affect the recent episodic memory, explicating the presence of a gradual gradient amnesia in some patients, whereas the whole MTL damage induces a flat and global retrograde amnesia.

Regarding the semantic memory, the MTT is in accordance with the SM: the hippocampal lesion induces a perturbation of the recent memory without affecting the remote one.

This is due to the fact that the multiple traces created by the memory reactivation, interlinked together, allow the “extraction” of factual characteristics of the episode and their integration with previous semantic memory. According to this theory, the factual components of the information acquired during an episodic event are segregated to the episodic component and are independently stored (as mentioned before the HPC is the site of spatial and temporal context but the cortical traces are context-free, in other words transformed in semantic ones). Thus, in accordance to SM, the semantic memory will temporarily depend on HPC that will be successively supported by the cortex alone (Fig. 16).

Thus, the common feature of the two models is that the reactivation of memories initiates a process of reorganization but the difference is where this organization takes place: in fact while for SM the reorganization process occurs in cortical networks, MTT predicts that reactivation should also lead to the generation of new traces within the hippocampus.

If the literature mainly agrees with the progressive hippocampal disengagement during the consolidation of semantic memories, some studies showed a flat gradient even for semantic memory (Moscovitch et al., 2005). This can be explained with an episodic aspect in the characterization of semantic memory. As a matter of fact, there is a dynamic interplay between these two types of memory and they can interact and influence each other (Winocur et al., 2010).



**Figure 16:** Comparison between SM (a) and MTT (b) on memory performance related to hippocampal status (Frankland and Bontempi, 2005).

#### v. The transformation hypothesis

A recent evolution of the MTT assumes that it is possible that the memory traces change their format during memory consolidation. According to this, it was shown that most of autobiographical memories lose their spatial and temporal details during the aging (Biedenkapp and Rudy, 2007; Furman et al., 2012; Levine et al., 2002; Wiltgen et al., 2010). The episodic memory, initially dependent from hippocampal-cortical network, is transformed in a semantic representation during its consolidation at cortical level. The corticalization of memory traces is correlated to a process of “decontextualization”, called also “semantization”, during the time, and the retrieval of detailed episodic memories is always dependent to HPC (Winocur and Moscovitch, 2011; Winocur et al., 2010).

Thus, the hippocampal disengagement during memory consolidation initially with episodic nature can be possible, but this disengagement reflects a semantization of the representation. Therefore, this transformation of memory trace explains the preservation of autobiographical memories in amnesic patients induced by hippocampal lesion: in these patients the memories will be deprived of detailed contextual characteristics, becoming qualitatively different compared to healthy subjects (Moscovitch et al., 2006).

This hypothesis is confirmed by several studies performed on rat models. Winocur (Winocur et al., 2007) tested the transformation hypothesis in two hippocampal-dependent tasks: social transmission of food preference (STFP) and contextual fear conditioning. In both task the memory performance of rats was tested at recent or remote time point in the same context of acquisition or in a different context. In tune with transformation theory, the change in context perturbs the retrieval of recent memory and not the remote memory, explainable through the decontextualization of the representation retrieved at remote time point.

Moreover, others studies performed in the same year showed that the remote memory can be *generalized* to different contexts, while the recent memory is more discriminative (since it richness in details), revealing that the contextual memory lose precision during the time (Biedenkapp and Rudy, 2007; Wiltgen and Silva, 2007).

In a recent study, Wiltgen (Wiltgen et al., 2010) demonstrated that animals able to discriminate two contexts showed a memory deficit after pharmacological inactivation of HPC after 14 days from encoding, conversely to others that are able to discriminate. These data showed that the HPC is

required for contextual memory retrieval. Contrariwise, if just the semantic component of initial memory is available, it can be retrieved by the cortical contribution.

#### **2.5.1.1. The debate on memory consolidation and last discoveries**

Thanks to new technology, in particular the optogenetic, it is possible to study with high precision and resolution the dynamic of systems consolidation of the memory trace, increasing the debate on systems consolidation theories and in particular the role played HPC during consolidation and retrieval.

One first advantage of this technique is that the inhibition of the target structure possesses a high precise temporal resolution avoiding the possibility of carrying out compensatory mechanism induced by long-lasting pharmacological inactivation or lesion that could bias the dynamics of the pathway.

As a matter of facts, Goshen and collaborators investigated the effect of the optogenetic inhibition of dHPC in the interaction with ACC during context fear conditioning task with the advantage of the temporal resolution of this technique (Goshen et al., 2011). A short optogenetic inactivation (5 minutes) of dHPC during the re-exposition to the fear context is able to impair the retrieval of the memory tested at 28 days, as well as 9 and 12 weeks after learning. A longer optogenetic inhibition, mimicking the pharmacological effect (duration of 30 min and starting 25 min before the beginning of the test) does not affect the retrieval of remote memory but it disturbs just the retrieval of the recent one, confirming the previous finding. Moreover, the short inhibition of CA1 impairs the c-fos expression in CA1 and ACC, while its longer inhibition impairs the c-fos expression in CA1 and increases the c-fos level in ACC. Therefore, this can support the hypothesis that prolonged inhibition of HPC induces a compensatory mechanism at cortical level, assuring the retrieval of the remote memory.

According to MTT and transformation theories, this study suggests that HPC is necessary to the retrieval of remote contextual information. However, the study performed by Goshen was able to show that HPC is implicated in the retrieval of remote memory. Thus, two possible pathways can mediate the retrieval of remote memory (Suzuki and Naya, 2011).

The first pathway, active by default, is represented by functional interactions between HPC and ACC, becoming both necessary for the retrieval of remote contextual memory. The second one is a compensatory pathway activated when HPC is blocked, leading to an increase of cortical activation (increase of c-fos expression) in order to assure the retrieval of remote contextual memory independently from HPC.

Another study demonstrated that the optogenetic inhibition of CA1 during context re-exposition impairs fear response (Tanaka et al., 2014), as well as the inhibition of DG or CA3, during the retrieval (Denny et al., 2014).

Thus, the blockade of reactivation of hippocampal index, during the period in which the fear response triggered by the context is represented at hippocampal-cortical level, is sufficient to block the reactivation of the whole hippocampal-cortical network.

Consequently, the short inhibition of CA1 during retrieval is sufficient to reduce the reactivation of cortical neurons (Ent, retrosplenial and perirhinal corteces). In other words, the hippocampal inhibition blocks the retrieval by preventing the cortical reactivation of the memory trace (Tanaka et al., 2014).

Conversely, the concept of cortical neuronal tagging (Lesburgueres et al., 2011; Tse et al., 2007) suggests that the memory trace is rapidly formed in the cortex at the moment of encoding. This trace is

still “immature” and needs to hippocampal-cortical reactivation to become mature thus allowing the behavioural expression of the task.

The hippocampal implication in the retrieval of a recent memory was artificially mimicked by optogenetic: in fact, the function generator of cortical activity mediated by HPC, during systems memory consolidation, can be mimicked by optogenetic firings enabling the selective activation of cortical neurons engaged by encoding. To mimic hippocampal-induced reactivation of cortical neurons, mice performing the context fear conditioning (Cowansage et al., 2014) were genetically modified to induce the expression of rhodopsin channel (ChEF), under c-fos promoter, in retrosplenial cortex, area specifically recruited during the encoding of this task, in order to manipulate optogenetically this cortical population at the moment of retrieval. After the fear conditioning to a first context and the relative recruitment of cortical and hippocampal neuronal population, the optogenetic reactivation of retrosplenial neurons in a neutral context (not associated to fear response) induced the retrieval of the fear memory. Moreover, interestingly, the same fear response in neutral context was obtained by artificial activation of retrosplenial neurons and simultaneous pharmacological inhibition of HPC.

Thus, this study showed that even if the HPC plays a fundamental role in memory stabilization at cortical level, an exogenous and artificial technique could bypass its implication by enabling the reactivation of cortical neurons implicated by encoding. In this case, it seems that the cortical memory trace is sufficient to retrieve the contextual fear memory.

In conclusion, even with the advent of new technologies, the contribution of HPC during cortical dialogue and after its role in memory retrieval is still under debate.

According to SM, HPC is the temporary custodian of the index that allows the coherent reorganization of the memory trace stored at cortical level (Frankland and Bontempi, 2005). Consequently, it is possible to suppose that the quality of memory retrieval induced by hippocampal reactivation does not change if the retrieval is induced by either physiological process or artificial technique.

Vice versa, MTT and transformation models propose that HPC permanently stores the spatial and contextual details of information, which are not present at cortical levels (Winocur and Moscovitch, 2011). Thus, the memory retrieved via HPC will be enriched by spatial and contextual details different from the recall of memory induced by direct (i.e. artificial) reactivation of cortical neurons, which will be semantic and generalized in nature (Wiltgen et al., 2010).

Actually, these systems consolidation theories differ in the role of hippocampal restitution of remote information and the nature of retrieved memory. In any case, the general consensus foresees the transitory hippocampal role in retrieval of semantic memories (Dudai, 2012; Squire and Alvarez, 1995; Squire et al., 2015; Winocur et al., 2010).

### **2.5.2. Synaptic consolidation**

Even if the synaptic consolidation will not be the subject of this thesis, we briefly introduce this concept (for reviews see (Kandel, 2001; Wang et al., 2006)).

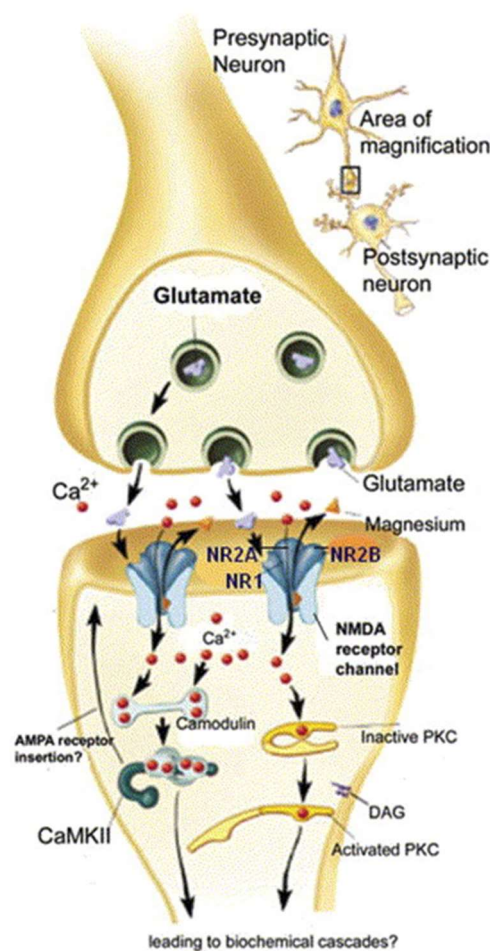
While systems consolidation includes the complex and enduring process that lead to a gradual reorganization of cerebral region subtending memory, implicating a time dependent shift in the circuits supporting memory recall, the synaptic reorganization is complete within hours and include the stabilization of changes in synaptic connection in localized circuits. This process involves assist to the



growth of new synaptic connection or a restructuration of existing ones (Frankland and Bontempi, 2005).

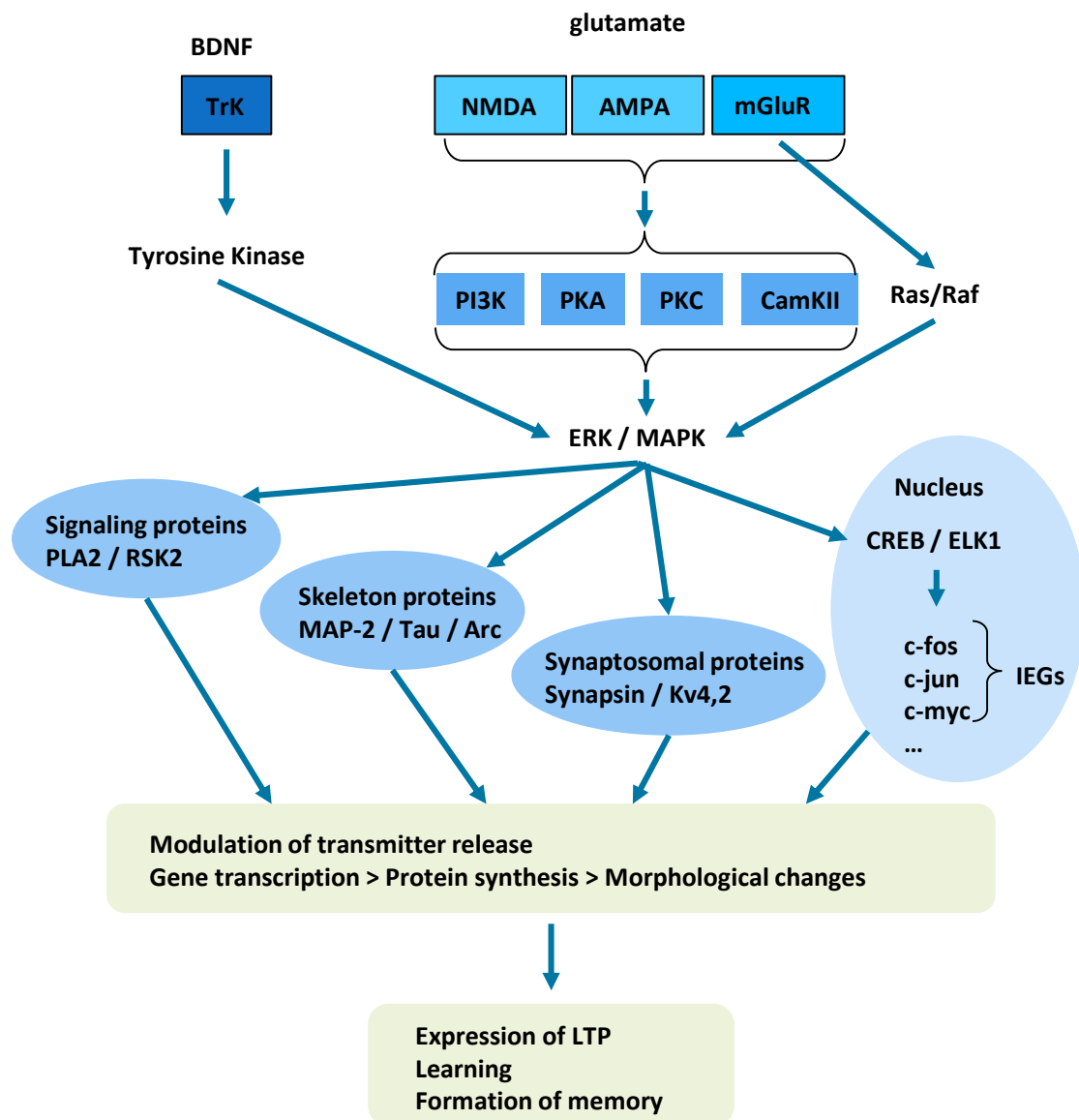
The synaptic consolidation is a rapid phenomenon that starts from the first min/hours following the encoding; as the name suggests it is the stabilization at synaptic level of the changes induced by encoding. Such changes include the formation of new synaptic connection and the restructuration of existing synapses (Dudai and Eisenberg, 2004). This process starts by recruiting the receptors localized at synaptic levels, and among these BDNF receptors, monoamines receptors and ionotropic glutamate receptors (iGluR) both AMPA and NMDA (Dudai and Eisenberg, 2004; McGaugh, 2000). Briefly, AMPA receptors are known to sustain the basal synaptic transmission while NMDA receptors are regulators of synaptic efficiency. As a matter of facts, NMDA receptors increase their activity when a simultaneous activity between presynaptic and postsynaptic neurons occurs (Hebbs' theory), inducing a massive entrance of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  (through ionotropic receptor and ions channels) and an extracellular diffusion of  $\text{K}^+$ , inducing in turn depolarization with the consequent entrance of  $\text{Ca}^{2+}$  (Fig.17). The  $\text{Ca}^{2+}$  entrance triggers the activations of transduction cascade leading to a reinforcement of the synapsis. Among these,  $\text{Ca}^{2+}$  activates the calmodulin-dependent protein kinase II (CaMKII) which plays an important role in memory formation (Mayford et al., 1996).

The postsynaptic activation of CaMKII leads to synaptic potentiation allowing the externalization of AMPA receptors on postsynaptic membrane and or the increase of its channel's conductance (Nicoll and Malenka, 1999a, b).



**Figure 17:** Key molecular actors implicated in synaptic consolidation (Wang et al., 2006).

This synaptic potentiation, called long term potentiation (LTP) is maintained by gene expression via complex mechanisms of transcription, protein synthesis which allows structural changes and strengthening of synapses (Kandel, 2001) (For a review (Lynch, 2004)). This process involves in particular the protein kinase A (PKA) and the mitogen-activated protein (MAP) kinase MAPK/ERK pathways (Abel and Nguyen, 2008; Frey and Morris, 1997; Sweatt, 2001) (Fig.18).



**Figure 18:** Schema of LTP pathway: ERK seems a point of convergence for several signalling cascades; modified from (Lynch, 2004). Abbreviations: BDNF, brain-derived neurotrophic factor; Trk, tyrosine kinases receptors; NMDA, AMPA and mGluR, glutamate receptors; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PKA, protein kinase A ; PKC, protein kinase C; CamKII, calmodulin-dependent protein kinase II; ERK, extracellular signal-regulated kinases; MAPK, mitogen-activated protein kinases; PLA2, phospholipase A; MAP-2, mitogen-activated protein-2; Arc, activity-regulated cytoskeleton-associated protein; CREB: cAMP response element-binding protein; c-fos, c-jun and c-myc, are immediate early genes.

Synaptic strengthening can be induced by a “synaptic tag”, a specific marker that permits the transport of newly-synthesized RNAs and proteins towards a correct site (Frey and Morris, 1997, 1998). Unfortunately, even if this mechanism has been showed to be fundamental in the consolidation of memory (Lesburgueres et al., 2011), its nature remains still unknown (Wang et al., 2006).

The perturbation of this step of consolidation is able to block the memory process. On the other hand, the same perturbations applied after this step will not affect memory formation.

## **2.6. The choice of the Social Transmission of Food Preference paradigm in the study of memory consolidation**

In order to pinpoint accurately the time-course of the hippocampal-cortical dialogue during the systems-level memory consolidation, we selected a behavioural paradigm particularly suitable for studying memory consolidation, that is the social transmission of food preference (STFP) , in which rats learn about the safety of potential food sources by sampling those sources on the breath of conspecifics:

1/ This task enables olfactory information to be encoded rapidly during one single interaction session identifying a precise acquisition phase without repeating learning sessions, leading to a rigorous control of induction of post-learning mechanisms implicated in the systems consolidation of the memory trace.

2/ It induces a robust and long-lasting memory. (at least 30 days).

3/ Its associative nature requires the HPC and specific cortical regions such as the orbitofrontal (OFC) and anterior cingulate (ACC) cortices, which are both, involved in processing olfactory associative information (Frankland and Bontempi, 2005; Lesburgueres et al., 2011).

In particular the memory trace is initially dependent by HPC, and during the time by ACC and OFC, showing that the consolidation of the associative olfactory memory is accompanied by a progressive contribution of neocortical areas during the post-acquisition period, according to the fact that the stabilization of memory trace is translated into a strengthening of cortical-cortical connections and requires an architectural remodelling of neuronal network that identify the memory trace (Chklovskii et al., 2004; McClelland et al., 1995).

4/ Having no spatial nature (Strupp and Levitsky, 1984), STFP restricts the functional implication of HPC in the consolidation process and non in the spatial navigation. As a matter of facts, the information of social interaction has a capital nature, in order to know if the food is safety or not; the information in fact is extrapolated independently from where the animal is placed. Thus the spatial learning is negligible comparing the importance of social context.

### **2.6.1. Ethological Principles of STFP**

The STFP task is based on an innate behaviour of the rodent that creates and expresses an associative natural memory, conversely to the majority of behavioural paradigms testing memory in which the animals are obliged to realize tasks typically designed for the experimental practice.

The STFP is based on ethological principles, such as the natural neophobia for the rodents towards the unknown food. In the natural context, the rodent is used to identify a new food source and then transmits his personal experience to others rats, becoming “pre-exposed” to that food. This pre-exposition is able to decrease the natural neophobia in the other rats. Thus, a rat that is been “pre-exposed to the pre-condition” will have a tendency to choose preferentially the familiar food compared to another unknown food (Posadas-Andrews, 1983).

Despite that, the simple odour exposition is not sufficient to increase such food consumption, inducing a fast forgetting of the information concerning this food. Conversely, if the information about the

safety food is acquired through an interaction with a conspecific, it will be considered “precious” for the rats, which will remember it. For this reason, the conspecifics are important “sources” of information in the colony, in particular transmitting the characteristics and the localization of the safety food avoiding the toxic one.

This social interaction and exchanges between rats have been studied from the 80<sup>th</sup> by Galef, who revealed the importance of the social interaction in the transmission of food preference in the colony. He was able to show that the social interaction induces the apparition of a food preference in Norway Rats, not dependent by a behavioural imitation since the “interaction” component was crucial for the behavioural expression.

### 2.6.2. Description of the STFP task

Since it is deeply described in the paragraph of Material and Methods, we briefly describe the principles of this task. The task starts with the *exposition* of a rat, called “demonstrator”, to a flavoured food. After this exposition, the demonstrator interacts with another rat, called “observer”. The *interaction* induce in the observer the formation of an association between the carbon disulfide (CS<sub>2</sub>) and the flavour of the food, both present in the breath of the demonstrator. During this phase the associative olfactory information is encoded. After the interaction, the observer rat performs the *test* session during which the rat is submitted to a choice between the familiar food (smell in the demonstrator breath) and another unfamiliar food, showing the preference for the familiar food if the consolidation and the retrieval of the associative olfactory memory take place.

In 20 years, Galef and collaborators showed the importance of some criteria of this task, here summarized:

- The social interaction is fundamental: comparing the food eaten by the rat interacting with the conspecific to the one of the rat simply exposed to the flavour, Galef and collaborators showed that the 30 min interaction with a demonstrator was sufficient to create a persistent food preference, while the simple exposition does not induce a food preference tested 15 min or 24h after the exposition. Thus, the presence of the conspecific is essential for the development of food preference. Moreover, the social interaction with another rat is important in the transmission of food information, making the food more attractive by the demonstrator rat (Galef, 2003; Galef, 1983).
- To further characterize the quality of the interaction, it has been tested whether the presence of a conspecific is sufficient or just the olfactory cues presented by the demonstrator are fundamental for food preference transmission. To reach this aim, Galef and colleagues try to compare the observers’ performance interacting with normal demonstrate to the one obtained by interacting with anesthetized demonstrators or rats dummies perfumed with the flavour of the food. Dummies demonstrators do not induce the food preference, while anesthetized and normal demonstrators produce the food preference (Galef and Stein, 1985). Moreover, they showed that the components of rat nose breath, such as carbon disulfide and carbonil sulfide (CS<sub>2</sub> and COS), are semiochemical compounds and can influence rats behaviours. As a matter of fact, rats puppets soaked with the flavour and the CS<sub>2</sub> induce the food preference in rats (Galef et al., 1988).
- The duration of social interaction and the numbers of interactions are important parameters for the food preference longevity: the 2 min interaction is sufficient to transmit the food

preference if tested immediately after it. Despite this, to produce a long lasting food preference an interaction of 15-30 min is required (Galef and Stein, 1985).

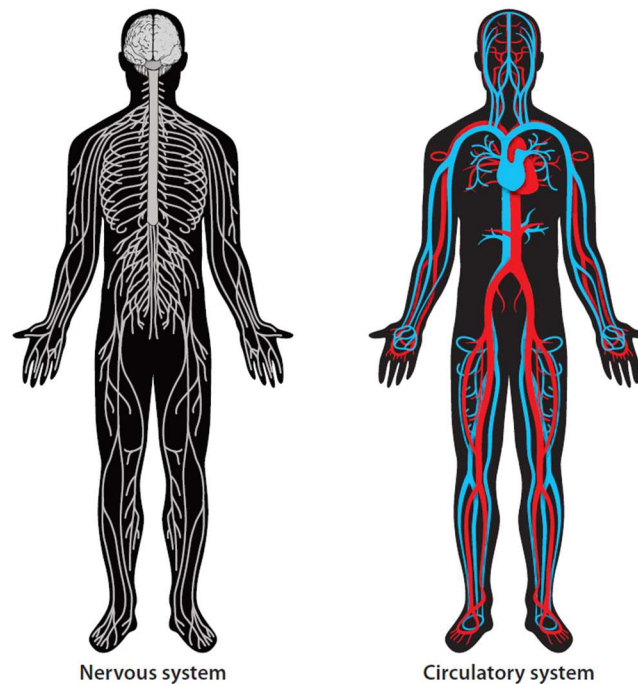
- The aged-matched (Galef and Whiskin, 1998) and non-familiar demonstrator (Galef and Whiskin, 2008) increases the strength of food preference. As a matter of fact, they showed that if the demonstrator is unknown the observer interacts more with it in order to identify it.

Of course this task has also some limits; the most important is that the food preference presented by the demonstrator is maintained if the observer is naïve to it. Moreover, if the food presented possess nutritive disadvantages the food preference disappears (Galef Jr and Whiskin, 2000).

## **Chapter 3: The cerebrovascular network**

### **Chapter 3: The cerebrovascular network**

Probably, the first image of the brain that comes up in mind is a neuronal assembly, implicated in transferring the information through different pathways. Of course it is one of its most studied functions. But brain is not composed just by neurons. The neurons are maintained functional by others systems such as the vascular network, which regularly supports all their actions and necessity (Fig. 19).



**Figure 19:** The common necessity of a functional circuit for the nervous and vascular system is the cause of their shared development features, specific guidance cues and cellular and molecular signalling events. The need of nervous and vascular system to respectively coordinate nutrient and information transfer is reflected by the interface of the neurovascular unit (Tam and Watts, 2010).

The cerebral vascularization and the blood flow are ones of the most important extraneuronal mechanisms implicated in brain functionality. As a matter of facts, if the cerebral blood flow (CBF) is interrupted, the cerebral functions fall down and in few seconds leading to irreversible cellular and functional damage (Hossmann, 1994).

The brain receives 15-20% of total cardiac output although it represents only about 2% of body weight, being the most highly perfused organ of the body (Attwell et al., 2010; Cipolla, 2009). It requires high basal metabolic rate and specific homeostasis but it possesses limited capacity to store the energy. This means that, in order to maintain its processes and regulate all its functions, it demands a constant and controlled exchange with blood flow, by which it is closely dependent (Dunn and Nelson, 2014). Therefore, the spatial and time coordination between the blood perfusion and the neurons metabolic request is a crucial step for brain functions.

The cerebrovascular network adapts its activity in order to provide energy and nutrients according to metabolic request of neuronal network; this mechanism is called functional hyperaemia and it depends on neurovascular coupling (for more details, see (Cipolla, 2009)), characterized by the interaction between endothelial cells of the capillaries, pericytes, astrocytes and neurons.

Moreover, to assure a constant and local blood distribution, the cerebrovascular network compensates the normal fluctuation of arterial pressure ( $P_A$ ) maintaining it in a range of 50-150 mm Hg, increasing the vasoconstrictor tone when the  $P_A$  rise up, inducing vasodilatation when the  $P_A$  decrease; this phenomena is known as autoregulation (Pires et al., 2013).

Consequently, there is a coherent interaction between cerebral vasculature and neuronal network during cognitive functions and memory process. Indeed learning, consolidation and the recollection of memory traces are followed by synaptic molecular changes, leading to morphological changes in dendritic arborisation in brain areas involved (Lesburgueres et al., 2011). But are the dynamics of vascular systems modified by this process? The aim of the thesis is to investigate whether there is a reorganization of vascular network during memory consolidation process. In particular, we focus on architectural modifications induced during the post-encoding period. Before explore the vascular modifications, a brief notion about cerebral circulation is required.

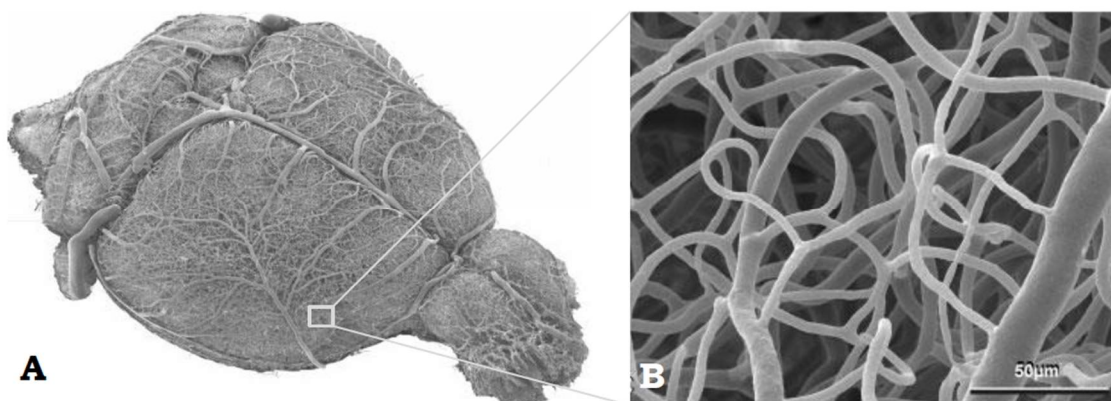
The brain seems to lack the survival advantages of the others organs, which are more tolerant regarding the decrease of CBF, but this limited autonomy is compensated by others mechanisms: firstly the ability to control cerebral perfusion with the humoral and neuronal influence on cardiovascular system (developed more in details in hypertension paragraph), secondly the autoregulation of arterial pressure in order to contrast the systemic fluctuation of pressure during the normal activities, and thirdly the regulation of the blood flow according to the metabolic request of neuron (Attwell et al., 2010; Tired et al., 2009). To understand these mechanisms is fundamental to analyse, respectively:

- The anatomy of cerebral circulation and the role played by the different actors in cerebral homeostasis
- How cerebral blood flow is assured (autoregulation)
- Cerebral adaptation to neuronal activity: neurovascular coupling inducing functional hyperaemia and hypoxia

### 3.1. Anatomy of Cerebral Circulation

#### 3.1.1. Cerebrovascular architecture

The vasculature developmental process in the brain presents some inter-species and intra-species differences, but despite all, the arterial vascular territories remains constant throughout the evolutionary process (Casals et al., 2011) (Fig. 20 and Fig. 21).



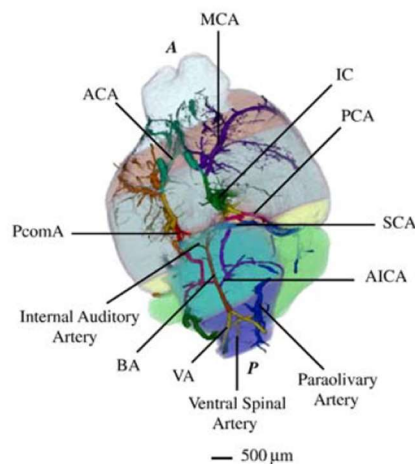
**Figure 20:** SEM images of vascular network obtained using polyurethane-based casting resin PU4iiy. A) Overview of the whole brain. B) Detail of the lateral cortex with the middle cerebral artery. Adapted from (Krucker et al., 2006).



Species	Major artery	Branches of major artery	Region vascularized
Human			
	Internal carotid artery	Middle cerebral artery	Frontal lobe to the parieto-occipital sulcus (anterior cerebral artery)
		Anterior cerebral artery	Superolateral face of hemispheres (middle cerebral artery)
	Basilar artery	Posterior cerebral artery	Midbrain, occipital and temporal lobe (posterior cerebral artery)
	Posterior cerebral artery	Posterior communicating artery	
	Anterior cerebral artery	Anterior communicating artery	
Rat			
	Internal carotid artery	Posterior communicating artery	
		Hypothalamic artery	
		Anterior choroidal artery	Lateral surface of the olfactory tract and cerebral cortex (middle cerebral artery)
		Middle cerebral artery	
		Anterior cerebral artery	
	Basilar artery	Posterior cerebral artery	Posterior portion of the arterial circle of Willis (posterior communicating artery)
		Vertebral artery	
	Posterior cerebral artery	Posterior communicating artery	

**Figure 21:** Cerebral regions supplied by the major encephalic arteries and their branches. Adapted from (Casals et al., 2011).

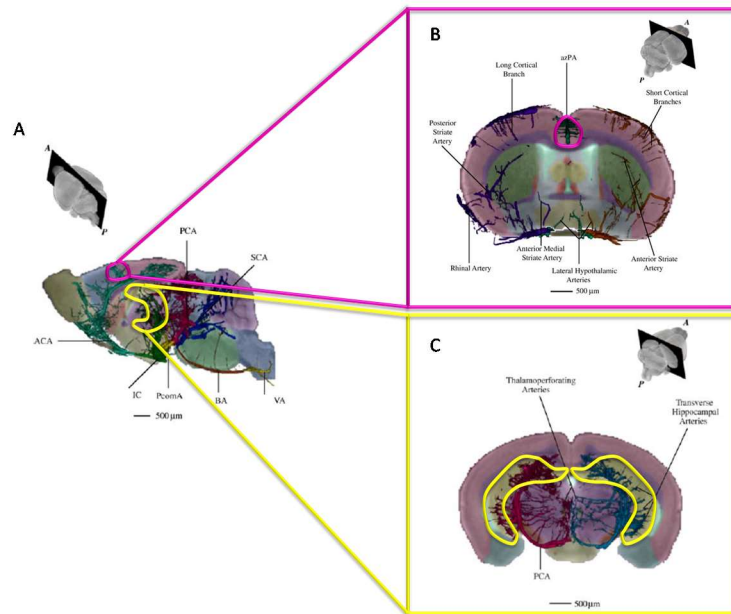
The arterial blood is supplied by two pairs of large arteries, which come from common carotid arteries, the internal carotids (left and right) and vertebral carotid arteries (left and right); the internal carotids perfuse the brain, while the vertebral carotids join it distally form the basilar artery. These two pairs of arteries join proximally other arteries constituting a complete anastomotic ring: the circle or polygon of Willis. It is composed of main arteries: anterior cerebral artery (left and right), anterior communicating artery, internal carotid artery (left and right), posterior cerebral artery (left and right) and posterior communicating artery (left and right); the basilar artery and middle cerebral arteries, supplying the brain, are not considered part of the circle. The circle of Willis represents a system of connections between principal vessels of brain, providing protection against possible occlusive events of a single vessel. In human as in rodents, this anastomotic loop allows low-resistance connections, providing the redistribution of the blood flow, as shown in Fig. 22.



**Figure 22:** Circle of Willis on mouse brain surface. Abbreviations: A, antero; P, posterior; ACA, anterior cerebral artery; MCA, middle cerebral artery; IC, internal carotid; PCA, posterior cerebral artery; SCA, superior cerebellar artery; AICA, anterior inferior cerebellar artery; BA, basilar artery; VA, vertebral arteries; PcomA, posterior communicating artery. (Dorr et al., 2007).

The cerebral arteries controlling the areas analysed in this thesis are the anterior cerebral artery (ACA) irrigating the ACC and the posterior cerebral artery (PCA) irrigating the HPC (Fig. 23), cerebral areas implicated in the STFP memory task. Since we study structural modifications in microvasculature, we do not deeply develop the arteries architecture description; in any case you can refer to the work

published by Dorr and colleagues (Dorr et al., 2007) who explore the three-dimensional cerebral vasculature of a mouse brain using MRI and micro-CT techniques.

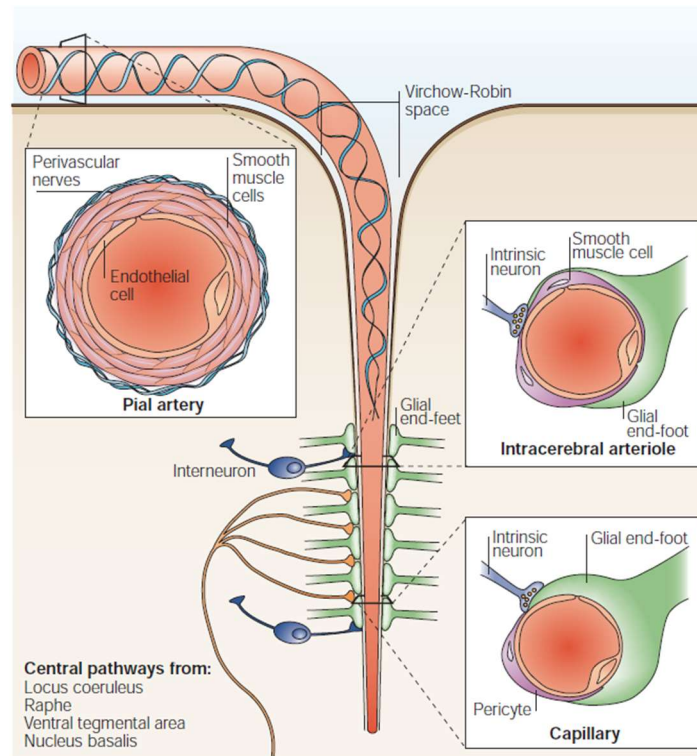


**Figure 23:** Slice planes showing cerebral arteries and their branches in anatomical location. A) Sagittal slice plane, midline, showing the ACC (pink) and HPC (yellow) irrigated by respectively ACA and PCA. B) Coronal slice plane showing the ACC irrigated by ACA branch: azACA C) Coronal slice plane showing the HPC irrigated by PCA. Abbreviations: A, antero; P, posterior; ACA, anterior cerebral artery; IC, internal carotid; PcomA, posterior communicating artery; BA, basilar artery; VA, vertebral arteries; SCA, superior cerebellar artery; PCA, posterior cerebral artery; azACA, azygos of the anterior cerebral artery. Modified from (Dorr et al., 2007).

The cerebral arteries branch into smaller arteries and arterioles which run superficially and then penetrate into the brain tissue (Iadecola, 2004; Jones, 1970; Joutel and Faraci, 2014) (Fig. 24).

The *pial vessels* are intracranial vessels localized between the pial and archnoid maters that run on the brain surface; they are formed by an endothelial cell layer, a smooth muscle cell (SMC) layer and a leptomeningeal external layer (adventitia). They are delimited by astrocytes and surrounded by cerebrospinal fluid (CSF). They possess a robust basal tone and receive perivascular innervations (extrinsic innervation) by the peripheral nervous system. The pial arteries form a network rich in collateral vessels, being insensitive to single vascular occlusion (no appreciable decrease cerebral blood flow), creating the second system of collaterals, together with the circle of Willis, responsible for the redistribution of blood flow.

They branch into pial arterioles and then, at Virchow-Robin space level, into penetrating arterioles and into parenchymal arterioles; these *parenchymal arterioles* (also called intracerebral arterioles) penetrate into brain parenchyma and their basement membrane is completely surrounded by astrocytes' end-feet. They receive intrinsic innervation (perivascular innervation) and they are composed by one single oriented layer of SMC. Conversely to pial vessels, intracerebral vessels are not largely branched, consequently an occlusion at this level results in a significant decrease in CBF (Nishimura et al., 2007). The penetrant arteries are supported by one or two neighbour venules, allowing another compensatory possibility of blood distribution (Guibert et al., 2012).



**Figure 24:** Architecture of cerebrovascular vessels and the differences in structural composition and connections; from (Iadecola, 2004).

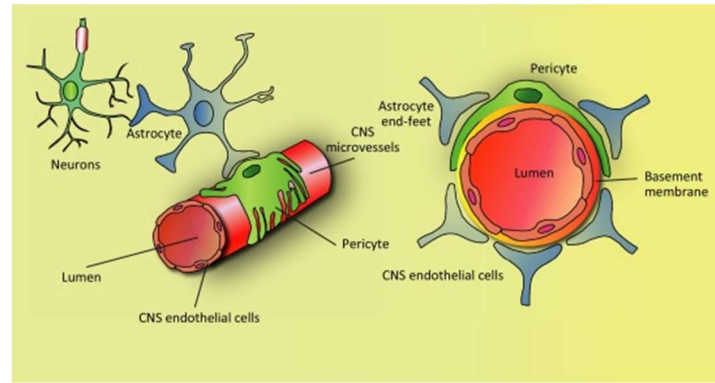
The arterioles branch in turn in *intracerebral capillaries* which create a dense intercommunicating network of specialized endothelial cells without muscular cells. It is the principal site of exchanges of oxygen and nutrients, being always perfused by blood. Their tone and blood flow is regulated by precapillaries arterioles and postcapillaries veins.

The endothelial cells (ECs) create a thin monolayer that cover the inner surface of the vascular tree; their functions is to integrate physical and neurohumoral signals from the blood and surrounding tissues to regulate different processes, such as vascular tone, cellular adhesion, inflammation, and proliferation (Boulanger, 2016).

The total construction of brain microvasculature is constant across the mammals, occupying the 3% of the brain volume (Kolinko et al., 2015). The capillary size is 7-10  $\mu\text{m}$  in average diameter, with an average intercapillary distance of 40  $\mu\text{m}$  (Duvernoy et al., 1983; Jucker et al., 1990). The capillaries are more dense in the grey matter than in the white matter correlating to a higher level of neural activity (Kolinko et al., 2015; Zhu et al., 2012) (Benderro and LaManna, 2014).

The capillaries density varies from region to region depending on the location and the energy consumption (Klein et al., 1986), which can be modified by physiological or pathological conditions. For example, chronic hypoxia increase vascular density through angiogenesis (Xu and Lamanna, 2006), being able to double its density after 3 weeks of chronic hypoxia. Moreover, hypertension can also cause vascular rarefaction and impair microvessels formation (Sokolova et al., 1985).

Cerebral capillaries are unique compared to other organs since their connections. They are composed by endothelial cells in contact with pericyte; both of them are surrounded by a basal lamina (formed by collagen type IV, heparin sulfate proteoglycans, laminin, fibronectin, and other extracellular matrix (ECM) proteins) which is continuous with astrocytes. Together with neurons, this assembly constitute the neurovascular unit (see paragraph « Functional hyperaemia» Fig. 25).



**Figure 25:** The cerebral endothelial cells create specialized structures in order to cooperate with neuronal network. On the right it is represented the Blood-Brain Barrier (BBB) localized in cerebral microvessels, composed by a continuous monolayer formed by non-fenestrated ECs, surrounded by the basement membrane, pericytes and astrocytes end-feet. This structure assure both the homeostasis and the signalling creating the neurovascular unit, on the left, allowing in turn the thigh coupling between vascular and neuronal signalling. From (Chow and Gu, 2015).

Interestingly, the specialized endothelium acts as a barrier allowing selective exchanges between blood and parenchyma: the Blood brain Barrier (BBB) (see next paragraph). In this next section we explore briefly the BBB and the components of neurovascular unit.

### 3.1.2. Blood Brain Barrier (BBB)

The existence of the barriers between brain and the blood was demonstrated by Ehrlich in 1885 by injecting Evan's Blue dye in tail vein of rats and finding that all organs except brain were stained.

He thought that it was due to a difference in cerebral tissue characteristics that avoid the adhesion of the dye. It was his graduate student Goldmann (Goldmann, 1913), who injected the dye in the CSF discovering that just the brain was coloured, assuming the existence of a barrier between brain and the blood, but there was no CSF-brain barrier. Nowadays, we know that also this was partially incorrect.

As a matter of facts, there are three interfaces which protect the neurons from circulating substances (i.e. pathogens, blood cells, albumin) and help to maintain the homeostasis (i.e. avoiding the fluctuation of electrolytes) for the neuronal functions: the blood-CSF interface, the blood-brain interface (BBB), and the CSF-blood interface (Zlokovic, 2008). The largest barrier is composed by the specialized cerebral endothelium, the BBB, and it is the barrier that we are going to focus on; the others barriers, just for mentioning them, are the blood-CSF barrier, composed by epithelial cells of choroid plexus, and the CSF-blood barrier formed by the avascular arachnoid epithelium which envelope the brain (Saunders et al., 2008).

The BBB is formed by cerebral ECs (not only at capillary level, but also in pial artery, arterioles and veins) possessing specialized structural and functional characteristics in order to protect the brain and the spinal cord. The unicity of this endothelium is due to 1/ the high concentration of tight junctions and adherens junctions (that are more typical of epithelium), which allows the selective passage of solutes, and 2/ the active transporters which control the passage of substances necessary to the brain. 3/ Moreover, ECs do not present fenestrations, possess a low rate of pinocytosis and present a high number of mitochondria associated with its high metabolic activity (Zlokovic, 2008). This system rigorously controls and restricts the brain-blood exchanges.

Since BBB is a wide topic of research, we are not going to details the system but we will try to give a general view of its functions.

The tight and adherens junctions connect ECs paracellularly, creating a continuous actin cytoskeleton forming a membrane with high electrical resistance retaining the ions in the vessel lumen (Zlokovic, 2008). The small molecules <400 Da with less than 9 hydrogen bonds can freely cross it through lipid-mediate diffusion (Pardridge, 2007). The **tight junctions** are composed by different proteins which role is to create a continuous electrical membrane that restricts the transport of molecules by blood to brain interstitial fluid and vice versa. The principal integral proteins are claudins, occludin, and junction adhesion molecules (JAM) but they include also accessory proteins like zona occludens proteins (ZO-1, ZO-2, ZO-3) and cingulin (Kniesel and Wolburg, 2000). The **adherens junctions**, as the name says, create and adhesive connections between ECs involving the binding of cadherin that joins the actin cytoskeleton via catenins. They interact also with ZO-1, influencing tight junction assembly. Cadherin is also important in maintaining endothelial integrity in quiescent vessels and for the correct organization in new vessels (Lampugnani and Dejana, 2007), regulating VEGFR-2 signalling (see paragraph “Angiogenesis”).

Oxygen, carbon dioxide and small lipophilic molecules are able to cross freely the BBB, but this membrane is impermeable to hydrophilic molecules like glucose, amino acids and other molecules essentials for life. Thus, ECs express apical transporters implicated in the capitation of nutrients from blood to neurons and basolateral channels which purpose is to inactivate and remove the toxic substances, allowing their efflux from the brain to the blood, together with inactivation and reuptake of neurotransmitters.

The transcellular and bidirectional transport across BBB is mediated by main categories of transports namely carrier-mediated transport, ion transport, active efflux transport, receptor-mediated transport and caveolae-mediated transport; for a review see (Zlokovic, 2008).

### 3.1.3. Pericytes

The pericytes, as the name suggests (peri, around; cyte, cell), are perivascular cells, adjacent to ECs, with which they share the basal membrane, discovered by the French scientist Charles Rouget (Rouget, 1874). They are composed by a cell body with a prominent nucleus and small cytoplasmic content but they possess several long process that wrap around ECs.

At the beginning, it was thought that pericytes possessed just a scaffolding role but then it was understood that they contribute to the stability of the vessel and release growth factors and matrix important for microvascular permeability, remodelling, and angiogenesis (for review (Bergers and Song, 2005).

As a matter of facts, they can communicate with ECs by direct contact (gap junctions) and via paracrine regulation; moreover, one single pericyte is able to touch several ECs thanks to cytoplasmic processes enabling the integration of the signals coming from both in the distal part of the vessel and other capillaries (Rucker et al., 2000) (Fig. 26).

Furthermore, they possess some characteristics in common to SMCs, included the expression of the contractile proteins such as  $\alpha$ -smooth muscle actin (currently proposed as a marker of pericytes in the brain), tropomyosin and myosin actin (Hirschi and D'Amore, 1996) which can provoke vasoconstriction and vasodilation in order to regulate vascular diameter and blood flow at capillary level (Rucker et al., 2000). The contractile function of pericytes can be moulded by different actors binding cholinergic and adrenergic pericytes receptors; in particular the  $\beta$ -adrenergic response leads to relaxation, whereas the  $\alpha_2$  stimulation produces contraction (Rucker et al., 2000). Moreover, pericytes functionality can be modified by the vasoactive molecules Angiotensin II and endothelin-1 and oxygen levels.



Pericytes density is different depending on the functionality of the vessel and the organs in which they are located, since their presence is determined by their functions. The ratio pericyte/EC in the brain is higher compared to all other organs (i.e. brain: 1:3 vs skeletal muscle 1:100) (Allt and Lawrenson, 2001). The reason for this higher density is that ECs interact with them and with astrocytes in the maintenance of BBB functionality and integrity.

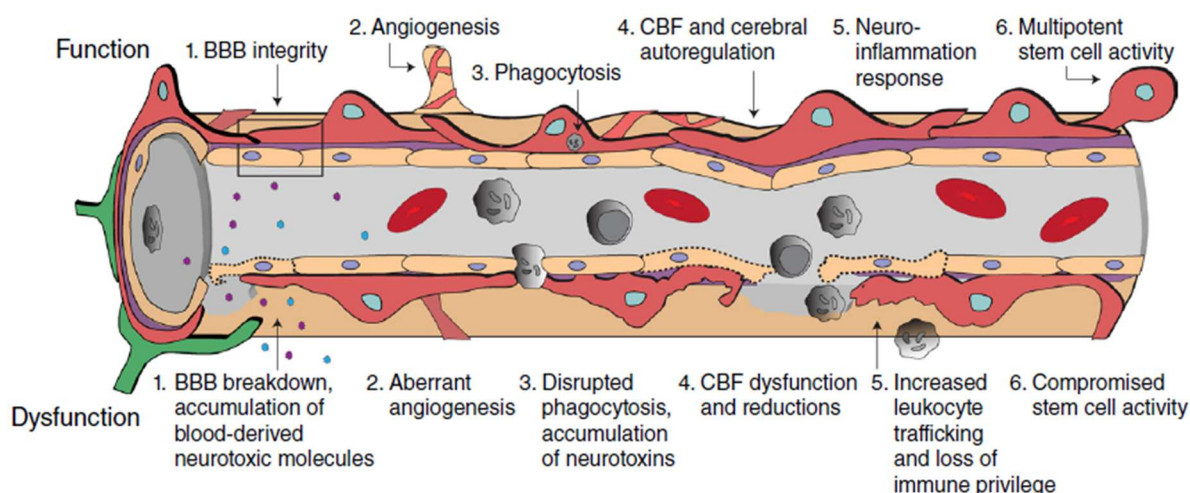
As a matter of fact, in vessel degradation induced by cerebral haemorrhage, pericytes can protect by hypoxia-induced BBB disruption (Hayashi et al., 2004).

Without entering in details, another cerebral pericytes function is macrophage-like phagocytic activity (immunological defence; for a review about this function see (Thomas, 1999)).

Furthermore, pericytes seems to have a role in vessel formation and angiogenesis, detailed in the paragraph “Pericytes role in angiogenesis”, being able to guide, similarly to tip cells, the ECs sprouting by releasing VEGF (Ozerdem et al., 2001; Ozerdem and Stallcup, 2003).

Moreover, once the newly formed sprout is proliferated and the ECs adhere to each other creating the new vessel, ECs secrete growth factors in order to both attract pericytes that envelope the vessel and to promote vessel stabilization.

For a recent review describing pericytes functions see (Sweeney et al., 2016).



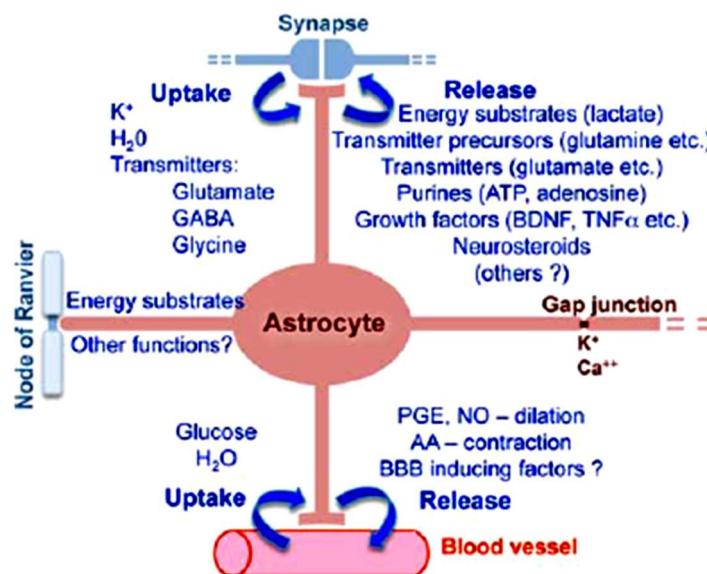
**Figure 26:** The pericytes are involved in several physiological functions summarized in the upper part of this drawing: The pericytes: 1. regulate BBB integrity controlling the junctions and the transcytosis across the BBB; 2. participate in angiogenesis process allowing a window of vascular plasticity (see paragraph “Pericytes role in angiogenesis”); 3. can perform phagocytosis and clearance of toxic metabolites; 4. contribute on CBF regulation acting on capillary diameter; 5. are involved in neuroinflammatory response allowing the leukocytes trafficking into the brain; 6. have multipotent stem cell activity. Their impairment affect all these process leading to the dysfunctions listed in the lowed part of the drawing. From (Sweeney et al., 2016).

### 3.1.4. Astrocytes

The astrocytes, from the Greek *astron* meaning stars and *kytos* meaning cell, were visualized for the first time in the late XIX<sup>th</sup> century and until quite recently the recognized astrocytes were considered as supporting glial cells in neuronal parenchyma and reactive cells responding to central nervous system’s insults, a process known as astrogliosis (Kimelberg and Nedergaard, 2010).

In the last 25 years, several functions were recognized to astrocytes in addition to inflammatory contribution, including a primary role in synaptic transmission in the neurovascular unit, in development, in regulation of CBF, in maintaining cerebral homeostasis, in metabolism and in BBB functionality (Sofroniew and Vinters, 2010) (Fig. 27).

All these functions are assured thanks to the structure of astrocytes; as a matter of facts, they make extensive connections both with vessels and with neurons, together with other astrocytes creating a communication network.



**Figure 27:** Schematic representation of astrocytes functions in physiological condition in central nervous system and its connections with the neurovascular components. From (Sofroniew and Vinters, 2010).

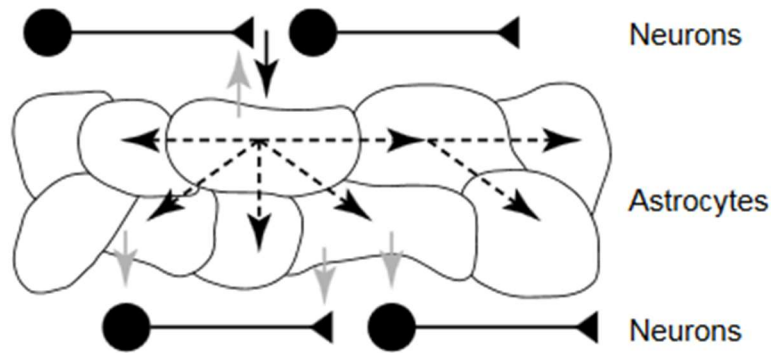
Regarding the astrocytes' role in BBB function, the settlement about them is still open since some BBB functions appear before the maturation of astrocytes during development; in any case, even if they do not participate to the structure of the barrier (role played by ECs (Reese and Karnovsky, 1967)), in vitro studies showed that astrocytes induce the BBB phenotype in ECs, including the up-regulation of tight junction proteins (Abbott et al., 2006; Bauer and Bauer, 2000). As in neurons, there is a cross-talk between astrocytes and ECs. In other studies, the astrocytes end-feet seem to regulate BBB properties through bone morphogenetic protein signalling, since the disruption of this mechanism lead to BBB leakage (Araya et al., 2008).

What is sure is that the extensive astrocytic-vascular contacts assure a bidirectional interaction, allowing the regulation of CBF. The astrocytes are able to produce and release several molecular mediators, like prostaglandins, nitric oxide (NO), and arachidonic acid (AA), that can increase or decrease cerebral blood vessel diameter and blood flow in a coordinated manner.

Moreover, they represent the interface between vessel and neurons, contributing in the adaptation of blood flow in relation to the levels of synaptic activity, as demonstrated by fMRI studies which detected changes in blood flow in response to visual stimuli; these changes were dependent on astrocyte function since the blockade of their glutamate transporters was sufficient to influenced the magnitude and duration of neuronal responses (Schummers et al., 2008).

Furthermore astrocytes can influence directly the neuronal activity at synaptic level regulating the release of glutamate (Glu), purines,  $\gamma$ -Aminobutyric acid (GABA) and serine. These substances are released through an intracellular increase of  $Ca^{2+}$  in astrocytes, altering the neuronal excitability.

This concept gives rise to the “tripartite synapse” (Araque et al., 1999) composed by presynaptic and postsynaptic terminals and the astrocyte. Neurotransmitters released by neurons induce intracellular increase in  $\text{Ca}^{2+}$  concentration (neuron-to-astrocyte signalling); this neuronal-induced increase in  $\text{Ca}^{2+}$  concentration is spread between neighbour astrocytes (astrocyte-to-astrocyte signalling), inducing the astrocytic release of Glu, which, in turn, increase the  $\text{Ca}^{2+}$  levels in adjacent neurons, influencing the neuronal electrical activity and modulating the synaptic transmission (astrocyte-to-neuron signalling) (Fig. 28).



**Figure 28:** Bi-directional communication between astrocytes and neurons. The neuron-to- astrocyte signalling is represented by black arrow, the astrocyte-to-astrocyte signalling by broken lines and the astrocyte-to-neuron signalling by grey arrows. From (Araque et al., 1999).

In addition to this direct effect, astrocytes can influence synaptic function through the release of growth factor, exercising a long-lasting effect on synaptic efficiency, exerting powerful influences on synaptic remodelling and pruning in the healthy adult brain.

Even if not totally understood, the role of astrocytes as a neurovascular interface, together with pericytes, is developed in the following session “Functional hyperaemia”.

### 3.2. Regulation of blood flow

The blood circulation is strictly dependent on heart contraction and is defined by a quantitative parameter that is the frequency (number of beats/minute) and a qualitative characteristic that is the rhythm (defined as regularity of heartbeats). But from a hemodynamic point of view, the blood flow can be compared to the passage of fluid in a tube, assuming that the flow is steady, laminar and uniform through thinned-walled in a non-distensible tube. This does not regard the big arteries since the thick wall and the microcirculation where flow is non-Newtonian.

In any case, the cerebral blood flow is directly proportional to the difference of inflow and outflow pressure ( $\Delta P$ , corresponding to the cerebral perfusion pressure, which is the difference between intra-arterial pressure and the pressure in veins) and inversely proportional to the resistance ( $R$ ) of the vessels and is governed by Poiseuille’s and Ohm’s laws, where  $R$  is dependent to blood viscosity ( $\eta$ ), length ( $L$ ) and radius ( $r$ ) of the vessel.

$$Flow = \Delta P \times R, \text{ with } R = \frac{8\eta L}{\pi r^4}$$

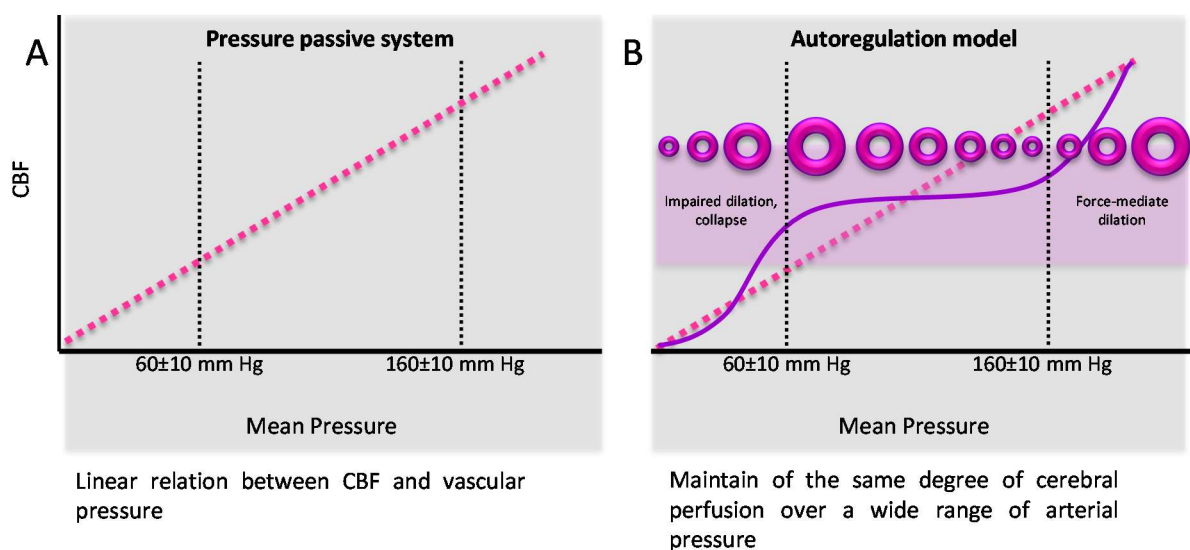


The radius of the vessel is very important since can strongly impact the blood flow; in fact, small changes in lumen diameter have significant effects on cerebral blood flow, allowing a fast change of regional and global cerebral blood flow (Ku, 1993). According to these laws, in a pressure passive system (Fig. 29 A), the blood flow is going to be linearly related to the mean arterial pressure. But this is not suitable for brain functioning, since it cannot be left at the mercy of oscillations in oxygen and energy. Thus it is fundamental to maintain a constant cerebral perfusion and this is possible thanks to the **arteriole vasodilation and constriction** of the pre-capillary arteriole (Fig. 29 B).

### 3.2.1. Myogenic response and arterial autoregulation

In fact in a normal physiological state, the blood flow is principally maintained constant by the large arteries and parenchymal arterioles (Cipolla et al., 2009; Faraci and Heistad, 1990).

#### Autoregulation of Blood flow



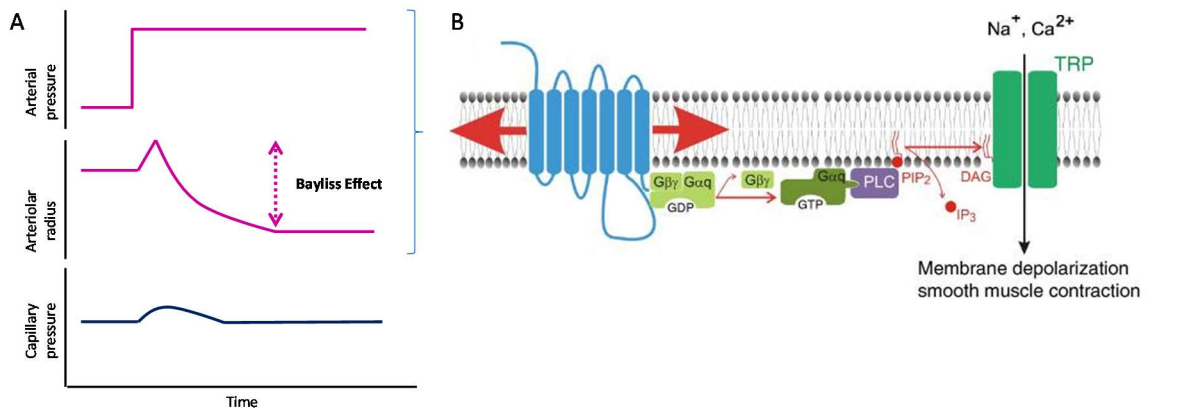
**Figure 29:** CBF in relation to artery lumen diameter. A) Pressure passive system not compatible with brain functions (pink dotted line). B) Autoregulation of CBF induced by myogenic response of cerebral arteries and arterioles (CBF is represented by the purple line). Dotted lines black lines represent the lower and upper limits of cerebral blood flow autoregulation. Pink circles symbolize the cerebral arteries. Pink band represent the constant CBF. Modified by (Pires et al., 2013).

The ability of the cerebral vessel to maintain a constant cerebral blood flow despite of changes in perfusion pressure is called **autoregulation** (Pires et al., 2013). The autoregulation maintain the cerebral perfusion pressure in a range of 60-160 mm Hg, limits beyond which the autoregulation is lost and the cerebral blood flow becomes dependent in a linear manner to arterial pressure.

The autoregulation is assured by the **myogenic response** of the SMCs of arteries and arterioles, which is the ability to change their myogenic tone in order to respond to fluctuation of intraluminal pressure, to maintain a constant blood flow.

The physiologist William Maddock Bayliss discovered over then 100 years ago (Bayliss, 1902) that SMCs of arteries and arterioles were able to increase their constriction in case of increase of pressure and to dilate in case of pressure decrease; this phenomenon is known as "Bayliss effect" (Fig. 30) (Voets and Nilius, 2009). This effect is counterintuitive if we think that in an elastic container, i.e. a balloon, the increase of pressure, in our case if we blow air in the balloon, leads to an expansion of the container, such as an inflated balloon; but cerebral vessel behave in the opposite way in order to firstly

maintain a constant CBF and secondly, but not less importantly, to protect downstream vessels and capillaries with low resistance from pressure insults. The myogenic response of the vessels is an intrinsic property of SMCs and strongly controls the autoregulation, even if the myogenic tone can also be influenced by vasoactive factors released from both ECs and perivascular innervation, affecting vascular resistance.



**Figure 30:** The Bayliss effect. (A) Increasing arterial pressure causes vasoconstriction at arteriolar level, a phenomenon known as the Bayliss effect. The resulting pressure at capillary level is less important, protecting it from pressure insult (B) Proposed mechanism for stretch-induced activation of TRPs in vascular smooth muscle membranes. Modified from (Voets and Nilius, 2009).

Autoregulation is maintained between the limits of 60-160 $\pm$ 10 mm Hg. Close to 60 mm Hg or to 160 mm Hg, arteries respectively decrease (vasodilation) or increase (vasoconstriction) their resistance to maintain a constant CBF. When the perfusion pressure decreases under the lower limit, as in the case of cerebral ischemia, the arteries lose the autoregulation capacity, leading to an impaired dilatation and artery collapse. Vice versa, when the pressure exceeds the higher limit, as in the case of vasogenic edema, a force-mediated dilation is induced (involving KCa channels or reactivity oxygen species), leading to an increase of blood flow (Cipolla and Osol, 1998).

The myogenic response is linked to the basal myogenic tone, which is characterized by a basal partial constriction at constant pressure, and the myogenic reactivity, in other words the alteration of muscular tone induced by a change of pressure (Osol et al., 2002).

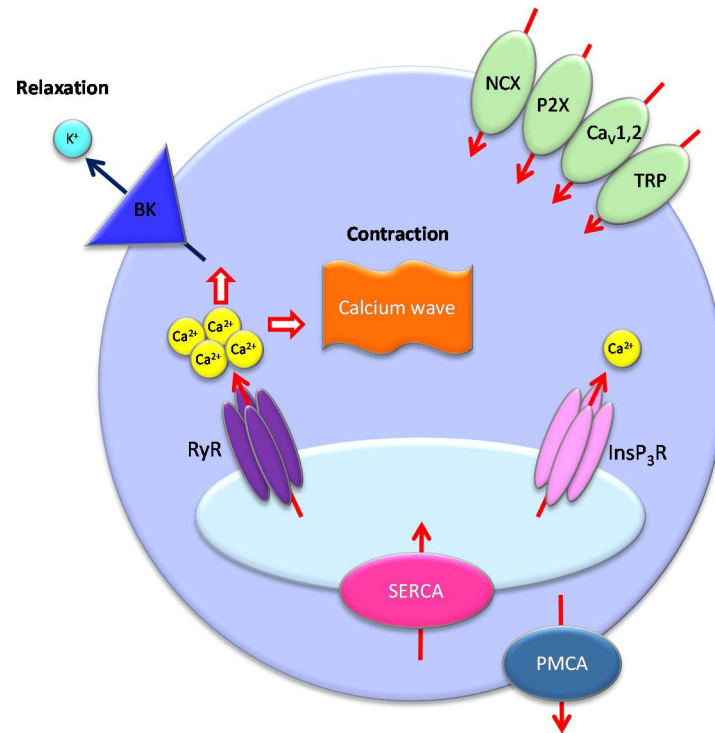
Briefly, when there is a change in pressure a system of transduction, involving stretch activated cation channels (including transient receptor potential channels, TRP), chloride channels and integrins (involved in mechanotransduction), induces depolarization in SMCs leading to an increase of intracellular Ca $^{2+}$  via opening the voltage-operated channel (Cav), which induces, in turn, the phosphorylation of myosin light-chain (MLC), leading to vasoconstriction (Knot and Nelson, 1998). Vice versa, the removal of intracellular Ca $^{2+}$  abolishes the myogenic response.

The vasoconstriction response induced by the development of myogenic tone is different by the one that we can attend in a vessel where the myogenic tone is already present, in other words it is unlikely that the stretch stimulus change enough the vascular diameter in a vessel under pressure. But in this phase Ca $^{2+}$  sensitivity is enhanced during myogenic reactivity.

In any case, the mechanisms implicated in the Ca $^{2+}$  signalling are very complex (for a recent review which describes the ionic signalling implicated in the control of cerebral blood flow see (Longden et al., 2016), in the following part we summarize the principal mechanisms implicated in the vascular reactivity in arterial SMCs.

The variation of intracellular  $\text{Ca}^{2+}$  concentration in SMCs of cerebral vessels can be triggered by different stimuli (stretch, neurotransmitters and astrocytes influences), and it is a key factor in the activation of contractile proteins and in turn in the regulation of the vascular tone.

In SMCs, intracellular  $\text{Ca}^{2+}$  is stocked in the sarcoplasmic reticulum (SR) and is maintained in this compartment by the Sarco-Endoplasmic Reticulum Ca-ATPase (SERCA) which actively transport  $\text{Ca}^{2+}$  from cytoplasmic compartment into the SR, where the  $\text{Ca}^{2+}$  binding proteins calreticulin and calsequestrin are able to trap  $\text{Ca}^{2+}$ , allowing a significant calcic storage (Wray and Burdya, 2010) (Fig . 31).



**Figure 31:** Calcium signalling in VSMC. At membrane level sodium-calcium exchanger (NCX), P2X receptors, Voltage-gated Calcium Channels (CaV) and TRP channels are responsible to  $\text{Ca}^{2+}$  entry in cytoplasmic compartments. PMCA and SERCA mediate the intracellular calcium decrease. Red and blue arrows indicate respectively calcium and potassium directions. Modified from (Berridge, 2008).

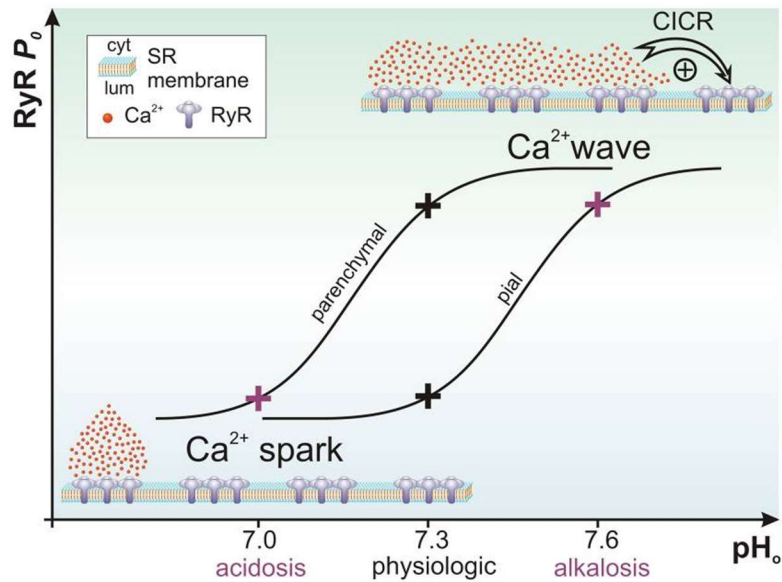
The  $\text{Ca}^{2+}$  release from SR to cytosolic compartment can be mediated by different channels localized on SR membrane, including inositol 1,4,5-trisphosphate receptor ( $\text{IP}_3\text{R}$ ) and ryanodine receptor (RyR) (Dabertrand et al., 2013; Morel, 2007).

Different vasoactive signals, like endothelin-1, acetylcholine (ACh), noradrenaline (NA) or serotonin (5-HT), induce the activation of  $\text{G}\alpha_{q/11}$  protein-coupled receptor, with the consequent stimulation of phospholipase C (PLC) and activation of inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) and of its receptor inducing  $\text{Ca}^{2+}$  extrusion. The level of liberation of this cation is dependent by the concentration of cytosolic  $\text{Ca}^{2+}$  and by the type of  $\text{IP}_3\text{R}$  involved (Berridge, 2008, 2009; Dabertrand et al., 2013).

The RyR exists in 3 different isoforms induced by different splicing: RyR 1, 2 and 3, all of them expressed both in pial arteries (Vaithianathan et al., 2010) and in cerebral microcirculation (Dabertrand et al., 2013). They can be activated SR  $\text{Ca}^{2+}$  load or, more importantly, by binding of  $\text{Ca}^{2+}$  on the cytosolic face of the channel. Moreover they can be activated by  $\text{Ca}^{2+}$  coming from either other plasmatic membrane channels or from RyR- or  $\text{IP}_3\text{R}$ -induced SR release; this mechanism is known as Calcium-Induced Calcium-Release (CICR). This activation can induce two phenomena: a transitory

local increases of  $\text{Ca}^{2+}$  (called sparks) or cytoplasmic calcium waves. The two mechanisms are really different inducing opposite response.

Sparks induce the activation of large conductance  $\text{Ca}^{2+}$ -activated potassium channel (BK) that allows the extrusion of  $\text{K}^{+}$  inducing hyperpolarization and consequently relaxation of the SMC. When the resting open state probability ( $P_o$ ) of adjacent RyRs is low, it will result in a local and transitory effect. Vice versa, if the RyR  $P_o$  is high, the  $\text{Ca}^{2+}$  local increase will induce, through CICR mechanism, the neighbored RyR activation (and probably the  $\text{IP}_3\text{R}$ ) with the consequent trigger of  $\text{Ca}^{2+}$  waves in the cytoplasmic compartment and activation of contractile proteins (Dabertrand et al., 2013) (Fig. 32).



**Figure 32:** The  $\text{Ca}^{2+}$  signalling via RyR in SMCs of arteries and arterioles. Relation between pH and open probability ( $P_o$ ). From (Dabertrand et al., 2013).

The cytoplasmic  $\text{Ca}^{2+}$  is complexed by calmodulin (CaM) protein, which activates the myosin light chain kinase (MLCK) that, in turn, induces the phosphorylation of the myosin. Phosphorylated myosin is thus able to interact with actin protein, inducing the sliding of light chain and heavy chain, resulting in the contraction of SMCs (Cole and Welsh, 2011).

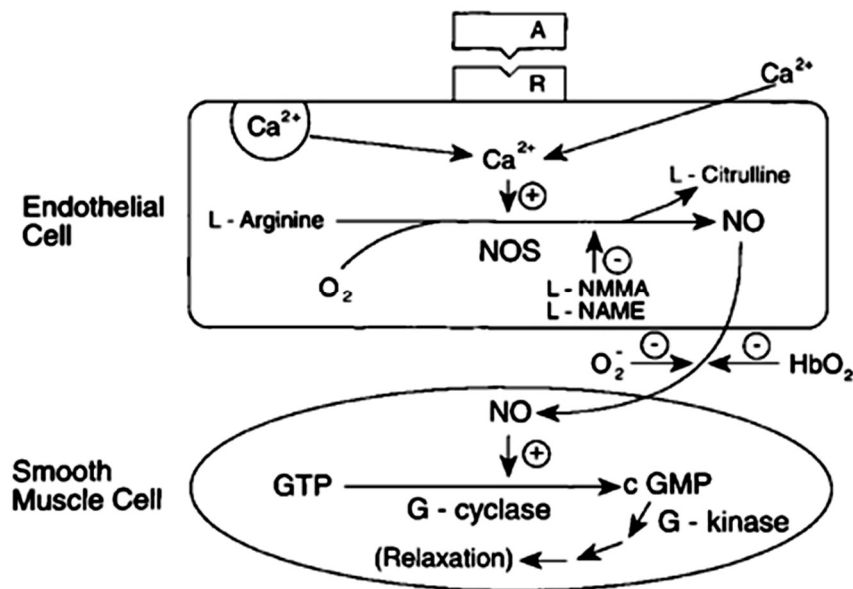
Successively, SERCA, plasma membrane  $\text{Ca}^{2+}$  ATPase (PMCA) and  $\text{Na}^{+}/\text{K}^{+}$  channels start to re-establish the initial cytosolic  $\text{Ca}^{2+}$  concentration.

### 3.2.2. Endothelial regulation of vascular tone

SMCs autoregulation, as said before, assure the CBF distribution and protect the down-stream vessels but have the ECs a role in tone regulation? The answer is of course yes, they have. ECs are able to influence the vascular tone and the cerebral blood flow through the release of several vasoactive mediators, including NO, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF).

Nitric oxide (NO) is one of the most important vasodilator actors, having a big impact on cerebral circulation, since the inhibition of its synthesis with such as l-nitro-arginine (l-NNA) causes significant endothelium-dependent vasoconstriction of cerebral arteries and arterioles (Faraci and Brian, 1994).

NO is produced by the oxygen-dependent transformation of the amino acid L-Arginine mediated by the enzyme NO-synthase (NOS) (Siragusa and Fleming, 2016). Cerebral NOS exists in three different isoforms: an inducible form (iNOS) and two constitutive isoforms localized in neurons (nNOS) and in ECs (eNOS). In ECs, NO, produced by eNOS, is able to diffuse through plasmatic membranes in SMCs, where activates the soluble guanylate cyclase (GC), causing production of guanosine 3',5'-cyclic monophosphate (cGMP) with the consequent phosphorylation of proteins, which in turn reduce the intracellular  $Ca^{2+}$  resulting in relaxation (Fig. 33).



**Figure 33:** Synthesis and molecular mechanism of NO in ECs and SMCs mediating relaxation. From (Furchgott and Jothianandan, 1991).

In cerebral ECs, the production of prostaglandin I<sub>2</sub> (PGI<sub>2</sub>), also called prostacyclin, can be activated by the hydrolysis of cellular lipid membranes, by of the calcium-dependent enzyme phospholipase A<sub>2</sub>, in arachidonic acid (AA). The AA is the substrate for cyclooxygenase (COX), lipoxygenases, and cytochrome P450 monooxygenases; in particular COX products can mediate vasodilation (PGI<sub>2</sub>, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), prostaglandin D<sub>2</sub> (PGD<sub>2</sub>)) or vasoconstriction (prostaglandin F<sub>2</sub> $\alpha$  (PGF<sub>2</sub> $\alpha$ ) and thromboxane A<sub>2</sub> (TXA<sub>2</sub>)). The PGI<sub>2</sub> is synthesized from PGH<sub>2</sub> by PGI<sub>2</sub> synthase, which can activate adenylate cyclase and increase cyclic AMP and protein kinase A in smooth muscle, causing vasodilation (Bogatcheva et al., 2005).

Finally, the mechanism by which EDHF induces vasodilation is not completely clear; some studies suggest that EDHF can be activated by basal activity or in response to an increase of  $Ca^{2+}$  induced by the involvement of small conductance  $Ca^{2+}$  activated K channels (SKCa) or intermediate conductance  $Ca^{2+}$  activated K channels (IKCa). The consequent hyperpolarization, transmitted to adjacent cells,

induces the closure of voltage-dependent calcium channels (VDCC), leading to vessel relaxation (Sandow et al., 2009).

### 3.3. Cerebral adaptation to neuronal activity: functional hyperaemia and hypoxia

The CBF is able to adapt its activity according to the metabolic request of the neurons, acting via two main mechanisms: firstly, to induce a fast adaptation to increase of energy request, the brain can increase the cerebral blood flow through modification of vascular reactivity (functional hyperaemia) that we are going to summarize in the following part; secondly, vascular network can adjust its functionality through structural adaptations, in other words changing its architecture via increasing the vascular density. The aim of this thesis is to analyse the functional implication of vascular network in memory consolidation, focusing on this second mechanism, analysed in the second part of this chapter.

#### 3.3.1. Functional hyperaemia

The autoregulation counteracts the effect blood pressure that occurs during the normal activities but the CBF is regulated according to the metabolic request of neurons. This phenomenon, as mentioned before, is called functional hyperaemia, and is nicely described in the review (Iadecola, 2004). The process by which functional hyperaemia happens in the brain involves a rapid communication between not only vessels and neurons, but also between different cell types integrating and sustaining this communication. Among the processes allowing the functional hyperaemia, the most important is the **neurovascular coupling** (Dunn and Nelson, 2014).

Nowadays the functional hyperaemia is well known but in the 1800s, the Italian physiologist Angelo Mosso discovered for the first time that an increase of neuronal activity was able to induce changes in brain volume and temperature. As matter of facts, he was able to analyse these changes in a man that has literally a hole in his head: a 45 years old construction worker Luigi Cane was hit by a brick, which had fallen down from a height of 20 m provoking a skull opening. Mosso was able to record in this way changes in blood volume following a question asked to the patient regarding what impression his wife had made on him the first time he saw her (Zago et al., 2009). Now, the gossip is that in some other publications is reported that the change of blood flow was induced by Mosso: “expressing the impression that his wife had made upon me when I first saw her. Cane did not speak. The blood to the brain increased immediately and the volume of the feet markedly diminished” (Iadecola, 2004). Independently by the interest of Mosso in Cane’s wife, in any case the effect was recorded and in the following years were developed others method measuring the CBF by Seymour Kety with nitrous oxide or by Neils Lassen and David Ingar, till the more recent techniques of PET and MRI.

How the neurons increase the CBF?

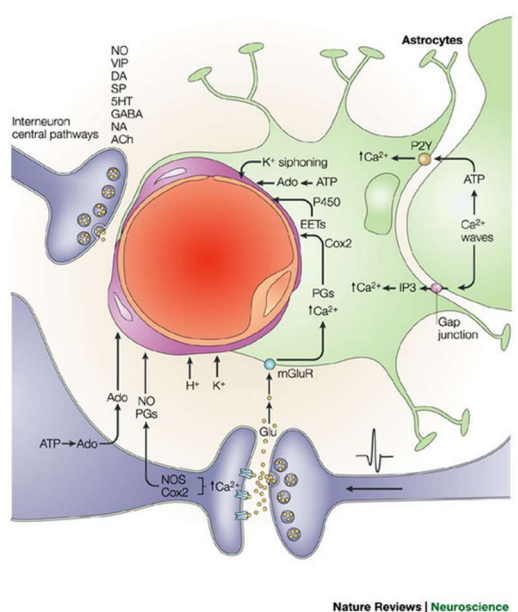
The neurovascular regulation of CBF is mainly at pial arteries and arterioles levels (Dunn and Nelson, 2014). The CBF is regulated by the release of vasoactive compounds, as you can see in Fig. 34, coming from different cellular types.

The production and/or release of vasoactive compound by neurons and others cell types are triggered by different stimuli. As mentioned before, it is known since many years that the increase of energy request, induced by the change in neuronal activity, results in decrease of oxygen and glucose, triggering the increase of CBF (Siesjo, 1978); in fact, hypoxia and hypoglycaemia are potent signal inducing vasodilators factors. However, changes in the hemodynamic appear 1-3 s after changes in neuronal activity while metabolic modifications are slower (Lou et al., 1987); consequently, there are

some other mechanisms participating in CBF regulation and the nature of this dynamic is more complex. Furthermore, some studies reported that the reduction of oxygen was so small, local and transitory to not be totally responsible of a general increase of CBF (for a review comparing the studies studying the “initial dip”, scilicet the deoxygenation inducing increase of CBF, see (Ances, 2004)). Finally, it was reported that functional hyperaemia can be modulated by different neurotransmitters (Lauritzen, 2005), as mentioned in the following part.

Although the imperfect coupling between cerebral oxygen consumption, the acceleration of blood flow and the glucose utilization lead to the idea that the initial increase of CBF is mediated by synaptic signalling more than energy deficit; anyway, some data show that the metabolic component is important: in fact, changes in the lactate/pyruvate ratio, reflecting NADH/NAD<sup>+</sup> ratio and in turns the energy and redox state of the cells ( $\text{NADH} + \text{H}^+ + \text{pyruvate}_{\text{intracellular}} \rightleftharpoons \text{NAD}^+ + \text{lactate}_{\text{intracellular}}$ ), induce a modification of CBF (Ido et al., 2001). In this study rats, undergoing to whisker stimulation and thus to an activation of somatosensory cortex, showed an increase of plasma lactate/pyruvate ratio concomitant to an increase of blood flow in activated somatosensory cortex; moreover, this increase was prevented injection of pyruvate (to lower lactate/pyruvate ratio), suggesting that NADH can act as a sensor for the CBF regulation, through the involvement of NO and protein kinase C.

Independently by the factors triggering the functional hyperaemia, the release and the diffusion of neuronal vasoactive substances is not sufficient to explain the rapid and region-specific increase of CBF. As a matter of facts, others cell types take part to the neurovascular coupling, namely interneurons, astrocytes and pericytes allowing the increase the rapidity and the efficiency of the communication (Fig. 34).



**Figure 34:** Vasoactive mediators released from neurons and glia during the neural activity. As described in the text these molecules can include ions that modify extracellular currents, Adenosin (Ado) derived from ATP catabolism, Glutamate influencing Ca<sup>2+</sup> and NO release, COX-2 and prostaglandins (PGs) and epoxyeicosatrienoic acids (EETs). From (Iadecola, 2004).

### 3.3.1.1. Astrocytes in neurovascular coupling

Astrocytes possess several strategic advantages that result in an efficient structure for the regulation of the CBF: firstly, their end-feet contemporaneously wrap the parenchymal arteries and project to neuronal synapses (tripartite synapses); secondly, vasoactive interneuron's have more contact with



astrocytes then with vessels; thirdly, neurotransmitter receptor uptake sites are expressed on astrocytes, influencing the signalling (Venkat et al., 2016).

Since the increase of CBF is rapid (less than 1s in neocortex (Norup Nielsen and Lauritzen, 2001)) and localized (less than 100µm in olfactory bulb, (Chaigneau et al., 2003)), it was assumed that the intervention of astrocytes and interneurons are required. So the neurons are responsible of the triggering of the neurovascular coupling, communicating the needs to vessels through astrocytes and interneurons (Banerjee and Bhat, 2007).

Since astrocytes are not electrically excitable, the traditional studies were performed with the induction of  $\text{Ca}^{2+}$  dyes in brain slices. Only recently it was possible to study *in vivo* the astrocytes influence of CBF, using multiphoton microscopy and  $\text{Ca}^{2+}$  markers (for a review of these studies see (Petzold and Murthy, 2011)). These *in vivo* and *in vitro* studies showed different pathway by which astrocytes influence the CBF, here in a following part we briefly summarized some of them but more mechanisms are explained in the reviews: (Dunn and Nelson, 2014; Iadecola, 2004; Petzold and Murthy, 2011; Venkat et al., 2016), and displayed in Fig. 35. Some studies showed that the stimulation of cortical astrocytes, achieved using direct or indirect techniques, induces  $\text{Ca}^{2+}$  waves, leading to either vasodilation or vasoconstriction of neighbour arterioles.

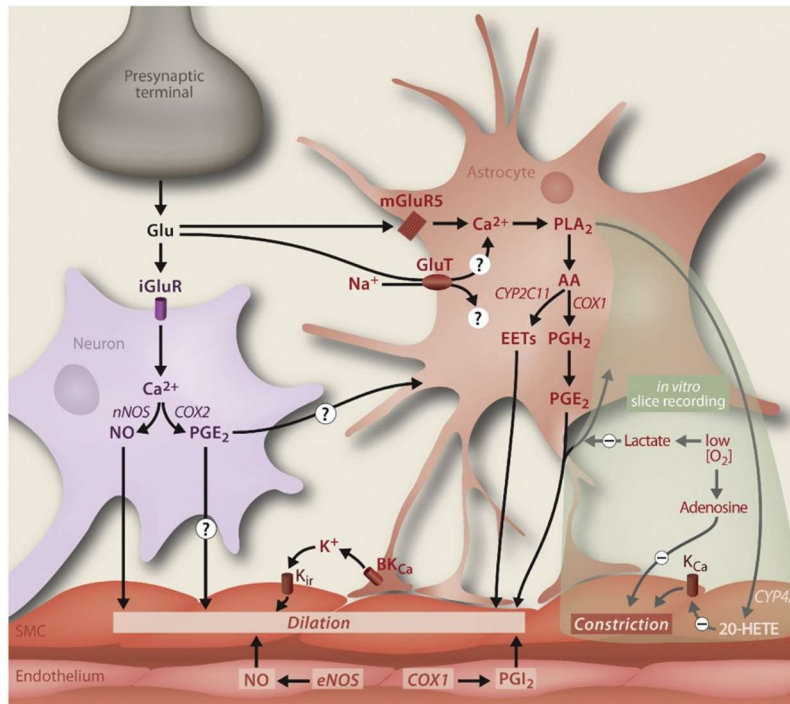
In particular, astrocytes can be stimulated by nearby neurons, activated by interaction of Glu with iGluR, inducing the increase of intracellular  $\text{Ca}^{2+}$ , stimulating nNOS and COX-2 activity with the consequent production of NO and PGE<sub>2</sub>, which induce vasodilation through astrocytes (even if this mechanism iGluR mediated is not really understood). Alternatively, the Glu released by neuron can activate directly the astrocytes either entering through Glu/ $\text{Na}^+$ -cotransport (but the pathways that eventually lead to vasodilation remain unknown), or binding the metabotropic glutamate receptor, mGluR (Attwell et al., 2010) (more probably mGluR5, coupled to Gq protein) of astrocytes which induces, via hydrolysis of membrane phosphoinositides, the production of IP<sub>3</sub>. The interaction of IP<sub>3</sub> with its receptor provokes the release of calcium from the SR, giving rise to  $\text{Ca}^{2+}$  waves which propagates either to others astrocytes till the perivascular compartment or directly inducing the production or release of vasoactive compounds. In particular, the increase of  $\text{Ca}^{2+}$  can activate phospholipase A<sub>2</sub> (PLA<sub>2</sub>) which in turn mediated synthesis of arachidonic acid (AA) and its transformation in PGE<sub>2</sub> through COX-1 activity or epoxyeicosatrienoic acids (EETs) through cytochrome P450 2C11 epoxygenase (CYP2C11). This mechanism is responsible to the vasodilation (Filosa et al., 2004; Filosa et al., 2006; Zonta et al., 2003).

Moreover another vasodilator mechanism can be triggered by the  $\text{Ca}^{2+}$  induced release of  $\text{K}^+$  through the BK, which provokes hyperpolarization of SMCs via inward-rectifier potassium channels (Kir).

Astrocytes can mediate also vasoconstriction by the conversion of astrocytic AA in 20-hydroxyeicosatetraenoic acid (20-HETE) (Mulligan and MacVicar, 2004) in SMCs by cytochrome p450 even if the mechanism is still unknown.

The system is more complicated by studies showing that vasoconstriction and vasodilation mediated by astrocytes are dependent from the level of NO (Metea and Newman, 2006), or  $\text{Ca}^{2+}$  (Girouard et al., 2010). Moreover, it has been shown that *in vivo* astrocytes are able to modulate vessels diameter depending from the oxygen extracellular concentration (Mulligan and MacVicar, 2004). In case of low oxygenation the astrocytes induce vasodilation of vascular network and, vice versa, in case of high concentration of oxygen the astrocytes mediate vasoconstriction response (Attwell et al., 2010).





**Figure 35:** Molecular pathways of neurovascular coupling and the astrocyte contribution, from in vivo and in vitro studies mentioned above (the pathways confirmed just in vitro studies are highlighted to the right). From (Petzold and Murthy, 2011)

### 3.3.1.2. Interneurons and neurons in neurovascular coupling

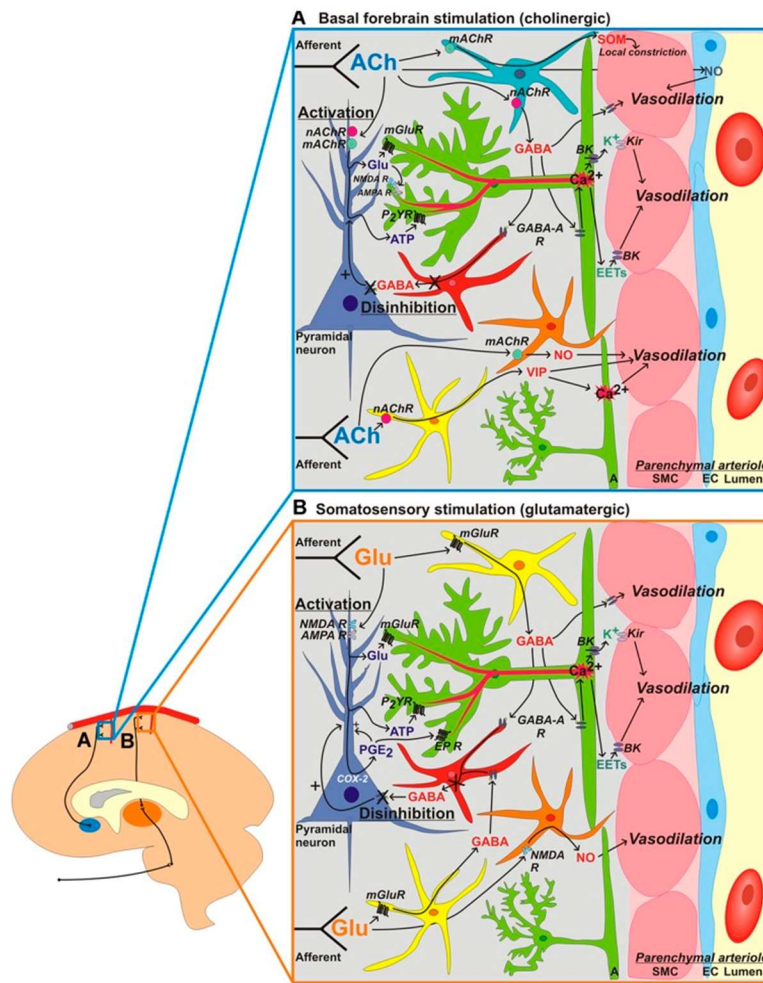
Both neurons and interneurons can influence directly or indirectly (via astrocytes) the vascular tone.

GABAergic interneurons can be recruited by signalling coming from forebrain, inducing the release of vasoactive compounds able to induce vasodilation or vasoconstriction by acting directly or via stimulation of neighbouring neurons or astrocytes.

Ascending afferent neurons, which come from subcortical areas, can project directly to intracerebral vessels, including parenchymal arterioles and capillaries, inducing cortical release of neurotransmitters that can be vasoactive per se. It is the case for ACh and 5-HT, which are respectively able to directly dilate or constrict the arterioles. The hyperemic response to ACh is not fully understood but it has been shown to induce vasodilation through activation of non-neuronal NO, probably eNOS (Fig. 36 A). Despite this, it seems that the major effect on CBF is induced by cortical pyramidal neurons. As previously said cortical pyramidal neurons can release Glu (Fig. 36 B), which can activate astrocytes via iGluR or mGluR. Moreover, Glu can bind NMDA receptors on NOS-expressing interneurons. Furthermore, Glu is able, through Ca<sup>2+</sup> mediated activation of enzymes both in neurons and in glial cells, to stimulate the production of NO (as mentioned in the previous paragraph) and metabolites of COX-2 (producing prostanoids and AA) P450 epoxygenase (producing EETs). Another neurotransmitter released by excitatory neurons is ATP, which can stimulate, through involvement of metabotropic P2Y purinergic receptors, PLC and IP<sub>3</sub>, Ca<sup>2+</sup> waves and vasodilation or vasoconstriction, depending from where and which kind of stimulus is applied. Moreover, neuronal metabolism can induce release of adenosine, a potent dilators of arterioles. Finally, some cations, such as H<sup>+</sup> and K<sup>+</sup> can modify synaptic transmission changing the extracellular ionic currents.

The system to control the CBF is very complex and we could not explore it carefully during this thesis, in any case some pathways are more detailed in (Attwell and Iadecola, 2002; Dunn and Nelson, 2014; Iadecola, 2004). The inhibition of any one of these molecules does not completely block the CBF, showing that the CBF regulation is organized at multiple levels by the cooperation of different

pathways. Furthermore the efficiency of inhibition is dependent by the cerebral region involved, e.g. the nNOS inhibition attenuates functional hyperaemia more in the cerebellum then in the other cortical areas (Yang et al., 2003a).



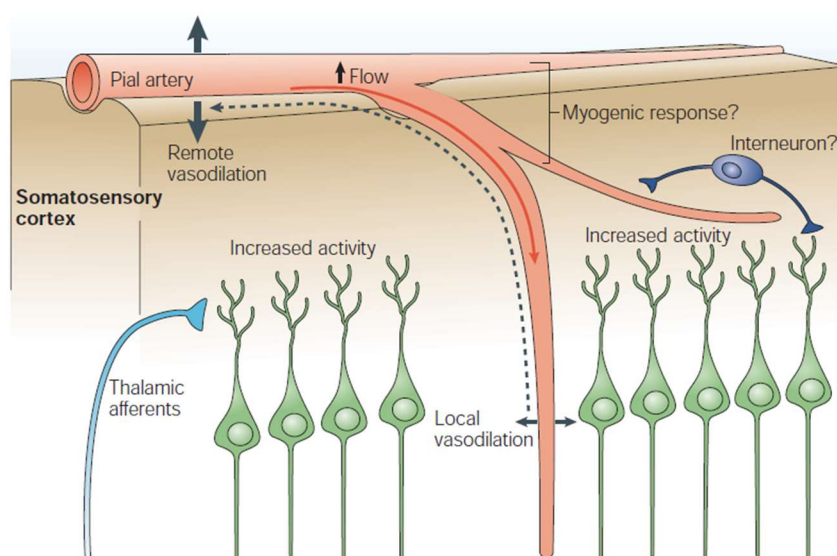
**Figure 36:** Cortical neurovascular coupling signaling mechanisms resulting in vasodilation and increase cerebral blood flow in response to activation of cholinergic basalocortical afferents via basal forebrain stimulation (A) or activation of glutamatergic thalamocortical afferents via somatosensory stimulation (B). Parenchymal arterioles are depicted at the right of each image. Blue, endothelial cell (EC); pink, smooth muscle cell (SMC); green, astrocyte (A); purple, pyramidal neuron; red, GABA inhibitory interneuron; yellow, vasoactive intestinal peptide (VIP)/choline acetyltransferase interneuron; orange, nitric oxide (NO) synthase/neuropeptide Y (NPY) interneuron; turquoise, somatostatin (SOM) interneuron. Neurotransmitters released from ascending afferent neurons are shown in blue for A and orange for B. Mediators derived from pyramidal neurons are shown in purple, interneurons in red, astrocytes in green, and vascular ECs in dark blue. R, receptor; mAChR, muscarinic ACh receptor; nAChR, nicotinic ACh receptor; mGluR, metabotropic glutamate (Glu) receptor; NMDA, N-methyl-D-aspartate; AMPA, -amino-hydroxy-5-methyl-4-isoxazolepropionic acid; BK, large-conductance  $\text{Ca}^{2+}$ -activated K channel; Kir, inward rectifier K channel; P2YR, P2Y receptor; EET, epoxyeicosatrienoic acid; EP, PGE2 receptor; COX, cyclooxygenase. From (Dunn and Nelson, 2014)

### 3.3.1.3. Pericytes in neurovascular coupling

As mentioned before, pericytes, localized in tight contact with ECs, are able to contract, regulating the capillary diameter via vasoconstriction or relaxation. *In vitro* studies showed that pericytes are able to induce vasoconstriction following the administration of noradrenaline and serotonin, or vasodilation with GABA, NO and adenosine. The ability to contract was misused also *in vivo* studies, but the lack of effect in CBF indicates that, even if the contractile regulation of pericytes in capillaries diameter exists, it have not a significant effect on CBF.

In synthesis, many pathways influence CBF regulation at many levels. Moreover, since pial arteries and surface arteries offer the major resistance site to the flow, are involved in CBF regulation. Thus, the vasodilation of downstream vessels does not change significantly the blood flow, unless the pial arteries are vasodilated. In other words, vasodilation of pial arteries is required to increase CBF (Iadecola, 2004).

The activation of whiskers stimulation produces an increase of CBF in pial arteries far from the site of stimulation (Cox et al., 1993). So the vasodilatory message is transmitted to upstream vessels by two main assumed mechanisms. The first one considers that the vasodilation signal, started in the region of neuronal activation, is transmitted by neurotransmitters but this process seems unlikely or less effective since pial arteries and arterioles receive innervation from autonomic and sensory ganglia localized outside the brain, without a direct link between neuronal pathways mediating the effect. It seems more probable that the information is transmitted by signalling within the vessels wall (intramural vascular signalling) through homocellular gap junctions and can propagate vasodilation in a retrograde way (Dietrich et al., 1996).



**Figure 37:** Propagation of vascular response induced by cortical activation showing a myogenic response of vessels irrigating non-activated neural territories, as well as possible interneurons involvement inducing an increase of blood flow. From (Iadecola, 2004).

In conclusion, it is clear that the hemodynamic control sites of CBF cannot be restricted to a cell type or a vessel type and its modulation to a single signalling pathway (Fig. 37). In fact, the signalling coming from neuronal activity induces the production of vasoactive compounds at neuronal or non-neuronal site (i.e. astrocytes). This release provokes vasodilation or constriction at local site involving arterioles or capillaries. Some local interneurons can corroborate the signal through the release of vasoactive molecules. The signal is thus transmitted to upstream vessels by intramural signalling.

The CBF is, in any case, maintained constant considering the whole cerebral structure by the resistance arterioles in order that there is no recall of blood from a “non-activated zone” to an “activated one” and all the areas are always functionally perfused.

Even if most of the time the variation of CBF is induced by neuronal activation, a study showed that the haemodynamic signal in the primary visual cortex in awoken monkeys is composed by two distinct components: the first one reliable to neuronal regulation induced by the stimulus, and the second one corresponding to a preparatory mechanism inducing a supplementary increase of flow independently

of visual input, anticipating the task repeated at regular intervals, like a kind of conditioned response of CBF (Sirotin and Das, 2009).

Of course, others actors plays a role in CBF regulation even in a long term interval, and the aim of this thesis is not to explore all of them.

In any case when the oxygen consumption increase the vascular network can increase its architecture in order to support neuronal activity. Thus, in the following part we are going to focus on hypoxic mechanism inducing an increase of vascular network via angiogenesis.

To complete the list, also carbon dioxide ( $\text{CO}_2$ ) has a deep impact on CBF; as a matter of fact hypercapnia causes marked dilation of cerebral arteries and arterioles and increased blood flow, via a probable direct effect of extracellular  $\text{H}^+$ , whereas hypocapnia causes constriction and decreased blood flow (Iadecola, 1992).

### **3.3.2. Hypoxia inducing angiogenesis**

Oxygen ( $\text{O}_2$ ) is necessary for the oxidative phosphorylation in order to maintain an efficient ATP production.

The oxygen diffusion distance in an oxygen-consuming tissue is very short (in the range of 20-100  $\mu\text{m}$ ), thus the adequate supply of oxygen within the tissue is closely dependent by vascular architecture (Zakrzewicz et al., 2002).

In our body, the request of oxygen depends from the balance between supply/consumption and retrieval/loss; moreover, different regions of our body have different needs of oxygen delivery. The oxygen levels are heterogeneously distributed within the body (21%  $\text{O}_2$  in the atmosphere corresponding to  $\sim 149$  mmHg, 14%  $\text{O}_2$  in the alveolar air, 12%  $\text{O}_2$  in the arterial blood and 5.3%  $\text{O}_2$  in venous blood, corresponding to  $\sim 100$ -85 mmHg (Fong, 2008)), but in our brain the interstitial tissue oxygen levels are lower and non-uniform, ranging from  $\sim 1$  to 5%, corresponding to  $\sim 30$ -50 mmHg (Sharp and Bernaudin, 2004). Thus, in a physiological condition, the cells are able to work under relatively low oxygen levels, referred as physiological hypoxia. However, the term hypoxia means a state of deficiency of available oxygen in the blood and in the tissue. Usually hypoxia can have three degree: mild, moderate and severe, but this classification is still under debate. In any case, the most part of studies consider a hypoxic condition when the arterial oxygen tension is lower than  $\sim 50$  mmHg. This degree of hypoxia is considered mild and normally it is equivalent to a pressure of 10% normobaric oxygen, or an altitude of  $\sim 5000$  m. Moderate hypoxia is considered when the arterial oxygen tension is between  $\sim 35$  and  $\sim 50$  mmHg (Xu and Lamanna, 2006).

The brain is particularly sensitive to the effects of hypoxia and the decrease of oxygen viability is related to several cognitive pathologies and neurodegenerative diseases, including Alzheimer disease (de la Torre and Stefano, 2000; Kolinko et al., 2015), showing the interconnection between the vessels (structures through which the oxygen arrives to neurons) and functional neuronal activity.

The modulation of CBF is mainly dependent to the oxygen level in the neuronal environment: thus the decrease of oxygen, hypoxia, induces an increase of extracellular lactate and of adenosine, resulting in vasodilation (Lee et al., 2011).

When the partial oxygen pressure ( $\text{PO}_2$ ) falls below  $\sim 50$  mmHg, the CBF increases (Masamoto and Tanishita, 2009). The hypoxic condition on animals can lead to different effects (see a summary of hypoxic effects Fig. 38) depending on the level and the duration of hypoxia.

(A) Major systemic adaptations to prolonged mild hypoxia

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1. Increased ventilation
  2. Core temperature fall
  3. Right shift of hemoglobin dissociation curve
  4. Bicarbonate ion secretion
  5. Increased packed red cell volume (hematocrit)
  6. Body mass loss
- 

(B) Acute effects of hypoxia on CNS

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1. Hemoglobin disoxygenation
  2. Decreased tissue  $P_{O_2}$  (left shift of histogram)
  3. Increased cerebral blood volume (vasodilation)
  4. Faster capillary mean transit time
  5. Increased cerebral blood flow
  6. Increased cerebral metabolic rate for glucose (CMRglu)
  7. EEG abnormalities
- 

(C) Major CNS adaptations to prolonged mild hypoxia

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1. Transient CBF increase
  2. Vascular remodeling
  3. Increased glucose transport across the blood–brain barrier and CMRglu
  4. Increased glycolysis to maintain tissue acid–base balance
  5. Decreased mitochondrial energy consumption
  6. Restoration of tissue oxygen tension profiles
- 

**Figure 38:** In vivo effects of hypoxia

In normoxic condition, the mammalian nervous system is maintained in a low-oxygen medium and the oxygen supply is governed by the “just sufficient” principle (LaManna et al., 2004). When the metabolic demand of neurons does not match with the  $O_2$  delivery, the acute hypoxia (normally considered lasting few minutes up to hours (Xu and Lamanna, 2006)) induces an increase in CBF via direct effects on vascular cells of cerebral arteries and arterioles. As a matter of fact, the drop of ATP induced by hypoxia opens ATP-sensitive potassium channel (KATP) on smooth muscle, causing hyperpolarization and vasodilation (Taguchi et al., 1994). Additionally, hypoxia rapidly increases locally NO and adenosine production, also promoting vasodilation (Golanov, 1997).

This mechanism is able to maintain the organ-specific and local adaptations altering the vascular reactivity and acting on metabolic activity of the tissue (LaManna et al., 2004).

Differently, a plethora of studies suggested that chronic systemic hypoxia (usually lasting weeks and months (Xu and Lamanna, 2006)) increases cerebral blood flow through different mechanisms (for others mechanisms of cerebral adaptation to mild chronic hypoxia see (Xu and Lamanna, 2006)); actually the increase of CBF is poorly understood and seems related to neurogenic component originating from the brain stem and NO regulation, but in any case the hyperventilation induced by hypoxia reduces the arterial  $CO_2$  pressure, by opposing the cerebral vasodilation. This limits the increase of blood flow.

Moreover, another limit is represented by the transport of oxygen from brain parenchyma to mitochondria, since is driven by the diffusion force (from capillary to tissue) which it is reduced because the arterial  $O_2$  pressure is still low in hypoxic condition it represents a limit (Xu and Lamanna, 2006).

But then, how is the brain able to assure neuronal functionality?



Hopefully our brain comes up with others mechanisms to support neuronal metabolic request: in fact among these mechanisms it has been shown an increase of capillary density, using different percentages of O<sub>2</sub> and duration of hypoxic condition (Boero et al., 1999; Dunn et al., 2004; Xu and LaManna, 2006). The increase of vascular density results in a decrease of both intercapillaries distance and diffusion distance (LaManna et al., 2004).

Among these studies, LaManna and collaborators showed that after three weeks of hypobaric hypoxia, in rat brains, the capillary density almost doubles and the average intercapillary distance decreases from 50 to 40 µm (LaManna et al., 1992; Lauro and LaManna, 1997), as a result of angiogenesis.

A recent study analysing the spatiotemporal dynamic of vascular network during chronic hypoxia, using in vivo two-photon microscopy on mice, reported that after 1-2 weeks of continuous hypoxia (8-9% O<sub>2</sub>) new sprouting vessels were detectable in the mouse cortex, especially in the cortical depth of 100-200 µm, indicating a spatial uniformity in this process among cortical layers (Masamoto et al., 2013).

Moreover, different kinds of cerebral vessels can be involved in hypoxia-induced angiogenesis: Boroujerdi and collaborators showed that mice exposed to mild hypoxia (8% of oxygen) for periods of 4, 7, and 14 days displayed the biggest increase in the number of medium to large size vessels, with some slight reduction in the number of small vessels, consistent with the notion that hypoxia triggers active remodelling of small vessels into larger ones (Boroujerdi et al., 2012).

Additionally, cultured brain-derived microvascular endothelial cells under hypoxic conditions (1% O<sub>2</sub>), isolated from rat brain microvessels, express angiogenic factors; in particular, hypoxia induced a rapid (less than 0.5 h) expression of hypoxia-inducible factor 1α (HIF-1α), without effect on cell viability. Furthermore, within 0.5 to 2 h of hypoxia induction the levels of vascular endothelial growth factor (VEGF) and endothelin-1 mRNAs and proteins were increased. In contrast, in the same study, hypoxic exposure of brain cultured ECs resulted in a significant decrease in tube length compared to control cultures. Thus, the pro-angiogenic switch seems not linear and more complex, suggesting that the expression of angiogenic factors is not sufficient for the development of new vessels, in these conditions (Luo et al., 2012).

Others angiogenic factors can be overexpressed during hypoxia, such as Angiopoietin 2 (Ang-2). Pichiule and collaborators showed, in the rat cerebral cortex, that the capillary density increased by 60% after 3 weeks of hypoxia; moreover, the capillary network progressively decreased to prehypoxic values after 3 week of normoxic recovery (deadaptation). Furthermore, Ang-2 expression in ECs was induced after 6 h till 14 days of hypoxia, falling down to control levels after 21 days of hypoxia. The expression of Angiopoietin 1 (molecule inducing vessels stabilization) and Tie-2 receptor (receptor of Angiopoietin 1 and 2) was not affected (Pichiule and LaManna, 2002).

These mechanisms matching the capillary density to O<sub>2</sub> tissue levels are not just induced by systemic hypoxia achieved by hypoxic chamber, but they are elicited during neuronal activity-induced hypoxia. In fact, they maintain the balance between oxygen availability and neuronal metabolic request when neuronal activity increases, such as cognitive functions and physical exercise.

For example, Black and colleagues showed that rats living in an enrich environment or with different conspecifics displayed an increase in capillary density compared to control rats; this increase was ascribed to an angiogenic mechanism triggered to compensate the metabolic request of the neurons (Black et al., 1987). It was moreover showed that increase of cortical dendritic density was detected in the same conditions, indicating that angiogenesis in an adult brain can occur in association with experience-induced increases in neuropil volume.

The increase of vascular density in association to a higher neuronal demand was shown after 1 month of motor training in adult rats (Isaacs et al., 1992). Another study performed on rats showed that during anaerobic physical activity, oxygen reduction leading to hypoxia affects brain cell metabolisms and induces the expression of HIF-1 $\alpha$  as well as VEGF, especially after 1 week of exercise, compared to 3 weeks (Flora, 2016).

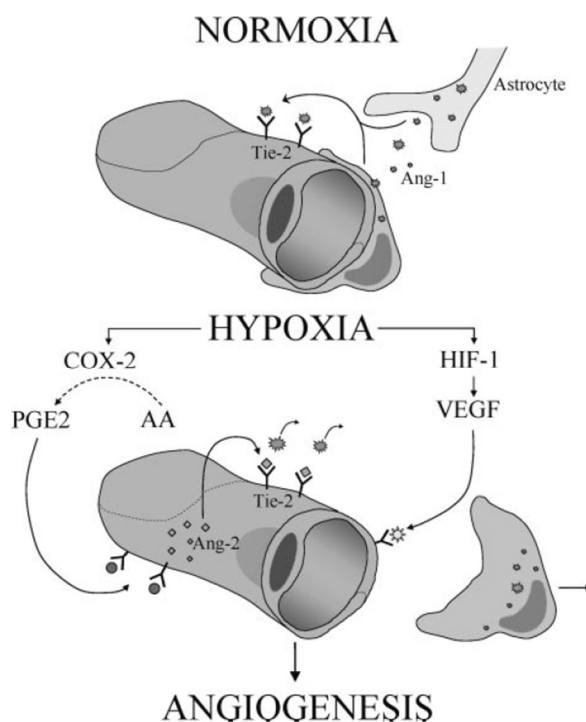
Hypoxic induced angiogenesis is moreover involved in tumour development and its dysregulation in cognitive pathologies, topic that we are not going to develop; for review (Krock et al., 2011).

In any case, among all the molecular mechanisms of hypoxic adaptation the most important is the one mediated by the induction of Hif-1 $\alpha$  and the interaction with angiogenic molecules in particular VEGF and angiopoietins (in particular the balance between Ang-1 and Ang-2).

In the following part we are going to briefly describe this pathway.

### 3.3.2.1. Hypoxia and angiogenesis triggering

Angiogenesis is the formation of new vessels from pre-existing vascular network. Angiogenesis, which will be successively described in this chapter, occurs mainly through the Hif-1 $\alpha$  activation and the related pathway; moreover, angiogenesis can be triggered by COX-2/Ang-2 dependent mechanism (Fig. 39), more detailed in the part regarding Hypoxia and angiopoietins: COX-2/Ang-2.

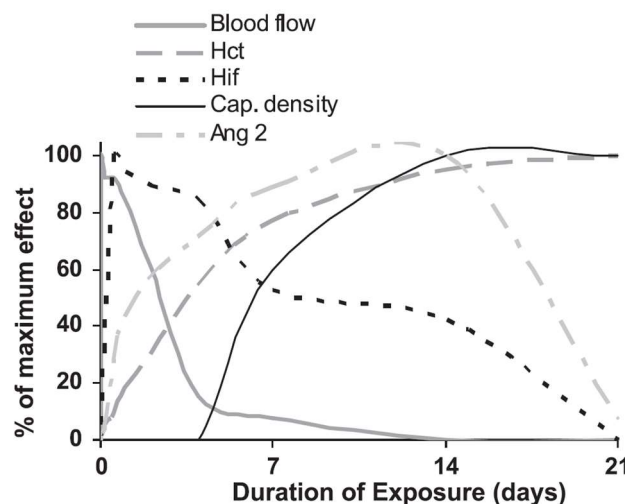


**Figure 39:** Mechanisms implicated in hypoxia-induced angiogenesis. In the normoxic situation, angiopoietin-1 (Ang-1) acting through the Tie-2 receptor on cerebral endothelial cells maintains structural integrity. During hypoxia, cyclooxygenase-2 (COX-2) is activated and arachidonic acid (AA) is converted to prostaglandin E2 (PGE2). PGE2 induces release of angiopoietin-2 (Ang-2) from endothelial cells, which occupies the Tie-2 receptor resulting in structural instability and falling away of the pericyte. This allows access of hypoxia-inducible factor-1 (HIF-1) upregulated VEGF to its receptors, stimulating the hypertrophy and hyperplasia of capillary angiogenesis (Xu and Lamanna, 2006).

The time course of the CBF response and of the increase of vascular network induced by hypoxia is summarized in the Fig. 40; these data come from different studies of LaManna's group.

Rats placed in hypobaric chambers from 1 to 21 days at a constant pressure 0.5 atm (equivalent to 10% normobaric oxygen) showed a rapid increase of CBF during the first days, falling down after 5 days (Xu et al., 2004). Moreover, after 3 weeks, the Ang-2 expression is upregulated in cortical ECs during the first two weeks, coming back to the basal level during the third week (Pichiule and LaManna, 2002). The increase of vascular network, the outcome of angiogenic process, is detectable already during the first week and is maintained during all the hypoxic condition. During deadaptation the vascular network decreases.

Moreover, rats undergoing to the same protocols showed a fast up-regulation of cortical Hif-1 $\alpha$  which halves after 4 days, coming to basal condition after 3 weeks.



**Figure 40:** Time courses of cerebral blood flow, Hif-1 $\alpha$ , capillary density, packed red cell volume (Hct), and angiopoietin-2 (Ang-2) in response to chronic mild hypoxic exposure. From (Xu and Lamanna, 2006).

These results show the time course of CBF and angiogenic process in systemic mild chronic hypoxia but during the neuronal activity induced by a memory task this kinetics may change.

### 3.3.2.2. Hif-1 $\alpha$

#### *Regulation of Hif- $\alpha$ concentration and activity*

Hif-1 $\alpha$  is one of the major regulators of oxygen homeostasis. This protein belongs to a family of proteins with basic helix loop helix-Per/ARNT/Sim structure (bHLH-PAS). It is formed by three O<sub>2</sub>-regulated  $\alpha$  subunits, responsible to 3 different isoforms, namely Hif-1 $\alpha$ , Hif-2 $\alpha$  and Hif-3 $\alpha$ , and a constitutively expressed  $\beta$ -subunit of Aryl hydrocarbon nuclear translocator family, including Arnt, Arnt2 and Arnt3. The  $\alpha$ -subunits are structurally homologous, and they are constituted by an N-terminal bHLH domain, which is responsible of DNA binding and specificity. The HLH and PAS domains are engaged in heterodimerization with the transcriptional partner Arnt (Krock et al., 2011).

Despite Hif-1 $\alpha$  and Hif-2 $\alpha$  are highly structurally similar, sharing many transcriptional targets binding the same DNA sequences, they have divergent functions thanks to unique patterns of expression and transcriptional activity. In fact while Hif-1 $\alpha$  is ubiquitously expressed, Hif-2 $\alpha$  expression is restricted to endothelial cells, type II pneumocytes of the lung, cardiomyocytes, macrophages, astrocytes, and the organ of Zukerlandl.



Hif-1 $\alpha$  or Hif-2 $\alpha$ -knockouts are lethal for the embryos, showing that each gene has unique functions. It seems that Hif-2 $\alpha$  promotes vessel maturation likely due to Hif-2 $\alpha$ -mediated expression of genes involved in adhesion (integrins  $\alpha$ 9 and  $\beta$ 2) and basement membrane formation (fibronectin) (Krock et al., 2011). In contrast, Hif-3 $\alpha$  might act as an antagonist of the Hif system.

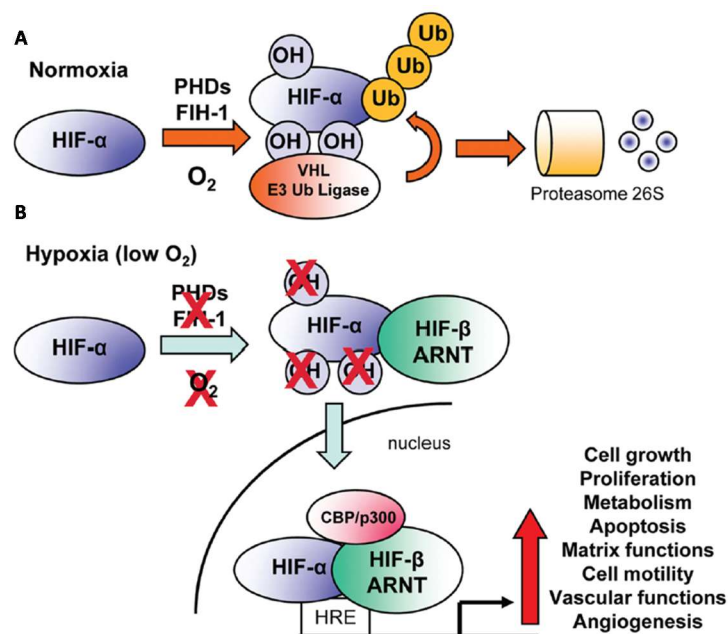
The  $\beta$ -subunit does not change based on O<sub>2</sub> level, but it is important in the hypoxia-induced transcriptional changes mediated by Hif heterodimer. Also Arnts family members are all structurally homologous, but just Arnt is the primary HIF- $\beta$  involved in the hypoxic response (Fong, 2008; Jain et al., 1998).

In normoxic environment, Hif-1 $\alpha$  is continuously produced but rapidly degraded. The activity of this protein is regulated by different enzymes (Sharp and Bernaudin, 2004).

One of the most important is the prolyl hydroxylase enzymes (PHD1-3), included in the Fe(II) and 2-oxoglutarate-dependent dioxygenase family. In normoxia conditions, both at nuclear and cytoplasmic level (Metzen et al., 2003), PHD enzymes, together with substrate O<sub>2</sub> and cofactor 2-oxoglutarate, hydroxylate Hif- $\alpha$  in on two conserved proline residues located within the Hif- $\alpha$  O<sub>2</sub>-dependent degradation domain (ODDD). The proline hydroxylation changes Hif- $\alpha$  conformation allowing the binding to the Von Hippel–Lindau protein (VHL) and others molecules (including elongin B and C, Cullin 2 and RBX1), acting as E3 ubiquitin ligase complex that ubiquitinates Hif- $\alpha$ , targeting it for proteasomal degradation (Fig. 41A).

Under hypoxic condition, the O<sub>2</sub> and 2-oxoglutarate are limited, diminishing the Hif- $\alpha$  hydroxylation and resulting in Hif- $\alpha$  accumulation and translocation into the nucleus. Once in the nucleus, Arnt subunit create a transcriptional complex with coactivator proteins (CBP and p300), hypoxia response elements (HREs), promoters and enhancers of target genes required in hypoxic response (Fig. 41B)

Interestingly, PHD enzymes are induced by hypoxia, suggesting a feedback mechanism avoiding the excessive Hif- $\alpha$  accumulation (Metzen et al., 2003).



**Figure 41:** Regulation of hypoxia-inducible factor (HIF) activity. Under normoxic (A) and hypoxic (B) conditions, detailed in the text. Abbreviations: OH: hydroxylation; Ub: ubiquitin; PHDs: prolyl hydroxylases; ODDD: oxygen-dependent degradation domain; HREs: hypoxia response elements; FIH-1: factor inhibiting Hif-1.(Krock et al., 2011).

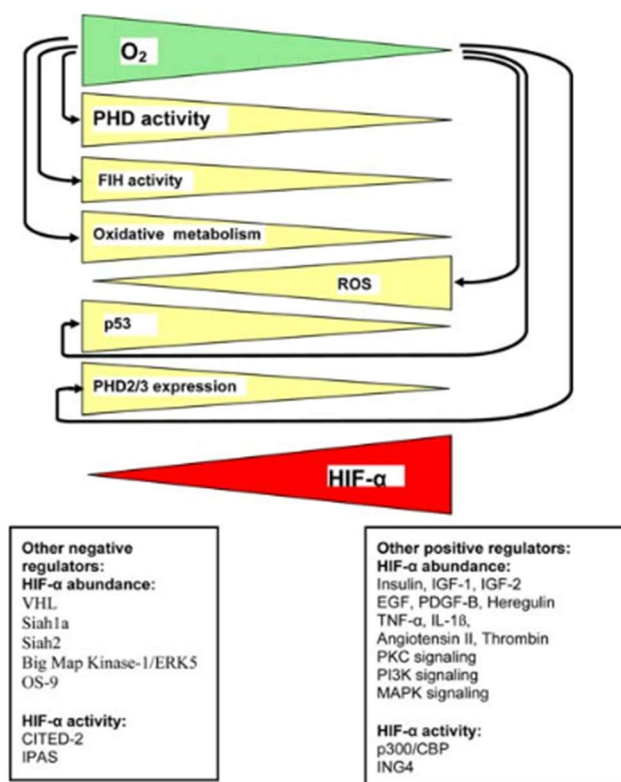
The ferrous ions, necessary for the link with PHD enzymes and for Hif- $\alpha$  degradation, can be sequestered by heavy metal ions such as cobalt (Jaakkola et al., 2001), inhibiting the degradation of Hif- $\alpha$ . Moreover, cobalt is able to stabilise Hif- $\alpha$  binding directly ODDD inhibiting both hydroxylation and the interaction between hydroxylate Hif- $\alpha$  and VHL (Yuan et al., 2003).

Hif- $\alpha$  activity can be mainly controlled by several post-translational modifications, such as ARD1 protein which promotes proteasome degradation of Hif- $\alpha$  by increasing the interaction with VHL though acetylation of lysine 532; moreover, factor-inhibiting Hif-1 $\alpha$  (FIH-1) can hydroxylate a conserved asparaginyl residue of HIF- $\alpha$  inhibiting the binding with the coactivator proteins (for a review(Sharp and Bernaudin, 2004)).

Hif- $\alpha$  is also modified by acetylation, phosphorylation, sumoylation, and s-nitrosylation. The aim of this paragraph is not to explore the post-transcriptional regulation of Hif-1 $\alpha$  in any case interesting review can fill this interesting part (Fandrey et al., 2006; Kietzmann et al., 2016; Krock et al., 2011; Lee et al., 2004c; Sharp and Bernaudin, 2004).

Even if Hif- $\alpha$  can be controlled by post-transcriptional mechanisms, Hif- $\alpha$  mRNA are influenced by oxygen (Sharp and Bernaudin, 2004); moreover Hif- $\alpha$  expression depends on others mechanisms oxygen independent, that we are not going to explore, despite are reviewed in (Fong, 2008).

In any case, even if O<sub>2</sub> is the most important regulator of Hif- $\alpha$  activity, others factors can influence Hif- $\alpha$  stability and functionality (Fig. 42).



**Figure 42:** The principal regulatory mechanisms of Hif-1 $\alpha$  expression and activity. The wider end of the triangles represents high concentration or activity, whereas the sharp end stands for the opposite. From (Fong, 2008).

Among these, the Ang-2 is able to increase Hif- $\alpha$  activity via the production of reactive oxygen species (ROS), which, decreasing the Fe<sup>2+</sup> availability and thus the PHD and FIH functionality, induce Hif accumulation (Cash et al., 2007).

As previously mentioned, Hif-1 $\alpha$  is produced continuously and accumulates in cells under hypoxia, while in normoxic condition is rapidly degraded (Sharp and Bernaudin, 2004) but the kinetics of Hif-1 $\alpha$  turnover depends from different conditions; an *in vitro* study (Chamboredon et al., 2011) reported that SV-40-immortalized human microvascular endothelial cells, exposed to hypoxia (1.5% O<sub>2</sub>) for 3–24 h, showed a rapid and transient increase of Hif-1 $\alpha$  protein levels, peaking at 3 h, compared to normoxic cells (19 % O<sub>2</sub>) where Hif-1 $\alpha$  was almost undetectable. During the hypoxic condition, a progressive decrease of Hif-1 $\alpha$  levels was reported even if it remained higher than basal levels at 24 h. Furthermore, hypoxia did not change Hif-1 $\alpha$  mRNA levels during the first 3 h, suggesting that hypoxia-induced increases in Hif-1 $\alpha$  protein are likely due to translational or posttranslational changes. By contrast, prolonged hypoxia progressively decreased Hif-1 $\alpha$  mRNA, diminishing till almost the 50% of the initial value at 24 h.

Another study (Stroka et al., 2001) performed of female mice underwent to hypoxic chamber (1-12 hours at 6% O<sub>2</sub>) showed that the basal normoxic expression (besides detectable) of Hif-1 $\alpha$  in brain, kidney, liver, heart, and skeletal muscle was further increased in response to systemic hypoxia but the kinetics of Hif-1 $\alpha$  expression varied among different organs. In the brain, however, Hif-1 $\alpha$  expression was proportional to the level of hypoxia; moreover, at 6% O<sub>2</sub> Hif-1 $\alpha$  reached the peak of expression at 5 h, coming back to basal levels after 12 h. Hif-1 $\alpha$  staining appeared in the neurons of the cerebral cortex and granular layer of the dentate gyrus and the hippocampus but contrarily to what reported in other studies (Chamboredon et al., 2011; Chavez et al., 2000), throughout the brain, ECs were negative. This result seemed due to undetectable levels of the protein, as a consequence of cell size regulation (Schmidt and Schibler, 1995). However, different studies reported that Hif-1 $\alpha$  is induced in hypoxic neurons, astrocytes, ependymal cells and ECs (Chamboredon et al., 2011; Chavez et al., 2000).

#### *Hif- $\alpha$ regulated angiogenic molecules and responses of vascular cells to hypoxia*

Hif-1 $\alpha$  regulates a broad array of gene in response of O<sub>2</sub> decrease (Fig. 43) which promotes both short term and long term adaptation. Despite this plethora of modification, Hif-1 $\alpha$  pathway has the common characteristic to facilitate the acclimation to low oxygen condition through vasomotor control, angiogenesis, erythropoiesis, iron metabolism, cell proliferation and cell cycle control, cell death and energy metabolism.

Among the short term adaptation Hif-1 $\alpha$  induce the upregulation of iNOS, allowing the production of NO and the consequent relaxation of vascular network, at least in cardiomyocytes (Jung et al., 2000).

Role	Gene product
Vasomotor control	Adrenomedullin (AM) Nitric oxide synthase 2 (NOS2) $\alpha$ 1B adrenergic receptor Endothelin 1 (ET1)
Angiogenesis	Vascular endothelial growth factor (VEGF) FLT1 (VEGF receptor 1, VEGFR1)
Erythropoiesis	Erythropoietin (EPO)
Iron metabolism	Transferrin Transferrin receptor Ceruloplasmin
Cell proliferation/ Cell cycle control	p21 (WAF1/CIP1) Insulin-like growth factor 2 (IGF2) IGFBP1, IGFBP2, IGFBP3
Cell death	NIP3, NIX
Energy metabolism	Glucose transporters 1 and 3 (GLUT1, GLUT3) Prolyl-4-hydroxylase $\alpha$ 1 Phosphofructokinase L Lactate dehydrogenase A Aldolases A and C Pyruvate kinase M Enolase 1 Hexokinases 1 and 2 Glyceraldehyde phosphate dehydrogenase (GAPDH)
Miscellaneous	Plasminogen activator inhibitor (PAI1) Transforming growth factor $\beta$ 3 (TGF $\beta$ 3) p35srj Haem oxygenase 1 Adenylate kinase 3 Phosphoglycerate kinase 1 Carbonic anhydrase 9 RTP801

**Figure 43:** Principal Hif-1 $\alpha$  target genes modifying several biological systems, among these the angiogenic pathway. From (Sharp and Bernaudin, 2004).

The long term adaptation induced by Hif-1 $\alpha$  is achieved via angiogenesis stimulation, though the regulation of proangiogenic genes, responsible to making Hif1- $\alpha$  the “master regulator of angiogenesis” (Krock et al., 2011), increasing vascular permeability, ECs proliferation, sprouting migration, adhesion and tube formation.

Hif-1 $\alpha$  induces the transcription of target genes binding the HREs, which presence has been reported concomitant to angiogenic molecules such as VEGF-A (Forsythe et al., 1996), VEGFR-1 (Marti and Risau, 1998), eNOS (Yu et al., 2005). In contrast, others angiogenic molecules, even if are not known to have HREs, are up-regulated by hypoxia or Hif-1 $\alpha$  overexpression, including genes encoding fibroblast growth factor (FGF) 2, platelet-derived growth factor (PDGF)-B, placental growth factor (PLGF), Ang-1 and -2, and angiopoietin receptor Tie-2 (Fong, 2008; Kelly et al., 2003).

Moreover, several studies demonstrated that the simple ectopic stimulation of Hif-1 $\alpha$  was enough to induce angiogenesis without others factors, for a review (Fong, 2008).

Briefly, but more developed in the next paragraph, ECs can create new vessels by sprouting angiogenesis but, since the ECs are physiologically quiescent, they require a signal to commence the angiogenesis; this signal is mainly represented by VEGF, a stimulating cytokine, allowing the detachment of ECs from the parent vessel and migrates into the neighbouring stroma. VEGF

expression is mainly regulated by hypoxia and moreover it is a direct transcriptional target of both Hif-1 $\alpha$  and Hif-2 $\alpha$ .

Moreover Hif-1 $\alpha$  participates to upregulation of matrix metalloproteinases, in particular MMP-2 (direct transcriptional target of Hif-1 $\alpha$ ), inducing degradation of the ECM.

Furthermore it regulates vessel branching through modulation of Notch signalling, an actor modulating the role of ECs on vascular remodelling (see next paragraph): Notch signalling pathway is complex but it seems to control ECs functions, allowing the selection of the EC which will guide the development of the new vessel and limiting the neighbouring ECs angiogenic response, in part by reducing the level of vascular endothelial growth factor receptors in endothelial cells (Fouillade et al., 2012; Phng and Gerhardt, 2009; Siekmann and Lawson, 2007).

It is notable, just to mention, hypoxia regulates the balance between ECs proliferation and degradation. In fact, hypoxia may also reduce ECs proliferation and cause apoptosis (through NF $\kappa$ B and Bcl-2 interaction, even if the mechanism is not fully understood (Matsushita et al., 2000)). Thus, the hypoxia outcome in ECs regulation can depend on the severity of hypoxia (Fong, 2008). In any case, as previously discussed, hypoxia through Hif-1 $\alpha$  effectively regulates several steps of angiogenic process (Fig. 44).

Pro-Angiogenic	Anti-Angiogenic
VEGF, Flt-1 (VEGF-R1), Kdr (VEGF-R2)	DLL1-4
Angiopoietin-1/2 (Ang-1/2), Tie-2	Vasohibin-1
ADM	Thrombospondin-1
FGF	Carbonic anhydrase-9 (CA-9)
PLGF	Regulator of G-protein signaling 5
PDGF-B	Angiostatin
SCF	Endostatin
Osteopontin	Canstatin
PAI-1	Interferons (IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ )
MMP, TIMP	
NOS	
COX-2	
Endoglin	
1B-adrenergic receptor	
Endothelin-1	
Semaphorin 4D	
Integrins, leptin	
Endosialin	
Adenosine A2A receptor	
Oxygen-regulated protein-150	
SDF-1	
Interleukins (IL-1, IL-2, IL-4, IL-6, IL-8, IL-10)	

**Figure 44:** Pro- and Anti-Angiogenic factors produced by the hypoxia-induced Hif-1 $\alpha$  expression Induced by Hypoxia/Hypoxia-Inducible Factors: ADM: adrenomedullin; Ang-1/2 : angiopoietin-1/2; COX-2 : cyclo-oxygenase 2; DLL : delta-like ligand; FGF : fibroblast growth factor; Flt-1 : fms-related tyrosine kinase 1; Kdr : kinase insert domain containing receptor; MMP : matrix metalloproteinases; NOS : nitric oxide synthases; PAI-1 : plasminogen activator inhibitor-1; PLGF : placenta growth factor; PDGF-B : platelet derived growth factor beta; SCF : stem cell factor; SDF-1 : stromal-derived growth factor; Tie-2 : TEK tyrosine kinase endothelial; TIMP : tissue inhibitor of metalloproteinases; VEGF : vascular endothelial growth factor; VEGF-R : VEGF receptor. (Krock et al., 2011).

Finally, hypoxia also activates pathways that do not depend on Hif-1, modulating the levels of neuroglobin and Ang-2. Ang-2 is upregulated by hypoxia in a non-Hif-1-dependent manner through upregulation of cyclooxygenase 2 (COX-2) (Pichiule et al., 2004), participating in the brain capillary

remodelling that occurs in the chronic hypoxic adaptation model (Pichiule and LaManna, 2002), more detailed in the part “Hypoxia and angiopoietins: COX-2/Ang-2”.

The aim of this paragraph is not to explore all the mechanisms through which hypoxia can induce angiogenesis but to introduce another fundamental molecule, induced by hypoxia playing an important role in angiogenesis: Ang-2; this protein represents our selective target to explore the dynamics of vascular plasticity during the consolidation of the olfactory associative memory.

In the following paragraph we are going to describe the mechanism through which Ang-2 is induced, introducing the concept of angiogenic process and the role played by the angiopoietins.

### **3.4. Angiogenesis**

The vascular system assures a correct and balanced distribution of blood throughout the body: insufficient blood supply can cause tissue ischemia in cardiovascular and other diseases, whereas in cancer the formation of new blood vessels offers to the expanding tumours an adequate access to nutrients and oxygen. Hence, the possibility to control the growth of blood vessels provides significant therapeutic opportunities (Fagiani and Christofori, 2013).

We can identify several types of vessels formation during physiological and pathological conditions; the three main cellular processes involved in vasculature formation are vasculogenesis, angiogenesis and arteriogenesis (Carmeliet and Jain, 2011).

During embryogenesis, the angioblasts differentiate into ECs, which create *de novo* vascular network; this process is known as vasculogenesis. Then, different signals regulate arterial and venous differentiation. The subsequent sprouting, starting from pre-existing network, ensures the expansion of vascular network, and this process is known as angiogenesis. The vessels increase their diameter, acquiring stability and control of perfusion, being covered by pericytes or vascular SMCs, through the process of arteriogenesis (Carmeliet and Jain, 2011).

In this thesis we focalize on angiogenesis, (from the Greek word Angêion, meaning vessel) the formation of blood vessels from existing vasculature (Adair and Montani, 2010).

The Scottish anatomist John Hunter was the first who provides the first insight into angiogenesis field which wrote “In short, whenever Nature has considerable operations going on, and those are rapid, then we find the vascular system in a proportional degree enlarged”, underlining the link between vascular and metabolic dynamics (Adair and Montani, 2010; Hunter, 1840). An important trigger for the modern knowledge of angiogenesis began with Judah Folkman, considered by many to be the father of the modern view of angiogenesis, who discovered that tumour growth is angiogenesis-dependent (Folkman, 1971).

Nowadays, we know that hypoxic-induced growth factors and the chemokines induce the ECs to change from their stable position in the vessel wall to a new network formation, until they are able to supply to the neuronal demand; after that they can come back to a quiescence state (Phng and Gerhardt, 2009).

It was extensively documented that the capillary growth (capillary length density) is proportional to the metabolic activity (mitochondrial volume density, which is considered the structural measure of the oxidative capacity of a tissue) in several tissues such as the heart, skeletal muscle and brain (Adair et al., 1990; Adair and Montani, 2010; Hoppeler et al., 1981; Krogh, 1919). Moreover it was shown, in different studies of endurance exercise training, that the increased of metabolic activity can promote

angiogenesis whereas its decrease, like in case of disuse atrophy and tenotomy in skeletal muscle, causes capillary rarefaction (also called capillary dropout, for a review see (Adair and Montani, 2010).

In adults, the vascular network rarely forms new branches but the ECs retain a high plasticity to sense and to respond to angiogenic signals. Changes in metabolic activity lead to proportional changes in angiogenesis and, hence, proportional changes in capillarity. In fact it is reported the existence of cerebral neurogenic niches which coexist with angiogenic areas, since they share common molecular pathways (Madri, 2009; Palmer et al., 2000; Yamashima et al., 2004).

Abnormal angiogenesis is also involved in pathologies such as tumour growth, metastasis, diabetic retinopathy and arthritis (Munoz-Chapuli et al., 2004), becoming a process of high interest; despite these pathways are deeply described, in this thesis we analyse the physiological process of angiogenesis, which has been extensively studied due to their therapeutic potential and their clinical implications.

As previously mentioned, oxygen plays a pivotal role in this regulation and tissue hypoxia is one of the main signals triggering an angiogenic response. Hemodynamic factors are critical for survival of vascular networks and for structural adaptations of vessel walls.

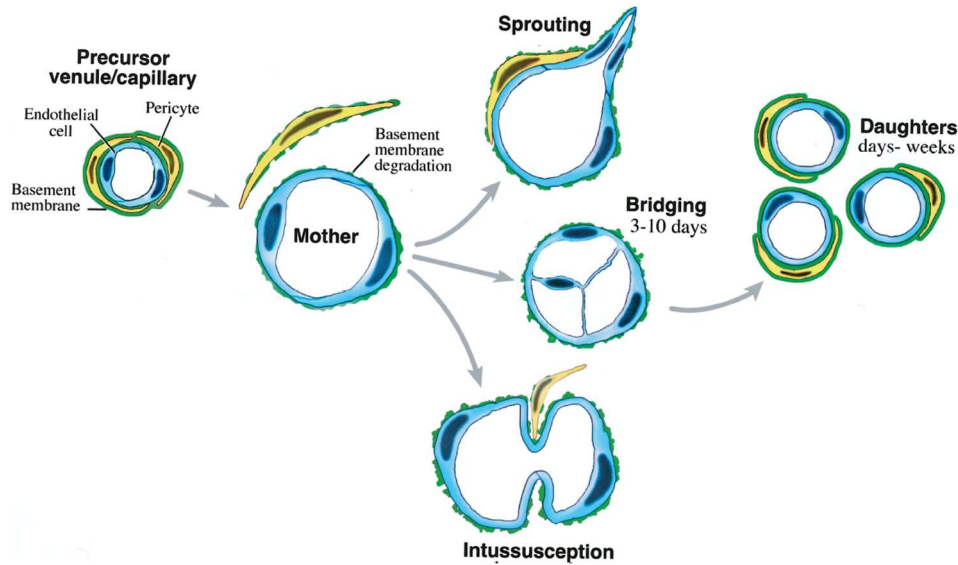
The angiogenesis can occur via different ways (Fig. 45), such as sprouting, bridging or intussusception, as reported in a study investigating the angiogenic process in adult mice and rats induced by VEGF injection in different tissues (such as skeletal muscle, myocardium, skin and peritoneal cavity) (Pettersson et al., 2000). These angiogenic forms are presented in tumour angiogenesis as well (Carmeliet and Jain, 2011).

In physiological angiogenesis, the bridging (projection of EC cytoplasmic processes into and across mother vessel lumens, creating a bridges within the lumen) and the intussusception (invaginations of connective tissue causing the slitting of the original structure into smaller branched structures, also called splitting angiogenesis) induce the vessel proliferation with low cellular proliferation, acting via ECM remodelling. These phenomena are thought to be faster and efficient compared with sprouting angiogenesis (Adair and Montani, 2010) since, initially, it only requires reorganization of existing ECs without necessity of immediate ECs proliferation and migration. This process is poorly understood due to the “recent” discover compared to the sprouting angiogenesis (Caduff et al., 1986) and to the difficulty of imaging method and scanning.

Conversely, sprouting angiogenesis is considered the main angiogenic process, creating new capillaries that are vulnerable if compared to the ones deriving from the others angiogenic process. These capillaries become, inter alia, the target for tumour angiogenesis.

In this thesis, thus we consider the sprouting angiogenesis, which involves an increase of ECs proliferation.





**Figure 45:** Different types of angiogenesis proposed in *in vivo* models. Modified from (Pettersson et al., 2000).

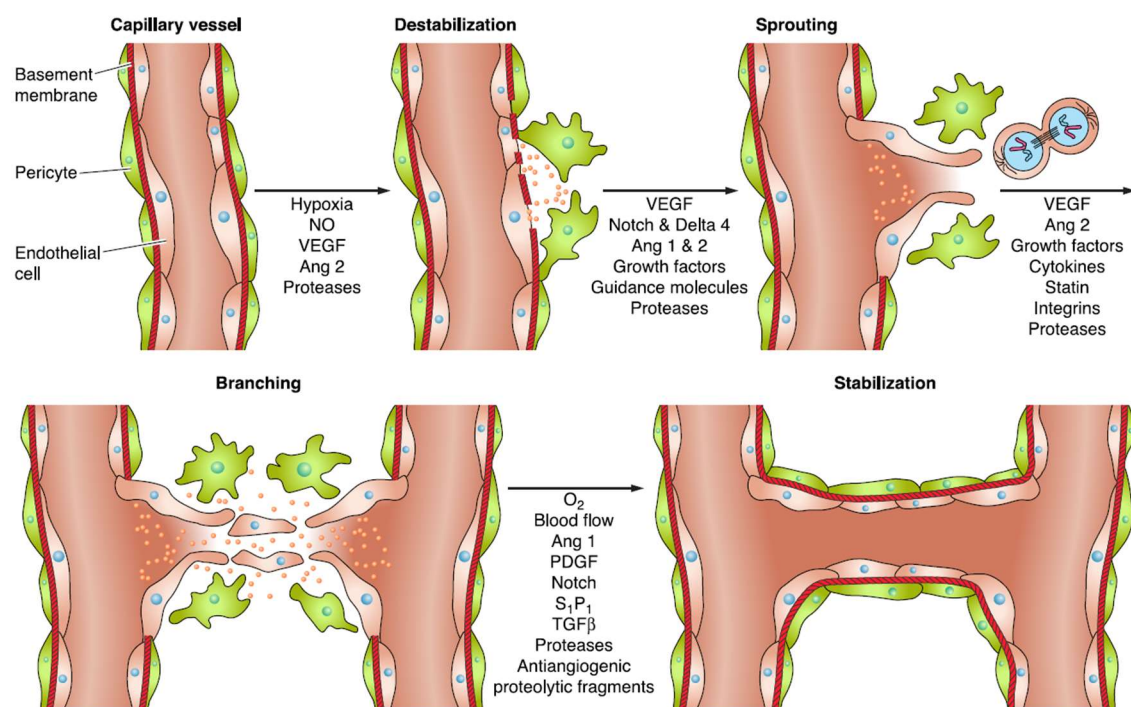
Before discussing the molecular players involved in this process we briefly describe the sequential steps of vessels branching and lumen formation.

In healthy adults, ECs possess long half-life and are protected against insults by autocrine signals (VEGF, Notch, Ang-1, FGFs). The function of vascular network is to distribute oxygen to the tissues, thus the ECs possess oxygen sensor such as hypoxia inducible factors that assure the re-adjustment of vessels shape in order to optimize blood flow.

Without stimuli, ECs are quiescent (phalanx cells, so called since their “cobblestone” aspect resembles the phalanx formation of ancient Greek soldiers (Mazzone et al., 2009)), forming an aliened monolayer interconnected by junction molecules like VE-cadherin and claudins. They are stabilized by pericytes, which inhibit ECs proliferation and release cell-survival signals such VEGF and Ang-1. As a matter of fact, only a 0.01% of all the endothelial cells of a normal adult are dividing at any given moment (Hobson and Denekamp, 1984). Moreover, labelling studies suggest that the ECs turnover takes from months up to 5 years and more (Foreman and Tang, 2003; Hobson and Denekamp, 1984; Woywodt et al., 2002); this ECs turnover rate increases during vascular trauma, angiogenesis, reaching the turnover rate of the bone marrow cells (Folkman, 1993).

When a quiescent vessel detects an angiogenic signal (such as VEGF, Ang-2, FGFs or chemokines, released by a hypoxic, inflammatory or tumour cell), triggering the angiogenic switch (Ribatti et al., 2007), the activation of the angiogenic cascade leads to a plethora of successive steps here summarized and reviewed in (Carmeliet and Jain, 2011; Geudens and Gerhardt, 2011) (Fig. 46).





**Figure 46:** Angiogenesis steps. Briefly, as described in the text hypoxia induces the production of NO and the expression of VEGF and Ang-1 and Ang-2, which interact with ECM proteases increasing the permeability of the capillary vessel wall. Destabilization then allows ECs to migrate and proliferate to form tubules, mediated by VEGF, Ang, guidance molecules, growth factors, cytokines, and degradation of the ECM. Maturation of the newly formed vessel is accompanied by increased expression of antiangiogenic factors, many released as a result of proteolysis. From (Clapp et al., 2009).

### *DESTABILIZATION and BRANCHING*

VEGF-A starts the process of vascular destabilization increasing the ECs permeability via phosphorylation of focal adhesion tyrosine kinase (FAK), leading to vascular endothelial Cadherin (VE-Cadherin) and  $\beta$ -catenin dissociation from junctions and an EC junctional breakdown (Chen et al. 2012).

The aim of this phase is to create a destabilizing medium to remodel the vascular wall; to obtain this environment, firstly the pericytes start to detach from the vessel wall (Ang-2 mediated), separating from the basement membrane by proteolytic degradation mediated by MMPs, basally slightly expressed but upregulated during angiogenic process; at cerebral level, the most abundant MMPs are the MMP-2, MMP-3 and MMP-9 (Ethell and Ethell, 2007). MMP-2 and -9 are upregulated during the tumour angiogenic switch but just MMP-9 seems to play a functional role. This molecule, in fact, degrading the ECM, allows the liberation of other angiogenic molecules from ECM. MMP-9 is also involved in angiogenic response following ischemia (Morancho et al., 2013).

The loss of ECs' junctions allows the formation of the nascent vessel; contemporaneously, the induced production of VEGF increases the permeability of ECs and promoting the plasmatic extravasation of proteins.

The plasmatic extravasation and deposition create a temporary ECM scaffold onto which ECs start to migrate in response to integrin signalling. Moreover, at the same time, the proteases induce the release from ECM of angiogenic molecules such as VEGF and FGF, creating and reinforcing the angiogenic environment.

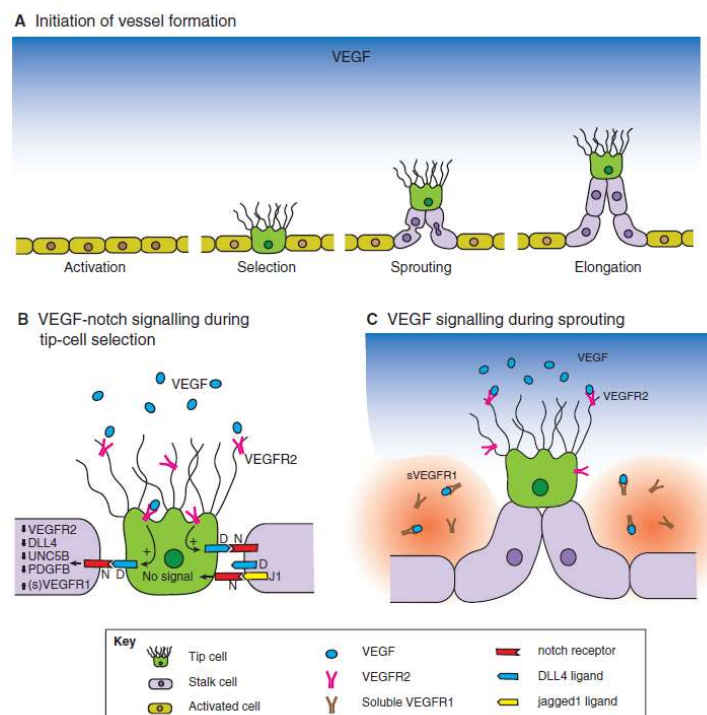
In order to create a functional vessel and to prevent ECs from moving randomly towards the angiogenic signal, only one type of endothelial cell, known as the **tip cell**, is selected to lead the tip in the presence of factors such as VEGF receptors, neuropilins (NRPs) and the Notch ligands Dll4 and JAGGED1, described below (Fig. 47).

Tip cell is followed by proliferating and differentiating cells, loosely covered by pericytes and smooth muscle cell. Conversely, the neighbours of the tip cell (**stalk cells**) assume subsidiary and subordinate positions (Carmeliet and Jain, 2011). It was shown recently that also tip cells can proliferate in some vascular beds in zebrafish (Nicoli et al., 2012).

### *Tip cell selection*

Tip cell and stalk cell are selected by a precise feedback loop between VEGF and Notch (specified in the paragraph “Notch mediated tip cell selection”): tip cells induce notch-mediated lateral inhibition by of stalk cell which leading capacity is prevented. This differentiation limits the number of outgrowing sprouts (Geudens and Gerhardt, 2011).

Briefly, it seems that the future tip cell produces more Dll4 and is less sensitive to Notch activity, being able to “dominate” the neighbour ECs. Thus, the Notch activation in stalk cells induces a VEGFR-2 inhibition, indirectly through Dll4 inhibition, reinforcing the role of the tip cell and reducing the number of others possible tip cells (Fig. 47) (Geudens and Gerhardt, 2011).



**Figure 47:** Tip selection process (Geudens and Gerhardt, 2011).

### *Tip cell branching*

The tip cells' branching is mediated by directional protrusion of filopodia and to the remodelling of cytoskeleton. The dynamic activity of the cytoskeleton depends from the myosin II contractility, which in turn, is determined by the physical and molecular property of ECM. It has been shown that the local downregulation of this protein induces the formation of lamellopodia which start the branching (Fischer et al., 2009). The direction of filopodia extension is in principle determined by the

polarization of the tip cell, dependent by Cdc42, a small GTPase of the Rho family (Etienne-Manneville, 2004), while, successively, is dependent by VEGF distribution (Ruhrberg et al., 2002).

## *MIGRATION and ELONGATION*

### *Tip cell guidance and stalk cell proliferation induce lumen formation*

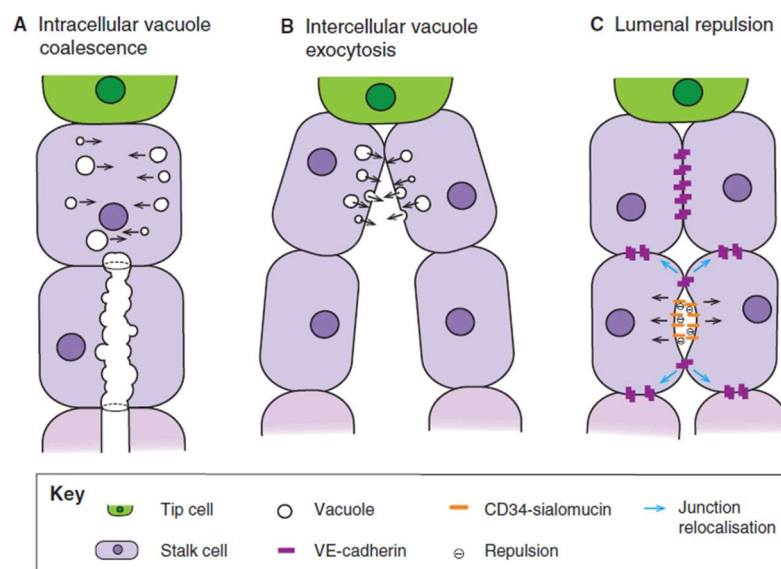
This sprout elongation is still dependent by the tip cell guidance, probably with a dynamic shuffling VEGFR/Notch/Dll4-mediated between ECs (Geudens and Gerhardt, 2011; Jakobsson et al., 2010) (see paragraph Notch mediated tip cell selection and guidance). This angiogenic environment is still corroborated, as said before, by the hypoxia-inducible program, driven by Hif-1 $\alpha$ , inducing gene transcription to increase both the ECs responsiveness to angiogenic signals and the angiogenic molecules production.

Stalk cells induce the formation of the body of the sprout and successively proliferate in order to create the building material necessary for the elongation of the vascular tube, responding to the surrounding different stimuli such as VEGF, Notch, NRARP, WNTs, PlGF and FGFs (Carmeliet and Jain, 2011), while tip cell are rarely proliferative (Gerhardt et al., 2003).

Interestingly, it has been demonstrated that stalk cells do not push the tip cell, but it is the tip cell that pull itself, interacting with the surrounding environment, towards the VEGF gradient (Geudens and Gerhardt, 2011).

The orientation of the stalk cell division is often perpendicular to the long axis of the vessel by which the sprout is growing and strongly influenced by VEGF gradient (Zeng et al., 2007) in absence of blood flow; in bigger perfused vessel the orientation of the growing vessel is determined by the shear stress sensed by PECAM1, VE-Chadrenin and VEGFR-2 (Tzima et al., 2005).

For big highly perfused capillaries has been proposed that the new growing vessel can be created by different mechanism: the first suggests is that the new lumen is formed by the coalescence of vacuoles of neighbour ECs (Fig. 48 A), while the second hypothesis suggests the exocytosis of vacuoles, in neighbours ECs, (Blum et al., 2008; Kamei et al., 2006) (Fig. 48 B). Another model of lumen formation was proposed based on mice and zebrafish in which the intracellular lumen seems to be created by the apical membrane repulsion (Fig. 48 C): VE-Chadrenin, which is required for the initial apical formation of the new vessel induces a shift of sialomucins, charged negatively, creating an electrostatic repulsion inducing the separation of the cells and the formation of the lumen.



**Figure 48:** Proposed models of lumen formation, specified in the text. From (Geudens and Gerhardt, 2011).

The new vascular network is, thus, formed by anastomosis and fusion of sprouts that must be stabilized to become functional.

#### *Anastomosis formation and connectivity*

The contact between sprouting vessels takes place between two sprout through a tip-tip contact (head-to-head anastomosis) (Blum et al., 2008) or between sprouts and functional vessel, involving only one tip cell ('head-to-side' anastomosis) (Betz et al., 2016) but the subsequent anastomosis between them is a process that is not completely understood; it seems that the anastomosis is often, but not always, accompanied by macrophages which might act as "bridge cell", helping the contact between the nascent sprouts (Checchin et al., 2006).

This process is driven by different molecules such as FGF, and in particular by interactions of Notch and Tie-2 and chemokine CXCR4 receptor, localised on macrophages, with their respective ligands Dll4, Ang-2 and SDF1, expressed in tip cells (Fantin et al., 2010; Geudens and Gerhardt, 2011; Strasser et al., 2010).

#### *STABILIZATION*

##### *Maturation and remodelling*

To create a perfused vessel a stabilization process is required, in order to stop the extravasation and seal the vascular structure. Firstly, myeloid bridge cells aid fusion with another vessel branch, allowing the blood to flow through (Fig. 46). After vascular connection, the vessels undergo to remodelling and either they are stabilized or they regress.

This mechanism, once again, is far to be fully understood, but it seems that pericytes play an important role. As a matter of fact some studies suggest that the pericytes mark the end of the plasticity window (Benjamin et al., 1998) and that their coverage of the vessel protect it from the regression, as reported in kittens retina development study (Chan-Ling et al., 2004). The pericytes recruitment seems to depend by different factors; among these we mention Ang-1/Tie-2 and Dll4/Notch pathway together with PDGF-B.

Ang-1, produced by ECs, induces the expression of chemokine MCP-1, which recruits the pericytes, which in turn produce factors like the tissue inhibitor of protease TIMP and Ang-1. The presence of Ang-1, moreover, stabilizes vascular endothelium (see paragraph “Angiopoietins”).

Furthermore, Notch signalling stabilises vessels inducing the expression of Notch regulated ankyrin repeat protein (NRARP) and the production of ECM component. NRARP creates a negative feed-back on Notch action, promoting in the same time the WNT/CTNNB1 signalling in stalk cells. This pathway supports vascular stability and prevents EC retraction (via proliferation induction and intercellular junctions improvement) (Phng and Gerhardt, 2009).

Notch can also stabilize the newly formed vessel by pericytes interaction, since Ang-1/Tie-2 signalling between pericytes and ECs is mediated via induction of Dll4 expression in ECs, even if the situation is not clear since in vivo Dll4 deficient mice did not show an impairment in pericytes recruitment (Geudens and Gerhardt, 2011).

The resultant of these process is that, under the guide of signals promoting the release of cell-survival signals and molecules inhibiting the angiogenic cascade, ECs resume their quiescent phalanx state and they are covered again by pericytes and SMCs. Protease inhibitors, in particular tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitor-1 (PAI-1), induce the deposition of a basement membrane and re-established the junctions to ensure the optimal flow distribution. If not perfused the vessels regress.

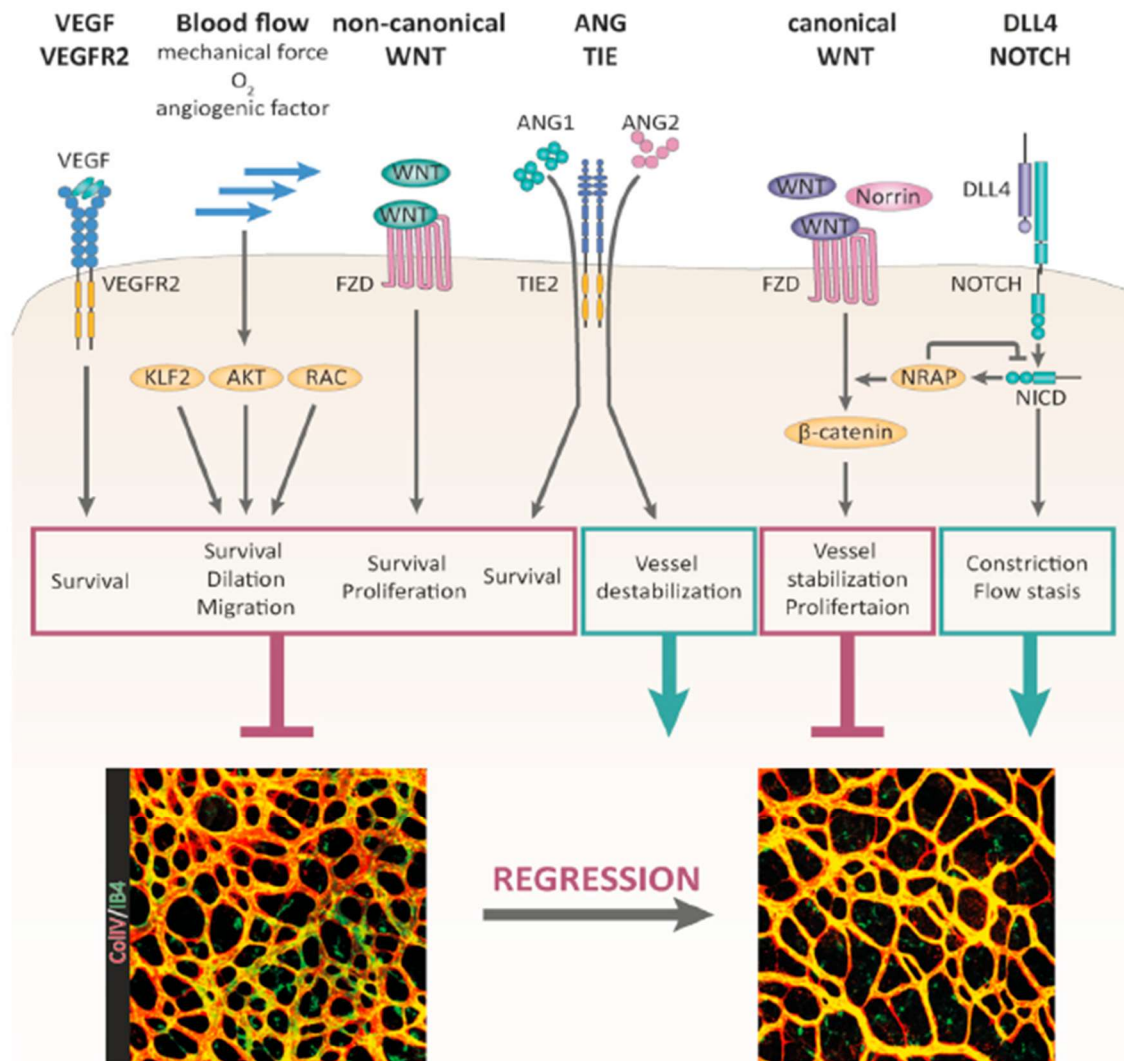
### *Pruning vessels*

The optimal vascular density is an important parameter since if it is reduced it cannot satisfy the metabolic request of neurons; vice versa some studies indicate that even an excessive vascular density can reduce the effectiveness of perfusion of the tissue (Cristofaro et al., 2013; Tirziu et al., 2012). Thus, when the vascular network is established, it can be remodelled to optimize the flow or to adapt it to changing demands in blood flow. The physiological regression of a subset of microvessels within a growing vasculature is called “vessel pruning” (Korn and Augustin, 2015).

It is still under debate if the vessel regression is due to either an active signalling pathway or the result of withdrawal of survival stimuli or the combination of the two mechanisms (Korn and Augustin, 2015).

There are parallel features between angiogenesis and reabsorption/pruning since this last can involve “reverse intussusception”, EC migration-dependent regression (the “opposite” of sprouting process) or apoptosis. While apoptosis has been implicated in the regression of larger blood vessels (Betz et al., 2016) and seems to be related to withdrawal of surviving factors such as VEGF, the destiny of little vessels is to prune and be reabsorbed by ECs.

Interestingly, not all the vessels are going to be reabsorbed but this event seems to be mainly dependent from both blood flow and Notch pathway, but also from O<sub>2</sub>, VEGF and Ang/Tie signalling (Fig. 49). For review see (Korn and Augustin, 2015; Ricard and Simons, 2015). For Ang/Tie signalling in pruning vessel see the part concerning the angiopoietins pathways.



**Figure 49:** Signalling Pathways involved in vascular regression. From (Korn and Augustin, 2015).

Shear stress in perfused vessels induces AKT signalling, resulting in Krüppel-like factor 2 (KLF2) activation and subsequent upregulation of nitric NOS and superoxide dismutase, promoting EC survival and NO mediated vessel dilation (Korn and Augustin, 2015). Using time-lapse imaging in the eye of zebrafish embryos, it has been shown that after an initial formation of a Y- shaped blood vessel branch, a rearrangement of ECs within the pruning blood vessel transforms a multicellular to a partially unicellular tube, a phenomenon known to be regulated by the blood flow (Kochhan et al., 2013). Moreover other studies analysing blood vessel pruning in zebrafish brains, mice retinae and airways showed that loss of blood vessel perfusion precedes blood vessel regression (Chen et al., 2012a; Lobov et al., 2011).

Another important regulator of the pruning destiny of ECs is Notch. The inhibition of Notch perturbs the normal pruning of capillaries in the developing retinal vasculature, as well as blood vessel regression after exposure to hyperoxia. Moreover the consequent loss of Notch-regulated ankyrin repeated protein (Nrarp) induces an increase in vascular regression due to a decrease in Wnt signaling-induced stalk cell proliferation. Furthermore, similarly, genetic alteration of Notch/Dll4 pathways reduces vascular pruning in developmental retinal vascular regression, confirming the involvement of



the Notch pathway in the control of vascular regression. This effect is associated to induction of genes encoding vasodilatory peptides (Lobov et al., 2011).

When the pruning process started, the lumen collapse and the remaining single EC migrates and incorporates into one of the main vessels (Ricard and Simons, 2015).

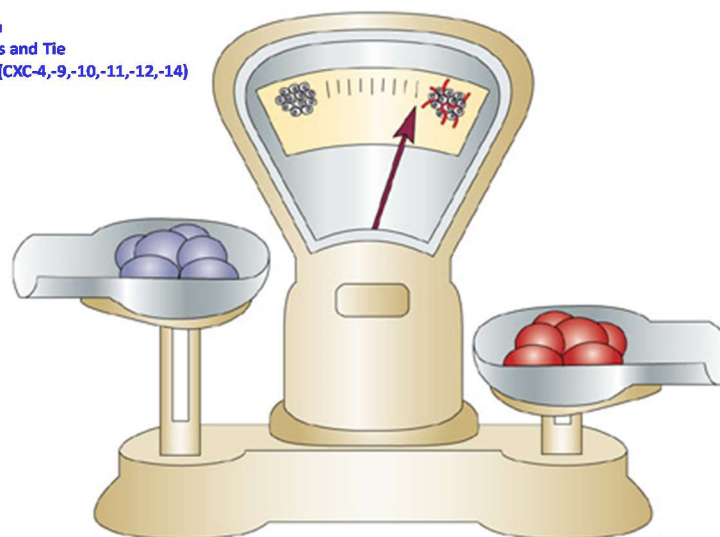
The complexity of angiogenic process requires a strict regulation, thus several angiogenic molecular pathways are involved, and reviewed in (Carmeliet and Jain, 2011; Krock et al., 2011) (Tab).

It is clear now that angiogenesis is orchestrated by a variety of activators and inhibitors. Some activators of endothelial-cell proliferation and migration are ligands of the tyrosine kinase receptor, such as VEGF, FGFs, PDGF and EGF. Among inhibitors we can list thrombospondin-1, which modulates endothelial-cell proliferation and motility and some statins.

Some of these actors are summarized in Fig. 50, and the most important are mentioned in Fig.51 with their relative functions.

#### Inhibitors:

Thrombospondin (TSP-1,-2,-3,-4,-5)  
Angiostatin  
Angioarrestin  
Angiopoietins and Tie  
Chemokines (CXC-4,-9,-10,-11,-12,-14)  
Endostatin  
INF- $\alpha$ , $\beta$ , $\gamma$   
PEDF  
IL 12  
TIMP-1  
DII1-4  
Vasohibin-1  
CA-9



#### Activators/promoters:

VEGF-A,-B,-C-D and VEGFR  
Angiopoietins and Tie  
PIGF  
VEGF  
FGF-1,-2  
HGF  
Hif-1,-2,-3  
PDGF-A,-B,-C,-D  
PLGF  
PG E1, E2  
TGF- $\alpha$ , $\beta$   
EGF  
LPA  
Chemokines (CXC-1,-2,-3,-5,-6,-7,-8)  
TGF $\beta$ -1,-2,-3  
Angiogenin  
EPO  
TNF (low levels)  
IL 8  
Osteopontin  
PAI-1  
MPP, TIMP  
NOS  
COX-2  
Endothelin  
Integrins  
Leptin  
A2A  
VE-cadherin

**Figure 50:** Angiogenic molecules responsible of angiogenesis pathways. Modified by (Bergers and Benjamin, 2003) and completed according to (Fong, 2008; Krock et al., 2011; Logsdon et al., 2014; Zetter, 2008)

The amount of “work” done by the ECs is considerable since they have to proliferate, produce molecules able to degrade the extracellular matrix, change their adhesive properties, migrate, avoid apoptosis and, finally, differentiate in new vascular tubes, and all this process is coordinated with mastery by different signalling pathway (Munoz-Chapuli et al., 2004).

Here we are going to define the most important mediators of this process such as VEGF and Notch signalling and Angiopoietins. Other molecules implicated in angiogenic pathways are reviewed in (Carmeliet and Jain, 2011; Clapp et al., 2009) and summarized in Fig. 51.

Angiogenic Molecules	Receptors	Functions
VEGF	VEGF-R1 (Flt-1), VEGF-R2 (Kdr)	Initiation of angiogenesis: <ul style="list-style-type: none"> <li>• Vascular permeability</li> <li>• Detachment of pericytes</li> <li>• Degradation of basement membrane</li> </ul> Neovessel formation: <ul style="list-style-type: none"> <li>• Endothelial cell proliferation and migration</li> <li>• Pericyte proliferation and migration</li> </ul>
Angiopoietin-2	Tie-2	Initiation of angiogenesis: <ul style="list-style-type: none"> <li>• Detachment of pericytes</li> <li>• Degradation of basement</li> </ul> Adaptation to tissue needs: <ul style="list-style-type: none"> <li>• Regression of neovessels due to lack of flow or presence of growth factor</li> </ul>
FGFs	FGFRs	Neovessel formation: <ul style="list-style-type: none"> <li>• Endothelial cell proliferation and migration</li> <li>• Pericyte proliferation and migration</li> </ul>
PDGF-B	PDGFR	Neovessel formation: <ul style="list-style-type: none"> <li>• Endothelial cell proliferation and migration</li> <li>• Pericyte proliferation and migration</li> </ul> Maturation: <ul style="list-style-type: none"> <li>• Attachment of pericytes</li> <li>• Deposition of basement membrane</li> </ul>
PLGF	VEGF-R1 (Flt-1)	Neovessel formation: <ul style="list-style-type: none"> <li>• Endothelial cell proliferation and migration</li> </ul>
Thrombospondin-1	CD36, CD47, Integrins	Neovessel formation: <ul style="list-style-type: none"> <li>• Endothelial cell proliferation, migration, adhesion, and motility</li> </ul>
Integrins	Extracellular matrix	Neovessel formation: <ul style="list-style-type: none"> <li>• Endothelial cell proliferation, migration, adhesion, and motility</li> <li>• Endothelial assembly and lumen acquisition</li> </ul>
SDF-1	CXCR4	Neovessel formation: <ul style="list-style-type: none"> <li>• Endothelial cell migration, adhesion, and motility</li> </ul>
DLL1-4	Notch	Neovessel formation: <ul style="list-style-type: none"> <li>• Endothelial cell proliferation, migration, adhesion, and motility</li> </ul>
SCF	c-Kit	Neovessel formation: <ul style="list-style-type: none"> <li>• Endothelial cell proliferation and adhesion</li> </ul>
Interleukins	Interleukin receptors	Neovessel formation: <ul style="list-style-type: none"> <li>• Endothelial cell proliferation and migration</li> </ul>
Angiopoietin-1	Tie-2	Maturation: <ul style="list-style-type: none"> <li>• Endothelial assembly and lumen acquisition</li> <li>• Attachment of pericytes</li> <li>• Deposition of basement membrane</li> <li>• Vessel maintenance</li> </ul>
Endothelin-1	Endothelin receptors (Et-A, Et-B)	Maturation: <ul style="list-style-type: none"> <li>• Vascular permeability, vasoconstriction</li> </ul>
Vasohibin-1	Vasohibin receptors	Maturation: <ul style="list-style-type: none"> <li>• Inhibits endothelial cell proliferation and migration</li> </ul>

**Figure 51:** Main Angiogenic Molecules, Their Receptors, and Functions. Abbreviations: CXCR4 : CXC chemokine receptor 4; DLL1-4 : delta-like ligands 1-4; FGF : fibroblast growth factor; FGFR : fibroblast growth factor receptor; Flt-1 : fms-related tyrosine kinase 1; Kdr : kinase insert domain containing receptor; PLGF : placenta growth factor; PDGF-B : platelet-derived growth factor beta; PDGF-R : platelet-derived growth factor receptor; SCF : stem cell factor; SDF-1 : stromal-derived growth factor; Tie-2 : TEK tyrosine kinase endothelial; VEGF : vascularendothelial growth factor; VEGF-R : VEGF receptor. (Krock et al., 2011)



### 3.4.1. VEGF and VEGFRs pathway

#### *VEGF*

Vascular endothelial growth factor VEGF and its receptors (VEGFR) are the predominant actors, which regulates the angiogenesis. The VEGF family consists of only a few non-redundant members. It was discovered in 1983, by Senger who describes it as a protein able to induce vascular leakage in the skin (Ferrara, 2009). VEGF (known as VEGF-A) is the major component, and it acts stimulating the angiogenic process, both in healthy conditions and during pathologies through the binding with VEGF receptor-2 (VEGFR-2). **VEGF-A** is not only the most expressed form of VEGF, but it is ubiquitously distributed in human (Nagy et al., 2007).

VEGF-A is a glycoprotein of 34-36 kDa that is presented as disulfide homodimer. The human gene is organized in 8 exons and 8 isoforms are obtained from RNA splicing: VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>162</sub>, VEGF<sub>165</sub>, VEGF<sub>165B</sub>, VEGF<sub>183</sub>, VEGF<sub>189</sub> and VEGF<sub>206</sub>. The different isoforms have different affinity in binding heparine and this determine the ability of the protein to accumulate in ECM or to diffuse. Soluble VEGF isoforms promote vessel enlargement, whereas matrix-bound isoforms stimulate branching. The most abundant protein in human is VEGF<sub>165</sub> that is highly affine to sulphate heparin and are mainly associated to ECs or ECM.

VEGF-A binds both VEGFR-1 and VEGFR-2 and its expression is stimulated mainly by hypoxia induced by Hif-1 $\alpha$ , which binds HREs in the internal part of VEGF promotor. The production of VEGF is also incremented by growth factors (EFG, IGF-1, FGF, PDGF), chemokines (IL 1, TGF $\beta$ , PGE2) or COX-2 activators.

VEGF-A, besides exerting a role in angiogenic process in ECs, is able to induce vascular permeability and to promote the survival of ECs inducing the expression of anti-apoptotic proteins; moreover recent studies have emphasized the potential role of VEGF as a neuronal protective factor implicated in neurogenesis, neuronal migration, neuronal survival and axon guidance, reviewed in (Mackenzie and Ruhrberg, 2012; Shen et al., 2016).

On the contrary the loss of VEGF, as well as VEGFR-2 deficiency, aborts vascular development.

Other VEGF proteins are VEGF-B, -C, -D and placenta growth factor (PLGF).

VEGF-B that is able to form heterodimer with VEGF-A, is expressed in several tissue (i.e. skeletal muscle, myocardium); it binds VEGFR-1 and it is involved in degradation of ECM and migration of ECs; anyhow its function is not deeply defined: it seems required for adult cardiac functionality but not for cardiovascular development or angiogenesis.

VEGF-C is produced by a precursor activated in extracellular space from proteases, which generate a homodimer with high affinity for VEGFR-2 and VEGFR-3; it is principally involved in the formation of vascular network during embryogenesis and later in linfoangiogenesis.

VEGF-D binds VEGFR 2 and VEGFR 3; it is expressed in several adult tissues, included vascular endothelium, heart and skeletal muscle; it possesses angiogenic characteristic in vitro and in vivo.

#### *VEGFR*

The VEGFR are a transmembrane tyrosine kinases receptors (RKT), composed by 750-amino-acid-residue extracellular domain, which is organized into seven immunoglobulin(Ig)-like folds (important for the binding and the specificity, for a review see (Olsson et al., 2006)) , a single transmembrane domain and an intracellular enzymatic (tyrosine kinase) domain activated by the growth-factor

binding, resulting in the transfer of phosphate groups onto tyrosine residues, for a review (Ferrara, 2004).

VEGFR exist in different isoforms, such as VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (FLT-4); VEGFR-1 and -2 are widely expressed by normal vascular ECs both in vivo and also in vitro, implying their preponderant function in the vascular system, but some studies reports their localization in other cells types like monocytes, macrophages, hematopoietic stem cells and neurons (Ferrara, 2009; Mackenzie and Ruhrberg, 2012; Olsson et al., 2006).

Despite VEGF binds to VEGFR-1 with higher affinity compared to VEGFR-2, it is mainly VEGFR2 that mediates VEGF signalling in endothelial cells, which is more expressed compared to VEGFR-1. VEGFR-1 signaling is still poorly known, and contradictory results have been obtained in different systems (Zachary and Gliki, 2001), but it was shown that VEGFR-1 is able to modulate positively and negatively VEGFR-2: the negative regulation is partially due to its nature of being the alternative splicing variant binding the VEGF and preventing the binding with VEGFR-2 (Olsson et al., 2006). VEGFR-3 is implicated in lymphatic and endothelial cell development and function.

Some studies suggest that VEGFR-2 biological action can depend from its subcellular localization: the arterial morphogenesis can be achieved if VEGFR-2 is located into intracellular compartment (Lanahan et al., 2010).

#### *VEGF biological activities*

In the following part we are going to point out our attention on angiogenic functions but of course VEGF participate in different pathways, for review (Hoeben et al., 2004; Olsson et al., 2006).

The activity of VEGFR is determined by the presence of ligand, in particular VEGF-A is upregulated in hypoxic condition by Hif-1 $\alpha$ . Moreover, both VEGFR-1 and -2 are upregulated during angiogenesis through a mechanism Hif-1 $\alpha$  induced-VEGF-A expression (Nagy et al., 2007), as well as during tumours.

The binding between VEGF and VEGFR-2 is the major extracellular signal that start angiogenic process: through dimerization and autophosphorylation the receptor is activated inducing the phosphorylation of different proteins, including PLC which directly activates IP3 with consequent increase of Ca<sup>2+</sup> (pathway described in the session regarding the vascular reactivity), activating in turn PKC. PKC stimulates the activation of extracellular signal-regulated kinases-mitogen activated protein kinases (ERK-MAPK) cascade (Munoz-Chapuli et al., 2004).

Furthermore, the activation of serine/threonine kinase AKT/PKB mediates the ECs survival and the increase of NO production through eNOS activation. Others studies suggest an implication of Ras/Raf mediated pathways, even if all the cascade is not fully understood (Olsson et al., 2006).

Once activated, ERK-MAP kinases, which translocate to the nucleus, activate the transcription factors involved in cell proliferation, such as Elk-1, c-Myc, c-Fos, Ets-1, SRF (For more details (Kanno et al., 2000; Wu et al., 2000)).

As previously described VEGF participates to several steps of angiogenesis process, in particular in the morphogenesis of the new vessel: VEGF-A is rapidly released to induce destabilization of vessel via FAK activation VE-cadherin and  $\beta$ -catenin dissociation inducing ECs junctions breakdown (Chen et al., 2012b). Successively, VEGF-A orchestrates tip selection and guidance promoting the migration of filopodia-studded endothelial tip cells and the proliferation of lumen-forming stalk cells (Geudens and Gerhardt, 2011) during the branching process, regulating the morphology and connectivity of capillary networks in the brain (Ruhrberg et al., 2002). Moreover VEGF promotes the expression of

several factors as Von Willebrand factor, integrin, interstitial collagenase, plasminogen activator (PA) and plasminogen activator receptor (PA-R) and increases the vascular permeability and fenestration, preparing a suitable angiogenic environment (Ucuzian et al., 2010).

The oxygenation of the tissue is an important factor exercising a negative feedback on paracrine VEGF production, helping the establishment of a quiescent state for the new vessels.

What is really interesting is the ability of ECs in inducing a controlled proliferation towards the tip cell.

This is possible through a fine interaction between VEGF and Notch which assures the selection of a tip cell that guide the sprouting toward the VEGF gradient and the inhibition of neighbour cells in order to become stalk cells, which follow the guiding tip cell and proliferating to support sprout elongation.

Briefly, VEGF pathway promotes filopodia formation, allowing the acquisition of the sprouting phenotype to the tip cell. Moreover, VEGF signalling induces expression of Dll4, which activates Notch signalling in the neighbour stalk cells, which down-regulates VEGFR expression, giving a non-sprouting quiescent phenotype to the stalk cell (Sewduth and Santoro, 2016).

In particular, Gerhardt's study on mice retinas shown that, in the tip cells, VEGF-A, released by astrocytes in response to hypoxia, induces the formation of dynamic long filopodia through interaction with VEGFR-2 in tip cell, to sense the environment for directional cues. The stalk cells produce less filopodia but start to proliferate when stimulated with VEGF-A. In other words, while VEGF-A presence guides the migration the tip cells, it induces a proliferative response in the sprout stalks. Moreover, while tip cell migration depends on a gradient of VEGF-A, proliferation is regulated by its concentration (Gerhardt et al., 2003). It is noteworthy, moreover, that tip cell can migrate without stalk proliferation and vice versa, but just the coordination of these two processes can establish a functional shaped sprout (Gerhardt et al., 2003; Geudens and Gerhardt, 2011).

But how is it possible to select the tip cell?

### **3.4.2. Notch mediated tip cell selection and guidance**

The tip cells and stalk cells show a different gene expression, leading to think that their specification is genetically determined. Some studies suggest that tip cell show higher levels of different genes inducing proteins expression, such as VEGFR-2, PDGFB, Dll4 UNC5B and MPP4 (Gerhardt et al., 2003; Geudens and Gerhardt, 2011), even if there is not a single gene or protein that can be used as an identifying marker (Fouillade et al., 2012; Geudens and Gerhardt, 2011).

The genetic predisposition of differentiation can be important in the first phase of tip selection but it is not the only actor playing in the tip cell guidance process; as a matter of facts other studies suggest a possible shuffling between stalk and tip cells: in vitro and in vivo studies propose that if the stalk cell is "better equipped", it will take over the guidance process, becoming the new tip cell (Jakobsson et al., 2010; Siekmann et al., 2013).

In any case, the specification of tip and stalk cell and the sprout guidance are strictly dependent by the Notch signalling, which is highly conserved at the evolutionary point of view (Fouillade et al., 2012; Phng and Gerhardt, 2009); Notch pathway is involved in several biological functions, such as cell fate specification, tissue patterning, and morphogenesis through effects on differentiation, proliferation, survival, and apoptosis, for general review see (Cai et al., 2016) and (Phng and Gerhardt, 2009).

Different Notch receptors were discovered in mammals, namely Notch1, Notch2, Notch3 and Notch4 and they are differently localized: as a matter of fact, Notch1 and Notch4 are mainly present in ECs while Notch3 is principally localized in SMC and brain pericytes (Fouillade et al., 2012; Joutel et al., 2000; Villa et al., 2001).

Notch receptor binds different ligands, among these Delta/Serrate/Lag-2 (DSL) ligands: Delta-like1 (Dll1), Delta-like3 (Dll3), Delta-like4 (Dll4), Jagged1 (Jag1), and Jagged2 (Jag2) (Fouillade et al., 2012). The Dll1, Dll4, and Jag2 were detected in ECs whereas Jag1 is expressed by both ECs and SMC, principally at arterial level (Fouillade et al., 2012; Villa et al., 2001). DSL and Notch receptors are transmembrane proteins with a large extracellular domain (ECDs) containing epidermal growth factor-like repeats (EGFR).

Notch is synthesized as precursor protein, cleaved in two different regions, a large ectodomain (NotchECD) and a membrane-tethered intracellular domain (NotchTMIC), taken together by non-covalent juxtamembrane heterodimerization. Subsequently to DSL binding, Notch is cleaved by ADAM metalloprotease and then by  $\gamma$ -secretase, inducing the release of the Notch intracellular domain (NICD) from the membrane. Once cleaved, the NICD translocated to cellular nucleus forming an active complex with transcription factor RBP-J $\kappa$  and co-activators including transcriptional activation of Notch targets such as the basic helix–loop–helix (bHLH) proteins Hairy/Enhancer of Split (Hes) and Hes-related proteins (Hey/HRT/HERP) (Fouillade et al., 2012).

For a clear and exhaustive review describing the Notch pathway in particular in SMC see (Fouillade et al., 2012) and for the coordinative role of Notch during angiogenesis in ECs see (Phng and Gerhardt, 2009). From these review is clear that Notch controls several steps during angiogenic process (not just tip cell selection), but despite it was ought to be mentioned, during this thesis we did not have the time to explore this molecule. .

Notch transcriptional activity and Dll4 action can be stimulated by Hif-1 $\alpha$ . Moreover, the activation of VEGFR2 by VEGF induces the expression of the notch ligand Dll4 in the tip cells.

The subsequent activation of notch by Dll4 in neighbour stalk cells downregulate their expression of VEGFR2 and Dll4 and upregulate VEGFR-1. Soluble VEGFR-1 produced by the cells immediately next to the outgrowing vessel branch sequesters VEGF molecules; this create a kind of corridor of higher VEGF levels perpendicular to the parent vessel, optimizing both the spreading of the vascular network and avoiding the contact with nearby emerging sprouts (Geudens and Gerhardt, 2011). Moreover subsequently to the VEGFR-2 downregulation, stalk cells become less sensitive to VEGF-mediated activation, limiting their ability to activate notch signalling in neighbouring cells, and making them more sensitive to other molecules such as PlGF (Carmeliet and Jain, 2011).

Furthermore, Jag1, selectively expressed in stalk cells, binding Notch, competes with Dll4 preventing its binding and promoting the nearby tip cell selection.

Another level of complexity is given by the fact that Notch is able to upregulate its own inhibitor NRARP in stalk cells (Carmeliet and Jain, 2011). NRARP is able to corroborate the Notch and WNT signalling. Notch activates WNT pathway in stalk proliferating cell; this binding is responsible to the stalk cell proliferation and subsequently vessel stabilization (Phng et al., 2009).

### **3.4.3. Tie receptors and angiopoietins signalling**

The angiopoietins and their receptors are important actors of the angiogenic process. We decided to focus our attention on Angiopoietin 2 signalling since its action is fundamental from the beginning of

the angiogenesis process; furthermore its ECs specificity, compared to VEGF, give us the opportunity of target the angiogenic process without interfering with neuronal functionality.

The activity of this pathway is strictly controlled both via ligand availability and via receptor phosphorylation activity that we are going to summarized below (Fagiani and Christofori, 2013).

#### 3.4.3.1. Tie receptors

In the 1990, during a study on tyrosine kinases involved in cardiogenesis and hematopoiesis (Partanen et al., 1992), two new tyrosine kinases receptors were identified on ECs and were called Tie-1 and Tie-2, where Tie stands for tyrosine kinase with immunoglobulin and EGF homology domains. Tie-1 and Tie-2 are considered **endothelial cell-specific receptors** with similar molecular weight of approximately 135 and 150 kDa respectively (Thomas and Augustin, 2009).

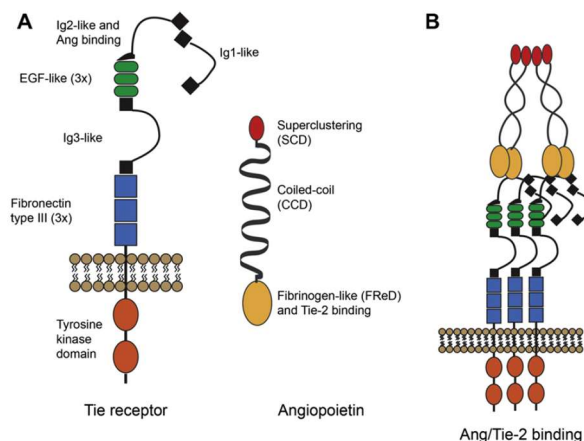
Both Tie-1 and Tie-2 are transmembrane receptors co-expressed in vascular and lymphatic ECs (Augustin et al., 2009); they are constituted principally of the N-terminal extracellular domain composed by two Ig-like domains (the ligands bind Tie-2 in the Ig2 domain), flanked by three Epidermal grow factor (EGF)-like motifs, then another Ig domain before three fibronectin-like repeats; this sequence is linked by an intramembrane domain and a smaller C-terminal intracellular part which is responsible of the catalytic action of the receptor. This last part is composed by a tyrosine kinase domain triggering intracellular transduction (Fig. 52 A).

Few years later, the endogenous ligands of Tie-2 were identified: angiopoietin-1 (Ang-1, ANGPT1), angiopoietin-2 (Ang-2, ANGPT2), angiopoietin-3 and angiopoietin- 4 (Fagiani and Christofori, 2013). In particular Ang-1 and Ang-2 bind Tie-2 with similar affinities and at the same site, becoming the central regulation core of the pathway (Fiedler et al., 2003)

Tie-2 is mainly expressed in vascular and lymphatic ECs (Tammela et al., 2005), but it is also expressed in non-ECs, such as the circulating haematopoietic cells (Puri and Bernstein, 2003) including a sub-population of monocytes (De Palma et al., 2005) and macrophages (Fantin et al., 2010), in human neural stem cells (Parati et al., 2002), in non-autonomic peripheral nervous tissue (Poncet et al., 2003) and in the nonvascular compartment of several tumour types (Lee et al., 2006). See the final part of “Role of Ang-1 and Ang-2 in blood vessels homeostasis and morphogenesis” for more details.

Tie-2 is constitutively expressed and phosphorylated in at least several organs such as brain, heart, lung, liver, and kidney in rats (Wong et al., 1997). A study showed that its distribution in normal microvascular endothelium differs between arteries, veins, and capillaries in mouse mesentery (Anghelina et al., 2005), with higher concentration in arteries and arterioles then in veins, even if the study of Wong did not detected this difference. Its expression can be increased by hypoxia and pro-inflammatory molecules (Willam et al., 2000).

Moreover its activity can be modulated by proteolytic molecules (Kim et al., 2011), such as MMP-14 and Epithin/PRSS14 either in constitutive manner or induced by VEGF via PI3K/Akt pathway; this proteolysis induces the cleavage of the 75 kDa soluble ectodomain (sTie-2) containing the extracellular binding site, process known as ectodomain shedding (Findley et al., 2007). In absence of angiopoietins, this mechanism may serve as an autoinhibitory pathway preventing Tie-2 autophosphorylation. Conversely, in presence of angiopoietins, Tie-2 intracellular part can undergo ligand-independent dimerization and phosphorylation (Kim et al., 2011) or can binds angiopoietins preventing the full Tie-2 activation (Findley et al., 2007).



**Figure 52:** Structures of Tie receptors and Angiopoietins, detailed in the text. From (Fagiani and Christofori, 2013)

After ligand binding, Tie-2 dimerizes or multimerizes, becoming trans-autophosphorylated at certain tyrosine residues in its C-terminal kinase domain (Fig. 52 B).

Tie-2 activation leads to recruitment of different molecules such as growth factor binding partner, the p85 subunit of PI3K, and Dok-R activating Akt, extracellular signal regulated kinase (ERK) (Thurston and Daly, 2012), and FAK (Kim et al., 2000). Moreover, Tie-2 activity can be counteracted directly via dephosphorylation by phosphatase such as vascular endothelial protein tyrosine phosphatase (VE-PTP), for more details see (Thurston and Daly, 2012).

Independently from the context and the ligand, Tie-2 can mediate cell survival, migration and maintenance of barrier function leading to vascular quiescence or angiogenic response.

Mice lacking Tie-2 gene are able to develop the primary vascular plexus but die between E10.5 and E12.5 due to an insufficient expansion and maintenance of primary vessels (Sato et al., 1995).

While Tie-2 is constitutively expressed by ECs, Tie-1 expression is strongly regulated (Augustin et al., 2009). Tie-1 still remains an orphan receptor since no ligands were detected to activate it. Thus, Tie-1 was proposed as regulator of Tie-2. As a matter of fact, it has been shown to heterodimerize with Tie-2 and to regulate its activity, even if the tendency of this regulation is not clear; it may act as negative regulator of Ang-1/Tie-2 interaction because Tie-2 phosphorylation increases when the Tie-1 ectodomain is cleaved (Hansen et al., 2010), but any effect was detected on Ang-2/Tie-2 interaction.

This receptor, although it is not required for angioblast differentiation in early angiogenic vessel growth, supports angiogenesis and EC proliferation at later stages (Partanen et al., 1996). Nevertheless Tie-1 deficiency causes embryonic lethality in mice (Sato et al., 1995). Thus, the lacking of Tie-1 or especially Tie-2 in mice is incompatible with life due respectively to loss of structural integrity and to vessels remodelling deficit (Sato et al., 1995).

#### 3.4.3.2. Angiopoietins

Angiopoietins have been identified as a family of secreted ~70 kDa glycoproteins (at monomeric state) primarily implicated in the development, morphogenesis and the stability of blood vessels (Thomas and Augustin, 2009). They are mainly formed by N-terminal signalling sequence ensuring protein secretion, followed by a small super clustering domain (SCD) that is responsible, together with the following central coiled-coil domain (CCD), for homo-oligomerization of the ligands (necessary for the activation of the receptor); this sequence is then connected, through a short linker domain, with

the C-terminal fibrinogen-related domain (FReD) required for binding to the second Ig-like motif of Tie-2 receptor (Davis et al., 2003; Fiedler et al., 2003). Once released they accumulate in the surrounding medium (Bogdanovic et al., 2006) (Fig. 52). The roles of these ligands with their receptors modify the vascular morphogenesis, including sprouting angiogenesis, vascular remodelling, vascular activation and vascular quiescence; others angiopoietins actions, such as the role in inflammation, or their involvement in tumour process are not treated in this thesis but for review see (Bupathi et al., 2014; Eklund and Saharinen, 2013; Fiedler and Augustin, 2006; Scholz et al., 2015; Thomas and Augustin, 2009).

As mentioned before in humans, three angiopoietins have been identified: angiopoietins 1, 2, and 4 (angiopoietin-3 is the mouse orthologue of Ang-4), and despite a similar structure, they differ in terms of expression pattern, efficiency for Tie-2 activation, and signalling outcome. The most studied are Ang-1 and Ang-2.

Ang-3 is expressed in several tissues in mice while Ang-4 is specifically present at high levels only in human lungs. Ang-4 activates Tie-2 receptor but the role of Ang-3 is not fully understood; it seems that are both agonists of Tie-2 receptor signalling, being able to induce angiogenesis *in vivo* (Fagiani and Christofori, 2013; Lee et al., 2004a).

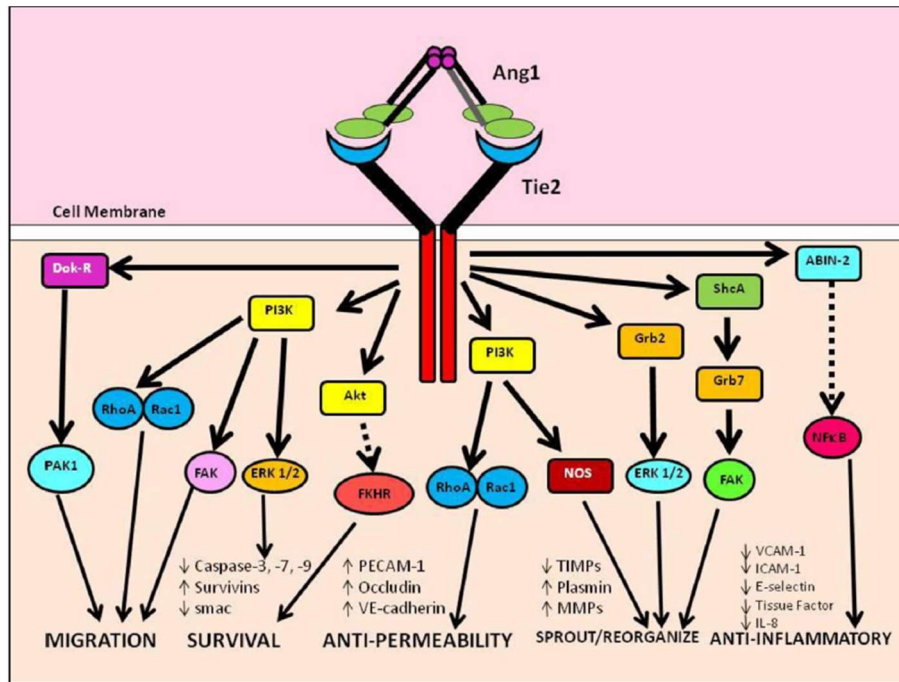
Both Ang-1 and Ang-2 form multidimeric structures (dimers, trimers and tetramers), and Ang-1 can create further assemblies into higher order multimers. Only the tetrameric or higher multimeric forms of Ang-1 activate Tie-2, while oligomeric Ang-2 is a weak context dependent agonist of Tie-2, and can antagonize Ang-1-mediated Tie-2 activation.

**Ang-1** is mostly expressed in **perivascular** cells such as pericytes, vascular smooth muscle cells, fibroblasts and tumour cells (Augustin et al., 2009; Wakui et al., 2006). In healthy adults, Ang-1 is **constitutively** produced at considerable levels but its expression can increase after hypoxia or in presence of VEGF-A and PDGF-B in pericytes and vascular smooth muscle cells, acting in **paracrine** manner.

As mentioned before, upon multimers Ang-1 binding, Tie-2 is phosphorylated and activated participating to tubule formation, EC migration, survival and most importantly helps the maturing endothelium to reach and maintain its barrier functions (Fagiani and Christofori, 2013). It is important to notice that Ang-1 is known to mediate both vessel stabilization and EC migration via Tie-2 activation. Furthermore, since Ang-1 presence after ischemic event, it has been proposed as neuroprotective factor; in fact in primary cultured neurons was showed that Ang-1 prevent neuronal apoptosis via PI3K activation (Valable et al., 2003); however, concerning *in vivo* studies, the Tie-2 expression in mature cortical neurons has not been detected and Ang-1 function was proposed to be mediated by integrin (Ward et al., 2005). This last action is dependent by the Tie-2 mediated decrease in adhesion proteins as VE-cadherin and Platelet endothelial cell adhesion molecule-1 (PECAM-1) and suppressing the dissociation of VE-cadherin from  $\beta$ -catenin (Gamble et al., 2000).

Implicated downstream pathways include the activation of PI3K/Akt which mediates antiproliferative and cell survival effects and induces NO production via eNOS stimulation (pathway implicated in Ang1-supporting angiogenesis action (Babaei et al., 2003). The Ang-1 mediated activation of Akt pathway assists ECs migration inhibiting forkhead transcription factor (FKHR), also known as FOXO1 (Daly et al., 2004) (Fig. 53).





**Figure 53:** Principal signalling pathways activated by the binding Ang-1/Tie-2. Abbreviations: PAK, p21-activating kinase; FAK, focal adhesion kinase; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular ligand-regulated kinase; FKHR, forkhead transcription factor; NOS, nitric oxide synthase; ABIN-2, A20 binding inhibitor of NFkB; NFkB, nuclear factor kappa B; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; smac, second mitochondrial activator of caspases; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases. (Moss, 2013)

These pathways are more detailed in the following part about the role of Ang-1 and Ang-2 in blood vessels homeostasis.

Differently to Ang-2, the Ang-1 binding with Tie-2 induces a rapid internalization of the receptor, which is degraded; this degradation may act as a turn off transduction signal. As a matter of fact in unstimulated HUVECs the half-life of Tie-2 was ~9 hours, while in presence of Ang-1 it was closed to 3 hours; differently, in presence of Ang-2 the half-life was ~7 hours. The difference between Ang-1 and Ang-2-mediated internalization mechanism seems due to the level of Tie-2 activation induced by these ligands (Bogdanovic et al., 2006).

Ang-1 deficient mice are similar to Tie-2 lacking mice, which a less severe gravity which bring to a retard in heart growth and severe vascular deficit leading the 90% of mortality (Suri et al., 1996).

**Ang-2** expression is strictly controlled; in adults physiological condition it is selectively **expressed in ECs** (Augustin et al., 2009; Fiedler and Augustin, 2006; Fiedler et al., 2004) and pericytes (Wakui et al., 2006) where active remodelling occurs; it almost absent in quiescent vasculature but it dramatically produced by ECs and up regulated in response to tissue hypoxia (Pichiule and LaManna, 2002), shear stress (biomechanical force acting on the vessels wall as a consequence of the tangential force exerted by the flowing blood).

It can be up regulated at transcriptional level by FOXO1, linking Ang-1 as a negative regulator of Ang-2 expression since Ang-1 acts as a FOXO1 inhibitor via Akt, as previously said (Daly et al., 2004).

Once produced, Ang-2 is stored in specialized endothelial storage granules, called Weibel-Palade bodies, which can release Ang-2 rapidly. In literature this stocking system seems more implicated in fast response of ECs to inflammation and coagulation (Saharinen et al., 2011) rather than angiogenic



answer that require longer transcriptionally driven programs. In fact, Ang-2 secretion has been shown to involve exosomes, small membrane vesicles transporting proteins, mRNAs and microRNAs with involvement of PK3I/Akt pathway (Ju et al., 2014). Vice versa, in quiescent endothelium, Ang-2 can be down regulated by Kruppel-like factor-2 (KLF2) (Augustin et al., 2009).

Once released, Ang-2 acts in **autocrine** manner on Tie-2 (a paracrine action is more linked to inflammation (Scholz et al., 2015)) but its agonist activity is much lower compared to Ang-1, thus is considered as partial agonist. Interestingly, in non-ECs where Tie-2 is introduced ectopically, Ang-2 induces similar Tie-2 activation compared to Ang-1, leading to think that there are other factors in EC that influence its agonist nature. *In vivo* Ang-2 affects EC stabilization, leading to weakened EC integrity. Thus, in presence of Ang-1 its action is competitive antagonist-like, displacing the more active ligand Ang-1.

It is clear that the angiogenic effect is finely regulated by the balance Ang-1/Ang-2. The effect of Ang-2 depends also from the presence of VEGF. As a matter of fact, the injection of Ang-2, without the presence of VEGF, induces endothelial cell death and vessel regression using the *in vivo* mice pupillary membrane model (Lobov et al., 2002). Thus Ang-2 promotes angiogenesis when VEGF is present, producing vessels destabilization, counteracted by Ang-1, while it promotes the vascular regression when VEGF is absent. Consequently, it is the ratio Ang-1/Ang-2 that is critical in balancing Tie-2 effects.

Ang-2 overexpressing mice show a severe vascular integrity deficit, closed to Ang-1 or Tie-2 lacking mice which support the hypothesis of the agonist Ang-1 and antagonist Ang-2 actions on Tie-2 (Reiss et al., 2007).

Differently, whole mouse Ang-2 deletion showed different results depending from the mouse background: mice with a mixed C57BL/6 and 129/J background were shown to die within two weeks after birth; while C57BL/6 survives until adulthood. Moreover, Ang-2 null mice showed defects and abnormalities in lymphatic system (Gale et al., 2002b).

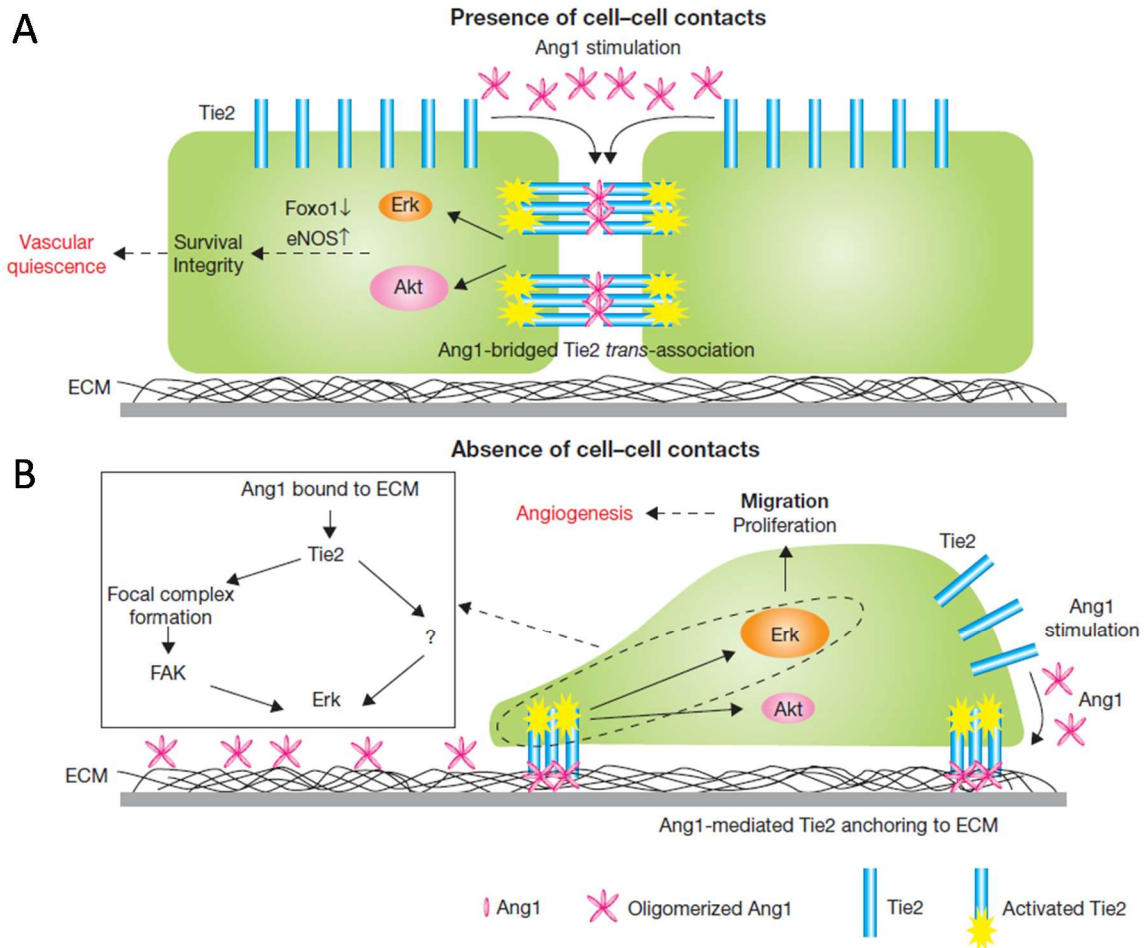
#### 3.4.3.3. Role of Ang-1 and Ang-2 in blood vessels homeostasis and morphogenesis

The regulation of Ang/Tie homeostasis is complex. Some studies demonstrate that angiopoietins determine the subcellular localization of Tie-2 in sparse (angiogenic) vs. confluent (quiescent) ECs, affecting the signalling outcome in angiogenic vs. quiescent endothelium (Saharinen et al., 2011).

The switch between quiescent and activated endothelium and Ang-2 action is a key aspect in angiogenesis triggering.

The angiogenic phase seems to be initiated by an increase in Ang-2 and VEGF, while the vessel maturation phase might be initiated by a relative increase in Ang-1 and a decrease in VEGF (Wakui et al., 2006).

*In quiescent ECs* upon **Ang-1** binding, the Tie-2 receptors cluster their kinase domains to **inter-endothelial cell junction**, into close proximity, establishing complex with other Tie-2 molecules of adjacent cells (homotypic Tie-2-Tie-2 *trans* associated complex); in this complex, the multimeric Ang-1 ligand can bridge Tie-2 receptors from neighbouring cells, in order to allow their phosphorylation and the activation of downstream mechanisms, preferentially activating Akt (also known like Protein kinase B) (Fig.54A, Fig. 56A).



**Figure 54:** Schematic representation of a proposed model for how Ang1–Tie-2 signalling is involved in both vascular quiescence and angiogenesis depending from the confluent conditions (A), resulting in formation of trans-association of Tie-2, or in the absence of cell–cell contacts, where Tie-2 forms a complex with ECM-bound Ang1 at cell–substratum contacts. From (Fukuhara et al., 2008)

In particular, Tie-2 stimulation activate the subunit p85 PI3K which activate Akt, in turn activating survival promoting pathways, such as eNOS, and suppressing apoptotic pathways like caspase9 and BAD (Bcl-2-associated death promoter). In addition Akt inactivates FOXO-1 which targets Ang-2 (negative feed-back loop of ECs on Ang-2 production) (Augustin et al., 2009).

All these actions are aimed to promote cell-cell adhesion, to increase ECs survival and to maintain a quiescent vessel situation.

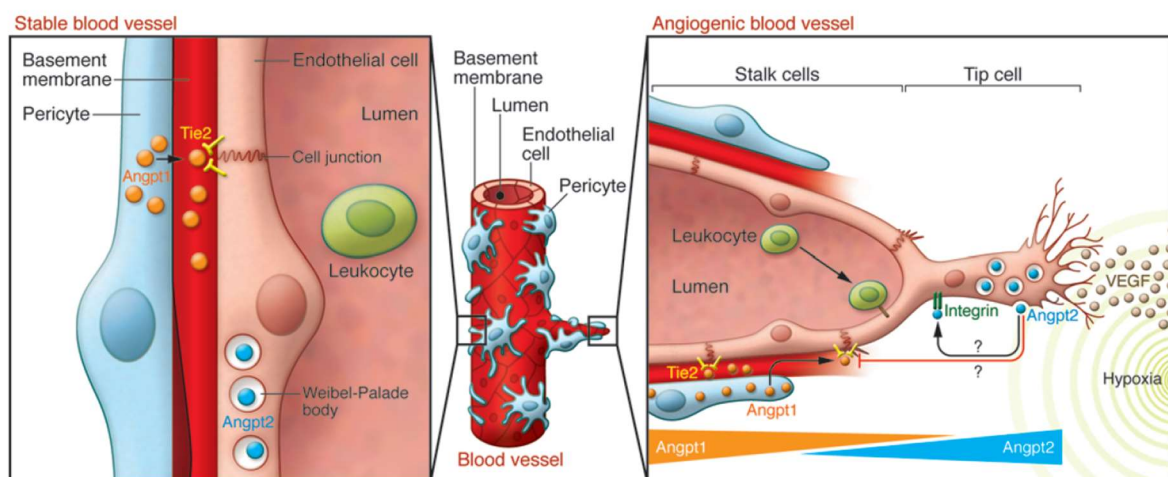
During angiogenic process, VEGF-A and the hypoxia induce the expression of **Ang-2** (Oh et al., 1999), that in presence of VEGF-A, is released making decrease the ratio Ang-1/Ang-2 at the level of tip cell (Fig. 55). Its competitive antagonist action in this condition leads the suppression of Ang-1 action, decreasing Ang-1-induced Tie-2 phosphorylation and disrupting PI3K/Akt survival signalling, allowing the endothelial response to **destabilization** and regression (Gale et al., 2002a) under the guidance of other angiogenic stimuli. Ang-2 has a fundamental role since it is able to binds Tie-2 without inducing signal transduction, acting as a destabilizing factor specifically restricted to ECs in areas of vascular remodelling (Maisonpierre et al., 1997).

Exerting its antagonist role, Ang-2 leads to vessels destabilization and pericytes dropout. As a matter of fact, together with VEGF synergic action increasing its production, Ang-2 is able to bind Tie-2 resulting in a dissociation of pericytes from vessels (Zhang et al., 2003a), a reduction of pericyte coverage and vessels destabilization (Ribatti et al., 2011), increasing permeability and vascular leakage. Consequently the unstable ECs lacking of pericytes can die (if VEGF is absent) or proliferate and migrate (in presence of VEGF as previously described).

Moreover, in vitro studies recently described that Ang-2 induces Tie-2 translocation to the specific cell–matrix contact sites localized at the distal end of focal adhesions, leading to impaired cell motility and weak cell–matrix adhesion, suggesting that the different in subcellular Tie-2 localization induced by Ang-1 or Ang-2 generates ligand-specific responses in the angiopoietin–Tie signalling pathway (Pietila et al., 2012; Saharinen et al., 2011). It seems that this Tie-2 distribution is also dependent by a lower oligomerization of Ang-2 (Pietila et al., 2012)

It is important to remember that Ang-1 is expressed in perivascular cells, such as pericytes, while Ang-2 is expressed in endothelial tip cells. Thus, the Ang-1 binds Tie-2 in stalk cells, associated with few pericytes, limiting the angiogenesis by inducing homomeric Tie-2 complexes across the cell-cell junctions, and thus mediating cell-cell adhesion, antipermeability, and cell survival (see paragraphs above).

Vice versa, Ang-2 regulates cell-matrix interactions in the growing vessels to facilitate sprouting.

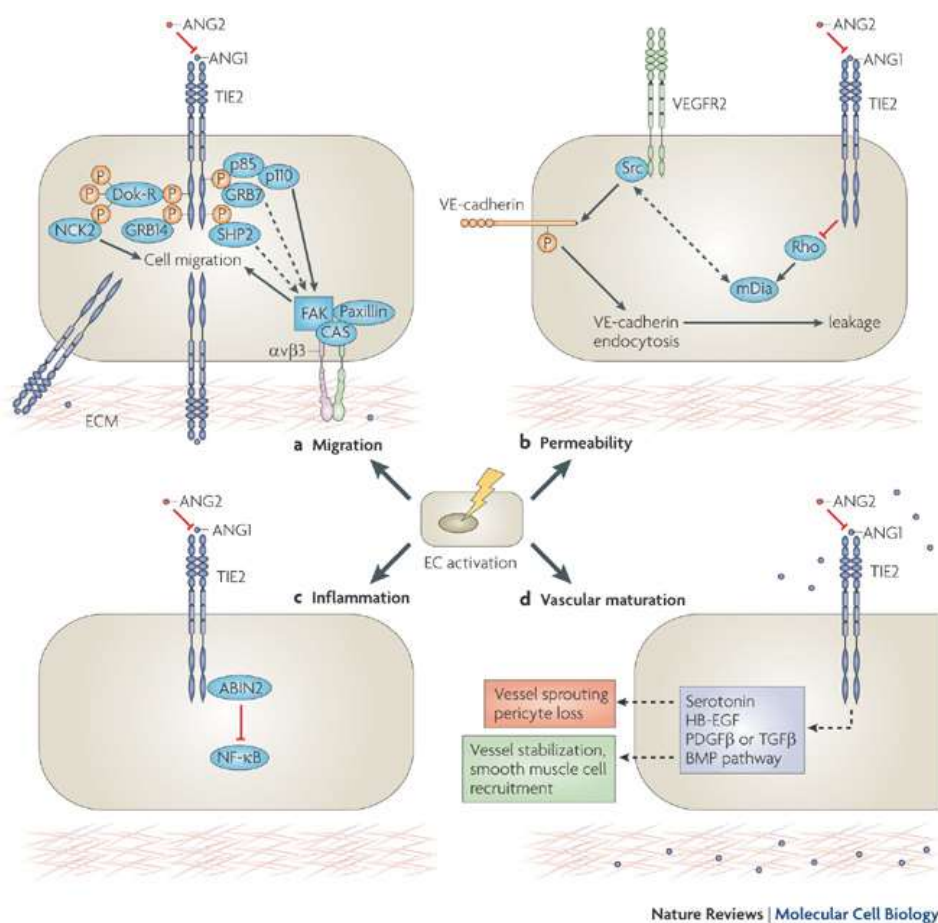


**Figure 55:** Angiopoietin (here indicated as Angpt) and Tie-2 coordination: The Angpt-Tie system in stable vessels and sprouting angiogenesis. In angiogenic blood vessel, in response to VEGF secreted by nearby hypoxic cells, Ang-2 is expressed predominantly in the tip cells of angiogenic sprouts, where it may regulate cell-matrix interactions by binding to integrins and connective tissue matrix. The Tie-2 receptor is expressed in the stalk cells, which become coated with pericytes and the basement membrane matrix that accumulates in between the cells in the stabilization phase of angiogenesis. Angpt1, from the perivascular cells, interacts with the Tie-2 receptor. In this context, Ang-1 is necessary for the stabilization of the newly formed vessels, for attenuation of angiogenesis, and for limiting the production of excess of connective tissue. From (Saharinen and Alitalo, 2011).

In activated non-contacting ECs, Ang-1 binds Tie-2 triggering ECs **migration** (Fig.54 B). The different signalling pathways of Ang-1/Tie-2 in quiescent and angiogenic process are due, as mentioned before, to the subcellular localization of the receptor, which is different in confluent versus sparse ECs. In the absence of cell–cell contacts, Tie-2 forms a complex with ECM, the ECM-bound Ang-1 (Saharinen et al., 2008), *at cell–substratum* contacts, and the formation of this complex with this localization preferentially activates the Erk pathway by inducing FAK-positive focal complex

assembly contributing to ECs migration and proliferation, thereby promoting angiogenesis (Fukuhara et al., 2008; Fukuhara et al., 2010). Thus, in other words, the different role of Ang-1 in the regulation of the balance between vascular quiescence and angiogenesis is correlated to the contacts of the ECs: in the presence of cell–cell contacts Ang-1 bridges Tie-2 at cell–cell contacts, resulting in trans-association of Tie-2. In contrast, in isolated cells, ECM-bound Ang-1 shifts Tie-2 localization at cell–substratum contacts. Furthermore, Tie-2, activated at cell–cell or cell–substratum contacts, leads to preferential activation of Akt and Erk, respectively.

Moreover, Tie-2 clustering at the cell rear was accompanied by polarized caveolin-1 location and microtubule organizing centre distribution in a manner typical of migratory cells, together with Dok-R activation (Beardsley et al., 2005).



**Figure 56:** Angiopoietin–Tie signalling during endothelial cell activation, detailed in the text (Augustin et al., 2009)

Furthermore, in activated ECs, Ang-1/Tie-2 complex can also limit the vascular **permeability** sequestering the non-receptor tyrosine kinase Src, which mediate the VEGF-induced ECs permeability. VEGF in fact activates Src which is responsible of the internalization of VE-cadherin. Ang-2, acting as antagonist, is able to counteract this mechanism (Fig. 56B).

The stabilizing role of the pathway during **vascular maturation** is not well characterized but it seems to involve Tie-2 signalling in peri-endothelial cells. In particular, possible modulatory molecules are endothelial heparin-binding epidermal-like growth factor (hB-EGF), that is implicated in SMC migration in a paracrine manner, hepatocyte growth factor (hGF), which induces SMC recruitment to

ECs, and serotonin has been recognized as Ang-1 downstream mediator of SMC recruitment to ECs (Fig. 56C).

Ang-2/Tie-2 is also implicated in vascular **pruning** in VEGF-deprived condition (Gale et al., 2002b; Korn and Augustin, 2015). As a matter of fact, in ovary model, Ang-2 showed a cyclic expression pattern correlated with waves of vessel regression (Goede et al., 1998). Moreover, Ang-2 and phosphorylated Tie-2 mRNA were overexpressed in tracheal regression during chronic airway inflammation (Tabruyn et al., 2010). Furthermore during pruning process, Tie-2 overexpression is induced by non-canonical WNT activation (Cornett et al., 2013; Korn et al., 2014).

In reality, this pathway is even more complex as described above and this complexity is well summarized in (Thomas and Augustin, 2009).

During this thesis we modulate Ang-2/Tie-2 pathway since is one of the step triggering angiogenesis sprouting. Moreover, we considered this molecule an interesting target since the specific localization of Tie-2 and Ang-2, compared to VEGF which action can affect neuronal activity (it is implicated in neuroprotection, neurogenesis and neuronal plasticity (Cao et al., 2004; Licht et al., 2011; Sun et al., 2003)).

As a matter of fact Tie-2 is considered an ECs specific receptor; recent publications showed effectively that Tie-2 has been identified in non-ECs such as in human neural stem cells (Parati et al., 2002), in neurons during the embryogenesis (Valable et al., 2003), in neuronal precursor in GD and subventricular zone (Androutsellis-Theotokis et al., 2010; Androutsellis-Theotokis et al., 2009) but not in mature neurons (Ward et al., 2005), or in non-autonomic peripheral nervous tissue (Poncet et al., 2003). Moreover, Tie-2 expression has also been reported in the nonvascular compartment of several tumour types, including leukaemia as well as breast, gastric, and thyroid cancers (Lee et al., 2006), in circulating haematopoietic cells including a sub-population of monocytes (De Palma et al. 2005) and macrophages (Fantin et al., 2010), and finally in cultured retinal pericytes isolated from bovine eyes (Cai et al., 2008) despite of the large majority of in vivo and in vitro publications did not find Tie-2 in pericytes.

#### **3.4.3.4. Hypoxia and angiopoietins: COX-2/Ang-2**

Moderate and prolonged hypoxia is able to induce an increase of capillary density at cortical levels both in mice and in rats (Benderro and Lamanna, 2011; Boero et al., 1999; Ndubuizu et al., 2010; Pichiule and LaManna, 2003). This increase is accompanied by an increase of angiogenic molecules, such as Hif-1 $\alpha$ , VEGF and Ang-2, EPO, suggesting the triggering of angiogenesis process. Moreover, synergistic expression of VEGF and Ang-2 during hypoxia is known to enhance the hypoxia induced angiogenic process (Dore-Duffy and LaManna, 2007; Pichiule and LaManna, 2002).

Different studies show that *hypoxia and Hif-1 $\alpha$  are able to increase the levels of Ang-2* in ECs (Kelly et al., 2003; Nilsson et al., 2004; Yamakawa et al., 2003b). In particular, Kelly and collaborators demonstrated that angiopoietins expression, together with PLGF and PDGFB expression, is induced by either exposure of primary cultures of cardiac and vascular cells to hypoxia or an adenovirus injection, which is able to constitutively encode an active form of Hif-1 $\alpha$  (AdCA5). Depending on the cell type, expression of angiopoietins was either activated or repressed in response to hypoxia or AdCA5. In particular, the expression of Ang-2 mRNA was increased in hypoxic ECs while decreased in hypoxic SMCs of pulmonary arteries. In all cases, there was complete concordance between the effects of hypoxia and AdCA5 indicating that Hif-1 $\alpha$  plays a major role in angiogenesis regulating the expression of genes encoding multiple critical angiogenic growth factors such as VEGF, PLGF, Ang-1, Ang-2, and PDGFB, but this expression depends on the cell-type considered (Kelly et al., 2003).

Moreover, a study performed to characterise the effects of *in vivo* hypoxia on Tie-2/Ang expression showed a clear correlation with the expression of Hif-1 $\alpha$  in different rats' organs, after 24 or 48 h of hypoxia (9-10% oxygen level). Hypoxia induces a rapid decline of Ang-1 and Tie-2 mRNA and proteins and an increase of Ang-2 mRNA (Abdulmalek et al., 2001).

Moreover as previously described, LaManna group showed an increase of CBF together with an increase of expression of Hif-1 $\alpha$ , Ang-2, Tie-2 and capillary density increased after hypoxia (Pichiule and LaManna, 2002; Willam et al., 2000; Xu and Lamanna, 2006; Xu et al., 2004). Moreover, several studies suggest a direct relation between COX-2 and Ang-2 since their induction and turnover profile is similar (Benderro and LaManna, 2014; LaManna et al., 2006; Pichiule et al., 2004). As a matter of fact a simultaneous increase of COX-2 is induced in the same hypoxic conditions following Ang-2 profile (Benderro and LaManna, 2014). Moreover, the increase of Hif and COX-2 suggests a synergic function of these proteins during the hypoxic-induced cerebral physiological responses, such as angiogenesis. Conversely, during the re-oxygenation period, while Hif-1 $\alpha$  VEGF and EPO come back to the basal levels, COX-2 and Ang-2 expressions are both maintained during hypoxic conditions. This suggests firstly that COX-2 and Ang-2 can have dual functions both in angiogenesis and in regression process of vascular plasticity. This profile is due to the fact that COX-2 is the molecule implicated in the direct regulation of Ang-2 (LaManna et al., 2006; Pichiule et al., 2004).

Cyclooxygenase is an enzyme converting the arachidonic acid in prostanoids molecules, including PGs, prostacyclin, and thromboxane. The latter are involved in different functions in the cardiovascular, gastrointestinal, urogenital, and nervous systems, in particular playing an important role in immunity, inflammation and resolution of inflammation responses. There are two more studied isoforms: COX-1 and COX-2, together a less studied third isoform, COX-3 (Gasparini et al., 2003). While COX-1 is a constitutive housekeeping enzyme that is ubiquitously expressed, COX-2 is an inducible enzyme, expressed following inflammation, infection and cancer. Moreover, COX-2 was also found to be expressed constitutively in areas not associated with inflammation, including the brain, thymus, gut, and kidney (Sasaki et al., 2004; Yamagata et al., 1993).

But how Ang-2 is upregulated?

For a long time this question was unsolved: it was clear that adaptive response to hypoxia was mediated by VEGF and Ang-2 but while Hif-1 $\alpha$  was well known to upregulate VEGF inducing the activation of HREs of different genes, the upregulation of Ang-2 was not well understood since it was discovered that it was mediated by prostaglandin E<sub>2</sub> which comes from increased endothelial cyclooxygenase-2 (COX-2) activity (Pichiule et al., 2004).

In particular it has been shown that the Hif-1 $\alpha$  inducers (such as CoCl<sub>2</sub>) did not affect Ang-2 levels indicating the possibility of existence of another hypoxia-driven mechanism in Ang-2 activation pathway. Furthermore blocking VEGFR, it has been shown that hypoxia-induced VEGF can help the Ang-2 action but it cannot account completely for Ang-2 expression. Finally, COX-2 inhibition decreases the levels of Ang-2 PGE<sub>2</sub> and PGI<sub>2</sub> and vice versa the administration of PGE<sub>2</sub> and PGI<sub>2</sub> were able to stimulate Ang-2. Moreover, in addition to this direct mechanism, as mentioned before, the Ang-2 expression can be indirectly modified by VEGF, FGF, TNF, Angiotensin II, and thrombin induce in cultured endothelial cells (Pichiule et al., 2004). Vice versa, Ang-2 can increase Hif-1 $\alpha$  activity via ROS sequestration of Fe<sup>2+</sup> availability limiting PHD and FIH functionality (Cash et al., 2007).

#### 3.4.4. Pericytes role in angiogenesis

A little parenthesis on pericytes' role in angiogenesis is necessary since the angiopoietins are expressed also in these cells (Ucuzian et al., 2010; Wakui et al., 2006).

The pericytes functions in vascular plasticity are related both to their incorporation in basal membrane, thus helping the stabilization of the neovessel, and to their role in regulating uncontrolled angiogenesis.

Pericytes are mainly recruited by ECs-produced PDGF during angiogenesis (even if Ang-1 can also participate to pericytes recruitment (Bergers and Song, 2005)), in particular via interaction of the isoform PDGF-B with PDGR $\beta$  receptor, while their differentiation seems mainly mediated by TGF- $\beta$ .

The pericytes participate to vascular stabilization; in fact, the ECs mitosis is decreased when they are associated with pericytes (Kutcher et al., 2007), via Rho GTPase activity. Moreover, it has been shown both *in vitro* and *in vivo* that pericytes express some survival factors, such as Ang-1 being associated with basement membrane formation, EC quiescence and endothelial leak resistance (Kutcher et al., 2007).

Moreover, pericytes participate to vessel stabilization limiting the branching and the migration of ECs, as showed *in vitro* via upregulation of PAI-1 in ECs (McIlroy et al., 2006). Furthermore, they produce TIMP-3 and induce the production of TIMP-2 in ECs, targeting MMP and ADAM proteinases decreasing the angiogenic potential in the surrounding environment. Finally, they participate to ECM deposition (Ucuzian et al., 2010).

The potential link between angiopoietins/Tie-2 and pericytes is underlined also by the fact that local overexpression of Ang-2 leads to pericyte loss as mentioned before, but some studies on Tie-2 null mice showed that pericytes were in any case recruited leading to think that Ang-1 and Tie-2 do not seem to be directly involved in pericyte recruitment, even if pericyte-derived Ang-1 has important roles in blood vessel formation and/or stability (Armulik et al., 2011). Moreover, Ang-2 has been found in pericytes surrounding angiogenic sprouting induced after subcutaneous implantation of a polymer disc in rats (Wakui et al., 2006).

Even if the pericytes role in angiogenesis process is not fully understood, a window of pericytes' absence marks the period vascular plasticity.

Some studies on corpus luteum suggest that pericytes participate to sprouting guidance by migrating ahead of ECs and expressing VEGF (Ozerdem and Stallcup, 2003). Moreover, since their vessel-embracing position, pericytes have been proposed in having a role in transferring angiogenic signals along the vessel length (Bergers and Song, 2005; Ribatti et al., 2011).

#### 3.4.5. Angiotensin

The angiogenic process can be also regulated by hormonal peptides that can be cleaved and thus converted in proangiogenic or antiangiogenic molecules (Clapp et al., 2009); among these the Renin-Angiotensin-Aldosterone system (RAAS) plays an interesting role in the angiogenic function, and since we used an antagonist of angiotensin receptor, namely losartan, to treat the SHRs, a little paragraph explaining its role is necessary.

The RAAS is mainly known in relation to its role in maintaining systemically and locally the control of the blood pressure and the homeostasis of body fluids. It has been a target for several drugs



developed in order to treat cardiovascular diseases, such as hypertension, renal diseases, cardiac hypertrophy and heart failure.

The proteins belonging to RAAS are the result of following enzymatic cleavage starting from the cleavage of angiotensinogen (AGT), produced by the liver, in an inactive decapeptide Angiotensin I (ANG I, different from Ang-1 which refers to Angiopoietin-1, unfortunately the abbreviations sometimes are not that clear) by the Renin (aspartyl protease) which is released by the juxtaglomerular cells of the kidney.

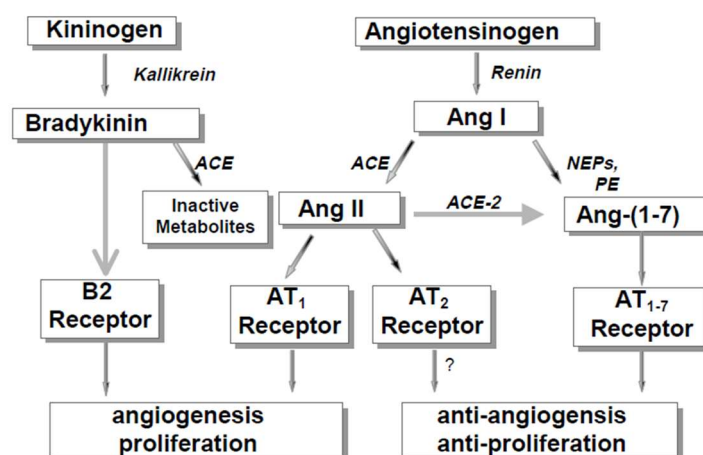
The circulating Angiotensin I is successively cleaved by angiotensin-converting enzyme (ACE) in the octapeptide Angiotensin II (ANG II, once again different from Angiopoietin-2, Ang-2), produced primarily in the pulmonary circulation.

ANG I and ANG II can be cleaved by other endopeptidase, such as neprilysin, prolylcarboxypeptidase (PrCP), and angiotensin converting enzyme-related carboxypeptidase (ACE2), producing the heptapeptide ANG-(1-7) or the nonapeptide ANG-(1-9).

ANG II binds two G protein-coupled receptor subtypes, namely AT1 and AT2, that both are abundantly expressed in the vasculature (Ribatti et al., 2007).

AT1 receptors are ubiquitously expressed and they are responsible for most known actions of ANG II, including vasoconstriction, aldosterone and vasopressin release, renal sodium and water reabsorption, sympathetic activation, augmented cardiac contractility, smooth muscle cell proliferation, vascular and cardiac hypertrophy, inflammation, and oxidative stress(Clapp et al., 2009).

In contrast, AT2 receptors are limited to brain, kidney, adrenals, uterus, ovary, and the cardiovascular system, and their activation leads to vasodilation, lower blood pressure, reduced cardiac and vascular hypertrophy, anti-inflammation, and suppressed growth, tissue repair, and apoptosis.



**Figure 57:** RAAS pathway and their implication in angiogenic pathway. From (Sane et al., 2004).

The RAAS opposite actions in blood flow homeostasis is reflected also in their angiogenic actions.

Concerning the role of ANG II in angiogenic response, there is a large debate since several publications showed opposite results.



As a matter of fact, the proangiogenic or antiangiogenic action of ANG II depends on the activation of different receptor subtypes and their opposite outcomes, the specific tissue, the animal model obtained, the physiological or pathological conditions, the kind of diseases studied and the proteolytic conversion of RAAS components (also ANG-(1–7) and des[ANG I]AGT influence angiogenic process) and reviews summarizing these results are (Clapp et al., 2009; Ribatti et al., 2007).

ANG II seems to have proangiogenic actions inducing vascular proliferation in several models used (chick embryo chorioallantoic membrane, the rabbit cornea, and the Matrigel model in mice) mainly via interaction with AT1, reviewed in (Clapp et al., 2009).

In fact, the block of AT1 reduces ANG II angiogenic effects (mouse Matrigel model, the rat ischemic hindlimb, and the electrically stimulated rat skeletal muscle); this can be due to the inhibition of ANG II-induced upregulation of VEGF, VEGFR2, Ang-2, and Tie-2 in endothelial cells and others angiogenic molecules (NO, bFGF, PDGF, IGF-I, EGF, HGF and TGF), reviewed in (Clapp et al., 2009) and (Ribatti et al., 2007). Vice versa, ANG II seems to have antiangiogenic action via AT2 receptor. In cultured ECs this action seems to be due to the inhibition of VEGF-induced endothelial cell migration and tube formation (Benndorf et al., 2003).

Interestingly for our work, it was shown that the inhibition of endogenous ANG II production or action, obtained via both ACE inhibitors and AT1 and AT2 blockers, including Losartan, stimulates angiogenesis in vivo (Walther et al., 2003). In fact, in this study, it was shown that, beside the inhibitory AT1 receptor function, these molecules promote an additional stimulatory AT2 receptor function, leading to angiogenic stimulation.

However, the contribution of each receptor subtype to the antiangiogenic effect of ANG II appears to depend on the chosen angiogenesis model.

### **3.4.6. Conclusion**

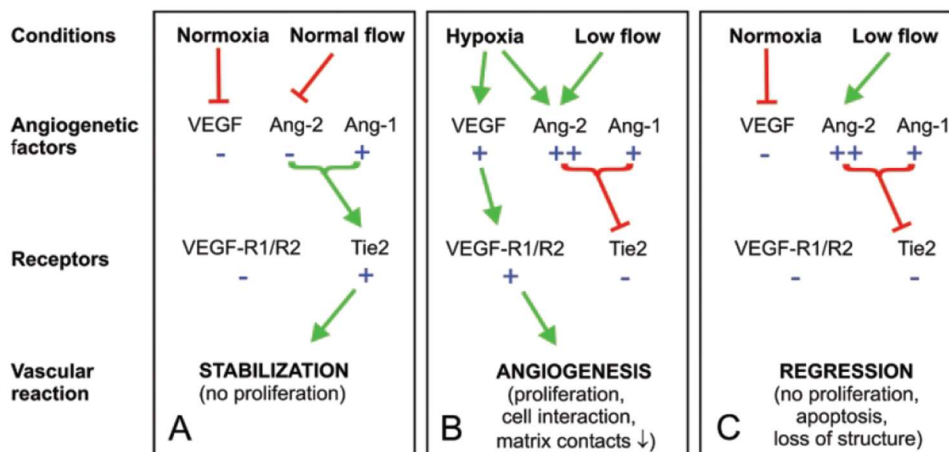
In conclusion, this chapter describes just a little part of the plethora of mechanism involved in angiogenesis process. In fact, the structural response of vascular network is determined not only by all the molecules involved and their combinations in angiogenic modulation, but also by changes in PO<sub>2</sub> and wall shear stress.

Anyway the most accepted version is that the angiogenic process is mainly influenced by the combined effect of VEGF and Ang-2/Tie-2 systems, which presence or absence drives different reaction patterns including stabilization, angiogenesis and regression, in combination of oxygen level and flow stress (Hanahan, 1997).

In a model proposed by (Hanahan, 1997) and reconsidered by (Zakrzewicz et al., 2002) it has been proposed that during normal conditions the VEGF and Ang-2 expressions are suppressed by respectively normoxia and CBF; the resultant action of Ang-1 on Tie-2 induces stabilization of the vascular network ruling the normal quiescence period in ECs (Fig. 58A).

When hypoxia and decrease of blood flow are detected, a huge signalling is triggered in order to re-establish the neuronal functional condition, leading to a modification of vascular reactivity together with the stimulation of angiogenic machinery; the result is the induction of VEGF and Ang-2 expressions and thus the promoting effect on angiogenesis: Ang-2 blocks the stabilizing effect of Ang-1 leading to pericytes dissociation and allowing the VEGF driving action in cellular proliferation and differentiation (Fig. 58B). When the normoxic condition is re-established, thanks to vascular

reactivity modifications and the newly formed vascular network, VEGF is suppressed. In the non-perfused vessels, Ang-2 can exercise its role in vascular regression (Fig. 58C).



**Figure 58:** Schematic representation of the key actors of angiogenesis to wall shear stress and partial pressure of oxygen. From (Zakrzewicz et al., 2002).

This schema underlines the fundamental role of Ang-2 in driving the angiogenic process: Ang-2 is converging point of angiogenesis pathway and its presence allows us to identify the angiogenic process since the initial phase.

These characteristics prompted us to choose it as preferential marker of angiogenic process and as target to modulate specifically the angiogenic pathway, also thanks to its specific role in ECs, compared to VEGF which action was shown to influence neuronal activity.

### 3.5. Vascular network and memory

To complete our excursus on the physiological angiogenic pathway we would like to mention some bibliographical notions underling the role of vascular network in memory process.

Several studies have been published concerning neurovascular impairment and cognitive dysfunctions both in case of neuronal damage, such as cerebral lesions (Armstead and Raghupathi, 2011), dementia and Alzheimer disease (Sagare et al., 2012), and in case of vascular dysfunction, like cerebrovascular injuries (Bastide et al., 2007), and vascular dementia (Carmeliet, 2003). Thus, even if the mechanisms are not always completely clear, it is an accepted opinion that the **vascular damage impairs cognitive functions**.

For example, memory deficit after stroke is a common phenomenon: close to 30% of stroke patients develop dementia within 1 year from stroke onset and this affects cognitive functions like attention, memory, language, and orientation. The most important post-stroke dementia is the vascular dementia, reflecting the link with cardiovascular disease (Al-Qazzaz et al., 2014). Another interesting review exploring the link between dementia and vascular dysfunction is (Iadecola, 2013)).

Moreover, concerning the blood pressure dysfunctions, it has been shown in human patients that age-related high BP reduces executive function and the processing of information (Bucur and Madden, 2010). Moreover long-lasting hypertension can induce Alzheimer's disease symptoms (Carnevale et

al., 2012; Carnevale et al., 2016; Glodzik et al., 2014; Kilander et al., 2000). This subject will be successively treated in the part concerning hypertension in the next chapter.

Also the age can impact memory functions via decrease of vascular density and plasticity as showed in the rat hippocampus (Ingraham et al., 2008). This study showed that, compared to young rats 4-month-old, 30-month-old rats had no difference in term of hippocampal vascular density at basal level, but they showed a significant impairment in hypoxia-induced capillary angiogenesis, lacking in vascular remodelling that can functionally sustain neuronal processing.

Others publications showed that Alzheimer's disease (AD), the most common type of dementia (nowadays AD is increasingly grouped together with vascular dementia (Iadecola, 2013)), is characterized by a decrease of CBF and vascular density in AD patients, often anticipating cognitive dysfunctions (Farid et al., 2011).

Moreover, a mice model of chronic cerebral hypoperfusion, induced by narrowing bilateral common carotid arteries, showed spatial memory deficit 1 month after surgery, accompanied by morphological changes at the neuronal, vascular and synaptic levels. In particular, this model reproduces a BBB disruption, neuronal apoptosis, axonal abnormalities, glial activation, BBB damage, amyloid deposition, and cognitive dysfunction, as in human AD (Wang et al., 2016b).

Furthermore, it has been shown that, together with the cerebral hypoperfusion, a deposition of amyloid  $\beta$ -peptide in cerebral vessel walls enhances the cognitive deficits associated with AD, probably via hypoxic dysfunctions (Dotti and De Strooper, 2009). Finally, statins administration restores memory deficit in mice model of Alzheimer tested in Morris Water Maze, via restauration of vascular reactivity (Tong et al., 2012).

More correlated to the purpose of this thesis, some studies interestingly showed that the restoration of vascular functions via **angiogenesis**-promoting treatments can re-establish, at least partially, the **memory deficit**: in particular Wang and collaborators showed that the intraperitoneal injection of VEGF for three consecutive days was able to ameliorate memory performance, tested in the Morris Water Maze paradigm, in a genetic model of Alzheimer disease mice. According to this study, the restoration of memory performance was due to the increase vascular network via angiogenesis since vascular proliferation and increase of vascular density was detected, counterbalancing the deficit of CBF detected in Alzheimer's patients (Wang et al., 2011). In any case, the neuronal effect of VEGF can bias memory results, since Zheng et al. also showed a neuroprotective VEGF effect after intraperitoneal injection in a mice model of amyotrophic lateral sclerosis (Zheng et al., 2007). VEGF is considered a neuroprotective factor and its action at neuronal level cannot be excluded in this study and in the other works described below (Mackenzie and Ruhrberg, 2012; Pati et al., 2009; Shen et al., 2016).

Another study demonstrated that the genetic upregulation of neuronal VEGF in a mice model of Alzheimer's disease was able to re-establish the memory decrease detected in T-maze task, possibly promoting the survival of cerebral ECs from the apoptosis often occurred in this pathology (Religa et al., 2013).

In the same way, the stimulated expression of VEGF and VEGFR induced by systemic pharmacological treatment with DL-3-n-Butylphthalide (Zhang et al., 2012) or EPO (Xiong et al., 2011) increases memory performance in ischemic spontaneously hypertensive rats tested in Morris Water Maze (Zhang et al., 2012) or rats underwent to traumatic cerebral lesion performing (Xiong et al., 2011). Once again, in Xiong study it has been proved that EPO treatment promoted memory recovery together with increased cell proliferation, angiogenesis and neurogenesis, thus it is difficult to understand whether the EPO/VEGF effect on memory was induced by neurogenesis, angiogenesis

or both. The same can be said for Zhang: the stimulation of VEGF together with the DL-3-n-Butylphthalide, which moreover protects cells from apoptosis, can represent a bias in the interpretation of memory rescue induced by angiogenesis.

If the literature about pathological conditions is abundant, the physiological and functional role of cerebrovascular network during cognitive and memory functions are less explored, in particular if related to angiogenesis.

In total, if you look for “angiogenesis and memory”, at least in the moment of the thesis writing, 271 results has been found and the almost the totality of them are related to pathologies, while just 2 results has been identified about “**angiopoietins and memory**” and both of them regard pathological situations.

The first study is about an alteration of angiogenic markers, in particular a decrease of Ang2, in Parkinson Disease patient with or without dementia, associated with BBB dysfunction, white matter lesions, and cerebral microbleeds (Janelidze et al., 2015).

The second study demonstrated that mice receiving an intracerebroventricular infusion of cord blood-derived endothelial colony-forming cells (Endothelial progenitor cells) after traumatic brain injury could repair the blood-brain barrier (BBB) and promote angiogenesis together with improvements in motor ability, spatial acquisition and reference memory tested by Morris Water Maze (Huang et al., 2013).

The literature exploring the **physiological angiogenesis and vascular functionality in memory** is limited. Anyway there are some studies demonstrated that rats exposed to enriched environments show an increase in cortical vascular network, via angiogenic mechanism, in order to compensate the increase of metabolic demand of neurons (Black et al., 1987; Black et al., 1991; Isaacs et al., 1992; Palmer et al., 2000; Wallace et al., 2011).

Greenough's group underlined that rats exposed to enrich environment, where the conspecifics and the toys were regularly changed, presented a higher vascular density in occipital visual cortex compared to the littermates living in classic home cage (Black et al., 1987).

According to this paper, the expanding cortical neuropil volume (not detectable after 10 days but clearly increase after 30 days) and the consequent increase of distance between the vessels and mitochondria can dilute the metabolic support for neurons. This situation triggers an increase of vascular network via angiogenic process (indirect evidence considering the CBF, the vessels diameter and the distance between capillaries) in order to decrease the vascular distance. This angiogenesis is accompanied by an increase of neuronal activity (increase of new synaptic connections and dendritic spines are induced by enrich environment) and metabolic needs (increase of mitochondria volume) (Black et al., 1991). The neuropil network increases after enrich environment, complex experiences or memory process is demonstrated in other paper such as (Beaulieu and Colonnier, 1989; Jones et al., 1997; Lesburgueres et al., 2011)).

Even if in the first 10 days of enrich environment the diameter of vessels were decreased, reflecting the formation of smaller new vascular network, after 30 days the vascular diameter was higher compared to home cage (Black et al., 1991).

Another group of rats which lived in enrich environment for 30 days and successively placed in home cages for other 30 days showed a decreased in cortical thickness indicating that not all the neuropil expansion is maintained; in this case the vascular distance decrease, but we cannot know if the vessels produced were preserved because the vascular density was not recorded, as we said before was an indirect measure of distance between capillaries and vascular diameter (Black et al., 1991).

In any case Greenough's group demonstrated that, while the angiogenic process is vigorous in 1/3-month old rats, the angiogenic response markedly decreased in 10 month old rats, even if an increase of cortical thickness was detected. In these rats no changes in vascular density was detected, but they showed a decrease of vessel diameter. This has been proposed to be due to the fact that the middle-aged animals were still installing immature vessels after 50 days, while the young adult (60 days old) animals finished the task in just 30 days. In contrast, 2 years old rats did not show either neuronal or vascular changes after enrich environment, suggesting an age related effect on cortical plasticity (Black et al., 1991).

Furthermore, in another study performed by the same group few years later, the neurovascular status was compared in female rats after 1 month of either physical repetitive exercise or complex motor learning, compared to rats maintained in inactive condition (Isaacs et al., 1992).

This study showed that in both cases the vascular network was increased. While the physical exercise group did not show a change in neuropil size, the motor learning group showed an increase of the volume of the molecular layer of the paramedian lobule.

The angiogenic creation of new vessels in these group was indirectly measured: in exercise rats the angiogenic answer was measured as decrease of vascular diffusion distance compared to both inactive and motor learning groups; since in learning rats there was expansion of molecular layer but an equivalent diffusion distance compared to inactive rats, the authors considered that these rats "must have added new blood vessels".

Thus, the angiogenic answer in physical training rats was associated with the increased metabolic demands, while the angiogenesis during complex learning was also induced by an increase of neuronal neuropil, showing that angiogenesis can occur in response to activation in the absence of changes in synapses in adult rat cerebellar cortex.

More than 20 years later, with more developed techniques, Wallace showed that the increase of vascular network, induced by enrich environment via angiogenic process, was preceded by an increase in neuronal and astrocytes growth (Wallace et al., 2011).

In particular, the density of astrocytic process and the thickness of visual cortex were increased after 4 days of enrich environment, while angiogenic process seemed to be detectable only successively, after 10 days. Thus, even the angiogenic process was not detectable as fast as the neuronal/astrocytes increase, it was not possible to exclude that a potential slight and localized angiogenesis could previously occur. Anyway, thanks to neurovascular coupling and the tripartite synapsis, it is possible that astrocytes can mediate the onset of angiogenesis in response to neural demand. Anyway, interestingly for us, this paper suggests that vascular functions can even limit the neuronal plasticity phenomena.

Another similar study showed an overlap of time course between neuronal and vascular plasticity localized in angiogenic niches at hippocampal level (Palmer et al., 2000). Female rats were injected with BrdU and allowed to survive up to 1 month before sacrifice. The examination of hippocampal structure, area of active proliferation known to generate new neurons throughout adult life, showed neuronal dividing cells associated with ECs, creating a cluster. In other words the neurogenesis was physically and temporally associated to angiogenesis in these zones.

Thus, these publications point out that memory process, associated to neuronal plasticity, can be accompanied by a vascular plasticity.

To segregate the functional contribution of angiogenesis and neurogenesis in memory process after physical exercise, Kerr and collaborators tried to block pharmacologically either neurogenesis or

angiogenesis in rats physically trained for 7 days, and test their visual spatial memory in Morris Water Maze (Kerr et al., 2010). Neurogenesis inhibition with AZT did not affect the increase of memory performance induced by exercise, whereas the treatment with SU5416, VEGFR-2 antagonist, induced a deficit in acquisition and retrieval of both recent and remote memory. But the use of a VEGFR-2 antagonist to block angiogenesis could be criticized since the absence of modification of the vascular network. Moreover, the inhibition of VEGFR-2 could also affect the neuronal plasticity. Anyway, this publication suggested that angiogenesis could be essential to sustain memory but the target used to modulate angiogenesis should be very vessel specific without effect on neurons. Thus we proposed in our study to use Ang-2 as this angiogenesis target.

In any case this study underlined the idea that the acquisition of a new memory, its retention and its retrieval can induce vascular modifications: these modifications regard not only the dynamics of vascular flow but also structural changes of vascular network.

Ambrose proposed that the neuroangiogenesis was an essential phenomenon during all the adult life, in order to adapt the microvessels to cortical functionality (Ambrose, 2012). As a matter of fact, during the time, the cognitive and memory impairments observed in elderly people have a common denominator: the decrease of angiogenesis. But are we sure that adult angiogenesis is possible?

The Ambrose's point of view was corroborated by Harb's study which interestingly followed the vascular changes in adult brains, using in vivo biphoton microscopy. Using this technique it was possible to follow the structural changes of vascular network from an early postnatal period to the advanced study of ageing (Harb et al., 2013).

Without surprise, postnatal mice showed a wide increase of sprouting, ECs proliferation and elongation, followed by an abundant increase in vessels pruning and sprouts elimination in order to refine the vascular network. Despite a clear microvascular remodelling decreasing over the mice life, it has been shown interestingly that in adult brain vascular plasticity was still present, including vascular regression and formation, representing the response to metabolic changes allowing the optimal neuronal functionality. Moreover, in young adult mice (3 month old) the vascular changes were increased by hypoxia (10% during 1 month), while this response was reduced in older mice starting from 4 months.

The Ambrose's and Harb's contributions underlined a possible functional role of vascular plasticity in cognitive process, accompanying neuronal adaptations.

Moreover, angiogenesis and neurogenesis coexist showing common molecular pathways (Madri, 2009; Palmer et al., 2000; Yamashima et al., 2004), showing a potential implication in memory process.

In fact, the contribution of vascular modifications and remodelling during memory consolidation is surprisingly not investigated.

The studies presented in this paragraph let us to hypotheses that memory consolidation can be accompanied by both a vascular architectural remodelling and a functional role of cerebrovascular network. Of course both these aspects are interesting, but for the moment we decided to focus our attention on the architectural changes induced in particular by angiogenesis.

In our study, we tried to unravel the still unknown contribution of vascular network during memory consolidation, considering physiological conditions (adult rats). Moreover we decided to corroborate our finding considering two models of vascular dysfunctions, such as hypertension and gravity changes. These deleterious conditions are known to alter vascular functions. The goal of our study, anyway, is not to study what are the perturbations of vascular network inducing the putative memory

deficit (despite the topic is very interesting and too wide for a thesis of 3 years), but we want to use these models as tools to underline the permissive role of vascular network in memory consolidation process.

Thus, to conclude this introduction we are going to briefly present hypertension and gravity changes to check their effects on vascular physiology. In particular, we are going to present the models that we choose, respectively the Spontaneously Hypertensive Rats (SHRs) and the hypergravity model, and the rational for their application in our study.

## **Chapter 4: The Arterial Hypertension (AHT)**



## **Chapter 4: The Arterial Hypertension (AHT)**

Among vascular diseases, arterial hypertension (AHT) is a current relevant medical care problem for different reasons. First of all, AHT is one of the most widespread human diseases in developed country; according to WHO (World Health Organization, 2013), close to 1 billion people suffer from hypertension and at least two-thirds are in developing countries. Since the increase of population's growth and ageing, the number of people with uncontrolled hypertension rose from 600 million in 1980 to nearly 1 billion in 2008, and the problem is growing: in 2025 it has been estimated that hypertensive adults will rise up to 1.56 billion. In France, more than 30% of the population is considered hypertensive (InVS studies, 2008). Hypertension is one of the most worldwide important causes of premature death and the worldwide leading cause of cardiovascular disease. Globally, cardiovascular diseases account for approximately 17 million deaths a year, nearly one third of the total and hypertension is responsible for at least 45% of deaths due to heart disease, and 51% of deaths due to stroke. AHT is one of the most important risk factors both in coronaropathy and in cerebrovascular accidents; it can induce directly congestive cardiac failure, aortic dissection and chronic renal failure (Danaei et al., 2009).

Secondly, this pathology is frequently discovered occasionally since the onset is mostly asymptomatic, inducing the WHO to call it the "silent killer", and many people do not realize they are suffering from AHT; for this reason, it is important to get blood pressure checked regularly, even in youngest patients who are not aware of own pre- or hypertensive status (only 23% of 15-44 year-old of tested people are aware of their hypertension status compared to 55% of 54-60 year-old people).

Moreover, the choice of medication and the patients' adhesion to the treatment is a critical step for the antihypertensive therapy. The several treatments of hypertension should be chosen according to the blood pressure profile, and often is required a combination of drugs. Moreover, one of the most important causes of therapy fail is the inconstancy in treatment following, since once the hypertension is diagnosed the therapy continues for the whole life.

While the causes of secondary AHT are well defined (nephropathy, renal vascular pathology, aortic coarctation, endocrine imbalance and iatrogenic), the causes of essential AHT (90-95%) are unknown (idiopathic and primitive), but it has been related to causal factors as the increase of sympathetic tone, the decrease renal capacity to sodium elimination, genetic factors, diet and life style (stress, sedentary). The AHT risk factors include age (> 55 for men and > 65 for women), smoke, dyslipidemia, familiarity for cardiovascular event in early age, obesity.

In most cases, AHT is contained between moderate levels for several years, being compatible with long survival rate, unless clinical complications mentioned before. Just in the 5% of cases, the blood pressure rapidly increases and induces death after 1-2 years the onset (malignant hypertension).

### **4.1. Blood pressure and regulation**

The blood pressure is a complex parameter, determined by the interaction of genetic, ambient and demographic factors that bring to an alteration of physiological regulation of blood pressure.

The blood circulation is strictly dependent to heart contraction and is defined by a quantitative parameter that is the heart frequency (number of beats/minute) and a qualitative characteristic that is the rhythm (defined as regularity of heartbeats). The blood pressure is produce by the blood flux,

pumped by the heart, through the vessels, and it is the difference between the pressures exerted by the blood on vessels wall and atmospheric pressure.

The arterial blood pressure ( $P_A$ ), often simply called blood pressure (BP), is defined in a range including a maximal pressure corresponding to systolic pressure and the lowest value representing the diastolic pressure. Systolic pressure is the blood pressure applied on arterial wall resulting from heart systolic contraction, when all blood contained in ventricles is pulsed within the vascular system. The diastolic pressure is the residual pressure in the arteries during heart diastolic relaxation.

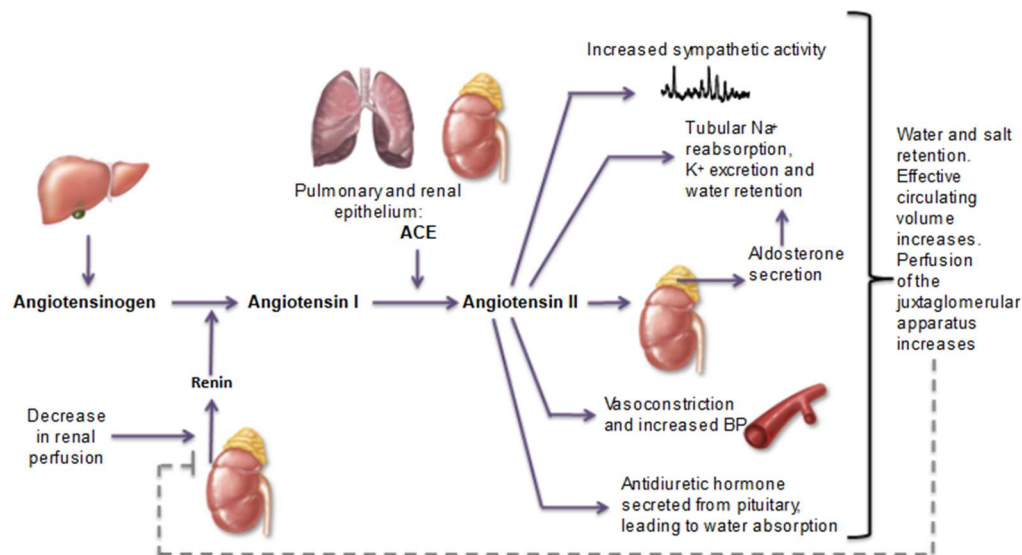
Even if there is not a predefined value of arterial pressure, diastolic and systolic pressures correspond to respectively 80 mm Hg and 120 mm Hg, even if they can fluctuate according to the circadian rhythm and social factors; AHT is defined when diastolic pressure is constantly higher than 90 mm Hg or the systolic pressure is constantly superior to 140 mm Hg (Chobanian et al., 2003).

The blood pressure depends from two fundamental hemodynamic variables: cardiac output and peripheral resistance. The cardiac output is influenced by hematic volume, which in turn it depends from sodium homeostasis. The peripheral resistance depends mainly from arteriolar resistances, related to the vascular lumen dimensions; this parameter hangs on the thickness of arteriolar wall and nervous and hormonal inputs that can exert vasoconstriction or vasorelaxation.

Briefly, the blood pressure can be regulated in short and long term.

The short term regulation includes baroreceptors activity and leads to fast modifications of BP: the increase of pressure stimulates baroreceptors with attenuation of the sympathetic outflow and resulting prevalence of parasympathetic system (negative chronotropic and dromotropic effects) modulating the peripheral vessels and the heart functions while, vice versa, a decrease in  $P_A$  unloads the baroreceptors and leads to increased sympathetic outflow, vasoconstriction (mediated by  $\alpha_1$  receptors on SMC), and increased cardiac output (mediated by  $\beta_1$ -receptors of heart resulting in positive chronotropic and dromotropic effects); for a review concerning the physiological baroreceptor reflex and its impairment in AHT see (Kougias et al., 2010)).

Regarding the long term regulation of blood pressure, the fundamental actor is the RAAS system, even if the system is much more complicated than described here. Briefly, the decrease of BP and consequently the kidney's blood flow induces an increase in renal production of renin which, in turn, increases the transformation of angiotensinogen in Angiotensin II (see previous chapter). Angiotensin II induces the increase of vascular resistance and the consequent increase of vascular pressure; moreover, it induces the secretion of aldosterone hormone, which increases  $\text{Na}^+$  and water retention with the consequent increase of blood volume and then blood pressure; furthermore it possesses a diuretic effect (Fig. 59).



**Figure 59:**RAAS control of blood pressure (Schrier RW, 1997.)

Since the regulation of blood pressure is not the core topic of the thesis, we decided to not develop this part, anyway for interesting review see (Atlas, 2007; Manrique et al., 2009; Mitchell and Navar, 1989; Montani JP, 2004).

In the following part we just mention the treatments commonly used to treat this pathology and describe what are the consequences of AHT on cerebral network affecting vascular architecture, BBB functions and vascular reactivity; after that, we analyse the relationship of AHT with cognitive dysfunctions and in particular with memory process; finally, we expose the most used animal rodent models reproducing this pathology, motivating our choice in choosing the SHR model.

## 4.2. Treatments

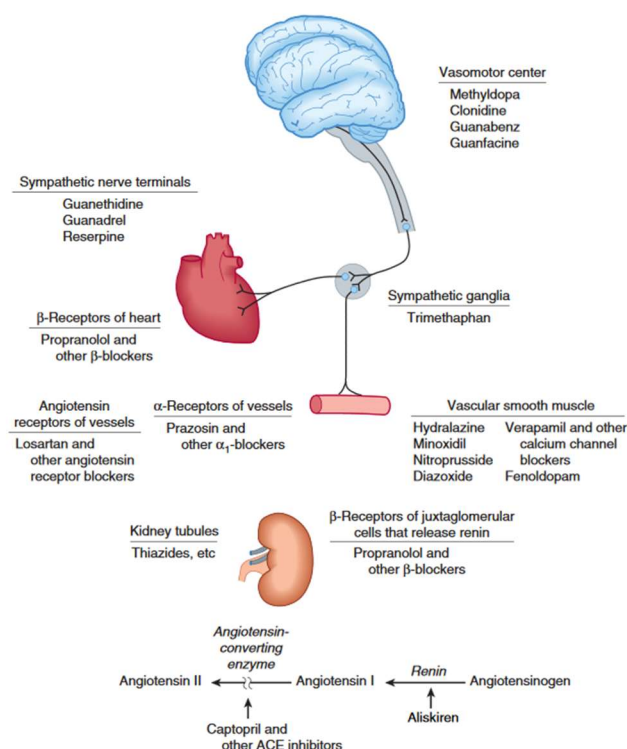
Nowadays, the most used antihypertensive drugs to treat AHT are the diuretics,  $\beta$ -blockers, central sympatholytic agents, calcium channel blockers, direct vasodilators, ACE-inhibitors and AT-1 blockers (Fig. 60).

The choice of the treatment is very important and, in the majority of the cases, more than a single drug is used efficiently to control the BP decreasing the dosage of drugs and thus the side effects related to them. The multiple therapies are also adopted in order to differently target the actions on cardiovascular systems: some drugs act on cardiac system, modifying the cardiac frequency or the cardiac output, and some others influence the vascular system, decreasing the peripheral resistance, the plasmatic volume and the RAAS pathway.

The diuretics increase the urine flow and  $\text{Na}^+$  excretion and are often used to restore quickly the volume or the composition of body fluids. The  $\beta$ -blockers (propranolol is the progenitor) can be divided in different classes depending the receptor selectivity (the most used are  $\beta_1$  blockers) but they mainly act reducing the cardiac frequency, the atrioventricular conduction and reducing and renin release. The central sympatholytic agents such as clonidine and  $\alpha$ -metildopa reduce the efferent input from vasomotor centre. The calcium channel blockers are used to block the  $\text{Ca}^{2+}$  influx in VSMC. Some of them induce vasodilation of resistance arteries (nifedipine, amlodipine), and some others show ionotropic, chronotropic and dromotropic negative actions (verapamil). The direct vasodilators

such as minoxidil, sodium nitroprusside and hydralazine are not frequently used since their side effects and the difficulty of dosage.

Finally, the ACE inhibitors (the progenitor is captopril) and AT-1 blockers (losartan is one of the most used AT-1 antagonists) modify respectively ANG II production and action. ANG II modifies the peripheral resistance inducing direct vasoconstriction, increase on  $\text{Na}^+$  reuptake, increase sympathetic system and release of catecholamine inducing a fast response in BP regulation. Moreover, ANG II can exercise a slow regulation of BP modifying renal functionality via  $\text{Na}^+$  reuptake, aldosterone release and sympathetic system. Furthermore, it seems that ANG II is involved in cardiac remodelling inducing cardiac hypertrophy.



**Figure 60:** Sites of action of the major classes of antihypertensive drugs. From (Katzung, 2012).

### 4.3. Effect of AHT on cerebral circulation

AHT is associated to different changes of cerebral circulation.

The hallmark of AHT is the increase of peripheral vascular resistances and this is the results of the alteration of the local structure of the vessel (narrowing of vascular lumen as results of vascular wall hyperthropy or lumen diameter decrease) and the modification of vascular network architecture (vessels rarefaction) and functionality (alteration of vascular reactivity, BBB dysfunctions and neurovascular coupling impairment).

*Changes in vascular architecture:*

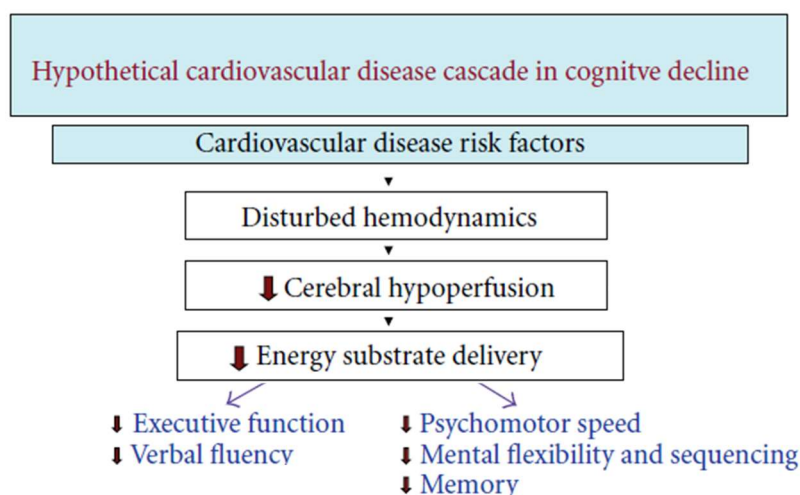
- *Vascular rarefaction:*

Microcirculation has an important role in the pathophysiology of hypertension. Peripheral resistance is determined primarily by small arteries (150–300  $\mu\text{m}$  diameter) and arterioles (10–150  $\mu\text{m}$  diameter).

The disappearance of capillaries and pre-capillary arterioles, a phenomenon known as microvascular (capillary) rarefaction, is a hallmark of hypertension (Humar et al., 2009). Moreover, hypertension induction in rat models provokes endothelial damage, followed by rarefaction within 3 days.

The phenomenon of microvascular rarefaction induced by AHT was linked either to arterioles/capillary occlusions, or to an effect of autoregulation due to the increase of pressure and the consequent prolonged vasoconstriction (Sokolova et al., 1985). Since the majority of the total vascular resistance is regulated by vessels that are less than 150  $\mu\text{m}$  in diameter, the vascular rarefaction could contribute to increased vascular resistance (Sane et al., 2004). A global vascular rarefaction is a phenomenon common to several models of AHT, such as SHR, DOCA-salt rats and renal AHT, with some differences concerning which kind of vessel (capillary vs pial artery) is affected in the different models.

The loss of capillary network and the consequent hypoperfusion can be the link between AHT and cognitive disease or vascular dementia in humans (Fig. 61) (de la Torre, 2012), even if artery rarefaction has not been reported; some studies demonstrate that chronic cerebral hypoperfusion in mice (Miki et al., 2009) and rats (Otori et al., 2003), via common carotid artery ligation or stenosis, induces white matter lesions that can be proportional to the severity of hypoperfusion (Shibata et al., 2004) and the cognitive deficit (Miki et al., 2009). Furthermore, in a mice model of small vessel disease (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)), it was shown that the rarefaction of capillaries in white matter is correlated to hypoperfusion and white matter lesion (Joutel et al., 2010).



**Figure 61:** Hypothetical model explaining how disturbed hemodynamic flow patterns inducing cerebral hypoperfusion and cognitive impairment. Modified from (de la Torre, 2012).

- *Vascular wall remodelling:*

The BP increase associated to AHT impacts the structure of vascular network and artery morphology (Mulvany, 2012; Pires et al., 2013) and the reduction of vascular diameter is associated to the increase of vascular resistance. The remodelling process can be eutrophic (increase of media-to-lumen or wall-to-lumen ratio without affecting in wall-cross section area), hypertrophic (increase wall area) and hypotrophic (decrease of wall area and wall-to-lumen ratio). The remodelling can interests the SMCs which increase their volume and it is accompanied by accumulation of ECM proteins, such as collagen and fibronectin, in the vessel wall (Faraco and Iadecola, 2013).

In AHT, this artery remodelling is a mechanism adapted by our organism in trying to reduce the stress created by the increase of blood pressure and in protecting the downstream vessels; thus, arteries increase their thickness and this process is often associated to decrease of lumen diameter and increase of wall-to lumen ratio.

The vascular remodelling in AHT can be linked to physic stress (hemodynamic effect) but also to the activation of RAAS system and dysregulation of  $\text{Cl}^-$  channels.

The increase of wall thickness induced by physic stress, which tries to protect vessels by BP increase, affects the hemodynamic regulation: despite some studies showed that the resting blood flow in AHT patients is not really altered, others researches demonstrated that old patients or not-well treated patients showed a reduction in CBF, especially in cerebral regions as occipitotemporal and prefrontal cortex and HPC (Pires et al., 2013).

The RAAS system is implicated in the long term regulation of BP (see above). Interestingly, the BP decrease obtained using  $\beta$ -blockers is not sufficient to ameliorate arteries' wall, despite they reduce the plasma levels of renin and thus decrease the production of ANG II (Blumenfeld et al., 1999), while the administration of ACE-inhibitors or AT1-blockers, drugs respectively blocking Angiotensin II production and action (see paragraph "Angiotensin"), in SHR is able to reverse the effects of hypertension on vascular structures, even if the mechanism subtending this result is not really clear.

The mechanism exercised by RAAS inhibitors mediating the restoration of vascular wall is linked both to the fact that ANG II and aldosterone are implicated in vascular remodelling via reactive oxygen species (ROS) production (Touyz et al., 2003), and to RAAS activation of MMPs (Galis and Khatri, 2002), responsible to movement and reorganization of SMCs (Pires et al., 2013).

- *Atherosclerosis and Small Vessels Disease*

The AHT is the leading factor for the atherosclerosis. It was reported that the rise of 10 mm Hg increases by 43% the chance to develop aortic atherosclerosis (Faraco and Iadecola, 2013).

The BP increase-induced perturbation of vascular architecture provokes the formation of arteriosclerotic lesions, in particular at the level of arterial bifurcations and sites of turbulence flow that can cause stroke via embolism and haemorrhage.

This mechanism is related to shear stress-induced expression of innate immunity receptors, macrophages, monocytes and inflammation (Sakamoto et al., 2001). Moreover, AHT is correlated to arteriosclerosis inducing SMCs loss, deposition of fibro-hyaline materials, lumen narrowing and vessel thickening (Faraco and Iadecola, 2013).

- *BBB breakdown:*

The BBB is a selective, physic, semipermeable and biochemical barrier that maintains the cerebral homeostasis.

It was shown that during AHT there is an increase of BBB permeability and a consequent decrease of homeostasis regulation, which can be attenuated with ACE inhibition (Nag and Kilty, 1997) in a rat model of chronic renal hypertension. BBB disruption was associated to vascular remodelling and inflammatory phenomena triggered; in fact, during remodelling the arteries are more dynamics, showing lumen reduction and increase of permeability due to an alteration of gap junctions.

Paradoxically, in this initial situation, hypertensive animals are more resistant to acute increase of BP, but not to chronic one (Mueller and Heistad, 1980). In fact, even if is not fully understood, chronic AHT seems to induce BBB breakdown through ROS and inflammation mechanisms (Sugamura and Keaney, 2011). The BBB disruption is decrease after treatment with ROS scavenger Tempol (Lochhead et al., 2012) or anti-inflammatory minocycline treatment (Yenari et al., 2006).

Anyway, since the most important BBB damage was found in rats (chronic renal hypertension) with really high levels of AHT (220 mm Hg), it is possible that the disruption can be due to the physic insult as well (Nag, 1996).

#### *Changes in vascular functions:*

- *Cerebral artery Autoregulation and myogenic reactivity during AHT:*

As described in the paragraph “Myogenic response and arterial autoregulation”, the autoregulation of CBF decrease the effects of fluctuation of  $P_A$  that can happen during the normal activity and living condition, allowing the maintenance of  $P_A$  between 60 and 160 mm Hg. Within this range, the increase or decrease of BP results respectively in constriction and dilatation of cerebral resistance vessels, assuring a relatively constant CBF (Faraco and Iadecola, 2013).

During the AHT this balance is lost (Fig. 62): if the BP goes above the autoregulatory limit, the normal myogenic constriction of SMCs is overcome by the excessive intravascular pressure, inducing a forced dilatation of cerebral vessels; this impairment in vasoconstriction decrease the vascular resistance inducing an increase of CBF (300–400%), known as autoregulatory breakthrough (Cipolla, 2009). Additionally, without the physiological restrictions, hydrostatic pressure of ECs increases, causing edema.

Likewise, the increase of vascular resistance and the failure of myogenic reactivity during AHT induce a shift to the right of the CBF autoregulation curve: thus, higher pressure is required to maintain the same blood perfusion, and this shift is often associated to a downward displacement of the curve. These alterations result in a decrease of cerebral perfusion.

Moreover, since the left limit of the CBF curve depends from the passive diameter of the vessel, and as explained before this is reduced in hypertensive patients, there is a high susceptibility to hypoperfusion that can bring to till the collapse of the vessel.

Furthermore, the *physiological increase in CBF induced by neuronal activation* is reduced in patients with chronic AHT and the cerebrovascular dysfunction correlates with cognitive deficits.

Anyway, there is still a debate concerning the *resting CBF*: as a matter of fact some authors report a reduction of resting CBF during AHT in human patients declining over the time (Efimova et al., 2008; Kitagawa, 2010), while others describe no changes in resting CBF of SHR and WKYs (Clozel et al., 1989; Dunn and Nelson, 2014; Harper, 1987; Sadoshima et al., 1986).

- *Compromise of ECs role in vascular tone:*

ECs can regulate vascular tone via different mechanisms, dependent or not from NO production.

As mentioned before NOS is able to produce NO from L-Arginine in ECs; NO diffuse in SMCs activating GC with creation of cGMP and PKG phosphorylation inducing in turn phosphorylation of contractile proteins and decrease of  $Ca^{2+}$  influx; moreover PKG activation provoke the

phosphorylation of IP3 receptors on sarcoplasmic reticulum and then inhibiting IP3 activation and thus avoiding  $\text{Ca}^{2+}$  release and inhibiting contraction (Pires et al., 2013). In AHT often the NOS/NO activity is reduced. This is can be due to a downregulation of NOS or a decrease of NO availability, resulting in vasodilation impairment.

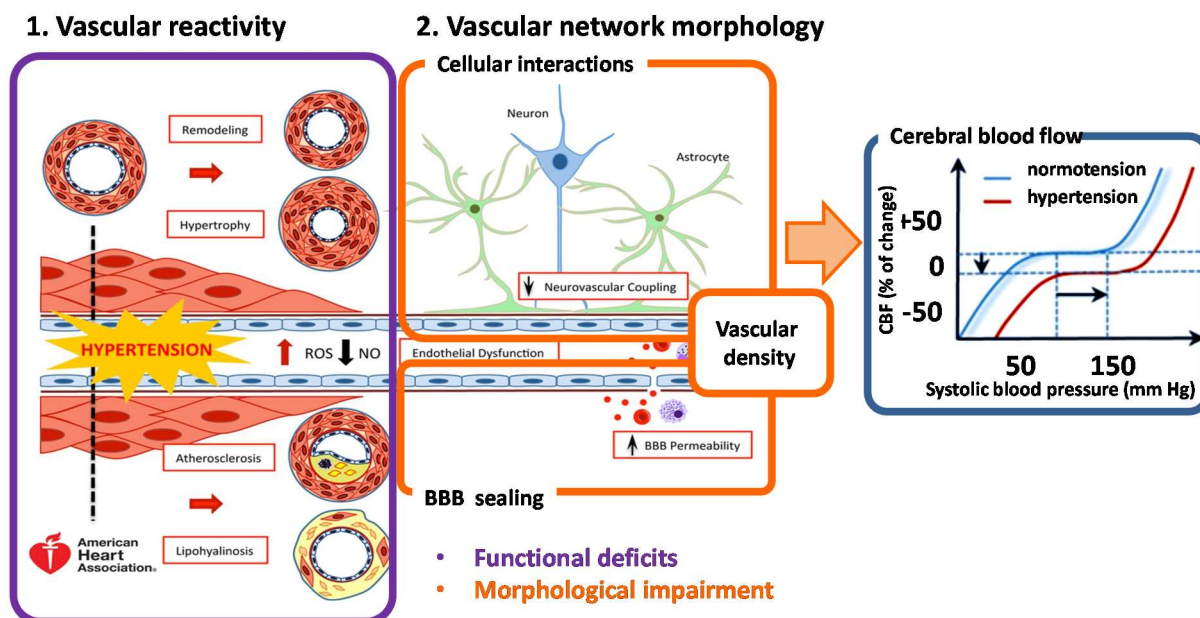
- *Neurovascular coupling dysfunctions:*

The ability of CBF increase in response to neuronal activity depends on neurovascular coupling. The neurovascular coupling is disturbed during AHT but is not clear if it is the direct consequence of AHT itself or it depends on AHT-induced RAAS dysfunctions. Some evidences showed that it is probably due to both phenomena (Dunn and Nelson, 2014). As a matter of fact the chronic infusion of ANG II disrupt neurovascular coupling before the development of AHT.

The putative mechanism thought which ANG II perturbs neurovascular coupling is linked to its interaction with AT1, which triggers ROS production via NADPH oxidase;  $\text{O}_2^{\cdot -}$  is combined with NO, especially coming from interneurons, decreasing its availability and decreasing neurovascular coupling activity, especially in ECs and adventitia of parenchymal arteries (Guan et al., 2006). Moreover, even in with a more strong effect, ROS production creates vascular oxidative stress leading to inflammation and disruption of the normal cellular functionality and structure (Dunn and Nelson, 2014).

Furthermore, during the progression of AHT, some studies showed pericytes degeneration, correlated to the increase of BBB permeability and astrocytes hypertrophy and fibrosis that can compromise the neurovascular communication (Arribas et al., 1999; Mulvany, 2012).

Finally, it is clear that AHT modify vascular functions, as summarized in Fig. 62.



**Figure 62:** Effect of hypertension AHT on cerebral blood vessels: it induces functional and morphological alterations described in the text, compromising the blood supply necessary for brain functions. Adapted from (Faraco and Iadecola, 2013).



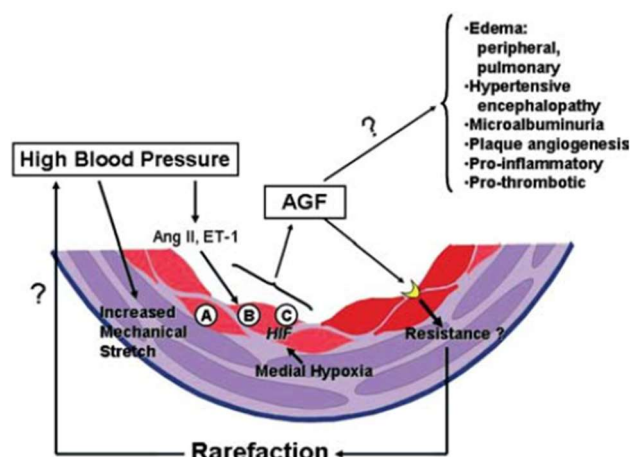
Despite we used an hypertensive model to prove the permissive plasticity of vascular network during consolidation, we are not going to further describe the AHT mechanisms of vascular architectural and functional remodelling; anyway, more detailed AHT-induced vascular deficits are mentioned in the review (Dunn and Nelson, 2014; Faraco and Iadecola, 2013; Pires et al., 2013)

But among these most cited effects of AHT angiogenesis is less mentioned, but since AHT affects several aspect of vascular functionality, what is about angiogenic mechanism during AHT?

#### 4.4. AHT and angiogenesis

In AHT, vessel rarefaction was observed. But, since angiogenesis can occurs when the increase of neuronal activity is not coupled with the increase of metabolic delivery, it is possible to think that during AHT angiogenic pathway can be perturbed.

One hypothesis sustains that angiogenic factors may be released in response to an increase of mechanical stretch or hypoxic condition in SMC. During the time, ECs acquire resistance to angiogenic factor activity, leading to lack of adequate angiogenesis, thereby promoting rarefaction, which could be a factor contributing to the development or severity of AHT and memory impairment (Sane et al., 2004) (Fig. 63).



**Figure 63:** Possible origins and actions of angiogenic growth factors (AGF) in hypertension. From (Sane et al., 2004).

Another putative mechanism of angiogenesis impairment was related to AHT-induced alteration of autoregulation mechanism: at the beginning, the increase of cerebral BP leads to acute vasoconstriction but since it cannot fully compensate the excess of perfusion the brain is still overperfused and thus overoxygenated. The prolonged hyperoxygenation leads to the downregulation of VEGF and other proangiogenic factors. The persistence of this situation induced a structural loss of microvessels and thus vessel rarefaction (Adair and Montani, 2010).

A recent study performed on hypertensive patients was aimed to analyse serum levels of angiogenic molecules, showing that AHT was associated with both the decrease of physiological levels of pro-angiogenic mediators (such as FGF, angiogenin, VEGF and IL-8) and increase of endostatin serum concentration which is an anti-angiogenic factor, showing a direct imbalance of pro-angiogenic and anti-angiogenic factors (Marek-Trzonkowska et al., 2015).

In some patients, the inhibition of VEGF induces or exacerbates hypertension (Sane et al., 2004; Yang et al., 2003b), while other studies displayed higher serum levels of VEGF and Tie-2 receptor (Felmeden et al., 2003; Filiz et al., 2015), showing the complexity of relationship between AHT and angiogenic factors. This last result has been proposed being dependent to secondary induced by endothelial cell injury since the high level of VEGF was concomitant to von Willebrand's factor (Felmeden et al., 2003; Sane et al., 2004). Finally, cross-sectional observational study showed that antihypertensive drug treatments increase capillary density in hypertensive subjects (Debbabi et al., 2006). Moreover, anti-angiogenic therapies can cause hypertension (Humar et al., 2009).

Some others studies on animal models, especially on SHR, suggesting a perturbation of angiogenic response during AHT are treated below.

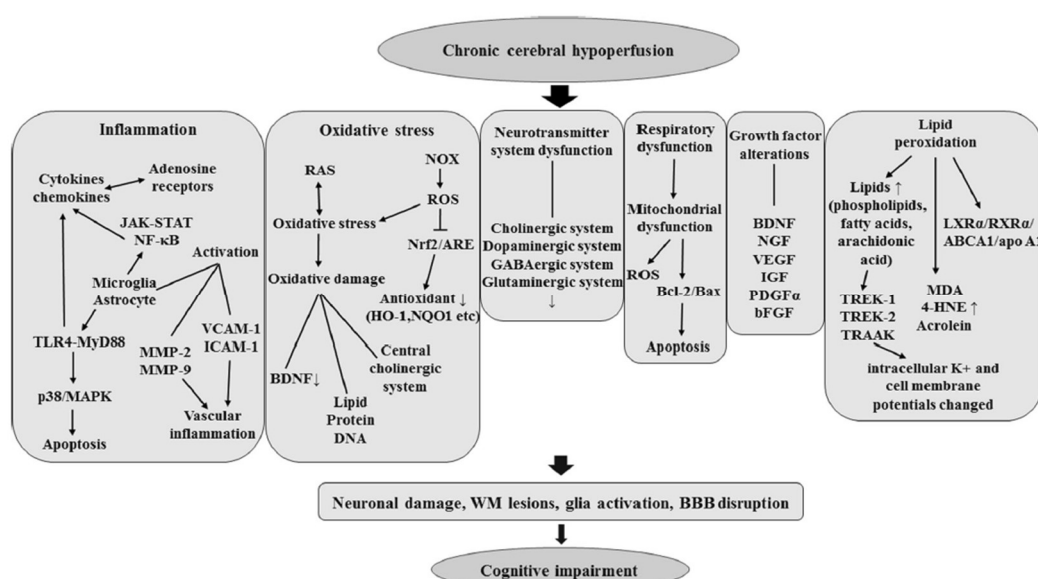
#### 4.5. AHT and cognitive impairment

AHT has been proposed as one of the most important risk factors able for inducing and/or increasing cognitive impairments, especially Alzheimer's Disease (AD) and Vascular Dementia (VD), although the underlying mechanism remains to be explored.

Both hypertension and cognitive impairments develop over a long period of time but hypertension typically precedes the onset of cognitive impairments (Wiesmann et al., 2013).

AD and VD are the most important causes of dementia and are characterized by cognitive and memory decline.

The most evident link between AHT and VD was thought to be represented by the stroke, in particular it has been proposed that multiple and discrete lesions, often present in hypertensive patients, can induce multiple stroke responsible to cognitive decline (multi infarct dementia). Anyway, with the use of brain imaging showed that the patients developing VD showed diffuse white matter lesions that can be link to the genetic pathology CADASIL, previously mentioned. As a matter of fact, the most important cause of VD is not stroke but the lacunar infarcts or white matter disease (Faraco and Iadecola, 2013; Wiesmann et al., 2013). The VD pathology was recently reviewed in (Iadecola, 2013) and (Du et al., 2016) and summarized in Fig 64.



**Figure 64:** Biochemical changes induced by chronic cerebral hypoperfusion explaining cognitive dysfunctions. From (Du et al., 2016).

Regarding AD, it has been demonstrated a relationship between AHT and accumulation of amyloid plaques, neurofibrillary tangles and brain atrophy (Skoog and Gustafson, 2006; Wiesmann et al., 2013). Actually the BP profile is not linear: at the beginning the AHT-induced increase of BP precedes the AD, but then the inability to maintain BP homeostasis, induced by the cerebral damage, leads to a decrease of BP, making difficult to treat the BP changes in the different phases of AD (Glodzik et al., 2014; Skoog and Gustafson, 2006). More details and some others epidemiological and experimental evidences showing the link between AHT and AD were recently reviewed in (Carnevale et al., 2016; Perrotta et al., 2016).

However, the sequence of events occurring between the induction of hypertension and the cognitive deficits are unknown; this is also due to the fact that AHT takes years to develop and AD requires decades to be discovered since the clinical signs appear in the late phase of disease.

It is difficult to find the mechanism responsible to AHT-induced AD, anyway it has been proposed that AHT inhibits vascular transport of brain A $\beta$  into the plasma and participate with Apo $\epsilon$ 4 in promoting amyloid deposition (Faraco and Iadecola, 2013).

Moreover, a recent interesting study showed that the surgical induction of AHT provokes Alzheimer's disease (AD) symptoms as amyloid deposition around vessels and memory impairment in mice performing Morris water maze (Carnevale et al., 2012). Moreover, they showed another AHT-dependent mechanism inducing  $\beta$ -Amyloid deposition: AHT upregulates the Receptor for Advanced Glycation End products (RAGE), responsible to the BBB transport of A $\beta$  into the brain, in hippocampal and cortical cerebrovascular network.

Despite the difficulties relied to the long-lasting pathology and multiple levels modifications, some candidate pathways, explaining the link between AHT and memory deficits, have been proposed.

Hypertension is characterized by modifications in vascular density and artery remodelling leading to vascular hypoperfusion, linked with a specific decrease of blood vessel network reactivity, affecting myogenic tone, autoregulation and intracellular signalling; all these factors can affect neurovascular coupling and thus neuronal activity, affecting memory process (Faraco and Iadecola, 2013; Jennings and Zanstra, 2009; Pires et al., 2013).

Moreover, hypertension can modify the blood brain barrier (BBB) creating extravasation of blood components and inflammatory processes. Therefore, a disruption mechanism of the BBB represents another probable link between hypertension and cognitive impairment. This hypothesis was also corroborated by the observation of extravasation of plasmatic molecules into the brain parenchyma in aged people as well as animal models and it is been suggested that this phenomenon is exaggerated in Alzheimer patients and models.

Human studies associating AHT with memory impairment are reviewed in (Birns and Kalra, 2009), showing that the majority of longitudinal studies demonstrated that BP increase is associated with memory decline, analysing the data of MEDLINE, EMBASE and Cochrane Library databases.

The majority of studies reported memory, learning, attention and time processing deficits during AHT (Birns and Kalra, 2009; Bucur and Madden, 2010; Fujishima et al., 1995); the memory impairment was detectable in both old and young patients and was related to the BP increase; the memory deficit was higher in old patients, displaying that age can exacerbate memory deficit (Fujishima et al., 1995; Kohler et al., 2014). Anyway some data showed that the link between memory impairment and blood pressure is not that clear since for example antihypertensive drugs reduce the risk of Alzheimer disease independent of blood pressure reduction (Kalra, 2014) or some studies fail to find a correlation between AHT and memory impairment reviewed in (Birns and Kalra, 2009).

It is clear that this topic needs to be further investigate; however the majority of these studies suggest that vascular impairment is at the origin of memory deficit, leading to the suggestion that antihypertensive treatment can be used as strategy to delay the apparition of neurodegenerative disease (Wiesmann et al., 2013). In the next paragraphs we describe SHRs model and the memory deficit associated to AHT.

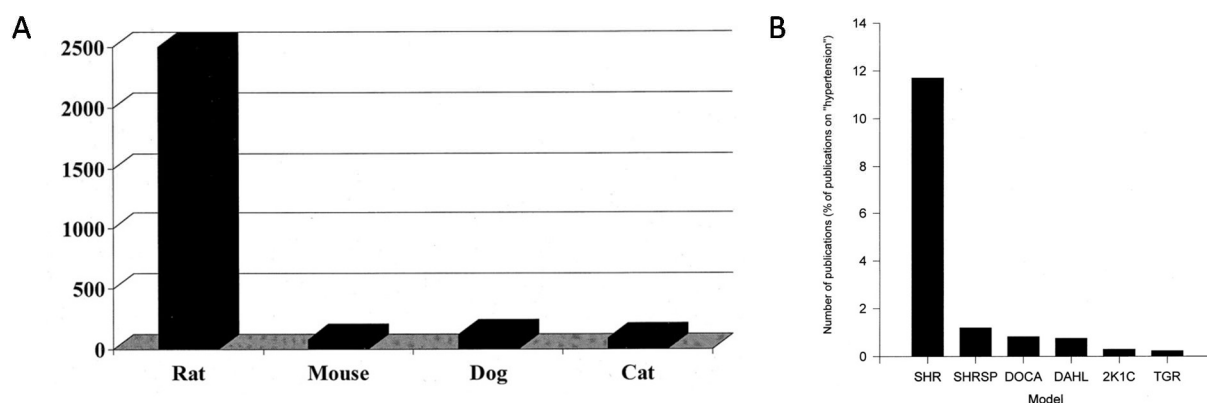
#### 4.6. Rodents' model of AHT: our choice in SHRs use

The use of hypertensive model in our study is aimed to understand how vascular impairment could influence memory consolidation process, through the modification of vascular and neuronal network, during different time-point of hypertensive process.

The importance of the vascular network in cognitive functioning is based in part on observations showing that disruption of the vascular system causes or accelerates cognitive pathologies (Carnevale et al., 2012; Faraco and Iadecola, 2013). This is the reason why we decided to investigate how cerebrovascular changes induced by hypertension are able to affect memory processes.

AHT affects different phases of memory process but the mechanism underlying it is not clear. To shed light on this relationship, we decided to explore the impact of vascular architectural changes induced by AHT during both recent and remote memory, in particular in areas implicated in memory consolidation. Over the time, several models of AHT have been created.

The first animal model used to study AHT was developed by Harry Goldblatt clipping the renal artery of a dog and inducing a secondary hypertension (Goldblatt et al., 1934). Several decades were passed from this study and the trend of publication using different models suggests an evolution toward rodents' models (Leong et al., 2015; Pinto et al., 1998) (Fig 65A).



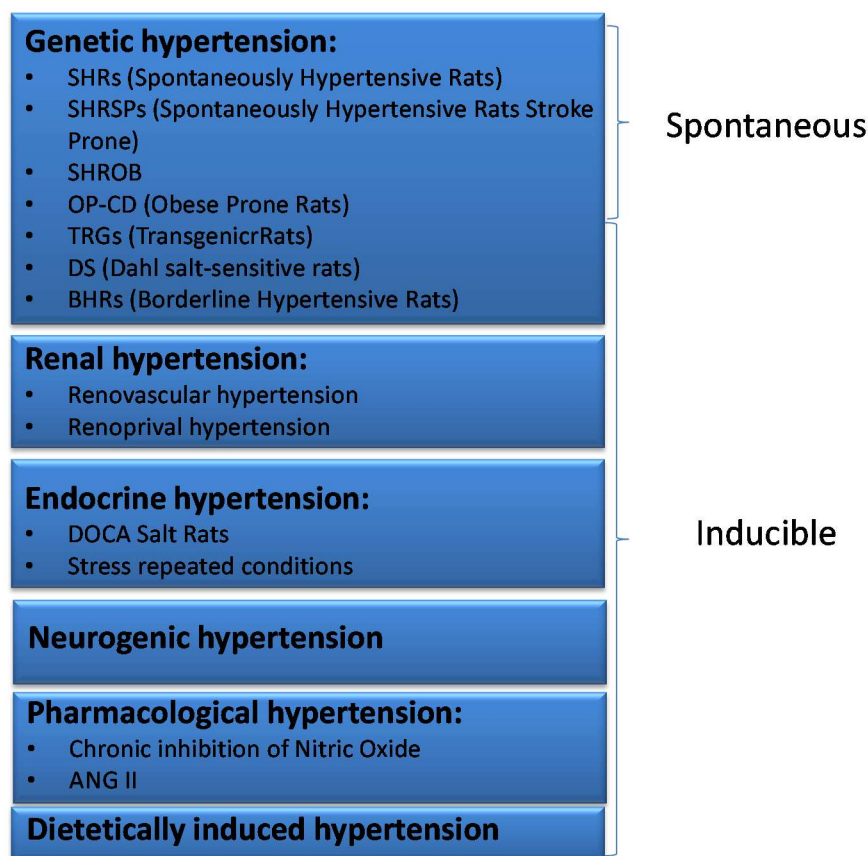
**Figure 65:** Choice of hypertensive models in preclinic research. A: number of papers published on hypertension in different species, rat, mouse, dog and cat from 1990-1997. B: Number of publications on a particular rat model of hypertension, as divided by the total number of papers on hypertension. From (Pinto et al., 1998).

Since AHT is a multifactorial disease involving different biological pathways, and it is influenced by genetic and environmental factors, different animal models have been developed in order to mimic the hypertensive responses observed in humans, to screen potential antihypertensive drugs, and to understand the aetiology, the development and the progression of hypertension (Leong et al., 2015).

In the past dogs were often used to study AHT but nowadays the most used model is the rat. This choice was influenced also by the finding of Okamoto and Aoki about a new rat model of AHT which did not require pharmacological or surgical intervention: the Spontaneously Hypertensive Rat (SHR)

model (Okamoto and Aoki, 1963). From that moment SHRs were used to test antihypertensive treatment, becoming the cornerstone of medical research in experimental hypertension (Leong et al., 2015) (Fig. 65B). Others strains have been developed in the following years but SHR remains the most studied and used model. This is also due to the fact that SHRs allow to study the development of essential hypertension, contributing to 95% of incidences and being associated with genetic influences, even though it represents only a particular type of genetic hypertension.

Without going deep in the details, we summarized the main AHT rats models in Fig. 66 and the different characteristics of these models in Fig. 67 (Dornas and Silva, 2011; Leong et al., 2015; Pinto et al., 1998).



**Figure 66:** Different rats' models of AHT.

## Spontaneous Rat Models:

STRAIN	Inbred	Controls	Characteristics	+	-
SHR	Wistar	Wistar Kyoto (WKY)	↑ BP: 10 weeks ; HTA: 100% rats in 7-15 weeks; ↑ Cardiac output->normalize + BP->vascular changes -> cardiac hypertrophy;	No treatments No surgery ↑↑ similarity of HTA in humans	Individual variations Hyperactivity Less anxiety
SHRSP	SHR	Wistar Kyoto (WKY)	↑BP in 10weeks + ↑ tendency to die from stroke Need to be fed at 7-8 weeks with 1% salt in the water to develop cerebral hemorrhage or infarction in 80% male over in 16-18 weeks	Animal model of spontaneous stroke/treatments	Extreme exemple Stroke
SHROB			Obese when fed high-fat diet HTA Metabolic Syndrome Nephropaty	Model HTA:Obesity	Diet Bias due to food ↓ Bias in STFP Elimination subpop non-responding-> ↑ n° Obesity
OP-CD	CD-SD	OR-CD	Obese when fed high-fat diet Obesity Metabolic syndrome HTA	Model polygenic obesity	Diet Bias due to food ↓ Bias in STFP Elimination subpop non-responding-> ↑ n° Obesity

## Induced Rat Models:

STRAIN	Inbred	Controls	Characteristics	+	-
DS	Sprague-Dawley	DR	Develop HTA with high NaCl diet Fulminant hypertension->die in 8 weeks Marked vascular and renal lesion	Model HTA/Renal failure	Mechanisms ? Nephrophaty Dayly food adm Bias due to food ↓and in STFP ↑ n° for stable range
TRG (mREN2)27			Fulminant hypertension 5 weeks Myocyte hypertrophy Endothelial disfunctions Nephron lesions	Model to study specific gene in pathogenesis HTA	No representative of human HTA
Renovascular HTA			Constriction of renal artery or both by clamp ↓perfusion->↑ANGII->↑PR->↑BP	Model NaCl/V	Surgery Spontaneous stroke
Renoprival HTA			Removal of 1or 2/3 kidney->↑BP	Model to studyRenoprival HTA	Surgery No representative of human HTA
DOCA salt rats			DOCA and high-salt diet↑ BP in 3 weeks ↓plasma renin activity Volume dependent HTA Oxidative stress	Model HTA/Volume	Develop severe HTA too quickly->no long term/stable/chronic studies Need surgery to↓renal mass ↑↑ NaCl No realistic model Dayly food adm Bias due to food ↓and in STFP ↑ n° for stable range
Stress Repeated condition			Stress repeated activate hypothalamic-pituitary-adrenalaxis, simpathoadrenal medullary system, RAAS, NA SN	Closed to humanmodel	Difficult to obtain Long period to obtain Different subtype
Neurogenic HTA			Denervation of sinoaortic baroreceptors Changes on BP heart rate and NA SN	Could be associated to other models	Surgery Norealistic models
Chronic I NO			Persistent HTA Renal injiury (glomerulosclerosis, glomerular ischemia) ↑↑periferical constriction	Model to stdy the role of NO	Chronical Oral Administration ↑ n°
AGN II			Infusion AGN II->slowing HTA	Model to stdy the role of RAAS	Chronic Infusion ↑ n° ↑ Need Salt diet
Dietetically induced hypertension			↑↑fat/salt	Model to study how diet can influence HTA	Dayly food adm Bias due to food ↓and in STFP ↑ n° for stable range Correlated pathologies

**Figure 67:** Summary of the characteristics of rat models of AHT (Dornas and Silva, 2011; Leong et al., 2015; Pinto et al., 1998).

Among these, our choice of using a spontaneous model derived from the fact that inducible models usually need long period to be obtained (daily drug or specific diet administration, surgery, BP measurements), a bigger number of animal and the elimination of subjects non-responding to the treatment or surgery. Moreover, differently from SHR, some other inducible or spontaneous models can develop more than one pathology (such as nephropathy, cardiac hypertrophy, heart failure, obesity, metabolic syndrome, diabetes ...) and the acute induction of AHT is less representative of human essential AHT.

Thus, in our study we decided to explore the effect of vascular dysfunctions on memory process using the SHR model. In this model, all rats develop a spontaneous and constant AHT (Tayebati et al., 2012). Contrarily to inducible models, SHR do not require long periods of treatment or surgery for developing a stable AHT, thus the number of animals required is significantly decrease. The stable AHT requires less frequent BP measurement. Moreover, the long-lasting and stable AHT is an important parameter allowing the study of the evolution of memory consolidation in parallel to vascular dysfunctions. Furthermore, the use of anti-AHT drugs on this model is well described in literature. Finally, compared to both inducible and spontaneous model, there is not comorbidity; moreover, the progression (onset and development) of the disease is more comparable to human AHT, avoiding fulminant crisis. The main difference from human pathology, that we have to take in consideration, is that SHR were selected, thus, the deliberate inbreeding induced a uniform combination of predisposing elements, differently to the human reality (Okamoto and Aoki, 1963).

Of course, as all models, represent only a kind of AHT and some disadvantages (behavioural alteration, the non-strictly inbred strain leading to individual variations in the genetic background of both SHR and particularly of their control strain (Dornas and Silva, 2011)) but a particular attention concerning these confounding effects was taken in order to minimize these factors and their putative bias on experimental results during this thesis.

Spontaneously hypertensive rats represent a well-established model for the study of the attention deficit hyperactivity disorder (ADHD) and the gold standard used to investigate the AHT and vascular blood disease (VBD), well-known risk factors for the development of memory impairments (Tayebati et al., 2012).

They were obtained in 1963 by Okamoto and Aoki who started to breed a male with a spontaneous hypertension and a female with high blood pressure coming from a stock of outbred Wistar rats from Kyoto University (Okamoto and Aoki, 1963). They selected furtherly the descendants based on the expression of AHT creating the segregation of this strain that uniformly resulted in 100% of the progeny having naturally occurring hypertension. In parallel, they segregated Wistar animals from Kyoto University with "normal" blood pressure, that were selected to be used as the same-genetic-background normotensive control of the Spontaneously Hypertensive rats: the Wistar Kyoto Rats (WKYs).

Due to a uniform genetic predisposition (Folkow, 1982), SHR are normotensive at birth and develop with aging a sustained hypertension (time-dependent increase of blood pressure) and brain damage (brain atrophy, particularly in hippocampus, modification and loss of neurons and glial cell, changes in blood brain barrier permeability) at six months (Tayebati et al., 2012). For these reasons, SHR result to be the most extensively strain exploited for the study of vascular disorders, in order to link the anatomical and neurochemical changes with the behavioural features.

SHR have a pre-hypertensive period in which the BP rises till 5-6 weeks of age, an early hypertensive stage lasting 2 months, and then steadily rise till 180-200 mm Hg reaching a stable AHT at 3-4 months, depending from bibliography source (12 weeks (Trippodo and Frohlich, 1981); 100 days

(Okamoto and Aoki, 1963), 15 weeks data provided by Janvier Lab, factory which delivered rats used in this study). They can develop over the time organ damage such as cardiac hypertrophy and renal dysfunction, but conversely to SHRSPs, SHRs have no tendency to develop strokes macroscopic atherosclerosis or vascular thrombosis (Okamoto and Aoki, 1963; Pinto et al., 1998).

SHRs showed behavioural alterations when compared to the WKYs. Such of alterations include hyperactivity, low level of anxiety, impulsivity, poor sustained attention (Sagvolden, 2000), impairment in learning and altered spatial and working memories (Gattu et al., 1997a; Gattu et al., 1997b; Meneses et al., 1996; Meneses et al., 2011a; Meneses et al., 2011b). SHRs are used also as a model of hyperactive rats and a model to study ADHD (Langen and Dost, 2011; Meneses et al., 2011a), as mentioned before. These behavioural features are commonly observed in animals modelling several neurodegenerative and neurological disorders as AD, Parkinson Disease, ADHD and schizophrenia (Meneses et al., 2011a).

Furthermore, neurochemical changes were described in this strain, such as decrease in ACh transmission, increase of NA activity, reduction of DA functions (Tayebati et al., 2012).

The precise phenotype of SHRs includes several differentially expressed genes, many of which are unknown. Some authors reports that SHR's AHT is polygenic in origin with at least 3-6 genes (Kurtz et al., 1990) and one of them one can be associated with the over-reactivity of RAAS (Yamori and Horie, 1977). Moreover, the BP profile in SHRs is also related to Y chromosome modifications (Ely and Turner, 1990; Johnson et al., 1995; Turner et al., 1991).

#### **4.5.1.Cerebrovascular morphology adaptation in SHRs**

During the time SHRs develop an increase of BP, followed by brain atrophy, loss of nerve cell, glia rarefaction and brain damage, characteristics sheared with human AHT, as well as vascular alterations (Tayebati et al., 2012).

- *Vascular rarefaction and remodelling*

In SHR strain a reduction of cerebral capillary numbers was observed in the reticular nuclei of the medulla oblongata and the pons, beginning from 3 months (Sokolova et al., 1985) and it was dependent from the level of hypertension (younger rats did not show any reduction); regarding pial arteries, some publication did not show a reduction of their number in 4 months old SHRs (Pires et al., 2013). The capillary rarefaction can be observed in SHRs starting from 5-6 weeks in cremaster muscle (Struyker-Boudier et al., 1988). Anyway, no rarefaction of cerebral arterioles in SHRs were detected by (Werber et al., 1990), leading the debate opened.

The decrease of wall-to-lumen ratio was observed in SHRs mesenteric and carotid arteries (Mulvany et al., 1978; Traub et al., 1995) and it may be associated also to the increase of the vascular response to constrictors agents; however, unexpectedly, in another derived strain, the Spontaneously Hypertensive Rats stroke prone (SHRSPs), it has been demonstrated that the large cerebral arteries possess lower contractile response, even if the thickness of the these vessels is increased, probably due to a subsequent mechanisms that try to reduce the vascular remodelling by decrease their responsiveness to vasoconstrictors agents (Vacher et al., 1996).



Anyway, in both models mentioned it has been shown that the pial (Baumbach and Heistad, 1989) and MCA (Dorrance et al., 2007; Rigsby et al., 2007) arteries have bigger thickness and smaller lumen section; this phenomenon has been found in others model of AHT (for a review see (Pires et al., 2013).

The SHRSP arteries remodelling were detected in 12 month old rats, despite the AHT is established at 3-4 month, suggesting that is an adaptive long term modification. Moreover they showed a different SMC orientation.

Architectural alterations of cerebral cortical network in SHRs was more evident in 6 month-old rats, in particular in frontal cortex were they displayed hypertrophy and lumen narrowing, while in HPC luminal narrowing was not associated to variation of vascular wall (Amenta et al., 1996; Sabbatini et al., 2001). Even if in the striatum the increase of vascular wall was not accompanied by lumen decrease over time, these authors showed a prominent lumen diameter reduction compared with normotensive WKYs.

In 12 month-olds SHRs the 3 months treatment with Captopril (ACE-inhibitor) reversed the hypertrophic remodelling in pial arteries (Dupuis et al., 2005). The same result was reported using the combination of Ramipril and Telmisartan at low doses (ACE-inhibitor and AT1-blocker, respectively) (Dupuis et al., 2010). The reversion of vascular wall damage induced by AHT was obtained with aldosterone antagonist Spironolactone in SHRSP (Rigsby et al., 2011).

- *BBB and barriers alterations*

The BBB dysfunction in SHR model is under debate, since some authors proposed that AHT affects CSF barrier compared to BBB. In 36 month-old SHRs, the composition of CSF was different compared to WKYs, confirming a CSF barrier deficit, assumed to be responsible for choroid plexus deficit (Gonzalez-Marrero et al., 2013).

Adult SHRs cerebral vessels are less susceptible to disruption of BBB induced by acute rise of BP, as mentioned before, compared to WKYs (Mueller and Heistad, 1980). Anyway, the increased presence of Aquaporine-4 expression at BBB level (regulating the cerebral water content and sensitive to brain injury) in frontal cortex of 6 month-old SHRs suggested an impaired water regulation that can be responsible, even if in part, to cytotoxic edema and brain lesions (Vizuete et al., 1999).

- *Changes in vascular autoregulation and myogenic reactivity*

The eNOS expression is decreased in SHRs compared to WKYs, provoking a decrease of ECs-dependent dilation (Yamakawa et al., 2003a). This deficit exacerbates ischemic attack, where a maximal dilatation of the neighbour vessels is required to circumvent the occlusion and minimize the necrotic area. The treatment of SHRs with Ciclostazol, which increases eNOS phosphorylation and then activity, was linked to an increase of CBF after ischemic attack and reduction of infarct size (Oyama et al., 2011).

Other ECs-mediated mechanisms in vascular tone regulation, impaired during AHT, are the EDHF that is responsible to vasodilation, and TRP channels. In SHRSP mesenteric arteries it has been shown an increase in IKCa even if the EDHF dilation is decrease, thus their role in AHT is not really clear (Giachini et al., 2009).

Regarding TRP channels, the regulation is complex: in SHR cerebral arteries TRPC3 expression is increased whereas TRPC1 is decreased and this is linked to the increase of vasoconstriction (Noorani et al., 2011).

- *Neurovascular coupling dysfunctions:*

Even if the neurovascular coupling has not been deeply studied in genetic models of AHT, it has been shown that the increase of CBF induced by administration of bicuculline (seizure inducer) in SHRs was reduced compared to WKYs, and this reduction was normalized using ACE-inhibitors (Cipolla et al., 2010).

Accordingly, another study showed that the somatosensory cortical increase of CBF induced by whiskers' stimulation in 5-8 month old SHRs was decreased compared to WKY. Moreover, the neurovascular coupling dysfunctions in 8 month-old SHRs were not reversed by 10-week treatment with verapamil or losartan, suggesting that probably others mechanisms, other than ANG II, may be involved at this stage of AHT (Blumenfeld et al., 1999; Bohlen, 1989).

Futhermore, during the progression of AHT, SHRs and SHRSPs showed pericytes degeneration, correlated to the increase of BBB permeability and astrocytes hypertrophy and fibrosis (Arribas et al., 1999; Mulvany, 2012). SHRs displayed neuronal loss closed to fibrotic astrocytes (Mulvany, 2012; Paiardi et al., 2009).

For more detailed information about the progression of cardiovascular autonomic dysfunctions in SHRs see (Lehnen et al., 2013).

#### **4.5.2.Cerebral microanatomy and neurotransmitter alteration in SHRs**

2 month-old SHRs showed a ventricular enlargement, a decrease of brain tissue and brain weight (Nelson and Boulant, 1981; Ritter and Dinh, 1986; Tajima et al., 1993) and 6/7 month-old SHRs displayed hypothalamic alteration and a decrease of neuronal density (Tajima et al., 1993).

The hippocampal volume is reduced in 6 month-old SHRs and the neuronal density at CA1 level is decreased (Bendel and Eilam, 1992; Johansson, 1986; Ritter and Dinh, 1986; Sabbatini et al., 2002; Sabbatini et al., 2001; Tajima et al., 1993). Moreover, data on cerebral glucose utilization showed a decreased consumption of glucose in grey matter indicating a decrease of neuronal activity in 6 months old SHRs (Johansson, 1986; Wei et al., 1992); this has been correlated to an higher disposition of SHR for cerebral ischemia (Grabowski et al., 1988).

A decrease of white matter volume was detected in 2 month-old SHRs (Ritter and Dinh, 1986; Tajima et al., 1993). The AHT-induced white matter deterioration, detected in SHRs, is a risk factor for stroke and is associated to cognitive impairment and gait disorders. Moreover, microglial reactivity in white matter was increased in 6 month-old SHRs, although it is not clear if the microglial hyperactivity induced the release of proinflammatory cytokines responsible to neuronal degeneration or if the increase of neurodegenerative process induced microglial activation.

Finally, astrocytes increase, associated to lesion-induced astrogliosis, was detected in 6 month-old SHR but not in younger SHR (2-4 months) and WKYs (Tomassoni et al., 2004).

To complete the panorama of cerebral dysfunctions in this strain, we briefly mention the neurotransmitter pathway. For more details, cholinergic, dopaminergic, noradrenergic and serotonergic alterations are reviewed by (Tayebati et al., 2012).

Dopaminergic and noradrenergic systems are responsible of behavioural alteration in SHR strain, in particular concerning hyperactivity, lower attention, inhibition deficit, and hyperreactivity to stress resembling, behavioural abnormalities of human attention-deficit with hyperactivity disorder (ADHD).

Dopaminergic reduction in PFC of SHR was correlated to ADHD, and increase of noradrenergic signalling in locus coeruleus, PFC, substantia nigra were correlated with the increase of BP (Tayebati et al., 2012).

The ACh is implicated higher brain functions (such as in attention, arousal, motivation and conscience) and a decrease of cholinergic functionality was associated to aging, VD and AD; this observation can represent another link between these pathologies and ATH, since a deficits in central nicotinic cholinergic receptors in SHR was detected, together with the observation of microanatomical changes.

ACh is also implicated in BP regulation and cholinergic neurons are localized in cerebral areas such as rostral ventrolateral medulla (RVL), the posterior hypothalamus, and the lateral septal area (Buccafusco, 1996; Kubo, 1998). The cholinergic activity in SHR is higher in RVL than controls and this can participate to the development or maintenance of AHT. Expression of vesicular ACh transporters (VACHT) in the frontal cortex, hippocampus and striatum of SHR is increased. The same increase of VACHT was found in human Alzheimer patients at early stages and in the hippocampus of individuals suffering of mild cognitive impairment. Moreover, some authors proposed that the cholinergic alteration in cerebral cortex and hippocampus may be responsible to impaired learning and memory functions (Kimura et al., 2000; Togashi et al., 1996).

#### **4.5.3.SHRs and angiogenic pathways**

As mentioned before there is not a large number of studies concerning the angiogenesis and the hypertension, even less regarding SHR and cerebral angiogenesis.

Concerning the mechanism triggering the angiogenesis, no important differences were detected regarding the tolerance to hypoxia in adult SHR compared to WKYs (Brooks et al., 1987), at least in myocardium.

In human Marek-Trzonkowska's results described above, a decrease of serum angiogenic molecules were detected in the hypertensive patients, tighter with vascular rarefaction, leading to think that the angiogenic process may be altered during hypertension.

Conversely to these results, at least at first sight, SHR showed a higher vascularization (both capillaries and microvessels with SMCs) of fibrin gel chambers implanted subcutaneously at dorsal level (Hudlett et al., 2005) compared to age-matched WKYs, together with a higher expression of FGF but not VEGF, letting the authors to conclude that the induced-angiogenic was higher in SHR and that the vascular rarefaction observed in the other studies was due to the hypertension effect on remodelling. Anyway, some observations were quickly presented by another author (Bobik, 2005) giving a different explanation to the Hudlett's study; according to Bobik and collaborators, the increased vascularization found in fibrin gel may reflect more the ability to respond of tissue injury more that the higher angiogenic response. Secondly, the SHR were just 1-2 months old (early stage of AHT; stable AHT is achieved at 4 months), thus it was difficult to conclude that observed higher angiogenesis was associated to AHT or to vascular rarefaction (Struyker-Boudier et al., 1988).

Anyway, other observations on mesenteric tissue of SHR showed that the alterations of angiogenesis and microvascular network associated with hypertension are more complex than just a loss of vessels. In small (classified as spanning an area less than 1  $10\times$  field of view) microvascular network SHR

showed a decrease in branching architecture closed to arteriolar anastomosis, compared to WKYs; but unexpectedly and less frequently the larger (classified as spanning an area greater than  $1 \times 10^4$  field of view) vascular network of SHR showed regions of dramatically increased vascular density (Murfee and Schmid-Schonbein, 2008).

Another study, sustaining angiogenic retard or impairment in SHR strain, assessed that after 14 days from subcutaneous sponge implantation, the level of angiogenic ingrowth in SHR was approximately half of those in age-matched normotensive WKYs or Sprague Dawley (SDs) rats: SHR angiogenic proteins MT1-MMP and kinase-insert domain-containing receptor (KDR) were downregulated compared to WKYs; furthermore, interestingly, the gene transfer of human VEGF<sub>121</sub> re-established both KDR and MT1-MMP expression and up-regulated ECs proliferation in SHR (Wang et al., 2004).

Furthermore, after diet-induced cerebral infarction (intragastric administration of high-sodium water for 7 consecutive weeks), SHRSPs showed upregulation of MPP9 together with an increase of number of blood vessels with discontinuous collagen IV expression. Moreover, the number of continuous collagen IV-positive blood vessels was lower in the infarct border zones compared to WKY and SHRSP drinking normal water, suggesting that MPP9 can be differently regulated during AHT (Hou et al., 2014).

A recent study showed that 5 month-old SHR have a hypertension-related inhibition of ischemia-induced angiogenesis. Briefly SHR and WKY were subjected to excision of the left femoral artery, inducing an ischemic response in the skeletal muscles. SHR showed a decrease of capillary density compared to age-matched WKYs after ischemic event; this could be due to both angiogenesis impairment and oxidative stress induced by AHT, which can accelerate cellular toxicity and exacerbate endothelial dysfunction leading to apoptosis. Moreover, the presence of angiogenic factors such as VEGF, eNOS and HIF-1 $\alpha$  was decreased in SHR rats after ischemic event. Furthermore, the same authors showed that therapeutic ultrasound treatment reverse angiogenic deficit. The mechanism by which this effect was achieved is still unknown but it seems to be related to increases Ca<sup>2+</sup> availability or upregulation of eNOS (Lu et al., 2016).

The angiogenic pathway in SHR can also depend on the area considered, for example SHR were able to create new vessels via angiogenesis following both short-term and long-term physical exercise in locomotor skeletal muscles but not in other kind of muscles; VEGF expression was detected just after acute exercise (Amaral et al., 2008). Anyway, another study showed a restoration of BP and angiogenesis functionality in SHR following physical exercise while non-trained SHR showed a basal vascular density and VEGF decrease compared to WKYs and trained animals. This study may suggest that, despite the basal level is different, the angiogenic response can be triggered (Fernandes et al., 2012).

Another group tried to unravel the link between hypertension and angiogenesis, analysing the effect of chronic hypoxia on SHR animals (10% O<sub>2</sub>). In young (5 weeks old) normoxic SHR the BP was higher compared to normoxic WKYs, but interestingly 7 weeks of hypoxia decreased the BP in both SHR and WKY. The decrease of BP was not accompanied by changes in cardiac output but to a decrease of peripheral vascular resistances. In adult rats (12 weeks old) with established hypertension, hypoxia-induced decrease of BP was detectable after 1 week. The return to normoxia for 2 hours did not reverse the reduction in BP excluding the short-term effect of hypoxia-induced vasoactive substances (especially of VEGF, potent vasodilator peptide) and suggesting an effect of the structure of the vascular network on blood pressure. Furthermore, despite, arteriolar density was not changed by hypoxia, capillary network in skeletal muscle and heart was increased, together with an increase of VEGF and VEGFR mRNA and proteins, suggesting that chronic hypoxia activated VEGF-A-induced angiogenesis and thereafter prevented both the BP rise in young and adult SHR (Vilar et al., 2008).

Concerning angiopoietins pathway, a study performed by Wang (Wang et al., 2002) tried to compare the angiogenic gene expression of Ang-1, Ang-2, Tie-1, Tie-2, or VEGF in SHRSPs, SHRs and WKYs, in basal condition and following the middle cerebral artery occlusion (MCAO) at cortical level. At basal conditions no differences were detected in any strain regarding any gene expression. The MCAO induced a decrease of Ang-1 expression in only SHRSPs and an increase of Ang-2 in SHRSPs and SHRs (even if a higher increase was detected in SHRSPs) compared to sham rats. Regarding the receptors, Tie-1 did not change following MCAO regardless of strain, while Tie-2 expression was reduced just in SHRSPs. VEGF was not modified by any condition. Clearly, some alterations of angiogenesis pathway were detected even if with a specific pattern regarding the different strains. Unfortunately, the age of these rats was not reported (adult rats). Another study anyway reported the same baseline condition in the level of Ang-1 and Ang-2 in 2 month-old rats (Rusai et al., 2011).

Vascular rarefaction can be due to either vessel destruction or insufficient angiogenesis; these studies give us the idea that angiogenic response (reparative angiogenesis ischemia-induced, exercise-induced or implantation-induced angiogenesis) can be altered.

Literature is not sufficient to have an exhaustive scenario about angiogenesis pathway in SHRs. Discrepancy in reported data show both that SHRs are capable to create new vessels if angiogenic process is triggered with different stimuli (Hudlett et al., 2005) (Fernandes et al., 2012) and that they present an impairment or a retard of this mechanism (Hou et al., 2014; Lu et al., 2016; Wang et al., 2004). Furthermore the angiogenic process can be trigger just in localized areas of interest or can concern different vessel types (Murfee and Schmid-Schonbein, 2008).

Anyway, all these works do not show a physiological response of angiogenesis induced by neuronal activity, despites they give us the idea that a link between AHT and angiogenesis impairment. Thus, this lack of information prompted us to investigate whether the angiogenesis pathway is impaired in the cortical areas subtending memory consolidation and if it can impact memory retrieval.

#### **4.5.4. SHRs and memory performances**

As mentioned, several clinical studies suggest impairment in memory process and cognitive functions in hypertensive patients and even much more studies have been performed to examine the relationship between blood pressure and cognitive performance in SHRs.

According to the majority of the studies (summarized in the review (Meneses et al., 2011a; Tayebati et al., 2012)), SHRs exhibit poorer performance in several memory paradigms such as conditional avoidance task at different ages (from 8 weeks to 50 weeks (Sutterer et al., 1980)), in a radial-arm maze task (12-, 48- and 65-week-old rats (Mori et al., 1995; Wyss et al., 1992)), in novel object discrimination. Associative learning and memory impairments are more remarkable during the progression of hypertension and aging, suggesting that they provoked similar and additive cognitive alterations.

Others studies, using different behavioural task such as water maze, autoshaping (Meneses et al., 1997) and open field tests (Heal et al., 2008; Wells et al., 2010), in 3, 6, 9, 12, 18, or 24 months (Meneses et al., 1997) or 3 month-old male SHRs (Heal et al., 2008; Wells et al., 2010), have shown an age-related impairment on learning, memory in this stain compared to the normotensive WKY rats. Moreover, SHRs and WKY rats displayed memory deficits compared to SD rats in the water maze task: in particular, SHRs exhibited deficits in dorsal striatum-related habit learning, whereas WKYs exhibited deficits in hippocampus-related spatial learning (Wells et al., 2010).

Anyway, 18/24-month-old rats showed the higher decrease of spatial learning and memory when challenged for radial arm maze task, compared to 3 month-old rats (van der Staay and de Jonge, 1993; Wyss et al., 2000). Moreover, the attention and learning deficits observed in SHR (Sagvolden et al., 1992; Soderpalm, 1989; Svensson et al., 1991; Turkkan, 1988; Wultz et al., 1990) were exacerbated by aging.

An interesting publication, concerning the time-dependent memory deficit in SHR, showed that 3 month-old SHR acquired the task more rapidly SD and WKY rats; moreover, aged-matched WKY were significantly slower in learning the spatial task than the two other strains. Conversely, the memory performance in 12 month-old SHR was significantly impaired compared to age-matched SDs, but remain significantly better than age-matched WKYs. In this study the memory performance was related to the number of errors, being susceptible to the potential bias of SHR impulsivity (Clements and Wainwright, 2007). Anyway, in accordance to (De Bruin et al., 2003), this paper showed that both the SHR and WKY rats displayed deficits in spatial learning when compared with the SD rats.

In SHR, the reversion of AHT is associated to an amelioration of memory functions. In fact, antihypertensive chronic treatments, such as captopril, were able to rescue the memory deficit observed in non-treated rats, together with an admelioration of BP profile (Wyss et al., 1992). Moreover, as documented in human hypertensive patients, non-treated SHR showed a local cerebral blood flow decrease in the cortex and thalamus, compared with normotensive rats together with a spatial memory and learning deficits in maze tests (Fujishima et al., 1995). This impairment was also associated to a decreased in cerebral glucose utilization in the medial septal nucleus, hippocampus, and other regions of the brain in 6-7 month-old rats (Johansson, 1986; Wei et al., 1992). Reduced cerebral blood flow, increased media thickness of the cerebral arteries and impaired cognitive function in SHR were improved by long-term antihypertensive treatment (Fujishima et al., 1995). The memory deficit was also linked to an aging- and hypertension-induced enhancement of brain damage and oxidative stress injury in the HPC of SHR, indicated by an increased presence of apoptotic cells and astrocytes (Li et al., 2016).

Moreover, 6 month-old SHR showed BP-related white matter damage, cognitive decline and neuroinflammation suggesting this strain as animal model of early-stage cerebral small vessel disease (Kaiser et al., 2014).

In the majority of the cases the memory impairment was higher in SHR compared to others strains, suggesting that the detrimental effects of hypertension are sufficient to induce the memory deficit, although age may exacerbate it (Goldstein et al., 1990; Hong et al., 1992).

The majority of the studies are in accordance to the fact that the memory deficit can be related to hypoperfusion due to vascular rarefaction (in turn due to vascular degeneration or angiogenic deficit, discussed below). In addition, some studies underlined an involvement of cholinergic alteration in memory impairment detected in SHR, since ACh is involved in learning and memory. As mentioned before the density of nicotinic receptors is reduced in several brain regions such as cerebral cortex, striatum, thalamus, and spinal cord of SHR compared to their normotensive cohorts (Khan et al., 1994; Kubo, 1998; Yamada et al., 1987). Finally, another study suggested a genetic influence (chromosome 4) on learning and memory deficit (Anselmi et al., 2016).

Although the memory deficits are partially or completely reversed by antihypertensive drugs (calcium channel blocker nimodipine in autoshaping task (Meneses et al., 1997)), the treatment for attention deficit with uridine and choline supplementation was able to improve water maze acquisition in both WKY and SHR (De Bruin et al., 2003). The most relevant studies sustaining this hypothesis are

reviewed in (Meneses et al., 2011a). Thus, the decrease of attention observed in this strain can impair the learning and decrease the strength of memory trace, affecting the memory performance score.

Anyway, the discrepancy in memory results showed in literature can be ascribed to the singular behavioural profile of SHRs strain: as a matter of fact more recent papers introduced the idea of an over-interpretation of memory deficit (Sontag et al., 2013), induced by the hyperactivity and impulsivity phenotype of SHRs.

For example, some authors showed that the locomotor activity appears to be a confounding factor in spatial memory tasks and should therefore be controlled. Using a holeboard, Sotang and colleagues (Sontag et al., 2013) studied the influence of the locomotor activity on the cognitive performance of SHRs. They were able to demonstrate that SHRs did not show spatial working memory and reference memory impairments; moreover, when the locomotor activity was taken into account the SHRs' working memory and reference memory were significantly better than in WKY rats, suggesting that SHRs were not a good model to study ADHD, since the memory deficit showed in the others publications were related to the activity profile and not to memory. Other examples explaining the potential bias of locomotor activity in the interpretation of memory tasks are reviewed in (Meneses et al., 2011a). Effectively, the majority of the memory tasks mentioned in the first part of the paragraph are related to locomotor activity. Thus in the choice of memory task, the locomotor and the impulsivity components should be reduced to not introduce a bias in the interpretation of memory result. This consideration strengthen our idea to use STFP to assess memory performance in SHRs; in fact, we decided to characterize the memory profile of SHRs using a task not strongly dependent from the locomotor activity, such all the maze or the recognition tasks.

Conversely, despite the majority of bibliography showed a memory impairment, few works reported that SHR exhibit better performance in several avoidance tasks (Campbell and Di Cara, 1977; Knardahl and Sagvolden, 1982; Randich and Maixner, 1981), and Morris Water Maze task (12 week-old (Widy-Tyszkiewicz et al., 1993)). The reason of this discrepancy is not clear but it may be related to the genetic derivation that regards SHRs and their genetic control WKYs, mentioned below.

Anyway, these publication are contrasted by others papers showing an impairment in habit recent and remote memory in SHRs, compared to WHY and SD, when they have to integrate information derived from sign and goal tracking like in autoshaping task (Meneses et al., 2011b); this deficit was ascribed to a PFC dysfunctions (Johnson et al., 1995). In contrast, when the working memory was assessed in the water maze with different strategic demands, the performance of SHRs did not differ from that of either of SD or WKY rats (Robertson et al., 2008).

In conclusion, the bibliography regarding SHRs suggests that the memory deficit detectable in SHRs depends on the type of memory considered and to the age of the rats. Nevertheless, to clarify the discrepant findings further studies are necessary, taking into account the choice of the control strain and the behavioural task, favouring the paradigms where locomotion and impulsivity are reduced. Surprisingly, any publication was found testing SHRs in STFP.

#### **4.5.5. Good control of SHR**

Deriving from the same ancestral outbred Wistar rat, WKYs are the closest and most used control strain for SHRs.

Despite this, the complicated breeding history of both SHRs and WKYs is responsible to the lack of a comprehensive understanding of the genetic background of different commercial substrains,

introducing a genetic heterogeneity and a large genetic divergence that is going to increase over the time, creating a difficulty in inter-strain and intra-strain comparison.

In fact, a recent study showed substantial behavioural and genetic differences among the WKY substrains, usually depending from the different vendors and breeders, being responsible of inconsistent and even contradictory findings (Zhang-James et al., 2013).

Moreover, rats differ in their emotional reactivity to behavioural stress (Meneses et al., 2011a). WKY rats show decreased locomotion and increased anxiety behaviour as compared to SHR in various behavioural models including the open field, in addition to differences in neurotransmission in different brain regions (Kaehler et al., 2004). More interestingly, as mentioned above WKYs showed deficits in spatial learning when compared with the SD rats (Clements and Wainwright, 2007; De Bruin et al., 2003).

Furthermore, WKYs are considered a model of depression (Lopez-Rubalcava and Lucki, 2000; Rittenhouse et al., 2002).

Thus, since our goal was to study an olfactory associative memory induced by social interaction (see paragraph “The choice of the Social Transmission of Food Preference paradigm in the study of memory consolidation”) we decided to use the Sprague Dawley strain (SDs) as control strain, commonly used in our laboratory.

Our conviction in this choice was corroborated by the work of Ferguson and collaborators which perform a behavioural comparison with elevated plus maze, in young (10 weeks) and adult (1 year) SHRs, WKYs and SDs, showing that the anxiety profile of SHR is closer to SD than WKY (Ferguson and Gray, 2005).

Furthermore, Wyss and colleagues described different rates of spatial memory decline in these three strains; despite we can imagine that the aging process is different in these strains (clearly represented by mean life span: SHR:18 months, WKY: 24 months, SD: 23 months (Ferguson and Gray, 2005)) both of those strains outperform the same-aged WKY; thus, we thought that the use of WKY strain as control can be a potential bias in behavioural interpretation (Wyss et al., 2000).

Finally it has been shown that WKY could present hypertension and display both hippocampal functioning deficits and signs of bilateral hippocampal cell loss and a deficit in HPC-related spatial memory (Wells et al., 2010).

In conclusion, despite we selected the SHRs to control the putative permissive mechanism of vascular network in the systems consolidation, others models can offer the possibility to induce a variation of vascular functions, allowing us to control the onset and the level of vascular perturbation, as described in the next chapter. The plethora of AHT-related models give us a wide choice, despite this, we decided to venture onto an atypical model, which has been shown to perturb the vascular function: the hypergravity.

The hypergravity induced by centrifugation allow us to precisely control the onset and the gravity of vascular modification depending on the duration of centrifugation and the level of hypergravity achieved; thus, this stimulating topic prompted us to study whether vascular modifications can impair memory consolidation.



## **Chapter 5: A ticket to space**

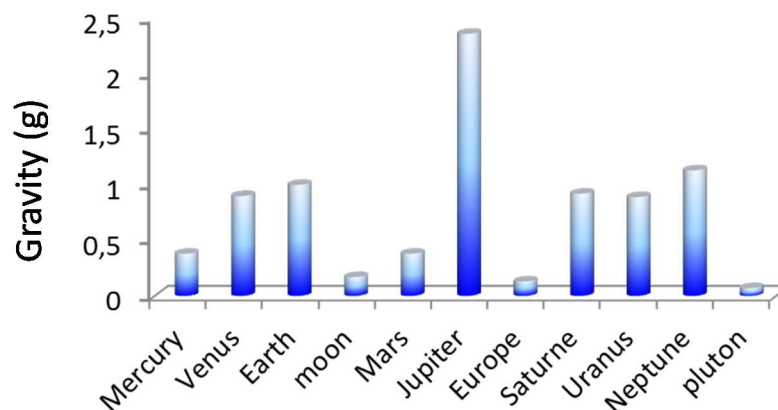
## **Chapter 5: A ticket to space**

As mentioned before the aim of this part is to use gravity alterations, known to affect vascular functions, as a tool to study vascular dysfunctions and their effects on memory.

The gravity is the force of attraction that exist between two any mass, particles or bodies. Plato and Aristotle believed that Planets and Stars were disposed on a concentric crystalline sphere centred on the Earth and they move “naturally” following a circular orb; they were not subjected to physical influence because they were considered as a ‘fifth element’ or ‘quintessence’. Controversially, the physical elements moved vertically, depending on their ‘heaviness’ or ‘gravity’; in particular, the heavy bodies move downward toward the centre of the universe because of their heavy nature. Conversely, light elements, such as fire, move upward toward the Moon. Thus in Aristotle's system heavy bodies are not attracted to the earth by an external force of gravity, but tend toward the centre of the universe because of an inner gravitas or heaviness. Hopefully, Isaac Newton in *Philosophiae Naturalis Principia Mathematica*, 1687, understood that the same force causing the fall of an apple to the ground keeps the planets in their orbs around the sun and the moon around the earth and is the same force that allows us to live on the surface of the earth without being thrown away. Newton's law of universal gravitation states that any two bodies in the Universe attract each other with a force that is directly proportional to the product of their masses and inversely proportional to the square of the distance between them:  $F = G \frac{m_1 m_2}{r^2}$ , where  $F$  is the force between the masses;  $G$  is the gravitational constant;  $m_1$  is the first mass;  $m_2$  is the second mass and  $r$  is the distance between the centres of the masses.

Gravity force is exerted permanently on all human being and it conditions their molecular, cellular and physiological organization; this happens not only on the Earth but also during spaceflights.

The life on Earth is influence by gravity force as shown in plant that are sensitive to gravity change s in a  $10^{-3}$  to  $10^{-4}$  G range (Driss-Ecole et al., 2008). All living species, exposed to spaceflight or changes in gravity force, as observed on other moons or planets (Fig. 68), show a physiological adaptation. If the Humanity wants to colonized Moon or Mars, a deeper knowledge concerning the effects of long lasting spaceflights and modified gravity on physiology is required. Furthermore, some studies developing altered gravity were aimed to understand the adaptation to internal fluid shifts and vestibular modifications.



**Figure 68:** Gravity level expressed in g on solar system planets and Moon. One of the Jupiter's moons, Europe, is also indicated because water has been recently found on this satellite and the possibility of life cannot be excluded.

During space-flights astronauts undergo to constant environmental modifications: a diminution of gravity (microgravity) caused by the increase of distance from the Earth, the different gravity accelerations that exerts the largest effect on physiology, the isolation and confinement that impacts the well-being of astronauts and the radiations (in particular secondary radiations produced by the impact of high energy protons, from the sun, with the materials of the rockets or the space station (Williams et al., 2009).

Even if the man has been able to adapt his life in space, the absence of gravity alters physiological systems in the short or long term. The acclimation (physiological and psychological responses to the space-flight environment) can be referred as short-term (hours to days) or longer-term exposures (days to months). A simplified summary of these changes is shown in the Fig. 69.

Physiologic effects	Launch	Duration of flight				Landing	Postflight period			
		24 h	48 h	2 wk	> 1 mo		24–48 h	1–2 mo	> 1 yr	
Fluid redistribution	<ul style="list-style-type: none"><li>• Redistribution of fluid to the torso and head</li><li>• 10% decreased fluid volume in the legs</li></ul>	<ul style="list-style-type: none"><li>• 17% reduction in plasma volume</li></ul>	<ul style="list-style-type: none"><li>• Gradual decrease in erythropoietin secretion, leading to a 10% decrease in total blood volume</li></ul>			<ul style="list-style-type: none"><li>• Orthostatic hypotension from pooling of fluids in the legs</li></ul>	<ul style="list-style-type: none"><li>• Return of normal fluid distribution</li></ul>			
Neurovestibular effects	<ul style="list-style-type: none"><li>• Space motion sickness</li></ul>			<ul style="list-style-type: none"><li>• Space motion sickness</li></ul>						
Muscle changes		<ul style="list-style-type: none"><li>• Gradual decrease in muscle mass by 20%</li></ul>	<ul style="list-style-type: none"><li>• Gradual decrease in muscle mass by 30%</li></ul>		<ul style="list-style-type: none"><li>• Muscle soreness and tightness</li></ul>		<ul style="list-style-type: none"><li>• Full recovery of muscle mass and strength</li></ul>			
		<ul style="list-style-type: none"><li>• Gradual decrease in muscle strength (up to 50% loss observed)</li></ul>								
Bone demineralization		<ul style="list-style-type: none"><li>• 60%–70% increase in calcium loss (urinary, fecal). Reduced parathyroid hormone and vitamin D production.</li><li>• Gradual loss of bone density (1%–2% per month)</li></ul>							<ul style="list-style-type: none"><li>• Complete or almost complete restoration of bone density</li></ul>	
Psychosocial effects	<ul style="list-style-type: none"><li>• Fatigue, sleep debt, isolation, emotional effects, stress to the astronaut's family, multicultural crew environment</li></ul>									
Immune dysregulation		<ul style="list-style-type: none"><li>• Possible reactivation of latent herpes viruses and impairment of cell-mediated immunity</li></ul>			<ul style="list-style-type: none"><li>• Numerous cellular and other changes leading to impaired immunity</li></ul>		<ul style="list-style-type: none"><li>• Gradual improvement in immunity (days to weeks)</li></ul>			

**Figure 69:** Timeline of physiologic acclimation and acclimatization experienced by astronauts from launch to after return to earth. From (Williams et al., 2009).

The first rapid phenomena (in the first two hours) that affect the astronaut is due to the change in spatial orientation and is called Space Adaptation Syndrome (SAS) or space sickness and provoke nausea, headache, lethargy, vomiting. This sickness is reversed within few hours but the most significant and serious effects regard the alteration of bone calcification inducing osteoporosis and muscle atrophy (Porte and Morel, 2012).

Together with a constant physical training that precedes the flight, the primary counteractions against microgravity-induced muscular changes are the exercise during space flight and rehabilitation after landing even if such arrangements are not sufficient for the long term spaceflight. Moreover physical exercise during space flight consumes valuable resources on the International Space Station including oxygen, water, food and crew-time. For these reasons, alternative countermeasures should be developed. Recently, dietary supplements were also proposed to compensate the metabolic disorders as the ectopic fat storage also observed in space (Bergouignan et al., 2011).

However, besides the deficits mentioned above, modification of gravity affects also the vascular physiology and the brain functions and this is the reason why, at the beginning of the thesis, our ambition has been to study a model able to recapitulate this symptoms.

Because cardiovascular functions and cognition must be preserved in humans undergoing to spaceflights, spatial agencies strongly support the research in these two fields. Since on the Earth, the gravity force influences body blood volume distribution (blood distribution is mainly concentrated in the lower part of the body), the absence of gravity induces a fluid redistribution shift towards the upper part of the body; consequently the blood pressure gradient assured on the Earth is affected, inducing a cardiovascular adaptation (Dabertrand et al., 2012). But what is the impact of these adaptations on cognitive functions?

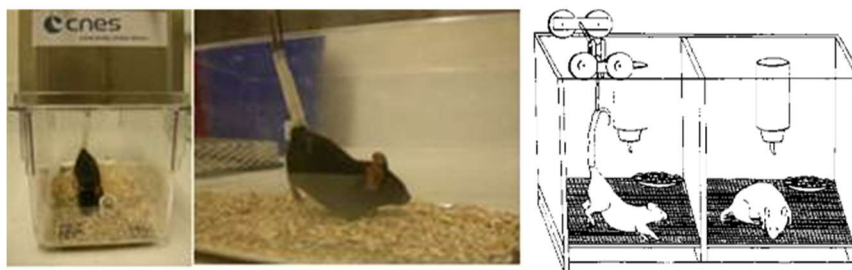
Being the number of studies on astronauts limited, our preliminary contribution was aimed to understand if memory process could be affected by hypergravity alteration. However, a plethora of questions remain unanswered. Could the memory be affected by gravity modifications? If so, which form of memory? What are the bases of the alterations?

Some studies tried to shed light on these questions, and in the following part we are going to firstly introduce how it is possible to achieve a model of altered gravity \on Earth, and secondly we will revised the literature concerning gravity alterations and its effect on memory, in particular via vascular adaptations.

### 5.1. Rodent models of gravity alteration.

There are different experimental designs to modify gravity. The best one but the most expensive is the spaceflight. From the first flight of Laika (1957) and Iouri Gagarin (1961), each year many space experiments have been done but, of course, not enough to produce statistically relevant results; for that reason laboratories on Earth are trying to achieve this goal.

In laboratories, several devices have been realized as hindlimb suspended or unloaded model, which model the unloading in the posterior part of the body and the fluid shift observed in space, mimicking the microgravity condition. This model is often used since it is able to reproduce the bone loss and muscular atrophy in the hindlimbs together with the fluid shift in the cephalic part of the body. However, the physical constriction can be considered an important bias in behaviour experiments affecting the performance, such as, for example, in a maze (Fig. 70).

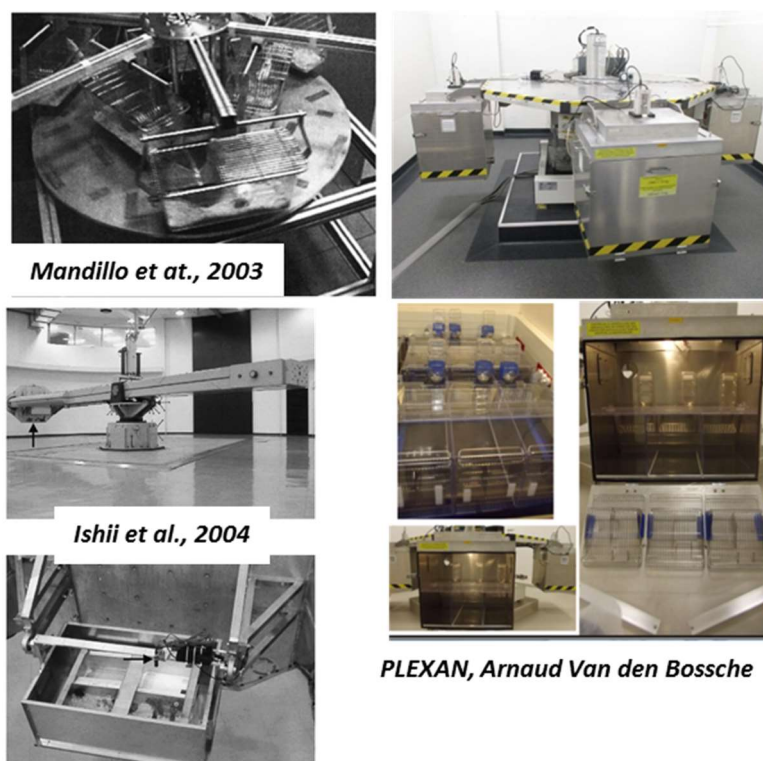


**Figure 70:** Models of tail suspension for mice (left) and rat (right).

In human the head-down bed rest or dry immersion are used to reproduce the lack of activity and the fluid shift with consequences on bones, muscles and for the cardiovascular system.

Another model used to study gravity changes is the hypergravity achieved by centrifugation.

This model has been developed based on the theory postulating that hypergravity (close to 2-3G) could produce the opposite effects to microgravity (Plaut et al., 2003; VanLoon, 2016). To study hypergravity modifications, during our study we used the device built by CNES and available in PLEXAN (University of St Etienne) because it offers the possibility to centrifuge large cohorts of animals. Moreover, the housing conditions during centrifugation are closer to the ones used in the STFP experiments (fig. 71).



**Figure 71:** Centrifugation devices used in previously mentioned publications (left, black and white) or by us in PLEXAN, St Etienne with inside view of gondola (right, in colour).

Despite some advantages, the interpretation of centrifugation results should take into account some considerations; the gravity can induce physiological effects on cerebral functioning depending on the level and the duration of hypergravity. Thus, in the choice of a short centrifugation period, that can mimic the acute effect of space-shuttles launch/landing, might be insufficient to induce observable effects. Vice versa, if we want to compare chronic protocols to the longer astronauts' spaceflights we could assume some potential bias induced by the non-equivalent life duration and by different biological development across species (Porte and Morel, 2012).

Moreover, the results are obtained in normogravity conditions, i.e. when the centrifuge is stopped. Thus, it is difficult to understand whether the effects relay on the hypergravity period or the change of gravity related to the stop of the centrifuge.

Furthermore, the level of centrifugation achieved is also important since it can affect the anxiety level of the animals (see the MS reported in the annex).

Finally, another difference between the human conditions during the spaceflight and the ground experimental model consists in the absence of spaceflights radiations, confinement, stress, lack of resources, sleep loss, fatigue, circadian desynchronization, work overload and others physiological adaptations as cardiovascular deconditioning that can cooperate synergistically altering the cognitive functions. In laboratory, it is difficult to reproduce all these parameters together. Nevertheless, the

centrifuge reproduces the confinement and the modification of gravity and a potential stress due to the acceleration of the centrifuge.

Despite the different factors participating to cognitive alteration during spaceflights, in our study we focused on gravity effects on memory and vascular systems; however, after the revision of the bibliography concerning memory and vascular modification, in the last part of the chapter, we present the possible effects of stress on memory since spaceflights, microgravity and hypergravity events present also stressful situations.

Concerning our project, the centrifugation represents both a protocol of gravity change and a modification of environmental conditions, known to modify vascular reactivity and brain functions. As seen for the hypertension model, we can imagine that the physiopathological adaptations of the vascular systems to hypergravity can be deleterious for the memory process.

## **5.2. Gravity changes, learning and memory**

In the total amount of 500 astronauts exposed to microgravity during spaceflights, no amnesia during and after these spaceflights has been observed, despite the memory processes could be finely or sneakily affected. In a study following the gravity induced modifications on the vestibular system it was reported that vestibular system also seems to play an important role in building and maintaining a mental representation of the world” (Mast et al., 2014). Then, gravity changes disturbing vestibular function can act on behavioural aspects such as emotions and memory. In fact, as reviewed recently (Bojados and Jamon, 2014; Mast et al., 2014; Porte and Morel, 2012), the vestibular system is implicated in spatial memory because the representation of space is required to identify the position between the subject and environment.

In Humans, studies about memory performances have been performed on astronauts but the results should be carefully interpreted because the experimental conditions are really different from each other (duration of spaceflight, memory tests,..), depending on the goal of the space mission. However, they revealed that motor behaviours is impaired and it can interfere with simultaneous cognitive task performance (Fowler and Manzey, 2000; Manzey et al., 1993, 1995; Manzey et al., 2000). Moreover, the perceptions of orientation (Dyde et al., 2009; Leone et al., 1995) and longitudinal body axis (Clement et al., 2007) are clearly altered during microgravity periods in parabolic flights. However, the mental rotation of three dimensional objects seems not affected (Leone et al., 1995); in contrast, recently, an altered 3D-images perception task has been reported by EEG at the beginning of the task (Cheron et al., 2014). Finally, modifications of grey matter are measured in volunteers exposed to bed rest during 30 days (Li et al., 2015).

In summary, experiments on humans showed that gravity changes are not able to abolish brain functions but they can modify some parameters of these functions as kinetics, duration, encoding. These parameters observed on highly trained humans as astronauts have been modeled in rats and mice to understand the fine mechanisms and the basis of gravity effects on memory.

In animal models, the effects of microgravity on memory appear controversial at first sight. For example, in rats centrifuged at 10-15G during 3-5 min, hypergravity induces spatial learning and memory impairment in Y and Morris Water Mazes together with neuronal apoptosis (Cao et al., 2007; Sun et al., 2009). Despite the intense exposure to high G, it is more reasonable to attribute these effects to intense acute physical and psychological stress, rather than hypergravity.. In another study, spatial learning and memory deficits have been revealed using Morris Water Maze in hindlimb suspended rats (Qiong et al., 2016), whereas this is not the case in rats after their early development in microgravity during spaceflight (STS90) (Temple et al., 2002). In this last study, a weak impairment is revealed on

the beginning of the learning phase (Temple et al., 2002). Finally, hypergravity-induced memory effects results to strictly depend on sex, duration of hypergravity exposure and animal species used (rat versus mouse) as reviewed in (Bojados and Jamon, 2014; Francia et al., 2004).

Hypergravity has been used to study two different effects: 1) to mimic a chronic perturbation of gravity, the opposite of microgravity induced by spaceflight, where hypergravity is applied chronically during days, and 2) as a countermeasure to limit the effects of microgravity, especially bone loss and muscle atrophy, in this last case, hypergravity is applied during a repetitive short period (1-2h/days).

The chronic hypergravity is principally used to disturb the early phase of brain development, so there are only few experiments on adult animals. After chronic centrifugation (14 days, 2G), only the initial phase of radial arm maze acquisition is affected and this delay in acquisition is rapidly compensated by hypergravity-induced hyperactivity (Mitani et al., 2004). Similar results were found in mice exposed 21 days to hypergravity and tested in Morris Water Maze (Bojados and Jamon, 2014). This last study, moreover, compared the effects of 2G to 4G hypergravity and confirms that the level of G is particularly important; at 3G-4G, the level of plasma corticosterone is significantly increased and behavioural exploration confirmed that mice were more stressed. So, it is not possible to exclude stress side effects. If one hour of centrifugation has no effects on the discrimination of new spatial arrangements novel object recognition, exposures to 2 hours or repetition of 1 hour during 5 days, 2G centrifugation alters this discrimination (Mandillo et al., 2003). But in this study, the device could influence *per se* the stress parameter and visual perception as shown in Fig. 71: in fact the use of transparent cages together with and the rotation of the centrifuge could influence the vision. This bias could explain the behavioural difference observed between both situation and “home cage” animals.

Sometime, the centrifugation can have even a beneficial effect on cognition, especially on flexibility, as assessed by water maze reversal test in young or adult CD1 mice treated 1h/day during infancy (Francia et al., 2004; Francia et al., 2006).

Finally, the social interactions were not significantly affecting in animals born in hypergravity (1.8G), just the self-grooming and latency before the appearance of cross-under events were decreased during social interactions (Thullier et al., 2002).

### 5.3. Gravity changes and neuronal function

The behavioural analysis of cognition is concomitant and/or associated with structural and molecular analysis of brain.

The c-Fos labelling reveals that gravity modifications induced the activation of the hypothalamic-hypophysis-adrenal axis suggesting effects on brain and on the stress pathways (Gustave Dit Duflo et al., 2000). High levels of hypergravity increase the neuronal apoptosis (Cao et al., 2007; Sun et al., 2009) and the incomplete filling of glutamate vesicles associated with a reduced exocytosis (Krisanova et al., 2009). In microgravity conditions, after 7 days (Spacelab3), the measure of radioligand binding indicated that the expression of 5HT1 receptor is increased in hippocampus whereas 5HT2, muscarinic, adenosine A1, GABA,  $\beta$ -,  $\alpha$ 1- and  $\alpha$ 2 adrenergic receptors were not affected (Miller et al., 1989). The cDNA arrays indicated that microgravity or simulated hypergravity affects the gene expression in brains structures involved in memory. In fact, after 2 weeks of simulated microgravity the gene encoding for the NMDAR1 -subunit of NMDA receptor which activation is at the origin of the induction of LTP- is upregulated (Frigeri et al., 2008). Hypergravity (1h/days during 5 days) increased also the hippocampal expression of the gene encoding SNAP-25 related protein involved in synaptic transmission. Interestingly, some genes encoding proteins closely related to stress response and control of pain are also affected (Del Signore et al., 2004).



Microgravity affects hippocampal neurons after 14 days of hindlimb suspension with a decrease of area, perimeter, synaptic cleft and length in CA1 neurons. However, the number of nodes, spines, and spine density were increased (Ranjan et al., 2014). But unfortunately these experiments have not explored the hippocampal-cortical connections and functionalities required for the memory tasks. Moreover, in CD1 mice exposed to hypergravity, the decrease of memory performances could be linked to the decrease of BDNF in hippocampus and to a decrease of NGF in frontal cortex (Francia et al., 2006) .

Recently, some studies have explored the role of oxidative stress in microgravity and hypergravity on brain functions. The spatial memory impairment measured in mouse exposed to hypergravity is reversed by antioxidant molecules but also by electroacupuncture (Feng et al., 2010). In fact, the simulated microgravity (21days) increased the oxidative stress and affects the expression of metabolome to regulate brain activity against oxidative stress (Wang et al., 2016c) and modified the expression of antioxidant genes (Sod1, Sod2) and their specific miRNA (miR-134 and miR-125b-3p) in the hippocampus but not in the cerebral cortex (Chen et al., 2016). This effect of hindlimb suspension is reversed with injection of mechano-growth factors known to protect neurons against death (Chen et al., 2016).

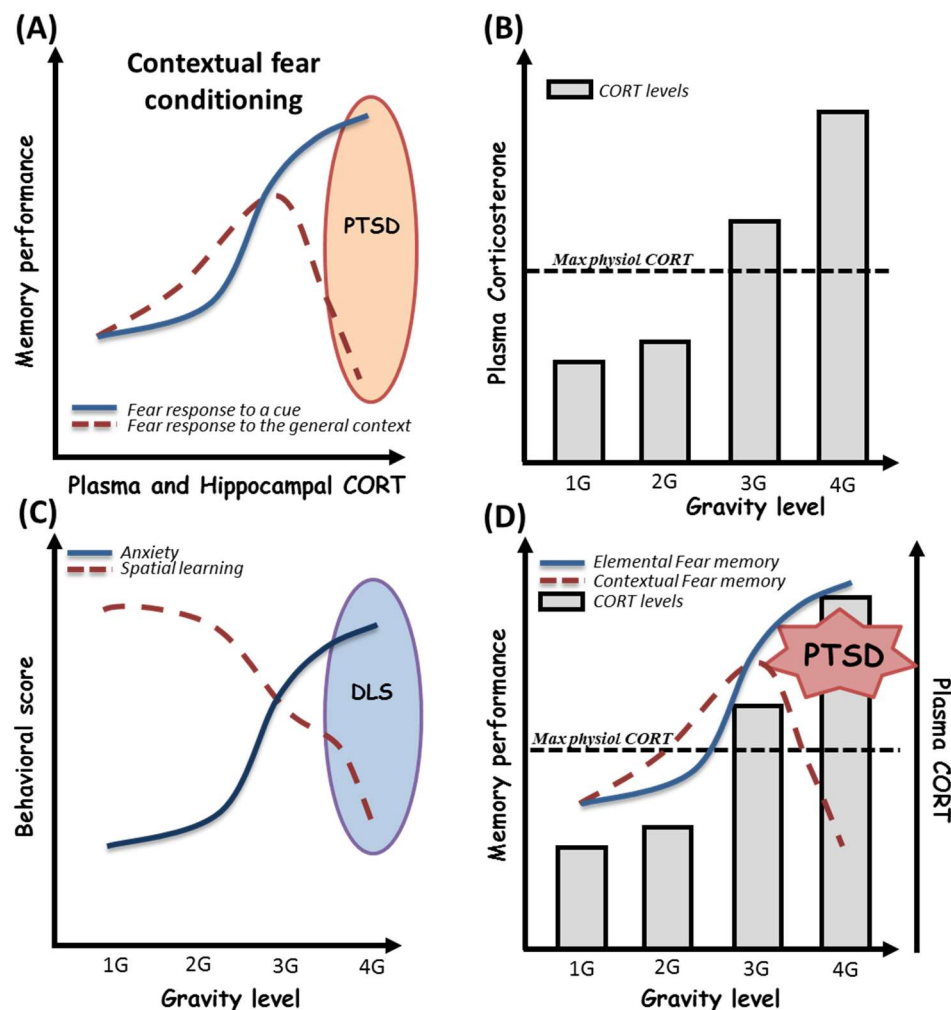
The long term potentiation is preserved in rat hippocampus exposed to hypergravity (Guinan et al., 1998), but 4G acceleration (during 48 h) induces the phosphorylation of AMPA receptors and increases the excitatory postsynaptic potential (EPSP) slope 1 h after tetanus stimulation, leading the authors intituling the paper “exposure to high gravitation forces induces long-term potentiation” (Ishii et al., 2004). They also suggested that this effect could be due to the action of stress hormones.

The situation during spaceflight can be even more complex depending on other factors including physics and psychological factors. For instance, radiations can affect memory; in particular in animal models, the cosmic radiations can accelerate the formation and deposit of  $\beta$ -amyloid (Cherry et al., 2012; Direk et al., 2013). The different phase of launch and landing and the modification of environment can be stressful, and then the stress effects on memory are evoked in the followed paragraph.

#### **5.4. Gravity and mood**

Stress, as well as anxiety and depression, dramatically modulate the encoding and retrieval of memory (Hur et al., 2016; Marchewka et al., 2016; Narme et al., 2016). The link between stress, gravity modifications and possible memory troubles have been recently reviewed (Porte and Morel, 2012). Briefly, increasing arousal promotes encoding and relevant retention of memory until a limit after which it becomes deleterious. The effects of anxiety and stress vary in function of the type of memory considered; very high levels of stress tend to alter relational memories (spatial, contextual) to the benefit of procedural ones (cued fear learning) (Packard, 2009), sometimes even leading to pathological profiles such as post-traumatic stress disorder (Fig. 72A) (Kaouane et al., 2012).

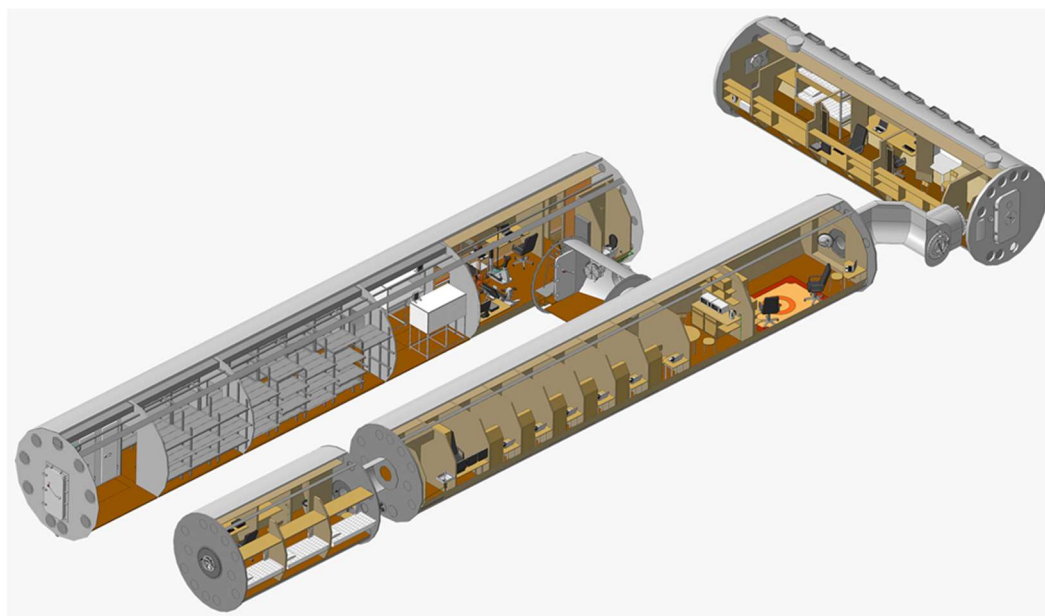




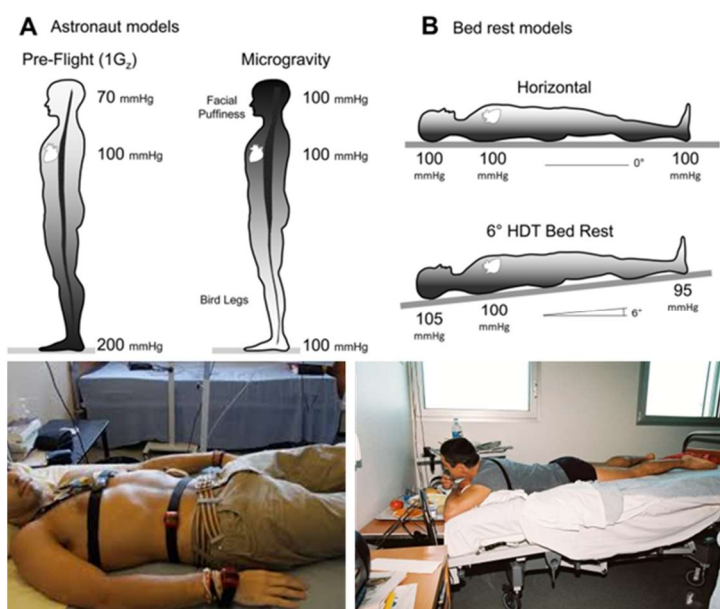
**Figure 72:** A) Effects of acute stress, as assessed by plasma and hippocampal corticosterone (CORT) levels, on contextual vs elemental fear memory. Globally, stress is associated with beneficial effects on both procedural cued and relational memories. However, in a given environment in which a negative event occurs, and under high emotional conditions, subjects can develop hypermnnesia for a salient cue of the environment associated to amnesia for the general context of the event. This paradoxical pattern, well described from the psychological point of view, has been named “Post-traumatic stress disorder” (PTSD). As depicted here, recently, Kaouane and colleagues modelled a PTSD-like syndrome in mice by increasing systemic and hippocampal CORT levels after contextual Pavlovian conditioning, demonstrating the importance of stress hormone concentration in the trauma. B) Effects of gravity level on plasma CORT in mice. Plasma CORT levels increase beyond the physiological maximal levels when mice are bred under more than 3G conditions by centrifugation (adapted and schematized from Guéguinou et al., 2011). C) Persistent effects of gravity level on anxiety and hippocampal learning in mice. Increasing levels of gravity (from 1G to 4G) dramatically increase persistent (>10 days) anxiety troubles associated to spatial learning impairments in the Morris Water Maze. We then hypothesize a possible depressive-like syndrome in mice bred under high gravity level conditions. D) Expected psycho-physiological pattern of mice bred under high gravity level conditions. High gravity level breeding conditions induce an increase in plasma and hippocampal CORT levels beyond the maximal physiological levels. As shown in this combined scheme, we hypothesized that these CORT levels could impair contextual conditioning during centrifugation. This could lead to a paradoxical pattern in which mice initially trained to associate a global environment with an electrical shock would display fear memory for a salient cue and amnesia for the context in its whole. In summary, 4G centrifugation associated with a negative event could induce PTSD-like syndrome in mice. From (Porte and Morel, 2012)

Mood disorders affect astronauts and can influence the crew’s mental stability (Carter et al., 2005; Kanas, 1998). Gravity, as well as confinement, loss of light-dark cycle, alteration of perceptions, the perception of the Earth and lack of physical activity, are probably implicated in the development of mental disorders. To study the segregation between gravity and other stressors, a crew should be totally isolated like in Mars500 (Fig. 73, a crew has been isolated during 500 days as in the module

reproducing a space module designed for missions to Mars) or Antarctic winter-over. The psychological effects of confinement have been recently reviewed and the increase of stress makers is confirmed in these set of experiments (Pagel and Chouker, 2016). The 6° head-down tilting bed-rest can also reproduce the lack of physical activity and the redistribution of corporal fluids (Fig. 74) (Hargens and Vico, 2016) but the effects on mood are not evaluated in the same manner because the subjects are solicited every day by the medical teams. Anyway, the subjects feel a decrease of their attention and periods of discouragement partially due to the repetitions of medical solicitation (for biopsies, travels to physical training devices,...) and the lack of activity.



**Figure 73:** Plan of Mars500 isolator (<http://mars500.imbp.ru/>).



**Figure 74:** A and B explanations of the fluid shift observed in real microgravity and in bed rest situation (from Hargens and Vico, 2016). At the bottom, two pictures of human in bed rest position, (from MEDES, the European space clinic where bed-rest experiments are conducted for ESA).

In young Sprague-Dawley male rats, the hindlimb suspension induces, after 2 weeks, a loss of sucrose preference coupled with the dysfunction of cardio-vascular tone due to sympatho-vagal imbalance (Moffitt et al., 2008). This anhedonia is sensitive to classical anti-depressors as Fluoxetine, an inhibitor of serotonin reuptake. The serotonin pathway is affected in microgravity and simulated weightlessness especially in hippocampus (Blanc et al., 1998).

Different studies showed that microgravity and hypergravity were associated with anxiety or stress. As previously mentioned, peri-adolescent CD1 mice and C57BL6j exposed to hypergravity (2G during 2h and 3-4G during 21 days, respectively) display anxiogenic profile tested elevated plus maze or light dark box testes (Francia et al., 2004; Gueguinou et al., 2012). The activation of hypothalamo-hypophyso-adrenal axis as well as the activation of amygdala after modification of gravity also suggests an increase of stress (Gustave Dit Duflo et al., 2000).

As also summarized in (Porte and Morel, 2012) “when a subject is submitted to non-terrestrial gravity conditions, he undergoes physiological adaptations that can evoke deregulations in mood, affect and arousal systems, resulting in the development of despair, anxiety– and/or depressive-like behaviours. From this line of evidence, and knowing that mood and arousal are direct modulators of memory acquisition and retention, one can consider the importance of studying these functions under modified gravity conditions”.

## 5.5. Effects of gravity modification on vascular physiology

In the previous chapter we have described how vascular dysfunctions can affect memory process, being considered a risk factor to the development of neurodegenerative disorders; since gravity modifications could affect spatial memory it is possible to assume that vascular function can be also involved in this phenomenon. **In human, during spaceflights the microgravity induced a corporal fluid shift or redistribution responsible for a cardiovascular deconditioning** (De Santo et al., 2001, Convertino et al., 1989, Norsk, 1992), reproducible in bed-rest experiments. The resulting effect of weightlessness is a vascular adaptation to the increase in cardiac output by reducing the systemic vascular resistance, which limits the increase of blood pressure blood pressure. This has an opposite effect in cerebral arteries (reviewed in Norsk and Christensen, 2009). During the first spaceflights, the blood concentration of norepinephrine have been measured indicating that the sympathetic response was exaggerated just after launch, returning to nominal values during the spaceflight, suggesting an adaptation more than alteration. In the landing phase the orthostatic pressure drop seems more deleterious and the cardiovascular mismatch seems more complex (Norsk et al., 2015). Moreover, long-term effects cannot be excluded; a recent retrospective study on astronauts indicates that the death due to cardiovascular disorder is significantly increased in the part of this population exposed to the deep space radiation, in physically and mentally trained men and women (Delp et al., 2016).

**The most important cardiovascular problem for astronauts is the orthostatic intolerance.** Retrospective study on astronauts exposed to microgravity during 7-18 days indicated that the orthostatic intolerance was observed if the arterial compliance was increased; contrary, if the compliance was decreased there was not orthostatic intolerance (Tuday et al., 2007). **In simulation of orthostatic intolerance experiments, the autoregulation of cerebral blood flow was not affected** (Rickards et al., 2007). A modification of cerebral arteries adaptation was measured during spaceflight (Gazenko et al., 1981). During the microgravity phase in parabolic flight, the blood pressure decreased as expected but the blood flow velocity in middle cerebral artery was not affected (Ogoh et al., 2015) whereas in bed-rest experiment it was reduced (Goswami et al., 2015). These results suggest that the cerebrovascular adaptation to fluid redistribution takes more time than in “systemic vessels”, confirming the measurement performed on astronauts of MIR. The vascular resistance alteration has

been decreased during the first 14 days and after day-24 it returned to normal values (Arbeille et al., 1996; Arbeille et al., 2001).

**The vascular adaptation to microgravity is due to modification in both endothelial and smooth muscle cells.** The animal models, even if the redistribution of fluid is not so important than in human (size, quadruped), are needed to understand the structural and molecular adaptations. In vascular field many or arteries and veins were studied after spaceflights and hindlimb suspension. Here, we focused on the effects of gravity alteration on cerebral arteries.

#### 5.5.1. Structural Modifications

**The effects of gravity modifications on vessel walls are controversial.** It seems depending on the model (human, rat or mice) (human, rat or mice) paradigm (spaceflight versus bed-rest or hindlimb suspension) and probably aging. This last point is poorly studied for the moment but both for memory and vascular alterations aging is an important factor (for example in the last century astronauts are more closed to 45-50 year-old for the first flight; now it is more closed to 35 year-old). For example, in human, during bed-rest (5 weeks) the lumen of femoral artery decreases without change of wall thickness and carotids are not affected (Palombo et al., 2015) whereas during spaceflight (6 months) carotids results more rigid (Hughson et al., 2016). **Studies describing the effect of gravity on artery wall shows similar the results to those observed in atherosclerosis.** After 7 days of hindlimb suspension, collagen increases in aortic wall (Tuday et al., 2007; Tuday et al., 2009) and the phenomenon worsen with the duration of suspension (Gao et al., 2009). Carotid lumen is decreased via an increase of glycocalyx (Kang et al., 2015). In middle cerebral artery, a decrease in elastin and increase of collagen are associated with a vascular hypertrophy (Lin et al., 2009) (Cheng et al., 2014; Looft-Wilson and Gisolfi, 2000). These results are presented as consequences of changes in reactivity, and the increase of pro-inflammatory factors implicated in vascular remodelling as Vcam and MPC1 (Liu et al., 2014).

#### 5.5.2. Regulation of the endothelial cell proliferation, a link with angiogenesis?

The adaptation of endothelial cells to microgravity and hypergravity was studied in culture placed in special devices for clinorotation, spaceflight or centrifuges. In vivo, the function of endothelium is tested through the contractile properties of the vessels.

As observed in vivo and in memory studies, the alteration of endothelial cell functions depends on the duration and force of gravity changes but also the origin of the cell appears determinant as reviewed recently (Maier et al., 2015). Indeed; the effects of gravity changes are different on endothelial cells from small or large vessels. The time course of the proliferation/differentiation balance is highly complex in simulated microgravity. The first hour of clinorotation can increase the proliferation of endothelial cells (Carlsson et al., 2002). In details, after 24h in clinorotation, iNOS is overexpressed, NO is increased, in 3D culture endothelial cells create tubes or necrotic aggregates (Siamwala et al., 2010), with AP1 transactivation (Wang et al., 2009) and modification of purinergic receptor pattern (Zhang et al., 2014b). After 48h, autophagosomes are formed via increase of expression of LC3 becline-1 (Wang et al., 2013b), first alterations of the cytoskeleton are measured and cells are more rounded (Janmaleki et al., 2016). During the first minutes to 10 days the synthesis of osteopontin and VEGF are modulated in opposite way to explain the different phases of proliferation and necrosis of the cells (Grimm et al., 2010; Infanger et al., 2004). Alterations of cytoskeleton are associated with increase of expression of pro-apoptotic factors (caspase-3, Bax1, p65,...) (Infanger et al., 2007) and

mitochondria disassembling (Morbidei et al., 2005) and finally apoptosis is clearly measured after 72h (Kang et al., 2011).

Angiogenesis should be affected since the increase of NO production, Timp2 Icam1 and Vcam expression and the decrease of proteasome activity observed in cultured cells can interfere with angiogenesis (Maier et al., 2015; Mariotti and Maier, 2008) (Muid et al., 2013).

### **5.5.3. Vascular reactivity adaptations**

The intravascular fluid loading and redistribution of fluid shift due to gravity changes influence the vascular reactivity in all vascular beds. Overall, the intravascular discharge causes an adaptation reducing vasoconstriction and sometimes the myogenic tone whereas fluid overload increases vasoconstriction. It is that why, many studies showed arteries differentially affected by spaceflight or hindlimb suspension, and also why the effects are globally in opposite ways. . The mechanisms involved affects the vasorelaxation induced by NO pathway in ECs and the vasoconstriction through the excitability and calcium signalling in SMC. The regulation of myogenic tone is still the subject of debate with conflicting results for the effect of spaceflight and suspension but also because its molecular bases are embedded in those balance vasoconstriction / vasorelaxation (Colleran et al., 2008; Looft-Wilson and Gisolfi, 2000). In cerebral arteries the results have been principally obtained in suspended animals and they are in perfect accordance with the theory claiming that the overload of fluid increases the vasoreactivity. Indeed, increase of vasoconstriction induced by depolarization, norepinephrine, angiotensin-II and serotonin measured in carotids, basilar, middle and posterior cerebral arteries correlates with the increases of AT1a and AT1b angiotensin-II receptors (Bao et al., 2007) CaV1.2 expression and L-type calcium currents (Xue et al., 2011), RYR (Morel et al., 2013).

The vasorelaxation drawn more attention thanks to its potential link with orthostatic pressure drop and intracranial hypertension related to perturbation of visual acuity. The decrease of efficiency of the NO pathway is measured in hindlimb suspended rat, increasing myogenic tone (Geary et al., 1998), via the decrease of eNOS expression (Prisby et al., 2006; Wilkerson et al., 2005). The repolarizing pathways are also affected by the increase of potassium conductance and channel (Fu et al., 2004; Xie et al., 2005; Xue et al., 2011; Xue et al., 2007). Surprisingly, the results of the experiment obtained during different spaceflights differ from each other. The recent BION-M1 experiment performed on C57BL6/N mice showed a decrease of depolarization- and thromboxane A2-induced contractions without modification of ACh-induced dilatation (Sofronova et al., 2015) confirming only partially the first results obtained with STS135 spaceflight (Taylor et al., 2013). The differences between the two spaceflights experiments could be due to experimental design (flight duration, radiations, return phase, sex and aging of mice) but also to the CO2 partial pressure. Indeed, the difference in pCO2 between ISS and Soyuz is closed to 100 fold. The duration of exposure to gravity change is also important for cerebral arteries, in human. Finally, an increase of apoptosis of smooth muscle cells is detected in cerebral arteries from hindlimb suspended rats, probably due to the increase of expression of calcium activated large conductance potassium channels (Xie et al., 2010).

### **5.5.4. Adaptation of cultured vascular smooth muscle cells**

In culture, microgravity (cells were placed in clinorotation or in specific device for spaceflights) modifies the expression pattern of purinergic receptors increasing P2X4 and decreasing P2X2 (Zhang et al., 2014b); moreover, it decreases the expression of RyR1 (Dabertrand et al., 2012), modifies the phosphorylation of myosin heavy chain (smMHC) and caveolin-1 (Spisni et al., 2006) and increases the destructure of cytoskeleton to increase apoptosis (Kang et al., 2013). So, the modifications

of P2X and RyR suggest an alteration of the calcium signalling linked to the decreased reactivity observed in arteries undergoing increase of blood pressure.

#### **5.5.5. Specific role for oxidative stress**

Oxidative stress induced by reactive oxygen species (ROS) is one of the most important pathways altering cell physiology. It can be triggered by stress, radiations and aging, all parameters linked with gravity changes and environmental correlates.

Angiotensin-II induced hypertension, mitochondrial dysfunctions and stimulates NADPH oxidases (Doughan et al., 2008). Hindlimb suspension as well as hypertension is characterized by a decrease in anti-oxidative function in mitochondria (Chrissobolis et al., 2008) (Dikalova et al., 2010). The increase of ROS is concomitant with the increase of expressions of Nox2 et Nox4 and the decrease of MnSOD/GPX-1. Drugs as apocynin (NADPH-oxidase inhibitor) or mito-tempo (anti-oxidative) decreased the effects due to hindlimb suspension (Peng et al., 2015; Zhang et al., 2014a). Finally, as well known, radiations increase the ROS production, then in space, the high level of radiations could also participate to the vasculature alteration via the increase of NO's trapping by ROS (Delp et al., 2016) (Ghosh et al., 2016).

#### **5.5.6. Adaptation of venous system**

It is not easy to study the physiology of veins, but the adaptation of vein to altered gravity should be important especially to explain the loss of peripheral vision observed in astronauts potentially induced by intracranial hypertension due to excessive pressure in cerebral veins. This theory has been established by comparison with the headache due to the altitude (Wilson, 2016) and the fact indicating that the venous volume is increased (except in calf vein) from the fifteen day of flight, in astronauts (Arbeille et al., 2015). Unfortunately, the effects of gravity alterations have not been studied in details and only in vena cava and mesenteric veins. Spaceflight induced a shift in the binding (Sayet et al., 1995) and decreased the norepinephrine-induced vasoconstriction (Behnke et al., 2013).

#### **5.5.7. Recovery**

After few hours of perturbation including tachycardia and increase of blood pressure (Fagette et al., 1995; Tarasova et al., 2001), the altered vascular parameters - by hindlimb suspension and spaceflights - return to normal values after 3 to 7 days after the recovery to a normal position (Morel et al., 1997; Xue et al., 2011) (Behnke et al., 2013) (Arbeille et al., 2015).

#### **5.5.8. Centrifugation as a countermeasure of microgravity-induced dysfunctions**

Daily applications of centrifugation are sufficient to restore a part of cardiovascular parameters (4% of the suspension duration, i.e. 1h/day during 28 days of hindlimb suspension). But if it is enough for cardiovascular system, bones and muscles alterations are still presents (Sun et al., 2004; Zhang et al., 2003b). The effects of this countermeasure is partial, especially in cerebral arteries in which the modification of L-type currents is not reversed (Xue et al., 2007) whereas the wall thickness returns to control values (Lin et al., 2009). It is noticeable that anti-hypertensive treatment with losartan reversed the effects of hindlimb suspension on cerebral arteries (Zhang et al., 2009).

## **GENERAL MATERIAL AND METHODS**

## **1. Ethical Consideration**

All experimental procedures adopted for *in vivo* studies were performed in agreement with the official European Guidelines for the care and use of laboratory animals (directive 2010/63/UE) and were approved by the Ethical Committee of the University of Bordeaux (protocol 50120140-A) and by the Ethical Committee of the University of St Etienne (CU15N02). All the experiments were conducted in order to minimize the suffering and the discomfort stress of the animals, and designed to reduce the number of rats used per group.

## **2. Animals**

The experiments were realized on adult Sprague Dawley rats (8 weeks of age, 225-250 gr) and Spontaneously Hypertensive Rats and Wistar Kyoto Rats bought at different ages (between 2 and 7 months regarding the experiments). All rats were provided by JANVIER Labs. Upon arrival in the animal facility, rats were habituated to their new housing conditions for a minimum of 3 days (2 rats per cage). Because some of our learning and memory procedures required specific testing and food deprivation procedures, all rats were then housed individually (1 rat per cage, 1 week before the beginning of the experiments) for the entire duration of the behavioural experiments. All rats were housed under standard conditions (22°C, 55% humidity, 12h light–dark cycle, lights on 7.00 am) with standard food and water *ad libitum*. Before the experimental protocol, each rat underwent a handling procedure in order to be habituated to the experimenter and to minimize as much as possible stress responses that would interfere with subsequent memory testing. This procedure consisted in removing the rat from its cage and holding it in the experimenter's hands until the rat felt comfortable.

## **3. Behavioural tests**

### **3.1. Food deprivation procedure**

The memory test procedure used in this thesis, named the social transmission of food preference task (see below the detailed description) is appetitive in nature and required the rats to undergo a partial and gradual food deprivation protocol in order to increase their motivation for food during the entire duration of the experiment. Rats were submitted to a partial food deprivation protocol to increase their motivation 3 days (Day-3) prior to the beginning of the memory task (interaction D0). The amount of food available in their home cage was gradually reduced to bring them to 85-90% of their baseline (*ad libitum*) weight and never more than 15%, as regularly performed in our lab. An adequate total food amount (about 15 g) was then distributed daily to maintain a level of food deprivation constant in all animals.

### **3.2. Social Transmission of Food Preference Task (STFP)**

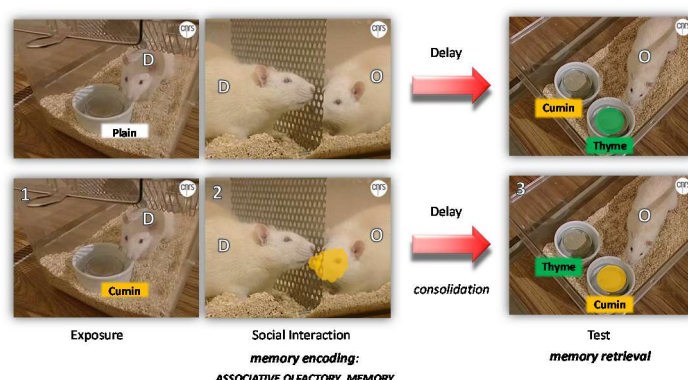
The social transmission of food preference (STFP) task involves an ethologically-based form of associative olfactory memory (Frankland and Bontempi, 2005) and the paradigm was performed according to what is described by Lesburguères et al. (Lesburgueres et al., 2011). This task, appetitive in nature, required a partial and gradual food deprivation which allows to increase rats' motivation for food during the duration of the experiment (see previous paragraph).



In the STFP task, within only one single interaction session of 30 min, rats encoded rapidly about the safety of potential food sources by sampling the odour of those sources on the breath of their littermates. Food deprived rats underwent a three-step procedure as illustrated in the figure below (Fig. 75).

To rule out the possibility of a taste bias in the SHR strain and to have the possibility to test the recent and remote memory on the same animals, two pairs of flavour were used: the cumin-thyme association and cocoa-cinnamon association. To simplify the explanation of the task we are going to describe the cumin-thyme association, but the same protocol is used for the second pair of odours. Just the percentages of spices change: instead using 0.5 % of cumin, it is possible to use 2% of cocoa, and instead of 0.75% thyme it is 1% of cinnamon.

The flavours associations used in our study are in accordance to the protocol described in (Lesburgueres et al., 2011).



**Figure 75:** The 3-step social transmission of food preference task. Above the STFP task when the demonstrator eat plain food (without flavour); below the STFP when the demonstrator eat flavoured food allowing the formation of the associative olfactory memory here detailed: (1) Exposure (30 min): a food-deprived demonstrator rat (D) eats cumin flavoured food (0.5%). (2) Social interaction (30 min): an observer rat (O) forms an association between the cumin odour and some constituents of the breath of the demonstrator rat. (3) Retention test (20 min): When submitted to a choice between cumin and a novel food (thyme, 0.75%), the observer rat expresses a memory of the association by preferentially eating cumin because it was present in demonstrator's breath, considered without danger and therefore safe to eat. Here in this example the association considered is cumin-thyme, but cocoa-cinnamon can be used respectively. Adapted from (Lesburgueres et al., 2011).

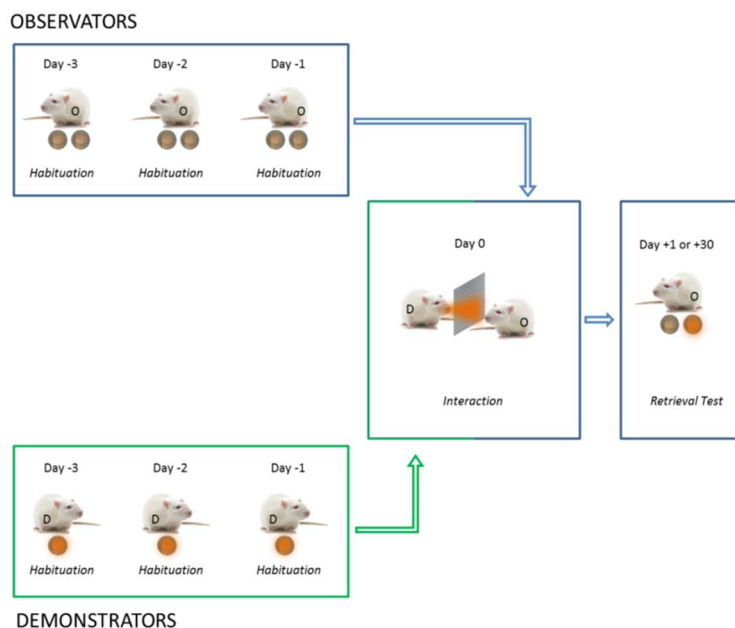
The task took place in the home cage of the animals. Rats called "demonstrators" were first presented with one cup (height: 4 cm; diameter: 7 cm carefully cleaned between each experiment to prevent olfactory cues) in their home cage and habituated to eating plain or flavoured powdered chow (0.5% cumin or 2% cocoa) for three days (30 min session). Observer rats were also shaped for three days to consume plain powdered chow from two cups placed in their home cage for 20 min. Cups were then weighed and additional food was given to reach a daily amount of food of about 15 g. These shaping and eating procedures minimized novelty-induced stress that would interfere with memory performance during the experimental procedure described below.

The detailed experimental procedure is described and illustrated (Fig. 76) below:

- 1) Exposure phase: Demonstrator rats were food-deprived and then habituated to eat plain or cumin (0.5 %, i.e. 0.5 g of cumin mixed in 99.5 g of plain chow; or 2 %, i.e. 2 g of cumin mixed in 98 g of plain chow) powdered chow for three days (30 min session). During these 30 min period, 40 g of powdered chow were available in a cup placed in the demonstrator's home cage. Water was removed from the cage. After the feeding session, the food was weighed, and the amount eaten

was recorded. Observer rats were also shaped for three days to consume plain powdered chow from two cups placed in their home cage.

- 2) Interaction phase: the demonstrator rat was moved to the observer's cage fitted with a stainless steel wire mesh divider (greed). Food-deprived observer rats and demonstrator rats were separated in opposite side of the cage for a 20 min interaction period, and then they were allowed to interact freely for another 10 min when the divider was removed. The demonstrator rat was removed from the observer's cage at the end of this 30 min interaction period. Observer and demonstrator rats were always unfamiliar with each other
- 3) Retention test: 1 or 30 days after the social interaction, respectively depending on the recent and remote memory test, food-deprived observer rats had to choose in their home cage between two cups containing a novel food (0.75% thyme or 1% cinnamon) or the familiar food that the demonstrator rat had consumed before interacting with the observer rat (0.5% cumin or 2% cocoa). After 20 min, cups were removed, weighed and olfactory associative memory performance was expressed as percentage of familiar food eaten (% cumin or % cocoa) using the following formula:  $(\text{amount of familiar food eaten} / \text{amount of total food}) \times 100$ . In addition, the total amount of food eaten was examined to control that all the groups had the same motivation for eating food.

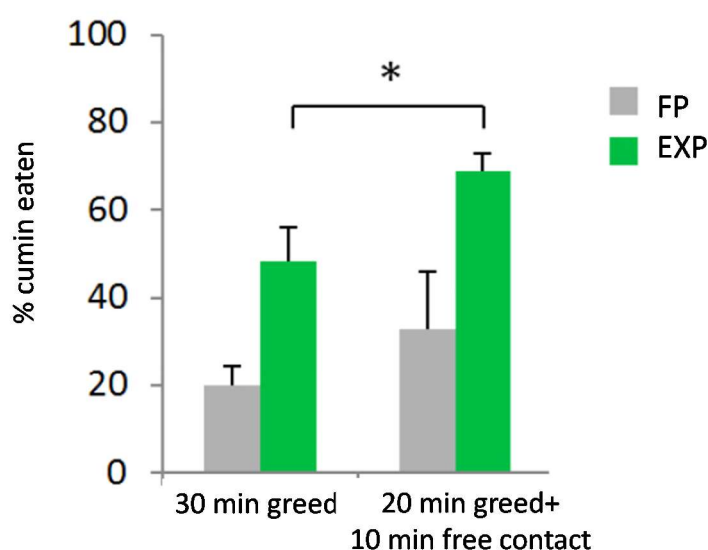


**Figure 76:** Details of the STFP paradigm. During the entire procedure, observer (O) and demonstrator (D) rats were food deprived and received a daily amount of food of 15g. Demonstrator rats were shaped 3days in a row prior to interaction day to eat plain or cumin powdered chow in cups. Observer rats were shaped 3 days prior to interaction phase with 2 cups of plain powdered chow placed in their home cage. 1 or 30 days following the interaction phase, observer rats were submitted to the retention test, upon completion they were euthanized 90 minutes after the end of the test. Brain were collected and processed for Fos immunostaining and Collagen IV labelling Alternatively, as specified for each experiment in the text, brains were dissected and the regions of interests were processed for biomolecular analysis according to the protocols given by the suppliers.

Flavour concentrations were chosen in pilot experiments to induce an innate preference for thyme or cinnamon (Lesburgueres et al., 2011). Because rats naturally prefer thyme over cumin (or cinnamon over cocoa) at the concentrations applied for the cumin/thyme flavour pair (or cinnamon/cocoa), the use of these two biased flavoured pairs permit to decrease the chance level at test and thus to optimize the possibility of detecting changes in memory performance across our various treatments. Indeed, interaction with a demonstrator that has eaten cumin powdered chow could reverse this innate

preference so that observers chose cumin over thyme (up to 80% of the total food eaten, chance level of ~20%). The same applies to cocoa and cinnamon.

In the experiment concerning Ang-2 multiple injection in SDs, we wanted to see an increase of memory performance but the classical STFP interaction (composed by 20 min with the mesh divider that segregate observers from demonstrators, guiding the tidy interaction through the holes of the greed, and 10 min without greed allowing the free explorations between animals) is sufficient to induce high level of memory performance making difficult the detection of memory amelioration. But, since the STFP task is based on the association between the odour detection and the social interaction, the decrease of social interaction could affect the quality of the retrieval. Thus, if the grid is not removed, the social interaction is not complete and it is possible to observe a decrease of memory performance during the retrieval (Fig. 77, Lesburgueres et al., 2011, Giacinti, Bessières Theses), as revealed by a lower quantity of cumin eaten by the observer rats interacted with the demonstrator feed with cumin. This strategy allows us to avoid that the memory performance reaches the maximal expression and to see an amelioration of the memory performance.



**Figure 77:** memory performance when the grid is not removed (30 min greed) compared to the normal protocol of interaction (30 min greed+ 10 min free contact). From Anaïs Giacinti thesis, 2015.

### 3.3. Open field (OF) and locomotor activity assay (LA)

Experiments were performed as described in (Ruzza et al., 2012), adapting the dimension of arena for rats size, on a subset of SHRs and WKYs during the light cycle (between 09.00 and 13.00) and 1 week after STFP in order to decrease the stress and to not influence memory performance of rats. Behavioural performance was recorded using the Noldus video tracking system (EthoVision XT5, Noldus Information Technology). Rats were positioned at the middle of the system which is composed by a PVC arena (100 cm x 100 cm) and opaque polycarbonate walls (Imetronic). The central zone of the arena was defined as the central square (50 cm x 50 cm). Rats' horizontal activity was monitored by a camera. Animals' locomotion was recorded for 30 min. The parameters analysed are the cumulative distance (cm) that the animal covered and immobility time (s, the animal is considered immobile when 90% of it remains in the same place for a minimum of 2.5 s).

For anxiety analysis, the first 10 minutes of recording session were taken in consideration (after 10 min rats get used to the arena) and the parameters measured were the time (s) spent by the rat

exploring the central zone, the number of entries in the central zone and the latency (s) to first entry in the central zone.

### **3.4. Odour threshold discrimination (OT)**

The odour threshold discrimination task was performed according to (Arbuckle et al., 2015), increasing the total time of recording up to 5 min.

Briefly, the rats were habituated in the experimental room for 1h and tested during the light cycle (between 09.00 and 13.00). 4 home cages, without bedding, were divided by opaque white paper in order to minimize the environmental cues. The cages were accurately cleaned between each experiment to prevent olfactory cues. The rat was allowed to explore the first cage for 15 min, before moving to the second one. This phase was repeated 4 times in the 4 cages. After this habituation, a filter paper contain the flavour at a specific concentration was introduced in the 4<sup>th</sup> home cage and the time spend by the rat exploring/sniffing the filter paper (s) was observed for 5 min. Behavioural performance were recorded using the Noldus video tracking system and analysed with the dedicated software. The concentrations used are cocoa 0%, 0.4%, 2% and 10%. The cocoa concentration of 2% was selected according to the one used in STFP. The others concentrations were calculated in order to have a 5 fold difference between all concentrations used.

### **3.5. Centrifugation protocol**

Before starting the experiment, rats were housed by 2 in standard cages with classical bedding, in a quiet room with constant temperature (22°C), 50% relative humidity, and a 12/12 h light-dark cycle. Food and water were provided ad libitum (Safe Diets A04). After a day of habituation in the specific cages designed for the centrifuge's gondolas, the centrifuge (COMAT Aérospace) was turned on to maintain a permanent level of hypergravity (2g) for 21 or 60 days. The centrifuge used had a radius of 1.4 m and four gondolas hanging on the periphery. Each gondola can accommodate up to three cages (1 rat/cage). All gondolas were equipped with a video surveillance system to control animals' condition and food/water stocks. Animals were provided with enough food and water for the whole duration of the experiments, thus the centrifuge was stopped once for the 21 days experiment and 6 times for the 60 days experiment for 30 min each time to weight the rats and change the bedding. Control rats were also placed into similar cage used in the gondolas and in the same room, to mimic experimental conditions, but were not exposed to centrifugation. The quantity of food eaten is evaluated as the difference between the available food before the centrifugation and the total food remained at the end of the centrifugation.

## **4. Euthanasia, tissue preparation and sampling**

**For immunohistochemistry labelling:** after 90 min the end of behavioural experiment (time for maximal expression of c-fos (Bisler et al., 2002; Zangenehpour and Chaudhuri, 2002), previously tested in our lab according to (Lesburgueres et al., 2011) ), rats were terminally anesthetized with of Sodium Pentobarbital (Ceva Santé Animale, 300 mg/kg), slowly (14 ml/ min) intracardiacally perfused with 200 ml of a solution composed by NaCl 0.9 % and heparin 2.8 ml/l (room temperature), 100 ml of NaCl 0.9 % (room temperature) and 350 ml of fixative buffer solution composed by Paraformaldehyde (PFA) 4 % in PB 0.1 M, pH=7.4 (4°C).

Rats were finally decapitated and their brains were removed. Serial coronal sections were obtained of all the brains (see detailed procedure for each experiment in the Fig. 78 below) and were collected in 4 wells and stored in a protective solution composed by sodium azide 0.02% in PB 0.1M, pH=7.4 or in cryoprotection solution if not quickly processed; all the sections contained 1 well were processed for immunohistochemistry.

For Evans' Blue experiment, rats were terminally anesthetized with of Sodium Pentobarbital, the brains removed and post-fixed with PFA 4%, before being cut.

Experiment	Post-fixation	Cutting Apparatus	Thickness slices $\mu\text{m}$
Pimonidazole injected rats	PB 0.1M over night	Vibratome	40
Edu/Coll IV/EB injected rats	PFA 4% 3 days; sucrose 3 days	Microtome	100
ONAS injected rats	PFA 4% over night; sucrose 3 days	Microtome	50
Ang-2 injected rats	PB 0.1M over night	Vibratome	40
Angiogenesis time course rats	PB 0.1M over night	Vibratome	40

**Figure 78:** detailed post-perfusion procedure to obtain the tissue preparation for each experiment.

Alternatively, for ELISA and Proteome profiler experiments, rats were terminally anesthetized with of Sodium Pentobarbital (Ceva Santé Animale, 300 mg/kg), the fresh brains were removed and the regions of interest were dissected and placed in tubes containing sample diluent solution (R&D system, DYC002) containing protease inhibitors cocktail 1% (Sigma, P8340) and 12 silica beads (diameter 3mm); samples were vigorously agitated with the minilys (Bertin technologies) during 30 s. Protein concentration was measured with  $\mu\text{Lite}$  (Biodrop) spectrophotometer and the associated BioDrop Resolution software, and stored in  $-80^{\circ}\text{C}$ . Proteins were used and prepared as described in the protocol given by the supplier.

Concerning plasma corticosterone, IgG, nitrite and nitrate arrays, blood were collected just after the cervical dislocation. After 10min, samples were centrifuged during 15 min at 1000 g and supernatants were collected and stored at  $-80^{\circ}\text{C}$  before assays that were performed following the supplier protocols.

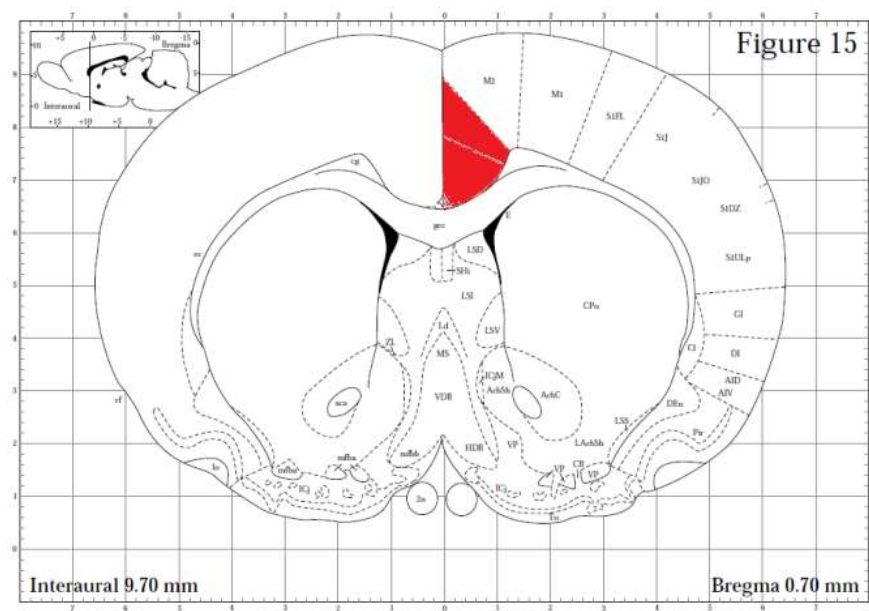
## 5. Surgery

All surgical procedures were conducted minimum 1 month before the beginning of behavioural protocols in order to avoid the discomfort of the rats and to reduce the induced inflammatory response that may affect the data analysis, as previously performed in our lab.

All the surgeries were made under isoflurane gas anaesthesia (i.e. guide cannula' implantation) according to the Ethical guidelines for long-lasting surgery. In absence of pain reflex, rats were placed on stereotaxic apparatus (Kopf Instruments) on warmed platform ( $\sim 26^{\circ}\text{C}$ , to reduce hypothermic response) and the incision of the skin was preceded by dermic application of xylocaine (xylocaine 5%, AstraZeneca) and antiseptic solution of betadine 10%. Ocrygel eye drops were applied during all surgery in order to avoid deterioration of corneas, frequent in albinos rats. Before guide cannulas placement, 2/3 screws were placed into cranial bones in order to assure a major adherence of cement helmet. Guide cannulas (l: 8 mm, ext  $\varnothing$ : 0.460 mm, int  $\varnothing$ : 0.255 mm) were implanted, as described after, in accordance to stereotaxic coordinates described in Paxinos and Watson atlas, 1998. A cement helmet incorporating the screws and guide cannulas was realized using methyl acrylate dental cement. Rats were monitored during a post-surgery care period.

In particular the region of interest targeted during this study is the Anterior cingulate cortex (Fig. 79):

Coordinates from bregma used AP (Anterior-Posterior) +0.2 mm, ML (Medial-Lateral)  $\pm 0.5$  mm DV (Dorsal-Ventral) -1.3 mm (guide cannula) or -2.8 mm (injection site).



**Figure 79:** Coordinates used for ACC cannulas implantations and injections.

## 6. Drugs and Injections

**Hypoxyprobe-1 or Pimonidazole** (Ref. HP6-200Kit, Hypoxyprobe Inc.) is a marker of hypoxia; it is composed by Pimonidazole hydrochloride revealed by mouse FITC-MAb antibody that binds to Pimonidazole adducts (composed by Pimonidazole linked to the thiol groups in proteins peptides and amino acids) in hypoxic tissues. The formation of adducts depends on the presence of redox enzymes in hypoxic cells. The Pimonidazole can detect hypoxia if  $pO_2 < 10$  mmHg. This is considered a very low amount of oxygen and is referred to as hypoxic tissue (more details on <http://www.hypoxyprobe.com/history-of-hypoxyprobe.html>).

**aCSF** (artificial CerebroSpinal Fluid) was used as vehicle for intracerebral injections and was injected in controls group of rats in order to mimic the procedure of injections. It is composed by 5mM of glucose, 125 mM of NaCl, 27 mM  $NaHCO_3$ , 2.5 mM of KCl, 0.5 mM of  $NaH_2PO_4 \cdot 2H_2O$ , 1.2 mM of  $Na_2HPO_4$ , 0.5 mM of  $Na_2SO_4$ , 1mM of  $MgCl_2 \cdot 6H_2O$  and 1mM of  $CaCl_2 \cdot 2H_2O$ .

**EdU** (5-Ethynyl-2'-deoxyuridine, Santacruz ref sc-284628A) is a thymidine analogue selectively incorporated into cellular DNA during S-phase, allowing the detection of cellular proliferation. EdU was injected intraperitoneally (61.6 mg/kg), in a volume of 2 ml/kg in order to facilitate the diffusion. The repeated injections were performed each  $\sim 12$  h (see protocol of injection in chapter 1). EdU was dissolved it in a solution of PB 0.1M and  $Na_2HPO_4 \cdot 2H_2O$  (27.6 g/l), adjusting the pH to 8.4 with a solution of PB 0.1 M and  $NaH_2PO_4 \cdot 2H_2O$  (35.6 g/l).

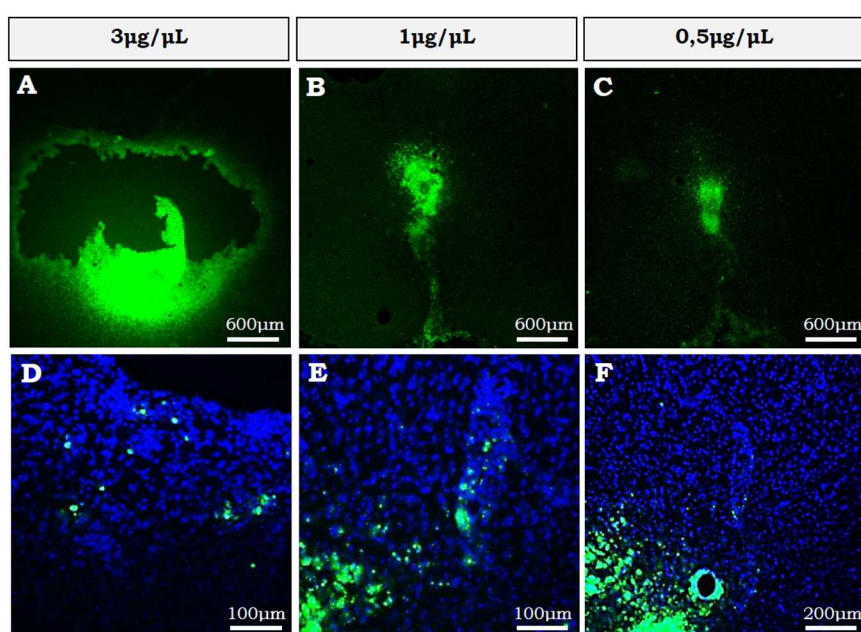
**Evans' Blue** (ref: E2129, Sigma) was dissolved in 0.9% NaCl (solution 2g/100mL) and injected 6 $\mu$ L/g of weight and i.v. injected 5 min before the decapitation. This solution was used to label the perfused vessels according to (Walchli et al., 2015) since it binds almost completely the serum albumin labelling the intravascular surface.



**Angiopoietin 2, Ang-2** (Recombinant Human Angiopoietin-2 Protein, ref: 623-AN/CF, R&D Systems), as explained in introduction, was used to induce local angiogenesis in presence of others trophic components. Exogenous Ang-2 was injected bilaterally intracerebrally at the final concentration of 100 ng/ $\mu$ L (dilution in aCSF), in a volume of 1  $\mu$ L for injection. The multiple injections of Ang-2 and the respective control aCSF were performed on two group of rats (food preference and experimental) during the early phase of associative olfactory memory consolidation induced by the interaction (Protocol specified in chapter 2).

**Antisense oligonucleotides and scramble:** Antisense oligonucleotides (ASON) directed against Ang-2 and their respective scrambles (SCR, same oligonucleotides used for ASON but in a different order, creating a sequence that does not codify for any known RNA in rat) were injected bilaterally in ACC according to the protocol specified in chapter 2.

The multiple injections of ASON and the respective SCR were performed on two group of rats (food preference and experimental) during the early phase of associative olfactory memory consolidation induced by the interaction, in order to analyse the impact of the angiogenesis manipulation on remote memory performances. In particular, ASON against Ang-2 (asAng-2) were used in order to selectively block the angiogenesis inhibiting the formation of Ang-2 protein. ASONs bind to the complementary mRNA by base pairing and induce the cleavage of targeted mRNA by ribonuclease H, an enzyme that degrades RNA in RNA–DNA duplexes (Kole et al., 2012). The sequence used was 5'-GCGTTAGACATGTAGGG-3' for ASAng-2 DNA and 5'-GACGCGAGTTGAGGTTA-3' for the scrambles, injected in the control group. These sequences were developed using BLAST technique and the sequences were synthesized by Eurogentec. ONAS and SCR have been provided of phosphorothioate backbone replacing the phosphodiester bond in DNA sequence in order to increase their stability. Moreover 6-FAM fluorophore (excitation 499nm, emission 519nm) has been added at of 5' level in order to detect their diffusion and distribution after injections. The concentration and the protocol used during this study was developed during the Ph.D. thesis of Anais Giacinti and based on (Boye et al., 2002; Dabertrand et al., 2012; Lee et al., 2004b; Neumann, 2000) studies. According to those, the dose of 1  $\mu$ g/ $\mu$ L of asAng-2 and the injection protocol used has been shown to not have toxic effect but a good penetration in the vascular network (Fig. 80).



**Figure 80:** Deleterious effects due to the too high concentration of ASON against Ang-2. Figure from Anais Giacinti thesis suggesting the concentration is 1  $\mu$ g/ $\mu$ L to circumscribe the diffusion of the molecule in the targeted brain area without damage in cerebral tissues.

#### *Justification of ASON choice:*

Other possibilities to inhibit Ang-2 synthesis via mRNA degradation, as ASON strategy, is the use of Small interfering RNA (siRNA) or a short hairpin RNA (shRNA) as used in the studies of (Bhandari et al., 2006; Marteau et al., 2011; Wang et al., 2013a) on mice.

Compared to siRNA, the advantage of ASON is that it is based on a protected DNA (see material and methods) being more stable compared to RNA. Moreover, the in vivo ASON used was already set up in the team (Dabertrand et al., 2012).

Compared to shRNA, the advantage of ASON and siRNA is that they produce the gene extinction just during a short period, allowing us to selectively target the consolidation phase without affecting the encoding during the STFP task. Moreover, shRNA needs to a viral vector to be integrated into the cells, while the others can directly injected in the area of interest. The disadvantage of ONAS is the necessity of multiple injections more traumatic for the animals. Despite this, the multiple injections protocol has been used several times showing that, if animals are sufficiently handled, the behavioural performance is not affected. Another disadvantage is the intrinsic but dose-dependent toxicity of these compounds produced by the chemical modifications induced in the base sequence in order to facilitate cellular transfection. Although this constraint, the dose of 1 µg/µl of ASON Ang-2 and the injection protocol used By Anais Giacinti during her thesis do not show toxic effect but assure the penetration in the vascular structure, based on (Boye et al., 2002; Lee et al., 2004b; Neumann, 2000).

## **7. Assays**

Elisa to quantify **Ang-2** (MANG20, R&D system) and **Ang-1**, (Ang-1 EK1295, Boster Immunoleader) were performed with proteins extracted from rat ACC following the instruction of suppliers. Protein were centrifuge 5000g 5min; protein dosage was performed using BioDrop µLITE System on the supernatant part just before the array and the samples were diluted to have similar protein concentration in each sample. The values obtained from Elisa assays were reported as concentration in 1µg of protein to be compared. All sample and standard were assayed in duplicate.

**Corticosterone:** The quantification of endogenic corticosterone in blood samples was performed using EIA kit DetectX Corticosterone (Arbor Assays) following the instructions of the supplier: samples and standards solutions were diluted (1:100) in the appropriate buffer and placed in the plate in duplicates.

**IgG ELISA assay:** The quantification of the IgG concentration of blood samples was performed with the (ab189578, Abcam) following the instructions of the supplier. The dilution of the samples is 1:1,000,000 with the assay buffer. All sample and standard were assayed in duplicate.

**Total nitric oxide and nitrate/nitrite** parameter assay kit was performed following the instruction of the supplier (Biotechnie, ref KGE001) on blood samples. All sample and standard were assayed in duplicate.

For all assays, the chemoluminescence was measured with the Optima apparatus (BMG Labtech).

**Proteome profiler** (ARY015, R&D systems) was performed just after the interaction in SDs and three days after interaction in SDs and SHRs; after ACC dissection in each sample was added 100 µl of PBS 1x and Protease inhibitors cocktail (10 µg/mL bacitracin , 100 µg/mL Leupeptin, and 10 µg/mL Pepstatin) and the tissue were homogenized at max speed for 30 seconds; protein dosage was performed using BioDrop µLITE System: precisely the samples were diluted at final concentration of 15000 µg/ml using PBS 1x and Protease inhibitors cocktail. Proteome profiler was performed



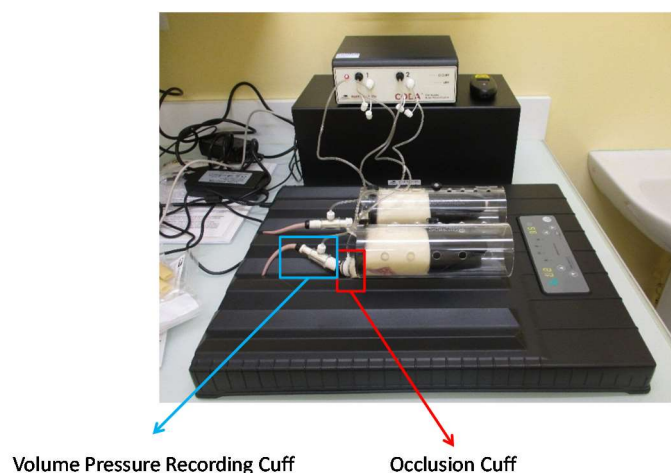
following the instruction Proteome profiler array the detection was made with LiCOR method (Ref.926-32230). The membrane were read with the odyssey IR-scanner (resolution 84  $\mu\text{m}$ ; quality medium; focus offset 0,0 mm intensities 1,5-3).

## **8. Losartan treatment:**

Losartan (Mylan 50mg) is an antagonist of angiotensin II receptor type 1 efficacious in rescue the blood pressure increase induced by hypertension in SHR (Demirci et al., 2005; He et al., 2014). The treatment was performed administrating this drug in SHR from the age of 2 months to the sacrifice of the rats (6/7 months old), in order to avoid the development of hypertension. 13-18 mg/kg/day of Losartan were dissolved in tap water, according to stability data provided by the supplier; the posology was intentionally increased during the treatment in order to assure the efficaciousness of the molecule in relation to the stage of hypertension (Demirci et al., 2005; He et al., 2014). The molecule administration and its efficaciousness on blood pressure were checked once for week.

## **9. Blood pressure measurement:**

The CODA system (Fig. 81) automatically performs multiple rapid, simultaneous measurements of different physiological parameters, such as systolic blood pressure, diastolic blood pressure, and mean pressure. The system is based on an occlusion tail cuff which is firstly inflated to impede the blood flow to the tail and then deflated slowly while a second tail cuff, incorporating the volume pressure recording (VPR) sensor, measures the physiological characteristics of the returning blood flow. As the blood returns to the tail, the VPR sensor cuff measures the tail swelling that results from arterial pulsations from the blood flow. Systolic blood pressure is automatically measured at the first appearance of tail swelling. Diastolic blood pressure is automatically measured when the increasing rate of swelling ceases in the tail. The measurements were done weekly, between 16h and 20 h, and the rats were placed in adapted cylinder, covered with dark blanket, on a term regulated plaque in order to minimize blood pressure variation anxiety induced and the discomfort of the rats. The blood pressure measurement and Losartan treatment was done with the help of veterinary student Annabelle Costet to check daily the health of the animals and adjust the treatment.



**Figure 81:** Coda system blood pressure measurement

## **10. Labelling**

The wash of slices was performed in phosphate buffer PB 0,1M for all the labelling. The blockade buffer was always composed by PB 0.1: 0.1g of BSA, 200 $\mu$ L of triton X100, 2mL of serum depending from the secondary antibody.

The vascular network was labelled with anti-**Collagen IV** (ref: ab6581, Rabbit polyclonal to Collagen IV Biot, dilution 1/250) recognizing the ECM synthesized by ECs (Bahramsoltani et al., 2014)) and thus identifying mature and neo-forming vessels. The secondary antibody used was Alexa, dilution 1/500, time incubation 1h30). The slices were boiled in trisodium-citrate buffer and permeablized 1 h in the blockade buffer, before the incubation with the first antibody and then with the secondary antibody. After that the slices were mounted on gelatin coating cover-slips with Fluoromount medium. To assess vascular proliferation and perfusion we performed a triple staining using Collagen IV, EdU and Evans' Blue. **EdU** labelling was performed using the information given by the supplier. EdU labelling was revealed using Click-iT® EdU Alexa Fluor® 488 Imaging Kit (ref: C10337): EdU is a thymidine in which the methyl group in the 5 position is replaced by a terminal alkyne group. This group is detected through a reaction with fluorescent azide in a Cu(I)-catalyzed [3+2] cycloaddition ('click' chemistry). This reaction is sensitive and fast. Moreover, the reagents used are 1/500th the size of antibody molecules, allowing a faster diffusion and high penetration in even thick slices, required for EB labelling. In addition, the reaction between ethynyl groups on DNA and fluorescent azides does not require denaturation of the samples required by BrdU labelling (citrate + high temperature), preserving the vascular network (Salic and Mitchison, 2008). **Evans' Blue** detection was made directly since its autofluorescence.

Finally **Pimonidazole** was revealed by mouse FITC-MAb antibody (dilution 1/100) according to the supplier information.

The labelling was performed with the help of Nathalie Biendon and the master students Laura Chaillot and Solene Dies.

**Analysis images:** The images concerning EdU, Collagen IV and Evans Blue labelling were acquired using confocal Leica SP5 microscope (488nm). All ACC and PC slices in 1 well were labelled and mounted; 2 images/animal/ACC were acquired with a zoom of 20x.

The quantification of vascular network labelled with Collagen IV was performed using NDPI Tools plugin (developed during the thesis of Anais Giacinti) and ImageJ software (Fig. 82).



**Figure 82:** Analysis of vascular network with ImageJ and Angiotool software: from left to right: original labelling acquired with the microscope, macro used in ImageJ software to skeletonize the vascular network, detection with Angiotool of vascular density and number of branching.

The Pimonidazole labelling was acquired with Hamamatsu Nanozoomer 2.0 (Bordeaux Imaging Center) on the entire ACC, PC and HPC (all slices in 1 well were labelled and mounted; the entire zone was acquired and quantified by Nathalie Macrez.

## ***11.Statistic***

The analysis of data was made using GraphPad Prism 6 software. For each group, data are expressed as mean  $\pm$  standard error of the mean (SEM) of  $n$  experiments. The Normal distribution of data was verified using Shapiro-Wilk test, in order to choose the correct statistic test. In all the comparison conducted, the 95 % confidence limits ( $p$  values  $< 0.05$ ) should be achieved in order to consider significant the differences observed. Data have been statistically analysed with Student's  $t$  test for unpaired data, one way ANOVA followed by the Tuckey's test, or two-way ANOVA (group/delay or strain/delay) followed by Tuckey's (in order to compare two groups by two, in case of normal distribution) post hoc test, as specified in table and figure legend.

## OBJECTIVES

## **Objectives of the thesis**

As highlighted in the introduction, neuronal and vascular systems closely communicate to coordinate their actions. Since the cerebral microvasculature is capable of adapting its activity in order to provide the adequate amount of energy and nutrients imposed by the metabolic demand of neuronal networks, it is reasonable to expect a dedicated vascular reorganization to couple energy supply to specific neuronal networks recruited during the various memory processes triggered by cognitive functions. We explored such a possibility and tested its validity by focusing our attention on memory consolidation, the process by which a memory trace acquires stability and persistence over time. While the formation and stabilization of enduring memories have been shown to require a temporary dialogue between the hippocampus and cortical regions that act as the permanent repository of remote, consolidated, memories, the functional contribution of the cerebral microvasculature to this dynamic process has remained unexplored.

In order to pinpoint accurately the time-course of the hippocampal-cortical dialogue during the course of systems-level memory consolidation, we selected a behavioural paradigm particularly suitable for studying memory consolidation, that is, the social transmission of food preference (STFP) task in which rats learn about the safety of potential food sources by sampling those sources on the breath of conspecifics. This task enables olfactory information to be encoded rapidly during one single interaction session and induces a memory which is robust and long-lasting. Its associative nature requires the hippocampus and specific cortical regions such as the orbitofrontal and anterior cingulate cortices which are involved in the processing of associative olfactory information. In a previous set of studies conducted in the team of Bruno Bontempi by Anaïs Giacinti during her Ph.D. (Giacinti, 2014), it was found that both the reactivity and architecture of cerebral vessels can be modified during memory consolidation. Changes in vessel reactivity revealed by ex vivo calcium imaging supported the successful expression of either recently or remotely acquired memories (increased reactivity of the posterior cerebral artery irrigating the hippocampus upon retrieval of recent memory; increased reactivity of the anterior cerebral artery irrigating the cortex upon retrieval of remote memory). Interestingly, the formation of associative olfactory memory after social interaction was also accompanied by a transitory increase in vessel density and number of vessel branching points in anterior cingulate cortex (ACC), suggesting the triggering of an angiogenic process.

Building upon these promising results, we designed a series of specific follow-up experiments aimed at unravelling the time course of angiogenic processes potentially triggered upon encoding of olfactory information in the STFP task. To this end, in the first chapter, we explored the putative mechanisms responsible for eliciting angiogenesis locally in the ACC, one likely candidate being the level of hypoxia generated by neuronal activation. Hypoxia levels were thus assessed using dedicated hypoxia markers. Moreover, we have measured the activation of angiogenic pathways in days following interaction primarily in the ACC which acts as a critical node within the broader cortical network supporting the formation of enduring associative olfactory memories in the STFP task. Many angiogenic factors have neurotrophic effects in adult cortex except for the angiopoietin pathway. The time course of angiopoietin 2 (Ang-2) expression required for angiogenic processes was followed by ELISA in the ACC. After identifying the peak of post-learning angiogenesis, we further established the presence of newly-born endothelial cells and determined whether new cortical vessels in the ACC acquired functionality.

Since the approaches mentioned above were only correlative in essence, we next designed specific causal experiments aimed at modulating angiogenesis specifically. In the second chapter, we thus hypothesized that blocking or enhancing cortical angiogenesis during the early phase of memory

consolidation should either impair or improve the subsequent formation of remote associative memory by impacting on the reorganisation of the neuronal networks supporting remote memory storage at the cortical level. To this end, we targeted Ang-2. Angiogenesis was inhibited by means of intracortical infusions of antisense oligonucleotides directed against Ang-2. These time-limited post-encoding infusions into the ACC during the early phase of memory consolidation impaired retrieval of remote memory in rats tested 30 days after social interaction in the STFP paradigm, indicating an inability to adequately form and/or retrieve remotely acquired information. Angiogenesis stimulation in the ACC was achieved by injection of the Ang-2 peptide during the early phase of memory consolidation, which resulted in an amelioration of remote memory in rats probed for memory retrieval 30 days following social interaction. Thus, we were able to identify early cortical angiogenesis as a crucial permissive mechanism underlying the subsequent formation of remote memories and their progressive embedding into cortical neuronal networks.

In a third chapter, we sought to determine the functional significance of the cortical angiogenic mechanism identified in physiological conditions by examining the potential deleterious effects of hypertension on the organization of recent and remote memories using the spontaneous hypertensive rat model (SHR) in which hypertension develops spontaneously over time. We first established a time-dependent impairment in these rats, with remote memory being selectively impaired, thus suggesting successful encoding but an inability to adequately stabilize and/or retrieve remotely acquired information. We took advantage of this memory profile to explore the status of vascular networks and found that the anterograde amnesia of SHR rats was associated with an impaired cortical angiogenesis. Importantly, we were successful in rescuing the memory deficit of SHR rats by either targeting hypertension in the form of a chronic treatment with the antagonist of angiotensin II receptor type 1 Losartan or by performing region-specific intracerebral infusions of Ang-2 during the early phase of memory consolidation, as previously applied to normotensive Sprague Dawley rats. Thus, these experiments established the functional importance of early cortical angiogenesis as a prerequisite to the formation of remote memories.

Finally, in a fourth and last chapter, we extended our analysis to the effects of gravity modification on the cerebral microvasculature. Since changes in gravity modify vascular reactivity, we tested the hypothesis that hypergravity could interfere with the memory consolidation process. Sprague Dawley rats underwent chronic exposure to hypergravity at 2G and impact of this environmental treatment on encoding, consolidation and retrieval processes was evaluated using the STFP paradigm. Hypergravity-induced changes in various physiological parameters (food intake, weight, stress) and inflammatory processes (circulating IgG) were also examined.

Overall, our integrative approach has enabled us to provide novel insights into the dynamics of the cerebral microvasculature during the course of memory consolidation. Our findings identify early cortical angiogenesis as a crucial neurobiological process underlying the formation and stabilization of remote memory. This vascular mechanism is further discussed in light of the existing knowledge on cerebral angiogenesis and a putative model incorporating the functional involvement of the cerebral vascular sphere to the dynamics of hippocampal-cortical neuronal interactions during the course of the memory consolidation process is proposed.



## **CHAPTER 1: Angiogenesis implication in the memory consolidation, correlative approach**



## **Chapter 1: Angiogenesis implication in the memory consolidation, correlative approach**

### **1.1. Introduction**

Systems consolidation refers to the post-encoding time-dependent processes assuring the transformation of encoded information from a fleeting form to a long term memory representations over distributed brain circuits (Frankland and Bontempi, 2005; Squire and Alvarez, 1995), in order to not be quickly forgotten (Lechner et al., 1999).

This progressive reorganization of neuronal circuits involved in long term memory formation could takes days and years but neuronal modification starts immediately upon encoding. As described in the introduction, synaptic and systems consolidations synergistically cooperate in parallel (Dudai, 2012; Dudai et al., 2015; Frankland and Bontempi, 2005; Squire and Alvarez, 1995). The synaptic consolidation is a fast process, occurring within the first minutes or hours after encoding, thereby inducing the formation of new synaptic connections or the strengthening of existent synapses (Dudai, 2012; Squire et al., 2015). On the other hand, systems consolidation is a slow phenomenon lasting weeks in rodents and years in human subjects depending on the kind of memory and the nature of the memory task.

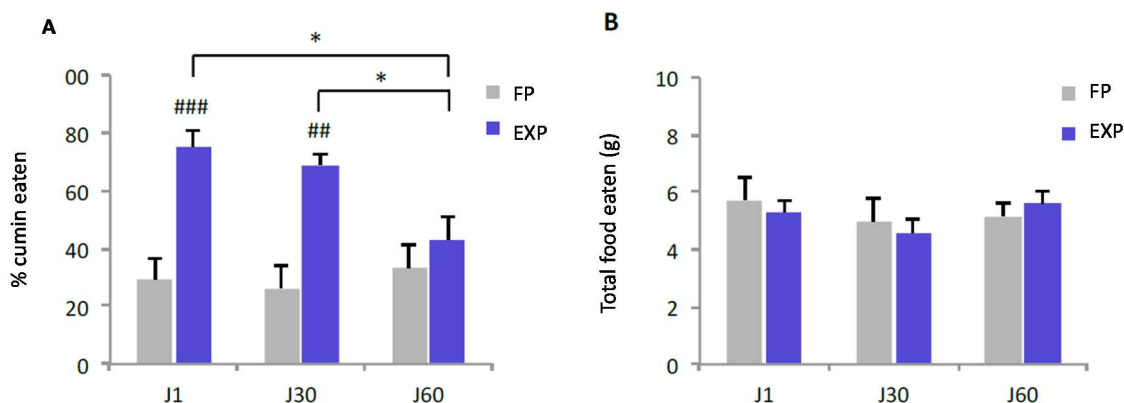
During this thesis we focused on systems consolidation, analysing the vascular architectural changes that accompany the brain circuitry and systems reorganization that supports the formation of long term memories during time (Dudai, 2012; Dudai et al., 2015; Frankland and Bontempi, 2005; Squire and Alvarez, 1995; Takeuchi et al., 2014).

According to the standard model, systems consolidation requires a transitory hippocampal-cortical interaction allowing the progressive remodelling of cortical neuronal networks to support the formation of remote memory traces (Bontempi et al., 1999; Frankland and Bontempi, 2005; Frankland et al., 2004; Lesburgueres et al., 2011; Maviel et al., 2004; Restivo et al., 2009).

In order to precisely identify the neurovascular post-encoding changes we selected the social transmission of food preference (STFP) (Galef and Stein, 1985; Galef, 1983), in which rats learn about the safety of potential food sources by sampling those sources on the breath of conspecifics. This task, developed by Galef, is based on the ethological observation that a rat exhibits enhanced food preference after a social interaction with a conspecific that had previously eaten the same food. This is because the food is considered to be without danger and safe to eat. In fact, after a single interaction with the demonstrator rat previously fed with cumin or cocoa, the rat observer encodes that this familiar food (cumin or cocoa) is without danger, forming the association between the odour and the safety of the food. After a delay during which this associative olfactory information is consolidating, the rat reverse its natural preference (thyme or cinnamon) to choosing the familiar food. In our experiments, these rats are called in the thesis experimental rats (EXP). Another group consists of animals interacting with a demonstrator rat fed with natural food without flavour, which thus will not form the associative olfactory memory showing their natural preference; these rats are referred to as food preference group, FP.

This association is rapidly encoded during one single interaction, identifying a precise acquisition phase leading to a rigorous control of induction of post-learning mechanisms implicated in the systems consolidation of the memory trace. Moreover, it induces a memory which is robust and long-lasting (at least 30 days; Fig.83), as reported by (Lesburgueres et al., 2011) and Benjamin Bessieres during his

Ph.D. As a matter of fact, by delaying the test it is possible to discriminate between a recent memory, tested 1 day after the interaction, and a remote memory, in our case tested 30 days after the encoding.



**Figure 83:** STFP results obtained during Bessieres Ph.D. thesis, 2016; performances of experimental rats (EXP) and food preference rats (FP) tested 1 day, 30 days and 60 days after encoding of the associative olfactory memory acquired during an interaction with a demonstrator rat precedently fed with cumin. A. Memory performance expressed by the percentage of cumin eaten (familiar food); B. Total food eaten by the rats during the test (g); ###p<0,01; ##p<0,001 versus FP; \*p<0,05 vs the same groups.

The associative nature of this task involves the HPC and the orbitofrontal (OFC) and anterior cingulate (ACC) cortices, both involved in processing olfactory associative information (Frankland and Bontempi, 2005; Lesburgueres et al., 2011). The consolidation of the associative olfactory memory is accompanied by a progressive contribution of neocortical areas during the post-acquisition period, accompanied by an increase of cortical spine density, confirming the findings that showed that the stabilization of memory trace induces a strengthening of cortical-cortical connections, requiring neuronal architectural remodelling (Chklovskii et al., 2004; McClelland et al., 1995).

Since the strong interaction between neurons and vessel during cognitive and memory process has been exposed, the interest of our laboratory was to investigate the neurovascular adaptation subtending the memory process. In fact, Anais Giacinti during her Ph.D. thesis has shown that both the reactivity (modification in cerebral artery reactivity irrigating the brain areas implicated in the hippocampo-cortical dialogue) and architecture of cerebral vessels can be modified during memory consolidation. In particular, the formation of associative olfactory memory was accompanied by a transitory increase in the vascular network in the anterior cingulate cortex (ACC), suggesting the triggering of an angiogenic process.

Thus, the aim of this thesis was to investigate the functionality of this vascular increase during the early phase of memory consolidation. First, we decided to strengthen the Giacinti's preliminary findings describing the nature of the correlation between the memory consolidation process and vascular increase in ACC.

To do that we explored the **hypoxia mechanism**, responsible for triggering angiogenesis (Boero et al., 1999; Dunn et al., 2004; LaManna et al., 2004; Masamoto et al., 2013; Pichiule et al., 2004; Xu and Lamanna, 2006) just after the interaction and the following day. Moreover, we investigated whether the angiogenic mechanism was triggered primarily in the ACC, which acts as a critical node within the broader cortical network supporting the formation of enduring associative olfactory memories in the STFP task, evaluating, furthermore, a general post-encoding **angiogenic signalling** through the use of a proteome profiler. To characterize the angiogenic involvement and the time window of this process,

we selected to study the **expression of Ang-2 protein**, via Elisa quantification, the key regulator factor in the angiogenic pathway (Augustin et al., 2009; Fagiani and Christofori, 2013; Pichiule and LaManna, 2002; Wakui et al., 2006). Finally, we tried to analyse the **final outcome of angiogenic mechanisms**, such as the ECs proliferation (quantifying the co-localization between EdU, marker of cellular proliferation, and ECs labelling with Collagen IV) and the **vascular network increase** (Collagen IV). We also tried to qualify the functionality of vascular network assessing the perfusion of newly-born vessels.

## 1.2. Experimental design and Results

### 1.2.1. Hypoxia induced in ACC by associative olfactory memory

The vascular network adapts its activity according to the metabolic request of neurons, in a process called functional hyperemia (Cipolla, 2009). If the neuronal activity is sustained, as in the case of memory trace formation, the metabolic demand of neurons does not match with the O<sub>2</sub> delivery, leading to local hypoxia (LaManna et al., 2004; Xu and Lamanna, 2006). This hypoxic condition can trigger several physiological responses, including angiogenesis. To detect the hypoxic post-encoding signal we have used i.p. injection of Pimonidazole, also named Hypoxyprobe, which reveals the hypoxic cells by specific immunostaining (see Material and methods) (Turlejski et al., 2016; Varghese et al., 1976). Before performing the experiment, we validated the protocol for immunostaining in the brain tissues of rats exposed to a hypoxic chamber, in collaboration with Jean-François Quignard (INSERM U1045, Université de Bordeaux).

Once validated, rats were divided in 3 groups (Fig. 84): the food preference (FP) and the experimental (EXP) groups (interacted with demonstrator rat fed with plain food and food containing cumin, respectively, as described in the material and methods chapter) and the home cage group. This control group was added to exclude a potential hypoxic effect induced by the injection and to determine if the manipulation of the rats and social interaction *per se* induced hypoxia in the regions of interest for the consolidation of associative olfactory memory. Pimonidazole was injected 1 hour before the euthanasia of the rats, allowing its incorporation in hypoxic cells.

The home cage group (HC) and a part of the experimental and food preference rats were injected on the same day as the interaction (more precisely 1 h after the end of interaction, Day 0, D0) and sacrificed 1 h after the injection. A second sub-group, composed by the second part of experimental and food preference animals, was injected 25 h after the interaction (Day 1, D1) and sacrificed 1 h after the injection. The second sub-group was added to investigate whether the hypoxic signal was sustained, suggesting the potential angiogenic triggering.

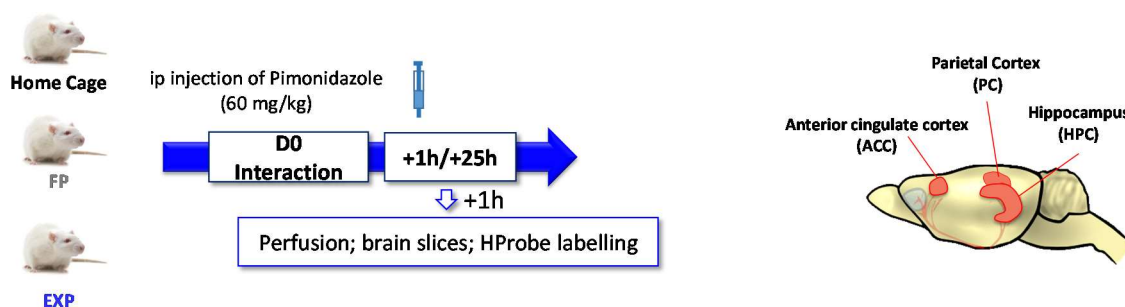
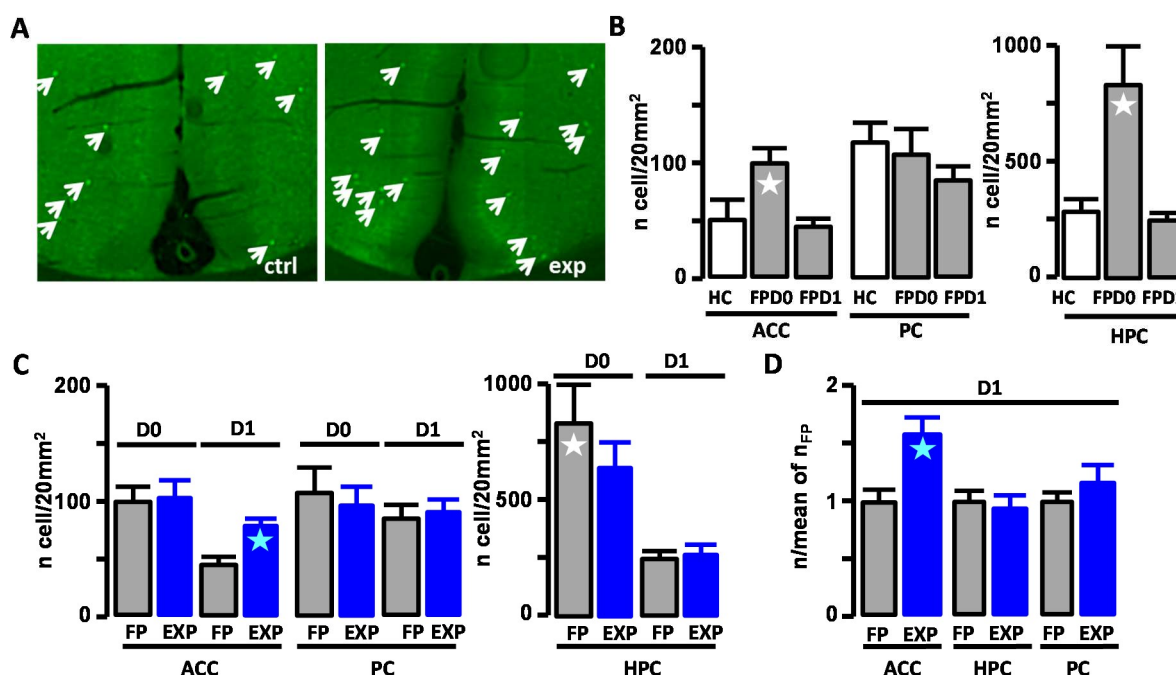


Figure 84: protocol for Pimonidazole injection and regions of interest of hypoxia quantification.

The immunostaining was acquired using Nanozoomer (Fig. 81A) in ACC, PC and HPC and the labelled cells were counted (Fig. 85B-D). The comparison between the food preference D0 group and the home cage group indicated that the number of cells labelled is increased in ACC and HPC 1h after interaction and is not affected in the parietal cortex (Fig. 85B). **This result showed that the interaction per se induced hypoxia both in ACC and HPC**, probably due to nonspecific components of the social interaction (novelty of the grid and first encounter with the demonstrator).

Twenty-four hours after interaction, the same comparison showed that the numbers of labelled cells were similar in both food preference D1 and home cage groups, in all studied structures (Fig. 85B), showing that the activation detected at Day 0 came back to the basal level after 1 day. The statistical analysis between the food preference and experimental groups indicated that the numbers of cells labelled with Pimonidazole was increased specifically in ACC at Day 1 (Fig. 85C). Moreover, we investigated the difference between the structures at Day 1 in order to see if the hypoxia signal was region specific (Fig. 85D). Due to the big difference in the number of hypoxic cells/mm<sup>2</sup> between the areas analysed, we compared the ratio of the number of labelled cells in experimental rats on the number of labelled cells in food preference rats to exacerbate the effect of the associative olfactory memory on hypoxia. **This result showed that, the day after the interaction, the increase of the number of hypoxic cells was only detectable in ACC of experimental rats** (Fig. 85D), suggesting a memory specific effect.



**Figure 85: Hypoxia measured with pimonidazole coupled to immunohistofluorescence.** A- typical immunostaining of cells in brain slices from rats injected with pimonidazole. B- number of labeled cells in 20mm<sup>2</sup>, in home cage condition (HC) and in food preference (FP) condition just after interaction (D0) and 1 day after interaction (D1), in ACC (one-way ANOVA, interaction  $F_{(2,15)} = 5.198$ ,  $p = 0.019$ ; post-hoc Tukey, D0 vs D1:  $p = 0.044$ ; D0 vs HC:  $p = 0.030$ ; D1 vs HC:  $p = 0.78$ ); in PC (one-way ANOVA,  $F_{(2,14)} = 0.596$ ,  $p = 0.56$ ) and in HPC (one-way ANOVA,  $F_{(2,15)} = 6.273$ ,  $p = 0.010$ ; post-hoc Tukey, D0 vs D1:  $p = 0.016$ ; D0 vs HC:  $p = 0.032$ ; D1 vs HC:  $p = 0.88$ ). C- number of labeled cells in 20mm<sup>2</sup>, in food preference (FP) and experimental (EXP) animals just after interaction (D0) and 1 day after interaction (D1), in ACC (two-way ANOVA, interaction:  $F_{(1,28)} = 3.96$ ,  $p = 0.056$ ; time:  $F_{(1,28)} = 0.69$ ,  $p = 0.41$ ; STFP group:  $F_{(1,28)} = 4.02$ ,  $p = 0.055$ ; post-hoc Tukey, D1-FP vs D1-EXP:  $F_{(1,28)} = 34.04$ ,  $p = 0.031$ , ★), in PC (two-way ANOVA, interaction:  $F_{(1,28)} = 1.449$ ,  $p = 0.24$ ; time:  $F_{(1,28)} = 0.08$ ,  $p = 0.78$ ; STFP:  $F_{(1,28)} = 0.022$ ;  $p = 0.88$ ), and in HPC (two-way ANOVA, interaction:  $F_{(1,27)} = 0.455$ ;  $p = 0.50$ ; time:  $F_{(1,27)} = 18.13$ ;  $p = 0.0002$ ; STFP:  $F_{(1,27)} = 1.196$ ;  $p = 0.28$ ; post-hoc Tukey, D0-FP vs D1-FP:  $p = 0.010$ , ☆). D- ratio of number of labeled cells in 20mm<sup>2</sup> in EXP rats on the mean of number of labeled cells in 20mm<sup>2</sup> in FP rats (two-way ANOVA, interaction  $F_{(2,46)} = 3.903$ ;  $p = 0.027$ ; structure:  $F_{(2,46)} = 3.962$ ;  $p = 0.026$ ; STFP group:  $F_{(1,46)} = 5.009$ ;  $p = 0.030$ ; ACC-D1-EXP vs ACC-D1-FP,  $p = 0.004$ , ★). Columns expressed means  $\pm$  sem.

### 1.2.2. Angiogenesis induced in ACC by the memory consolidation

The sustained hypoxia measured in the ACC in observer rats interacting with a demonstrator fed with cumin, induced by encoding of the associative olfactory memory, is probably the trigger signal to induce angiogenesis. To investigate this point rapidly, we used proteome profiler array the same day of interaction (D0) and 3 days after (D3) to check if this process is still present a few days after memory encoding (protocol described in Fig. 86).

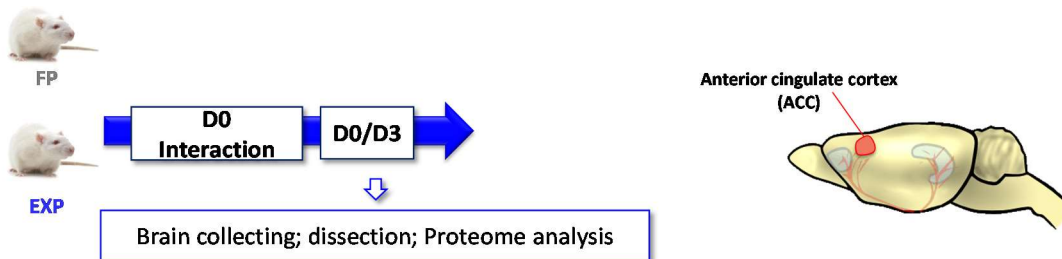
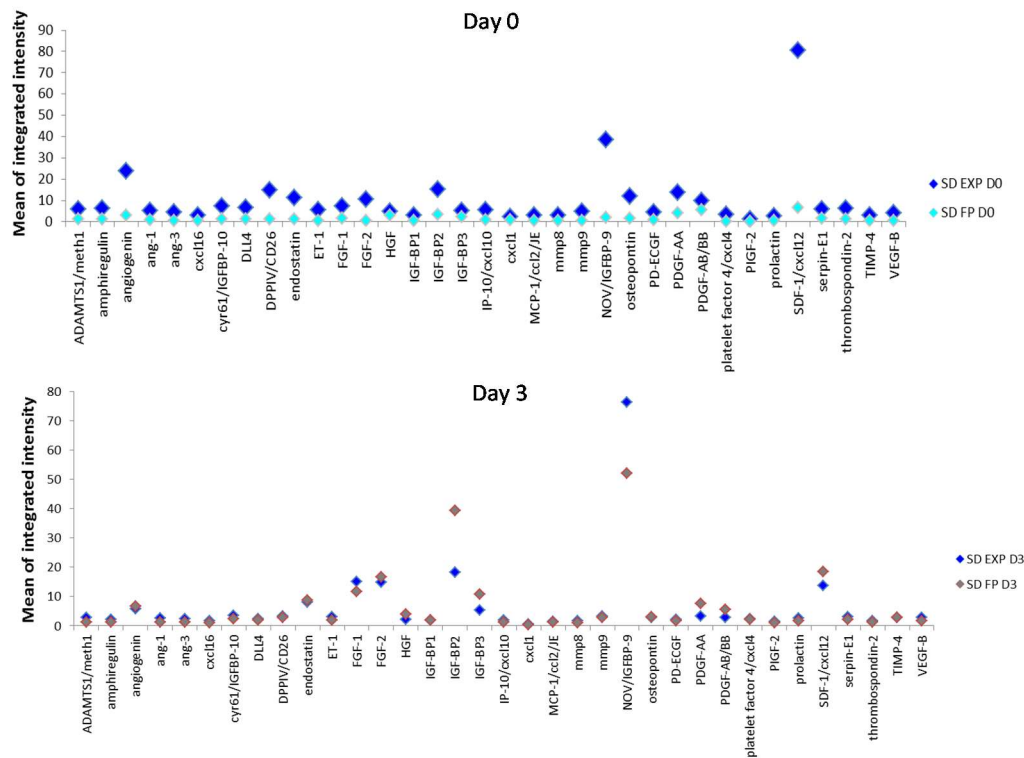


Figure 86: Protocol used in proteome profiler analysis and region of interest

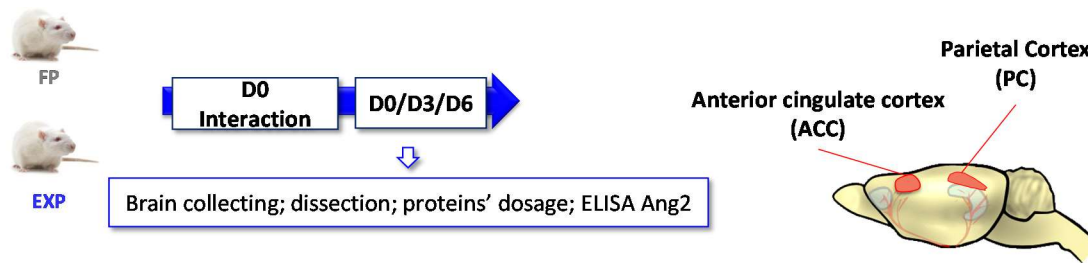
The Proteome Profiler Array Kit is a membrane-based sandwich immunoassay, dotted with different antibodies directed against several proteins known to induce, control and inhibit the angiogenesis process. This array is principally used in cultured cells and cancer angiogenesis but we validated the use also in physiological angiogenesis. Proteins were extracted from ACC of the observer rats from both food preference and experimental groups and pooled to be hybridized on proteome profiler membranes spotted in duplicate with antibodies to specific angiogenic target proteins, then revealed using chemiluminescent detection reagents. The signal produced was proportional to the amounts of analyte bound. The fluorescence emitted by dots on both membranes was quantified using a dedicated macro of Image J software and the mean of duplicate values was calculated. As shown in Fig. 87, in the experimental group, the angiogenic factors dotted on the membrane were more expressed than in the food preference group at D0 (90 min after interaction) as well as D3, despite a time-dependent global decrease in the number of angiogenic proteins expressed.



**Figure 87: Mean of integrated intensity observed on the angiogenesis proteome profiler array performed at Day 0 (above) and Day 3 (below).**

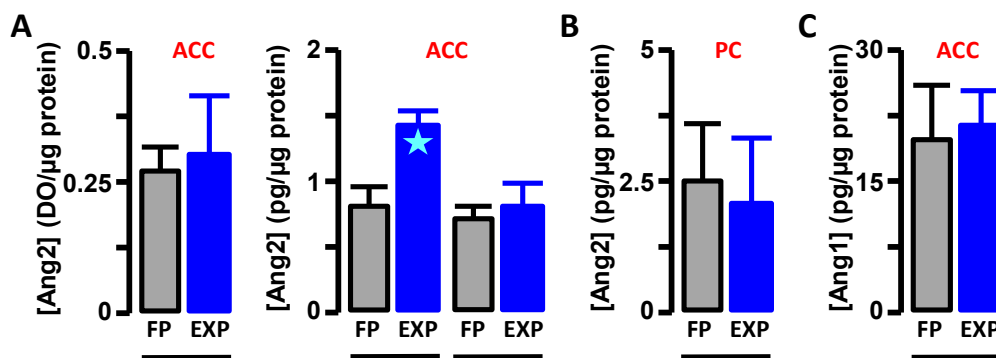
The results suggested a time-dependent regulation of angiogenic factors i.e. the angiogenesis factors were not regulated in the same manner at D0 and as at D3 (for example if NOV/GFBP9 is increased at both time points, IGFBP2 and PDGF-AA are increased at D0 and decreased at D3, Angiogenin, DPPIV/CD26 and SDF1/cxcl12 are upregulated at D0 but not as D3 in experimental rat). Anyway, **this result suggested that the angiogenesis in ACC is activated in the same time window of the hypoxia and the process is probably persistent for a few days after the interaction.** However, this general analysis of angiogenic factors appears not sufficiently specific in revealing its vascular impact: if it can reveal several regulations of angiogenic factors, it is not totally vessel specific and we are not able to individualize the results (one array per rat is not possible since there is not enough protein/sample).

Therefore, we focused on the angiopoietin pathway, as in an in mature adult brains Ang-2 appeared as a highly vessel specific, pro-angiogenic factor that could probably be detectable by ELISA and western blot. We have chosen solid phase ELISA in order to precisely quantify rat Angiopoietin-2 in tissue lysate. The rats were sacrificed at different time points during the post-encoding phase (Day 0; Day 3; Day 6; Fig. 88). The concentration of Ang-2 proteins in ACC and PC lysates, from individual rats of both food preference and experimental groups, were tested in duplicate following the supplier instruction. The Ang-2 protein expression was quantified using Optima plate reader and analysed with the associated software.



**Figure 88: Protocol used in ELISA quantification analysis and regions of interest**

Our data revealed that the Ang-2 expression in ACC, the same day of interaction, is not different between food preference and experimental rats; interestingly, Ang-2 concentration is increased in the ACC of experimental rats compared to food preference at D3, whereas it comes back to the control level at D6 (Fig. 89A). The Ang-2 concentration is not affected in PC by the task (Fig. 89B). Moreover, at D3, the Ang-1 concentration also revealed by ELISA in the same animals was not affected (Fig. 89C).



**Figure 89: time course of Ang-2 synthesis in brain measured by ELISA..** Mean of Ang-2 concentration A-in ACC (D0: t-test,  $t_8 = 0.550$ ,  $p = 0.60$ ; D3: t-test,  $t_{24} = 2.606$   $p = 0.0155$ ; ★; D6: t-test,  $t_{10} = 0.530$   $p = 0.61$ ) , B- in PC (t-test,  $t_{10} = 0.599$   $p = 0.56$ ), Mean of Ang-1 concentration in ACC revealed by ELISA (t-test,  $t_7 = 0.389$   $p = 0.71$ ). Columns expressed means ± sem.

These results indicated that Ang-2 concentration is specifically increased at D3 in ACC of rats which interacted with the demonstrators fed with cumin, suggesting that the memory consolidation of the associative olfactory information is coupled with angiogenic proteins expression.

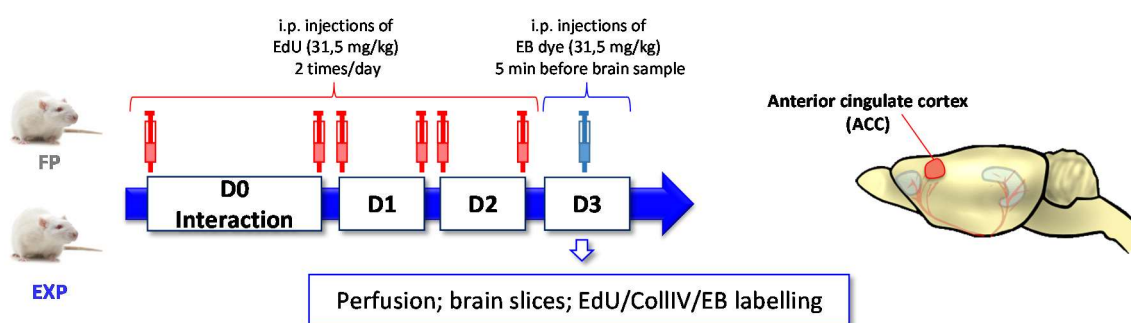
### 1.2.3. Outcome of angiogenic process in ACC during memory consolidation

To verify that the increase of Ang-2 concentration and activation of angiogenic pathways are efficient, we investigated the outcome of the angiogenic process such as the vascular proliferation in ACC brain slices from both food preference and experimental groups. Moreover, we decided to investigate the functionality of the newly formed vessels, analysing their perfusion. To reach this aimed, we designed a triple labelling, analysing the localization of different makers: EdU (marker of cellular proliferation), Collagen IV (labelling vascular network) and Evans' Blue (index of perfusion). At this stage of investigation, the angiogenesis process seems to be long lasting during the three first days after interaction.



To reveal new formed cells, we performed multiple injections of EdU on the same day and during 2 days after interaction, euthanizing the animals at Day 3 (Fig. 90). EdU is incorporated during DNA synthesis over the cell proliferation process, thus, revealing proliferative cells in the brain slices. Our choice in using EdU, instead of the classical BrdU staining in revealing cellular proliferation, is based on the more efficient revelation protocol using a chemical linkage with a fluorophore instead of immunostaining. To detect the perfused vessels, Evans Blue dye was injected just before animal sacrifice. The co-localization of EdU labelling with the Collagen IV immunostaining reveals the newly formed vessels.

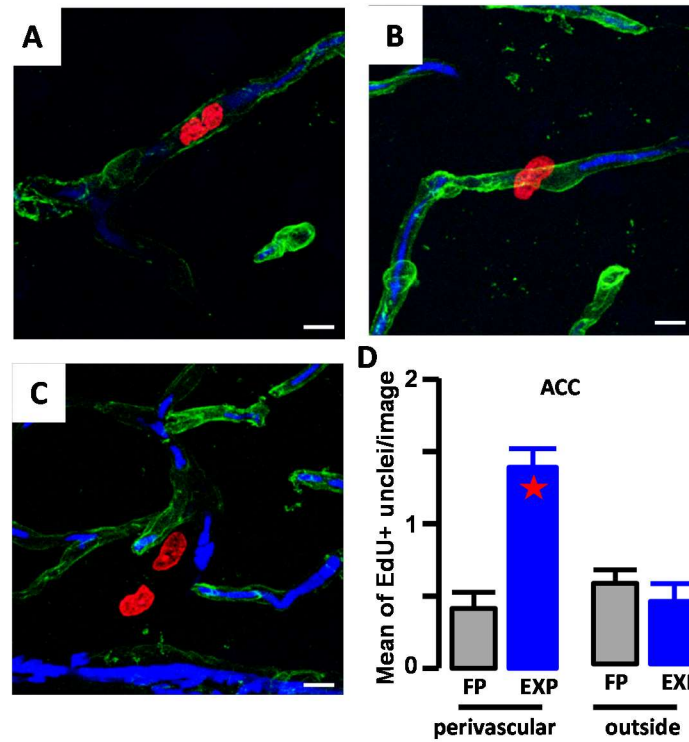
ACC slices of experimental and food preference groups were labelled; in particular, the vascular network was revealed by anti-collagen-IV antibody, the endothelial cell proliferation was revealed using Click-iT reaction (developed in materials and methods) and the functional vascular network was revealed by the auto-fluorescence of Evan's Blue.



**Figure 90: protocol for EdU and Evan's Blue (EB) dye injections to reveal the cell proliferation with co-localization of blood vessels by collagen-IV immunostaining.**

As illustrated in Fig. 91, EdU positive cells associated to anti-collagen-IV antibody were localized within and in contact with the vessel (Fig. 91A-B), but also outside the vessels (Fig. 91C). The proliferative cells outside the vessels were excluded from the counting. The EdU positive cells were counted and the mean of EdU positive cells in the ACC per image per rat was calculated and reported in Fig. 91D. Thus, the number of EdU positive cells associated with the vessel wall was significantly increased in the experimental group in comparison with the food preference group, whereas the number of EdU positive cells not associated with the vessels was similar in both groups.

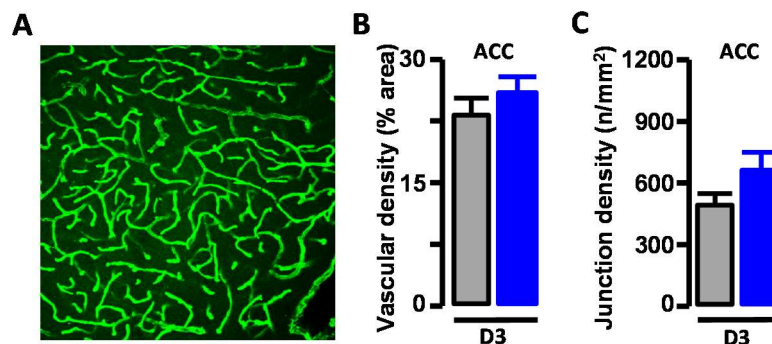




**Figure 91: proliferative cells in ACC labelled with EdU, collagen-IV and Evan's Blue.** A-C- typical images of labelling in which nuclei labelled with EdU (red) are associated with vessels labelled with collagen-IV (green). When the vessels are perfused they contained Evan's Blue (blue). **D-** Number of EdU positive nuclei pairs observed in situations illustrated in A and B per image (perivascular) (t-test,  $t_8 = 4.998$ ,  $p = 0.001$ , ★) and in C per image (outside the vessel periphery) (t-test,  $t_8 = 1.354$ ,  $p = 0.21$ ). Columns expressed means  $\pm$  sem.

Successively, we detected the auto-fluorescence of Evan's Blue spread within the vascular network: we qualitatively observed that the proliferative vascular cells were localized in perfused vessels, suggesting their functionality.

Finally, we wanted verify whether the angiogenesis process increased the vessel density and the branching (as described in introduction (Xu and Lamanna, 2006)). The Collagen IV immunolabelling specifically highlights the extracellular matrix of brain vessels. The immunostaining images analysis with Angiotool, dedicated software to quantify the vascular density and the density of junction, was performed on 2D images obtained with SP5 confocal microscope [objective: x10, image: 512x512 pixels, scanning: 32 lines average, iris: 1 $\mu$ m, zoom: 1.7, z step: 2 $\mu$ m, 24 stacks, (Fig. 92)].



**Figure 92: vascular density and branching in ACC from rat injected with EdU.** The immunohistofluorescence labelling of Collagen-IV analysed with Angiotool was performed on rat killed 3 days after interaction. **A-** typical image obtained after compilation of confocal images, treated with Angiotool to determine vascular and junction densities **B-** vascular density expressed as the area of the collagen-IV on the total area of the image, in ACC (t-test,  $t_9 = 0.980$ ,  $p = 0.35$ ). **C-** Vascular

branching expressed as the number of junctions per mm<sup>2</sup> in ACC (t-test,  $t_9 = 1.819$ ,  $p = 0.10$ ). Columns expressed means  $\pm$  sem.

The statistical analysis did not reveal any difference between food preference and experimental groups, suggesting that the vascular density is not increased by angiogenesis, despite the fact that a slight increase in branching points can be noticed. This experiment was reproduced more than 3 times, and in each experiment differences close to 10% and 15% were found in terms of vascular density and junction density, respectively. Moreover, this result confirms those obtained by Anaïs Giacinti obtained by a similar quantification of vascular network using images of the staining of endothelial cells with RCA-lectin. Considering the low number of proliferative cells we could not expect a great increase of vascular density, induced by a physiological phenomenon.

To further explore this mechanism, we decided to determine the cortical angiogenesis time-course induced by the consolidation of the associative olfactory memory. We scarified several groups of experimental and food preference rats at different time point following interaction, in order to quantify the density of vascular network and number of branching. Unfortunately, the quantification is still ongoing but preliminary results showed an increase of vascular density and branching between D1 and D3.

### 1.3. Discussion

The formation of enduring memories requires a transitory hippocampal-cortical interaction to enable the progressive remodelling of cortical neuronal networks involved in the long-term stabilization of remote memory traces (Bontempi et al., 1999; Frankland and Bontempi, 2005; Frankland et al., 2004; Maviel et al., 2004). According to the standard model of systems-level memory consolidation, using cellular imaging coupled to invasive approaches consisting in region-specific inactivation, Lesburguères and colleagues (2011) showed that the OFC and ACC are required for the retrieval of an associative olfactory remote memory (tested 30 days after encoding in the STFP paradigm). These cortical regions exhibit higher neuronal activity upon remote memory retrieval together with an increase in the complexity of the neuronal networks, as shown by a higher number of dendritic spines compared to rats undergoing recent memory testing. Parietal cortex is neither involved in the retrieval of remote nor recent associative olfactory memories (Lesburgueres et al., 2011). For this reason, we decided to consider it as a control region in our analyses. Moreover, a previous set of studies, conducted in the team, showed that the reactivity and architecture of cerebral vessels were modified during memory consolidation (Anaïs Giacinti, Ph.D. thesis, 2014). In particular, the formation of associative olfactory memory after social interaction was accompanied by a transitory increase in vessel density and branching in cortical regions supporting the memory trace, suggesting the triggering of an angiogenic process.

#### Hypoxic signal triggering angiogenesis

To further explore this result, we analysed one crucial mechanism triggering local angiogenesis, namely the hypoxic signal induced by neuronal activation (Boero et al., 1999; Boroujerdi et al., 2012; Dunn et al., 2004; LaManna et al., 2004; Masamoto et al., 2013; Pichiule et al., 2004; Pichiule and LaManna, 2002; Xu and Lamanna, 2006).

Using the Pimonidazole hypoxic marker (Turlejski et al., 2016; Varghese et al., 1976), which covalently binds the thiol group of protein depending on the presence of redox enzymes in hypoxic cells, we showed that the level of hypoxia was elevated preferentially in the areas implicated in the processing of associative olfactory memory shortly (1 hour) after the social interaction, suggesting a

memory-specific effect. This effect was however not statistically significant when compared to food preference controls, possibly because of the nonspecific components of the social interaction (novelty of the grid and first encounter with the demonstrator) which may have masked the hypoxia related to the memory-specific component of the task. To rule out the incidence of these potential confounding factors at Day 0, we propose to pre-expose the observer animals with a demonstrator fed with plain powdered chow in order to habituate them to the general layout of the interaction procedure (presence of the demonstrator and the grid). We can expect, after the second interaction, that the cells labelled by Pimonidazole in the ACC or hippocampus should primarily be those specifically activated by the processing of the associative olfactory memory. The most significant result concerns the persistence of hypoxia 24h after the social interaction specifically in ACC, suggesting that hypoxia-dependent mechanisms, once triggered, can persist in this cortical structure. Moreover, to strengthen this result, we propose to show that Pimonidazole-labelled cells also express c-fos to underline that hypoxic cells are predominantly activated neurons (Gualtieri et al., 2013).

Several studies showed co-localization between Pimonidazole and Hif-1 $\alpha$  (Gualtieri et al., 2013; Sluimer et al., 2008). Moreover, as reported in the general introduction, Hif-1 $\alpha$  is implicated in the downstream regulation of angiogenic processes. These results point to early hypoxic events taking place within the ACC that elicit an angiogenic mechanism supporting the progressive reorganization of neuronal networks induced during the course of the memory consolidation process, therefore strengthening the concept that this cortical region acts as a critical node within the broader cortical network supporting the formation of enduring associative olfactory memories in the STFP task.

### **Angiogenic pathway and vascular modification induced by memory consolidation**

We found that an angiogenic process is engaged after memory encoding, between D0 and D3 post-social interaction (data from proteome profiler). Encouraged by these results, we investigated the presence of Ang-2, one of the key regulator factors of angiogenesis produced from the onset of the angiogenic process (Augustin et al., 2009; Fagiani and Christofori, 2013). Ang-2 expression in ECs has been shown to be prompted 6 hours after hypoxia induction (Pichiule and LaManna, 2002), and the angiogenic phase is correlated with an increase in Ang-2 and VEGF (Wakui et al., 2006), while the blood vessel maturation phase is associated with a relative increase in Ang-1 and a decrease in VEGF (Wakui et al., 2006). Moreover, another important advantage of this molecule is represented by its vascular specificity compared to other growth factors, such as VEGF that acts and is expressed within endothelial cells but also in neurons and astrocytes, (Mackenzie and Ruhrberg, 2012; Shen et al., 2016) in adult brain. In our study, the memory testing-induced Ang-2 overexpression measured in the ACC was absent in the parietal cortex indicating that the Ang-2 increase was region-specific and was related to the consolidation of associative olfactory memory. The increase in memory-induced Ang-2 production was absent just after the social interaction but significantly overexpressed at Day 3, contemporaneously to a lack of Ang-1 increase. This suggests that Ang-2 can promote angiogenesis by producing vessel destabilization and by antagonizing the action of Ang-1 (Maisonpierre et al., 1997). Moreover, the long-lasting presence of Ang-2 suggests a transcriptional regulation, excluding that the Ang-2 protein detected come only from the release of Weibel-Palade bodies (Ju et al., 2014; Saharinen et al., 2011). The increase in the Ang-2 protein level returns to the basal level at Day 6, revealing a transitory process and suggesting that angiogenesis is related to the early phase of memory consolidation to potentially support the dialogue between HPC and ACC, as suggested previously for the neuronal component (Lesburgueres et al., 2011).

We further established that the formation of the memory trace induces a significant increase in the level of expression of proliferative cells within and around the vessels. This result could be confirmed

by an additional co-staining with anti-RECA antibody (recognizing the rat endothelial cell antigen localized on the cellular membrane of mature ECs) and anti-collagen-IV antibody (recognizing the ECM synthesized by ECs (Bahramsoltani et al., 2014)), in order to label respectively the luminal and the matrix sides of the endothelium (Duijvestijn et al., 1992). Moreover, our results showed that the detected new cortical vessels could also be perfused with Evans Blue suggesting that these vessels are functional (Walchli et al., 2015).

Finally, together with an enhanced cellular proliferation, a small trend towards an increase of vascular density was specifically observed in the ACC of the same rats during the early stage of the memory consolidation process. The increase in the ACC vascular network did not reach a significant difference 3 days after interaction; nevertheless, the increase of 10 % of vascular network and 15 % of branching points between experimental and food preference rats has always been detected in the 3 different subsets of our experiments, confirming the preliminary data obtained by Anais Giacinti. Thus, our opinion is that the vascular network is potentially increased, but our system of detection is not perfectly adapted to detect tiny effects. In fact, we have used software dedicated to tumour angiogenesis known to be very different to physiological induced-angiogenesis. This difficulty in quantification can be solved moving from a 2D to a 3D quantification, as suggested recently (Kolinko et al., 2015). Another explanation, concerning the difficulty in detecting a memory-induced vascular increase is that the newly formed vascular network can replace the old, less functional, vessels. These old vessels may be less perfused, degenerating and disappearing via a pruning process (Korn and Augustin, 2015). We propose to increase the memory trace strength via multiple interactions in the same day (Benjamin Bessieres, Ph.D. thesis, 2015), presuming that the wider neuronal activity and then the more extensive vascular angiogenesis process triggered can facilitate the detection of this effect.

The ACC takes in charge the remote memory several weeks after social interaction. Thus, we can suggest that early angiogenesis can assist the initial maturation of the neuronal cortical network that is going to subsequently support progressive maturation and stabilization of the memory trace. To validate this concept, we have performed invasive approaches, to either inhibit or potentiate angiogenesis, to establish the functional value of angiogenesis during the early phase of memory consolidation.

## **CHAPTER 2: Implication of angiogenesis in the memory consolidation, invasive approach**

## **Chapter 2: implication of angiogenesis in the memory consolidation, invasive approach**

### **2.1. Introduction**

In the previous chapter we showed that the consolidation of the associative olfactory memory may trigger a transitory angiogenic process resulting in time-dependent changes in the microvascular architecture in the ACC, together with the presence of newly-born endothelial cells. Furthermore the analysis of the hypoxia generated by neuronal activation, one of the mechanisms triggering angiogenesis, revealed a sustained region-specific increase of hypoxic environment in ACC. These results convinced us that angiogenesis is required in the early phase of consolidation of the associative olfactory memory.

Since the previous findings are only correlative in nature, we decided to move towards a more decisive invasive approach aimed at modulating angiogenesis specifically, thereby analysing the impact on memory consolidation. To reach these aims, we targeted Angiopoietin 2 (Ang-2) because of its vascular specificity compared to VEGF in the adult cerebral cortex, as demonstrated by the various studies defining the cellular distribution of Ang-2/Tie-2 molecules (Mackenzie and Ruhrberg, 2012; Shen et al., 2016). Its specificity allows us to target the vascular network without affecting neuronal activity, and thus getting rid of a confounding factor in the interpretation of memory performance. The goal of this set of experiments was to prove that the modulation of angiogenesis achieved by either a stimulation or an inhibition of Ang-2 was able to affect memory consolidation, and in particular to respectively enhance or decrease the remote memory retrieval.

The role of Ang-2 in the angiogenic process is to destabilize the vascular network enabling the sprouting of the new vessel (Fagiani and Christofori, 2013); its prompt action in angiogenic process represents an important advantage since it allow us to modulate from the early stages of angiogenesis.

Ang-2 is known to be one of the actors participating in the formation of the new vascular network; despite this, several studies showed that it is key point in the angiogenic pathways; as a matter of facts, *in vivo*, the exogenous administration of Ang-2 has been shown to boost the angiogenic process in presence of VEGF (Lobov et al., 2002); moreover, *in vivo* exogenous administration of Ang-2 induces vascular remodelling allowing the vascular proliferation and sprouting angiogenesis in presence of VEGF. The angiogenic action depends also on the Ang-2 concentration injected. Nag and collaborators showed that high dose of Ang-2 induce BBB breakdown and ECs apoptosis (Nag et al., 2005).

Thus, based on these studies and preliminary data obtained injecting Ang-2 in OFC during Anais Giacinti's Ph.D. thesis, we decided to modulate angiogenic pathways using a strategy of multiple injections in the ACC.

On the contrary, the pharmacological blockade of the action of Ang-2 hampers the development of tumour angiogenesis. In particular, L1-7(N) (by Amgen; (Brown et al., 2010; Mazziere et al., 2011; Tabruyn et al., 2010)), the antibody 3.19.3 (by MedImmune, (Coxon et al., 2010; Herbst et al., 2009; Oliner et al., 2004)), both inhibiting specifically Ang-2/Tie-2 interaction, and AMG 386 (by Amgen; (Coxon et al., 2010; Herbst et al., 2009; Oliner et al., 2004)), blocking simultaneously Ang-2/Tie-2 and Ang-1/Tie-2 interactions, confirm their anti-angiogenic action on ECs, prompting the use of these drugs in the clinical phase as an antitumor strategy. Concerning the choice of Ang-2 blockade different strategies can be used. Since the molecules above cited are not available, another strategy was adopted: we decided to block the production of Ang-2 protein using antisense oligonucleotides

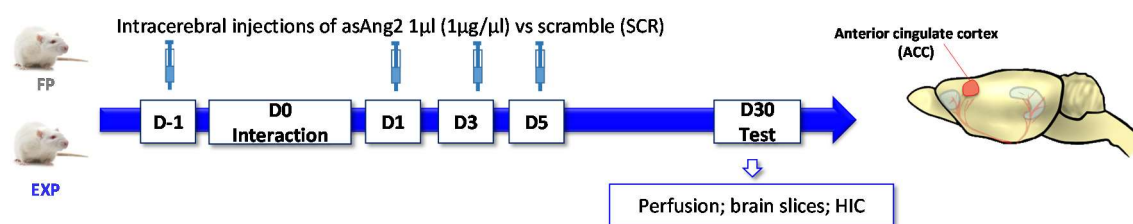
(ASON) directed against our target, the Ang-2, as used by Giacinti during her thesis (see material and methods “Justification of ASON choice”).

## 2.2. Experimental design and Results

### 2.2.1. Inhibition of memory consolidation by injection of ASON targeted Ang-2 in ACC

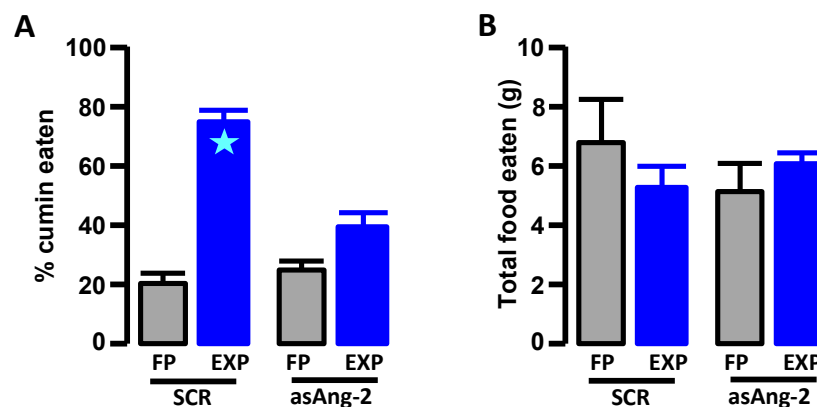
To inhibit the angiogenic pathways, Ang-2 has been chosen as the central key factor. Previously, an ASON against Ang-2 (asAng-2) was designed and the best usable concentration was determined as reported in Anaïs Giacinti’s thesis and in the material and the methods section.

The rats were bilaterally injected as described in the timeline protocol (Fig. 93) with 1 µg/µl of ASON against Ang-2 (asAng-2) or the scramble version of the asAng-2 (SCR). The repeated injections protocol was set according to the previous experience of the team (Dabertrand et al., 2010; Dabertrand et al., 2012) and was performed to ensure the presence of ASON during the early phase of the memory consolidation phase, corresponding to the angiogenesis period as defined by the correlative approach (Ang-2 concentration measured by ELISA). The injections were performed once every 2 days to avoid a toxic effect of ASON whilst assuring its expression in vascular network. The choice of beginning the injection protocol before the encoding was done based on the fact that the half-life of Ang-2 mRNA is of 3 hours in normoxic conditions and 5 hours in hypoxic conditions, but the protein can be detected till 24 hours after hypoxic stimulation (Pichiule et al., 2004). Moreover, if it is stocked in Weibel-Palade bodies the half-life of the protein is 18 hours. Thus, in order to reduce the presence of Ang-2 at the moment of the encoding we preferred to start the injection the day before interaction.



**Figure 93:** protocol for ASON and SCR injections.

The memory performance was tested 30 days after encoding, as reported in Fig. 94. Injections of asAng-2 were able to impair the remote memory retrieval in observer rats interacting with demonstrators fed with cumin, yet without modification of the total food eaten, thus the differences in memory performance between the groups were not a consequence of food intake motivation.

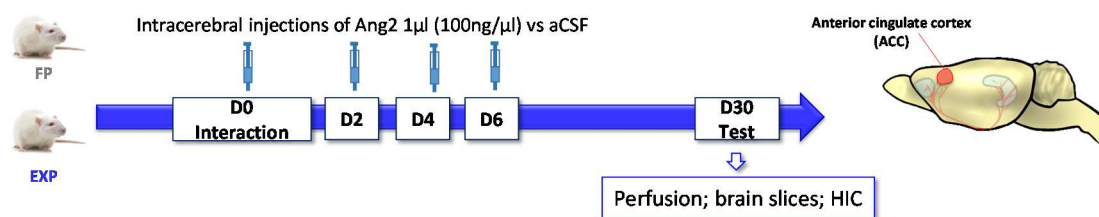


**Figure 94: effects of intracerebral injections of ASON directed against Ang-2 (asAng-2) and SCR on associative olfactory memory.** A- % cumin eaten (two-way ANOVA, interaction  $F_{(1,19)} = 21.18$ ;  $p = 0.0002$ ; treatment:  $F_{(1,19)} = 10.81$ ;  $p = 0.004$ ; STFP:  $F_{(1,19)} = 57.9$ ;  $p < 0.0001$ ; post-hoc Tukey SCR-FP vs SCR-EXP  $p < 0.0001$ , ★; aCSF-EXP vs ang2-EXP  $p < 0.0001$ , ★). B- total food eaten (two-way ANOVA, interaction  $F_{(1,19)} = 2.014$ ;  $p = 0.17$ ; treatment:  $F_{(1,19)} = 0.282$ ;  $p = 0.60$ ; STFP:  $F_{(1,19)} = 0.277$ ;  $p = 0.60$ ). Columns expressed means  $\pm$  sem.

**This result showed that the infusion of asAng-2 during the early phase of memory consolidation impairs remote memory in experimental rats (D30).**

### 2.2.2. Stimulation of memory consolidation by injection of exogenous Ang-2 in ACC

To complete this first result and validate the hypothesis about the angiogenesis role in memory consolidation, we performed bilateral multiple injections of 100 ng/ $\mu$ l of Ang-2 or aCSF (control group) in ACC of rats after encoding. The repeated injection protocol is described in Fig. 95. The exogenous Ang-2 administration begun just after social interaction, in order to increase the angiogenic process from the beginning of the post-encoding phase. The choice of injections each 2 days was done to minimize the physical stress of the injection and according to previous experiments performed in the lab.

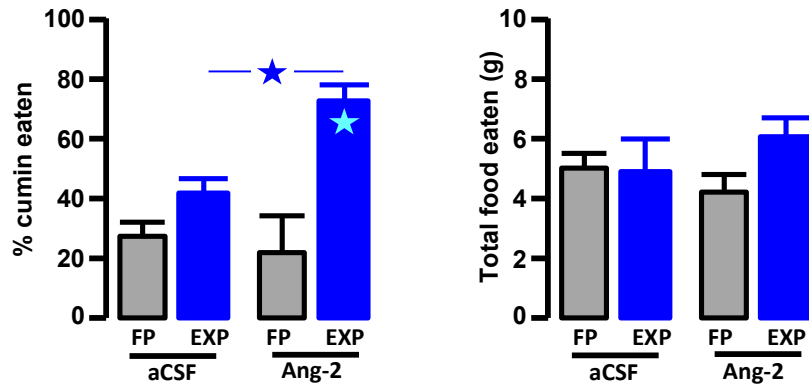


**Figure 95: protocol for Ang-2 and aCSF injections.** The rats were tested 30 days after encoding

The interaction protocol used for this experiment was different from the others, since the greed dividing the observers from the demonstrators was left during all the time of interaction (30 min). This strategy allows us to avoid that the memory performance reaches the maximal expression in aCSF rats and to see the supposed amelioration of the memory performance in treated rats. In fact, after the classical STFP interaction (20 min with greed that divide observers and demonstrators, guiding the tidy interaction through the holes of the greed, and 10 min without greed allowing the free explorations between animals), the memory performance is so good that it is difficult to see any possible amelioration. Thus, as described in Material and methods, if the grid is not removed, the memory trace strength is diminished and the memory performance during the retrieval is decreased, as revealed by a lower quantity of cumin eaten by the observer rats interacting with the demonstrators fed with cumin.

Thirty days after the social interaction, the retention test indicated a correct memory retrieval in Ang-2 injected experimental rats compared to Ang-2 injected food preference rats; interestingly the memory performance of Ang-2 injected experimental rats is significantly increased in comparison to the one measured in experimental rats injected with aCSF (Fig. 96A). Moreover, the total food consumed by all groups during test is similar, indicating no confounding effect of motivation and that the food intake is not modified by cerebral injections (Fig. 96B).

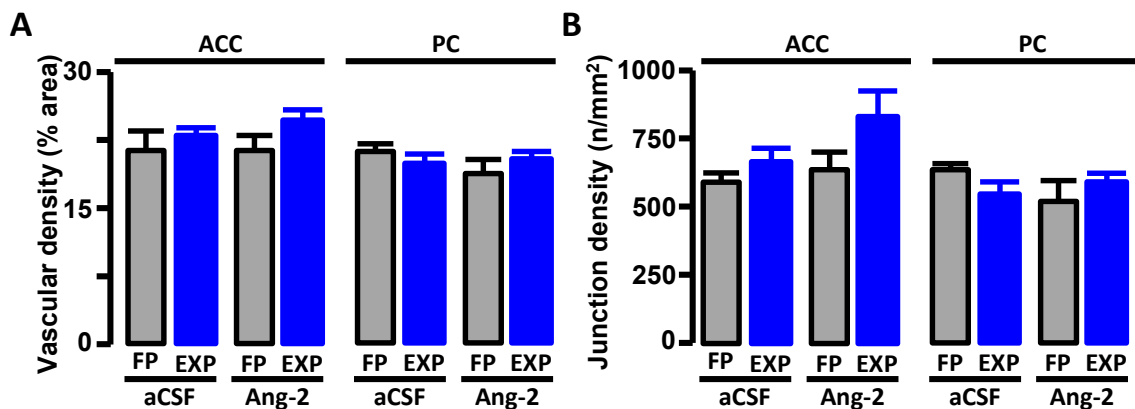




**Figure 96: Effects of intracerebral injections of Ang-2 and aCSF on associative olfactory memory.** **A-** % cumin eaten (two-way ANOVA, interaction  $F_{(1,16)} = 11.13$ ;  $p = 0.0042$ ; treatment:  $F_{(1,16)} = 6.585$ ;  $p = 0.021$ ; STFP group:  $F_{(1,16)} = 32.87$ ;  $p < 0.0001$ ; post-hoc Tukey ang2-FP vs ang2-EXP  $p = 0.0007$ , ★; aCSF-EXP vs ang2-EXP  $p < 0.0001$ , ★). **B-** total food eaten (two-way ANOVA, interaction  $F_{(1,16)} = 0.819$ ;  $p = 0.37$ ; treatment:  $F_{(1,16)} = 0.044$ ;  $p = 0.84$ ; STFP group:  $F_{(1,16)} = 1.438$ ;  $p = 0.24$ ). Columns expressed means  $\pm$  sem.

### Angiogenesis stimulation achieved by injections of Ang-2 during the early phase of memory consolidation ameliorates remote memory in experimental rats (D30).

Finally, the analysis of the vascular network was performed using Collagen IV immunostaining and vascular density and branching were quantified in each group of animals using Angiotool software. After a retrieval test at D30, both the vascular density and the junction density were not significantly increased in experimental groups as well as by the injection of Ang-2. Despite a very tenuous effect in experimental group compared to food preference group was detected; the memory-induced vascular increase was multiplied by two in the Ang-2-injected rats in comparison with aCSF-injected rats (Fig. 97).



**Figure 97: effects of Ang-2 and aCSF injections on vascular density and branching.** The both parameters were measured on immunohistochemistry of collagen-IV at D30, after the retrieval test, and analysed with Angiotool. **A-** Vascular density expressed as the area of the collagen-IV on the total area of the image. labelling in ACC (two-way ANOVA, interaction  $F_{(1,17)} = 0.304$ ;  $p = 0.58$ ; treatment:  $F_{(1,17)} = 0.385$ ;  $p = 0.54$ ; STFP group:  $F_{(1,17)} = 1.628$ ;  $p = 0.22$ ) and in PC (two-way ANOVA, interaction  $F_{(1,18)} = 2.378$ ;  $p = 0.14$ ; treatment:  $F_{(1,18)} = 0.864$ ;  $p = 0.36$ ; STFP group:  $F_{(1,185)} = 0.0003$ ;  $p = 0.98$ ). **B-** Vascular branching expressed as the number of junction per  $\text{mm}^2$  in ACC (two-way ANOVA, interaction  $F_{(1,16)} = 0.302$ ;  $p = 0.59$ ; aCSF vs Ang-2:  $F_{(1,16)} = 1.879$ ;  $p = 0.19$ ; STFP group:  $F_{(1,16)} = 2.919$ ;  $p = 0.10$ ) and in PC (two-way ANOVA, interaction  $F_{(1,18)} = 3.016$ ;  $p = 0.09$ ; treatment:  $F_{(1,18)} = 0.444$ ;  $p = 0.51$ ; STFP group:  $F_{(1,18)} = 0.157$ ;  $p = 0.69$ ). Columns expressed means  $\pm$  sem.

In this chapter, **our results confirm, by an invasive approach, that the angiogenesis is a permissive mechanism to help the formation of the consolidation of the associative olfactory memory trace and validate that Ang-2 participated in this mechanism.**

### 2.3. Discussion

In the previous chapter, we found that consolidation of an associative olfactory memory is concomitant with the triggering of an early and time-dependent angiogenic process, which induces changes in the microvascular architecture of the ACC. Accordingly, we detected the presence of newly-born vascular cells in this cortical region involved in the formation and retrieval of remotely acquired information, as well as spatial or non-spatial memories (Bontempi et al., 1999; Frankland et al., 2004; Lesburgueres et al., 2011; Maviel et al., 2004; Takehara et al., 2003; Teixeira et al., 2006). Furthermore, the analysis of hypoxia generated by memory-induced neuronal activation, one of the mechanisms triggering angiogenesis (Boero et al., 1999; Dunn et al., 2004; LaManna et al., 2004; Masamoto et al., 2013; Pichiule et al., 2004; Xu and Lamanna, 2006), revealed a sustained region-specific increase of the hypoxic environment in the ACC. To examine whether this transitory angiogenic process is necessary for the formation and subsequent storage of remote memories at the cortical level during the course of the memory consolidation process, we have chosen to adopt a causal strategy aimed at modulating specifically cortical angiogenesis this in order to highlight its functional importance as a permissive mechanism responsible for the progressive embedding of the memory trace within cortical networks..

Antisense oligonucleotide strategies were classically used to inhibit the translational machinery leading to a selective target (Kole et al., 2012), as to reduce the angiogenesis pathways (Park et al., 2016). Here for the first time, we have used ASON against Ang-2 to inhibit the memory-induced angiogenesis. The Ang-2 target was selected based on its crucial role in promoting the angiogenesis when VEGF is present, producing pericytes dissociation from vessels (Zhang et al., 2003a) and vessels destabilization (Ribatti et al., 2011), increasing permeability and vascular leakage. Ang-2 expression is strictly controlled. In adults physiological condition it is selectively expressed in ECs (Augustin et al., 2009; Fiedler and Augustin, 2006; Fiedler et al., 2004) and pericytes (Wakui et al., 2006) where active remodelling occurs. It's almost absent in quiescent vasculature but it dramatically produced by ECs and up regulated in response to tissue hypoxia (Pichiule and LaManna, 2002) and shear stress (biomechanical force acting on the vessels wall as a consequence of the tangential force exerted by the flowing blood). Ang-2 activates molecular cascades underlying vascular destabilization required for angiogenesis (Hakanpaa et al., 2015), the final outcome being a more powerful and coordinated vascular signalling, resulting in a more efficient, dense and stable vascular network (Fagiani and Christofori, 2013). Our findings show that repeated intracerebral injections of antisense oligodeoxynucleotides directed against Ang-2 (asAng-2), infused into the ACC during the early phase of memory consolidation, are efficacious in impairing retrieval of associative olfactory remote memory probed 30 days after social interaction in the STFP paradigm. Thus, while Ang-2 is not the only actor involved in the modulation of angiogenesis (Carmeliet and Jain, 2011), its selective inhibition of expression was sufficient to impact remote memory performance. Conversely, we explored whether angiogenesis stimulation could improve remote memory in experimental rats. To achieve this, we performed repeated infusions of exogenous Ang-2 peptides into the ACC during the early phase of memory consolidation. This treatment was successful in improving remote memory. The absence of between-group differences in the total amount of food consumed during the retrieval test indicated that both asAng-2 and Ang-2 treatments did not affect the motivation of rats to eat powdered food, thus pointing to memory-specific effects of these two treatments. Furthermore the lack of significant differences between aCSF-injected animals and non-treated animals used in other

experiments indicates that the constraints associated with the intracerebral injection procedure (contention, placement of intracerebral cannulas, infusion of the vehicle), did not interfere with the ability of the rats to perform in the STFP task.

Taken together, our behavioural results suggest that early angiogenesis in cortical networks acts as a permissive mechanism which could regulate the subsequent development of molecular and structural modifications in neurons underlying the formation and maintenance of enduring memories. Indeed, converging evidence have shown that the formation of remote memory is associated with an increase in spine density; according to Lesburgueres and colleagues, the consolidation of the associative olfactory memory is accompanied by a cortical increase of spine density, detectable since Day 1, in regions such as OFC (Lesburgueres et al., 2011); the progressive increase of dendritic spine growth in ACC was also detected during the consolidation of a contextual fear memory or spatial memory (Frankland et al., 2004; Maviel et al., 2004; Restivo et al., 2009; Vetere et al., 2011). Moreover, the stabilization of the associative olfactory memory is accompanied by a progressive surface redistribution of cortical NMDA receptors (Benjamin Bessieres, Ph.D. 2016), known to be involved in the induction and regulation of synaptic plasticity (Lau and Zukin, 2007; Mayford et al., 2012).

In order to strengthen this possibility in our own behavioural model, experiments are underway to examine the status of the architecture of neuronal networks (synaptophysin labelling as a presynaptic marker of synaptogenesis) and neuronal activation (c-fos labelling) upon remote memory retrieval in these rats in order to correlate the observed memory impairment and enhancement to a decrease or increase of neuronal activity and network complexity in the ACC and other connected cortical regions, respectively.

As suggested in the previous chapter, the increase of vascular network density induced by the consolidation of associative olfactory information generated by the STFP paradigm quickly returns to a basal level a few days after memory encoding, suggesting a transitory role of this angiogenic mechanism initiated during the course of the memory consolidation process. However, the possibility remains that the observed slightly increased density in the vascular network within the ACC could persist longer due to the action of Ang-2. In order to investigate whether the vascular proliferation initially boosted by Ang-2 was potentially maintained, a quantification of the architecture of the vascular network of the ACC was performed in Ang-2 injected rats 30 days after the social interaction. The quantification of vascular density and number of vessel branching points did not reveal significant differences between groups, even if a tendency towards an increase, particularly in the number of vascular branching points was apparent in Ang-2-injected rats. Importantly, Ang-2 repeated injections in food preference groups injected with aCSF or Ang-2 did not affect vascular density per se.

To further characterize the efficacy of Ang-2 injections (does Ang-2 increase vascular network in term of vascular density?) and the duration of this vascular network increase (does Ang-2 slow down the vascular regression?), additional experiments will be needed to examine the vascular network just after the end of Ang-2 injections and at different time points thereafter.

Antisense oligonucleotides, including asAng-2, are typically designed to inhibit the production of proteins, in our case the Ang-2 protein (Kole et al., 2012). In our experiment, asAng-2 injections inhibit remote memory retrieval probed several weeks after the last injections of asAng-2. However, we still have to demonstrate the efficacy of this treatment by showing a lack of increase of density of the vascular network in the experimental group injected with asAng-2 just after the end of asAng-2 injections, a time window for which we know that increased density in the vascular network occurs.

Finally, we have to establish the specificity of asAng-2 or Ang-2 injections, their direct effects on ECs proliferation and on the Ang-2 expression. In light of our results, it is possible to predict that the Ang-

2 or asAng-2 injections will increase or decrease the ECs proliferation and Ang-2 expression in the ACC of experimental rats, respectively.

In conclusion, our results suggests that the basal state of the vascular network in ACC is not optimal, since Ang-2 injection in the early phase of memory consolidation is able to slightly increase its density at Day 30, supporting and enhancing the expression of remote memory. The data obtained suggest that the function of Ang-2 and its associated vascular increase can be useful to support the dialogue between hippocampus and cortex that is required to enable the embedding of remote memories within cortical networks and this despite the apparent transient nature of the angiogenic process. In the topic about memory consolidation, memory forgetting is also an important parameter (Hardt et al., 2013). Thus, it will be very interesting to test if transitory early angiogenesis induced by Ang-2 injections can somehow slow down memory decay by not only facilitating memory expression at the time of retrieval but perhaps also by strengthening its informative content during memory formation.

The effects of Ang-2 on angiogenesis are thought to be modulated by VEGF. When injected in the absence of VEGF and of an angiogenic environment, Ang-2 induces endothelial cell death and vessel regression (Lobov et al., 2002). Conversely, Ang-2 promotes angiogenesis when VEGF is present by producing vessels destabilization prior to inducing vessel sprouting. Thus, we show that blocking the expression of Ang-2 in experimental rats is sufficient to interfere with the formation and/or the expression of remote memory, presumably blocking angiogenic mechanism, pointing to the importance of Ang-2 as a key molecular factor underlying vascular changes induced

Furthermore, given the specificity of Ang-2 on vascular network (Augustin et al., 2009; Fiedler and Augustin, 2006; Fiedler et al., 2004; Wakui et al., 2006), we can exclude a direct effect on neuronal activity in our conditions. In fact, in vivo neuronal Ang-2 action has been reported just during development (Marteau et al., 2011) in neuronal progenitors (Liu et al., 2009) or fibroblast differentiation (Lee et al., 2007) but not in adult cortical neuronal network. According to the described dynamic of vascular changes during the course of memory consolidation, it is possible predict that delaying the injection of Ang-2 after the angiogenic peak should not affect remote memory expression. To clearly show this time course, we would like to inject Ang-2 and asAng-2 after the angiogenic peak to show the absence of remote memory enhancement or memory perturbation, respectively. These experiments are still underway.

## **CHAPTER 3: Spontaneous hypertensive rat is a model of consolidation impairment**

### **Chapter 3: Spontaneous hypertensive rat is a model of consolidation impairment**

#### **3.1. Introduction**

Systemic hypertension is described as one of the most important risk factors for neurodegenerative disorders leading to memory impairments, such as Alzheimer's disease (Carnevale et al., 2012; Hazar et al., 2016; Valenti et al., 2014). Several hypotheses are proposed to link hypertension to neuronal dysfunctions (for review, (Iadecola, 2013)). Hypertension induces endothelial and smooth muscle dysfunctions potentially responsible for vascular remodelling, atherosclerosis, impairment of vascular reactivity and blood brain barrier, alterations of cell interactions between capillaries and glial cells, all these effects triggering decreases of brain perfusion and exchanges to affect neuronal and glial functions (Faraco and Iadecola, 2013). Moreover, some studies proposed that hypertension was also characterized by a capillary rarefaction (Humar et al., 2009; Sane et al., 2004; Sokolova et al., 1985). Finally, the deleterious effects of hypertension are linked to the chronicity of the pathology.

Despite that, there are no direct studies aimed to unravel the potential effects of hypertension on memory consolidation via a potential deficit in cerebral angiogenesis. Thus, we decided to select a hypertensive model in order to study the impact of vascular impairment, in particular a putative angiogenesis deficit, on memory consolidation.

To reach this goal we decided to select the Spontaneously Hypertensive Rats (SHRs) which, due to an uniform genetic predisposition, develop hypertension over time (Okamoto and Aoki, 1963; Tayebati et al., 2012). This model was used as a complementary approach to explore the functional contribution of neurovascular networks to cognitive processes. This model has been widely used in hypertension studies since its discovery (Okamoto and Aoki, 1963; Pinto et al., 1998); we decided to use this model because, contrarily to inducible models, SHRs do not require treatment or surgery to develop a stable AHT, reducing the number of animals used. Moreover, the long-lasting and stable AHT allows the study of the evolution of memory consolidation in parallel to vascular dysfunctions. Finally, compared to both inducible and spontaneous models, there are no other comorbidities and the progression (onset and development) of the disease is more comparable to human AHT, avoiding fulminant crisis detectable in other models of rodents' hypertension (Dornas and Silva, 2011; Leong et al., 2015; Pinto et al., 1998).

The angiogenic status in this strain is still under debate since some studies find angiogenic impairment (Lu et al., 2016; Murfee and Schmid-Schonbein, 2008; Wang et al., 2004) and others lack to find differences in angiogenic pathways compared to other strains (Amaral et al., 2008; Hudlett et al., 2005; Murfee and Schmid-Schonbein, 2008; Wang et al., 2002).

Moreover, despite the contradictory bibliography found on this strain about memory (reviewed in (Meneses et al., 2011a)), it seems that these rats exhibit lower memory performances compared to control strains (Meneses et al., 2011a; Mori et al., 1995; Sutterer et al., 1980; Tayebati et al., 2012; Wyss et al., 1992). These memory performances were exacerbated during the time (Heal et al., 2008; Meneses et al., 1997; Wells et al., 2010) (van der Staay and de Jonge, 1993; Wyss et al., 2000), but it is difficult to segregate whether this effect is due to the progression of the pathology or to aging, considering that they provoke similar and additive cognitive alterations. In any case, the major decrease of memory performance was observed in spatial learning and memory in 18/24-month-old rats (van der Staay and de Jonge, 1993; Wyss et al., 2000).

Since the potential bias introduced by the behavioural phenotype of this strain, including hyperactivity (Meneses et al., 2011a; Sontag et al., 2013), in the interpretation of results concerning memory test in this strain, we used for the first time the STFP paradigm in SHR strain, minimizing the locomotor activity component.

Because hypertension develops progressively, independent groups of SHR rats were tested at 4 months (beginning of stable hypertension) and 6 months (established level of hypertension) on the recent (tested at Day 1) and remote (tested at Day 30) memory performance. During this phase, both cocoa-cinnamon and cumin-thyme associations were used, in order to exclude odour significance of a particular pair or in the taste preference of SHRs. We thereafter selected the cocoa-cinnamon association to perform the following experiments since we noticed a smaller data dispersion using these flavours. The memory performances of SHRs were compared to the ones of Wistar Kyoto Rats (WKYs), the classical control strain (Okamoto and Aoki, 1963; Tayebati et al., 2012), and Sprague Dawley Rats (SDs) used in the previous part of the thesis.

Moreover we characterized the behavioural phenotype of SHRs to unravel potential confounding factors such as olfactory sensitivity (odour detection threshold) and initial memory strength over the interpretation of impaired memory profile in SHRs, as well as the locomotor activity and anxiety profiles.

To further characterize the putative permissive function of vascular networks and angiogenesis during memory consolidation processes, we adopted the same approaches used before in the first two chapters on SD. Firstly, using a correlative approach, we investigated the angiogenic profile in ACC, 3 days after STFP interaction. Moreover, to solve the riddle of vascular dysfunction and memory consolidation impairment we adopted two invasive approaches: the pharmacological reversion of AHT through chronic treatment with Losartan, an antagonist of angiotensin II receptor type 1, and the restoration of angiogenic pathway through region-specific intracerebral infusions of Ang-2 during the early phase of memory consolidation.

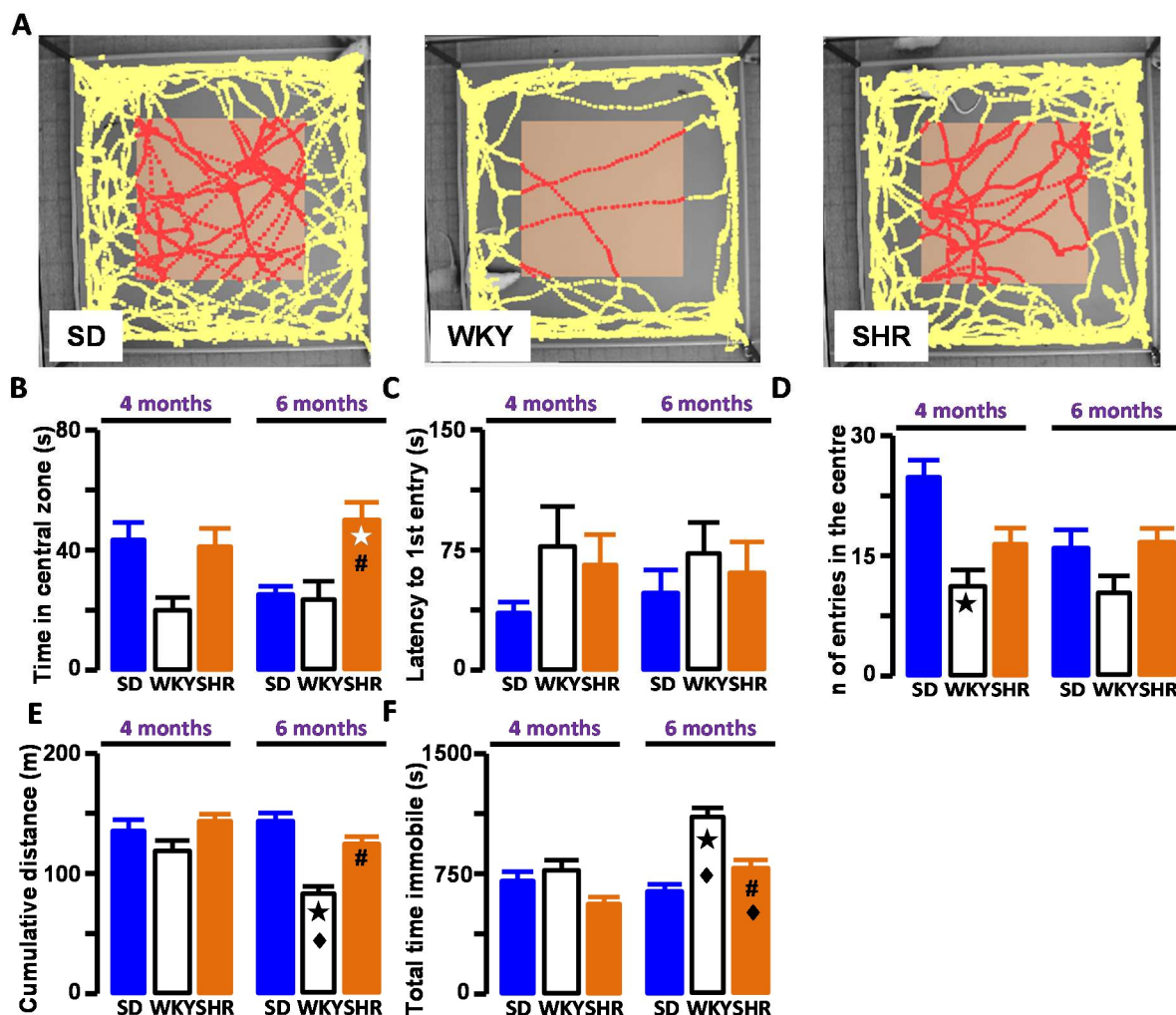
## **3.2. Experimental Design and Results**

The STFP task was used to characterize the putative impairment of memory consolidation in the SHR strain; anxiety and locomotion, taste preference, thresholds of odour detection and social interactions have been carefully analysed to circumscribe potential bias. As mentioned in the introduction, hypertension is established in the early adult life of the SHR and then it becomes stable. We have used animals at two different ages to determine if the chronicity of the pathology plays a role in the memory consolidation process.

### **3.2.1. Behavioural analysis of the SHR strain, comparison with WKY and SD strains**

Before testing the memory performance of SHRs with the STFP task, we have characterized the SHR behaviour in comparison with WKY and SD rats to determine if SD could be used as SHRs' control. SHRs have been previously proposed as a hyperactive and hypo-anxious strain in comparison with WKY, the classical control strain from which SHRs are derived; this was especially important for us as these parameters can modulate social interaction and food intake. To compare the anxiety and locomotor activity in the 3 strains, we used an open field task and analysed the exploration behaviour of the rats (Fig. 98). The anxiety of the rats was measured during the first 10 minutes of the presence of the rat in the arena by the analysis of the time spent by the rat to explore, the latency of the first entry and the number of entries in this zone. Our results indicated that the 6 month-old SHRs spent

more time in the central zone than WKY and SD rats (Fig. 98B) and WKY rat entered less often in the central zone than SD rats (Fig. 98D). The locomotor activity was evaluated using both the cumulative distance that the animals covered during 30 min and the immobility time. The SD and SHRs have similar locomotor activity at 4 and 6 months (Fig. 98E); but SHRs presented an increase of the total immobility time due to aging (Fig. 98F). Finally, the WKY rats spend more time immobile and move less than both SDs and SHRs (Fig. 98E-F).



**Figure 98: Behavioural comparison between Sprague Dawley (SD) Wistar Kyoto (WKY) and SHR in open field (A,B,C,D) and locomotor activity (E,F).** A- Typical path travelled by the rat in 10 min. B- Time spent in the central zone during 10min (two-way ANOVA, interaction  $F_{(2,75)} = 2.822$ ,  $p = 0.065$ ; age:  $F_{(1,75)} = 0.169$ ,  $p = 0.68$ ; strain:  $F_{(2,75)} = 8.166$ ,  $p = 0.0006$ ; post-hoc Tukey SHR-6M vs WKY-6M  $p < 0.019$ , #; SHR-6M vs SD-6M  $p = 0.006$ , ☆). C- Latency to the first entry in the central zone during 10min (two-way ANOVA, interaction  $F_{(2,75)} = 0.163$ ,  $p = 0.85$ ; age:  $F_{(1,75)} = 0.027$ ,  $p = 0.87$ , strain:  $F_{(2,75)} = 1.712$ ,  $p = 0.19$ ). D- Number of entries in the central zone during 10min (two-way ANOVA, interaction  $F_{(2,75)} = 2.096$ ,  $p = 0.13$ ; age:  $F_{(1,75)} = 2.952$ ,  $p = 0.09$ , strain:  $F_{(2,75)} = 8.062$ ,  $p = 0.0007$ ; post-hoc Tukey SD-4M vs WKY-4M  $p = 0.0056$ , ★). E- Cumulative distance travelled during 30 min (two-way ANOVA, interaction  $F_{(2,75)} = 3.867$ ,  $p = 0.025$ ; age:  $F_{(1,75)} = 6.818$ ,  $p = 0.011$ , strain:  $F_{(2,75)} = 12.81$ ,  $p < 0.0001$ ; post-hoc Tukey SHR-6M vs WKY-6M  $p = 0.003$ , #; WKY-6M vs WKY-4M  $p = 0.0029$ , ♦, SD-6M vs WKY-6M  $p < 0.0001$ , ★). F- Total time immobile during 30 min (two-way ANOVA, interaction  $F_{(2,75)} = 8.517$ ,  $p = 0.0005$ ; age:  $F_{(1,75)} = 14.90$ ,  $p = 0.0002$ , strain:  $F_{(2,75)} = 18.78$ ,  $p < 0.0001$ ; post-hoc Tukey SHR-6M vs WKY-6M  $p < 0.0001$ , #; WKY-6M vs WKY-4M  $p = 0.0006$ , ♦; SHR-6M vs SHR-4M  $p = 0.0021$ , ♦; SD-6M vs WKY-6M  $p < 0.0001$ , ★). Columns expressed means  $\pm$  sem.

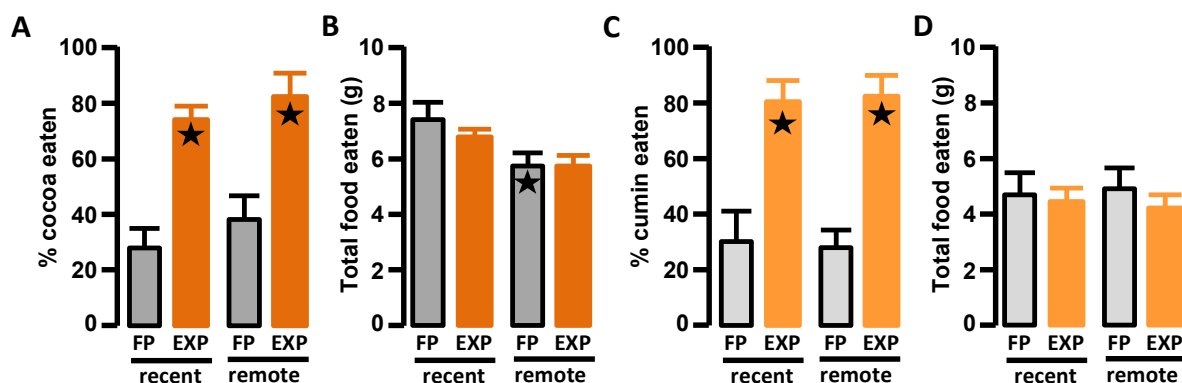
Taken together, these results suggest that (1) the SHRs and SDs are not different in terms of locomotor activity; (2) the increase of time in the central zone during the first 10 minutes confirm that SHRs could be less anxious than the others strains. This last weak effect is not confirmed by



the analysis of others parameters, suggesting that it is probably not sufficient to alter the quality of social interactions.

### 3.2.2. Performances of SHRs in STFP task

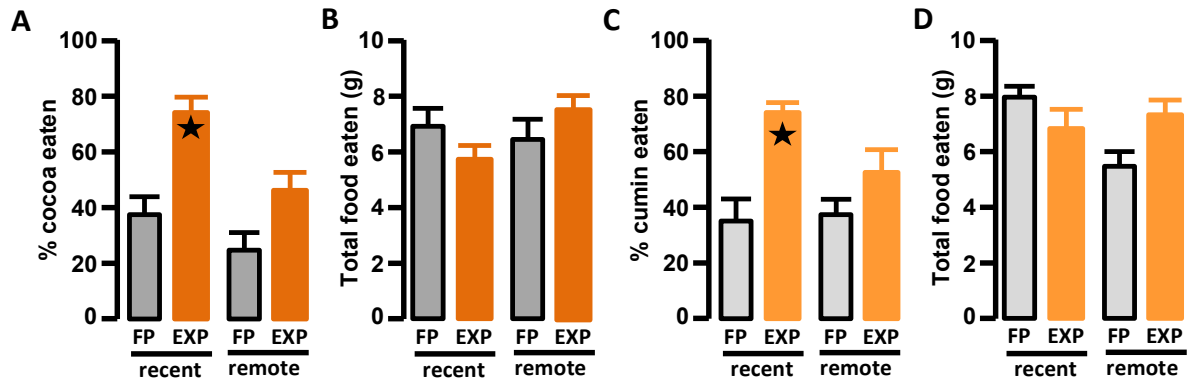
The 4 month-old SHRs were first tested in the STFP task using two different pairs of odours: cumin/thyme and cocoa/cinnamon. As illustrated in Fig. 99, the observer rats of the experimental group ate significantly more food containing cumin (Fig. 99A) or cocoa (Fig. 99C) than those of the food preference group 1 day and 30 days after the social interaction.



**Figure 99: recent and remote memory evaluated with STFP task on SHR 4 months old.** A- % cocoa eaten (two-way ANOVA, interaction  $F_{(1,24)} = 0.062$ ,  $p = 0.80$ ; recent vs remote:  $F_{(1,24)} = 2.479$ ,  $p = 0.13$ ; STFP groups:  $F_{(1,24)} = 39.26$ ,  $p < 0.0001$ ; post-hoc Tukey recent FP vs EXP:  $p = 0.0006$ , ★ ; remote FP vs EXP:  $p = 0.0015$ , ★). B- Total food eaten (two-way ANOVA, interaction  $F_{(1,24)} = 0.585$ ,  $p = 0.45$ ; recent vs remote :  $F_{(1,24)} = 11.37$ ,  $p = 0.0025$ ; FP vs EXP:  $F_{(1,24)} = 0.639$ ,  $p = 0.43$ , post-hoc Tukey FP recent vs remote:  $p = 0.035$ , ★). C- % cumin eaten (two-way ANOVA, interaction  $F_{(1,17)} = 0.058$ ,  $p = 0.81$ ; recent vs remote:  $F_{(1,17)} = 0.0029$ ,  $p = 0.96$ ; STFP groups:  $F_{(1,17)} = 46.83$ ,  $p < 0.0001$ ; post-hoc Tukey recent FP vs EXP:  $p = 0.0009$ , ★ ; remote FP vs EXP:  $p = 0.0007$ , ★). D- Total food eaten (two-way ANOVA, interaction  $F_{(1,17)} = 0.3226$ ,  $p = 0.58$ ; recent vs remote:  $F_{(1,17)} = 0.014$ ,  $p = 0.91$ ; STFP groups:  $F_{(1,17)} = 1.115$ ,  $p = 0.30$ ). Columns expressed means  $\pm$  sem.

**This result showed that the recent as well as the remote memories were not affected in 4 month-old SHRs.**

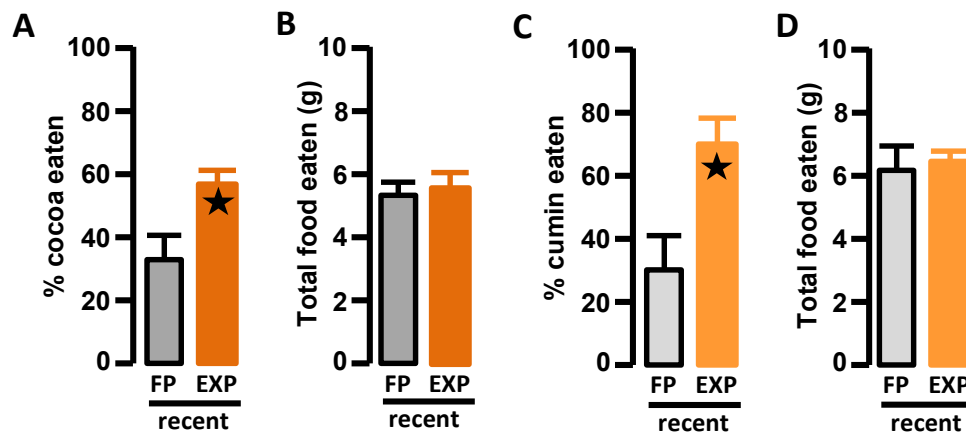
We then performed the experiment with 6 month-old SHRs, in order to be sure that the hypertensive symptomatology was well established. We chose not to select older rats to limit the confounding effect of aging that can *per se* be responsible for memory deficit as used in the previous studies (Diana et al., 1994; Wyss et al., 1992). The observer rats of the experimental group ate significantly more food containing cumin or cocoa than those of the food preference group during the retrieval of the recent memory at Day 1 after interaction (Fig. 100A and C, recent). Moreover, the quantity of cumin and cocoa eaten by experimental rats was not different, showing an absence of taste preference. Nevertheless, there is no difference in the % of cocoa as well as in % of cumin eaten by the food preference and experimental groups when the retrieval test is made 30 days after the social interaction (Fig. 100A and C, remote). Furthermore, the total food eaten by the rats was similar in all the groups compared, indicating no confounding effect of motivation. These results indicated that the recent memory is intact but the remote memory is affected.



**Figure 100: recent and remote memory evaluated with STFP task on SHR 6 months old** A- % cocoa eaten (two-way ANOVA, interaction  $F_{(1,52)} = 2.018$ ,  $p = 0.16$ ; recent vs remote:  $F_{(1,52)} = 13.13$ ,  $p = 0.0007$ ; STFP groups:  $F_{(1,52)} = 24.88$ ,  $p < 0.0001$ ; post-hoc Tukey recent FP vs EXP:  $p = 0.0002$ , ★; recent EXP vs remote EXP:  $p = 0.0035$ , #). B- Total food eaten (two-way ANOVA, interaction  $F_{(1,52)} = 4.122$ ,  $p = 0.047$ ; recent vs remote:  $F_{(1,52)} = 1.518$ ,  $p = 0.22$ ; STFP groups:  $F_{(1,52)} = 0.0035$ ,  $p = 0.95$ ). C- % cumin eaten (two-way ANOVA, interaction  $F_{(1,46)} = 3.057$ ,  $p = 0.087$ ; recent vs remote:  $F_{(1,46)} = 2.869$ ,  $p = 0.097$ ; STFP groups:  $F_{(1,46)} = 19.60$ ,  $p < 0.0001$ ; post-hoc Tukey recent FP vs EXP:  $p = 0.0003$ , ★). D- Total food eaten (two-way ANOVA, interaction  $F_{(1,46)} = 7.644$ ,  $p = 0.0081$ ; recent vs remote:  $F_{(1,46)} = 3.308$ ,  $p = 0.075$ ; STFP groups:  $F_{(1,46)} = 0.6477$ ,  $p = 0.42$ ; post-hoc Tukey recent FP vs remote FP:  $p = 0.0091$ , #). Columns expressed means  $\pm$  sem

These results suggest that, in 6 month-old SHRs, the consolidation of remote associative olfactory memory is impaired without affecting the encoding since the recent memory is not impaired.

The remote memory retrieval was performed 30 days after encoding, when the SHRs are 7 month old. Thus, it can be speculated that the observed remote memory deficit is related to aging, a factor weakening the strength of the encoded memory trace, which can thus potentially cause a faster decay of the memory trace. Thus, to prove that the deficit in remote memory retrieval performance observed in SHRs (encoding 6 months and retrieval at 7 months) was related to a problem of consolidation and not to a problem of encoding, we tested in the same rats the recent memory at 7 months using the other pair of flavours (Fig. 101).



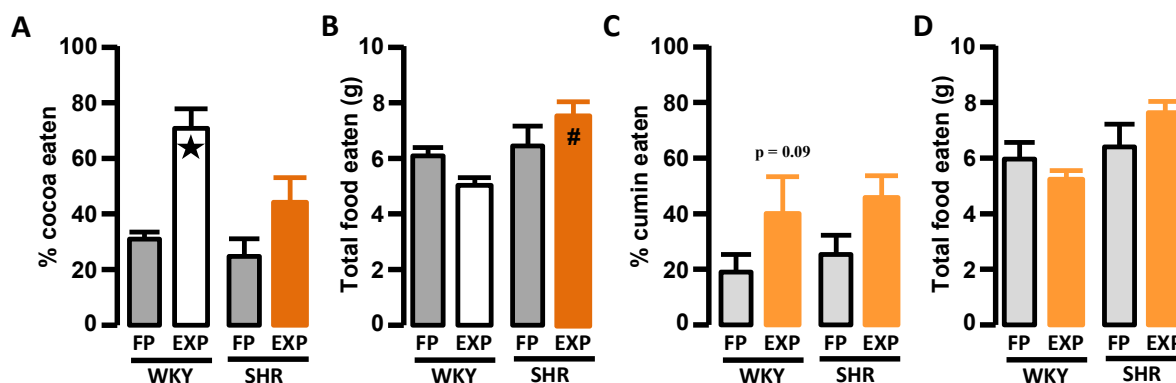
**Figure 101: recent memory tested in the STFP task in SHR 7 months old** A- % cocoa eaten (t-test,  $t_{17} = 2.376$ ,  $p = 0.029$ , ★). B- Total food eaten (t-test,  $t_{17} = 0.3862$ ,  $p = 0.704$ ). C- % cumin eaten (t-test,  $t_{16} = 2.535$ ,  $p = 0.022$ , ★). D- Total food eaten (t-test,  $t_{16} = 0.1826$ ,  $p = 0.857$ ). Columns expressed means  $\pm$  sem.

The recent memory performance of these rats was intact at 7 months, independently by the flavour pair used. The total food eaten by the rats was similar in all groups showing a similar motivation in food intake.

**These data suggest that the recent memory in 7 months old rats was preserved, excluding that an age-related deficit of encoding can alter the remote memory performance, weakening the strength of the encoded memory trace and potentially causing a faster decay.**

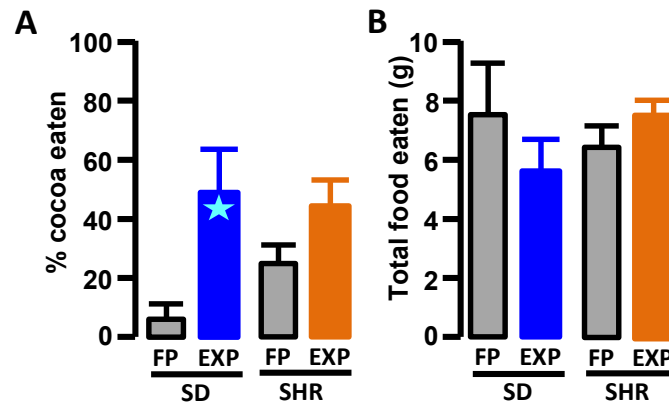
### 3.2.3. Validation of the memory consolidation impairment in 6 month-old SHRs

To be sure that the remote memory consolidation deficit detected in 6 month-old SHRs was not due to age, despite unlikely, or to the genetic background, the performances in STFP task of SHRs were compared with those obtained with the genetically closer strain WKY (Fig. 102). The memory performance was analysed 30 days after the social interaction. The experimental WKY rats, but not SHRs, ate significantly more cocoa-flavoured food than those of the food preference group (Fig. 102A), showing an intact remote memory. The same tendency ( $p = 0.09$ ) is showed in the cumin experiment (Fig. 102C). The quantity of food eaten in experimental WKY was slightly lower than in SHRs, suggesting a potential decrease in motivation in food intake.



**Figure 102: Remote memory evaluated with STFP task on WKY and SHR 6 months old** A- % cocoa eaten (two-way ANOVA, interaction  $F_{(1,34)} = 1.47$ ,  $p = 0.23$ ; strain:  $F_{(1,34)} = 3.757$ ,  $p = 0.061$ ; STFP groups:  $F_{(1,34)} = 14.43$ ,  $p = 0.0006$ ; post-hoc Tukey WKY FP vs EXP:  $p = 0.031$ , ★). B- Total food eaten (two-way ANOVA, interaction  $F_{(1,34)} = 2.477$ ,  $p = 0.12$ ; strain:  $F_{(1,34)} = 4.661$ ,  $p = 0.038$ ; STFP groups:  $F_{(1,34)} = 0.00042$ ,  $p = 0.98$ ; post-hoc Tukey WKY EXP vs SHR EXP:  $p = 0.032$ , #). C- % cumin eaten (two-way ANOVA, interaction  $F_{(1,33)} = 0.0069$ ,  $p = 0.93$ ; strain:  $F_{(1,34)} = 0.6348$ ,  $p = 0.43$ ; STFP groups:  $F_{(1,33)} = 5.973$ ,  $p = 0.02$ ; post-hoc Tukey WKY FP vs EXP:  $p = 0.09$ ). D- Total food eaten (two-way ANOVA, interaction  $F_{(1,33)} = 1.995$ ,  $p = 0.17$ ; strain:  $F_{(1,34)} = 4.738$ ,  $p = 0.036$ ; STFP groups:  $F_{(1,33)} = 0.0611$ ,  $p = 0.81$ ). Columns expressed means  $\pm$  sem.

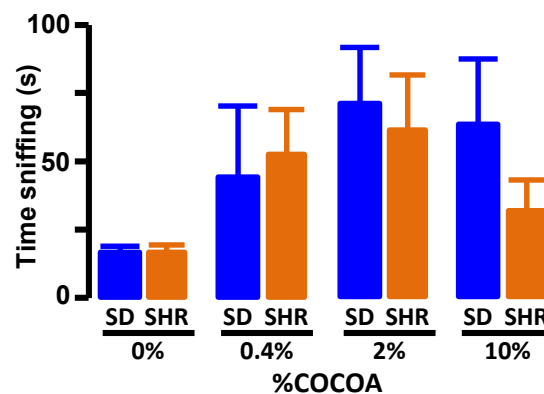
This last result suggested that the remote memory of WKYs could be dependent either on the odour relevance of a particular pair of flavours, or on the taste preference, differently from SHRs. Since the behavioural phenotype of WKYs (in terms of activity, anxiety and taste) remains mainly unclear, we thought to use SDs as a control strain of SHRs, according to the literature (Meneses et al., 2011a). Thus, we compared the remote memory performance of 6 month-old SDs to the age-matched SHRs. Since the association cumin/thyme has been already shown efficient in SDs, we extended this result using the pair cocoa/cinnamon. As expected, the SDs experimental group ate more cocoa than the food preference group in a retrieval test performed 30 days after the social interaction, showing that the remote memory was not affected in 6 month-old SD, whereas it was impaired in SHRs (Fig. 103). Moreover, the amount of food eaten in the different groups was similar, showing that motivation does not affect the memory performance results and suggesting that the difference of food eaten in the SHR/WKY comparison was probably due to a difference in WKY behaviour.



**Figure 103: remote memory in SHR and SD both 6 months old.** A- % cocoa eaten (two-way ANOVA, interaction  $F_{(1,32)} = 1.432$ ,  $p = 0.240$ ; strain:  $F_{(1,32)} = 0.238$ ,  $p = 0.62$ ; STFP groups:  $F_{(1,32)} = 10.55$ ,  $p = 0.0027$ ; post-hoc Tukey SD-FP vs SD-EXP  $p = 0.078$ , ★; SHR-FP vs SHR-EXP  $p = 0.137$ ). B- Total food eaten (two-way ANOVA, interaction  $F_{(1,32)} = 2.803$ ,  $p = 0.10$ ; strain:  $F_{(1,32)} = 0.296$ ,  $p = 0.63$ ; STFP groups:  $F_{(1,32)} = 0.239$ ,  $p = 0.63$ ). Columns expressed means  $\pm$  sem.

Furthermore, we recorded these interactions to quantify the nose-to-nose contacts between observers and demonstrators in order to assure that the memory encoding strength is the same in the two strains. The analysis of this parameter is still undergoing.

The odour threshold discrimination for cocoa of SD and SHRs was tested via the ability of the rat to detect a filter paper containing the cocoa flavour in a known environment. After a habituation phase to the experimental arena, the filter was introduced and the exploration/sniffing time (recorded during 5 min) was quantified as index of odour threshold discrimination. Both SD and SHRs have similar odour threshold to discriminate the cocoa flavour (Fig. 104).

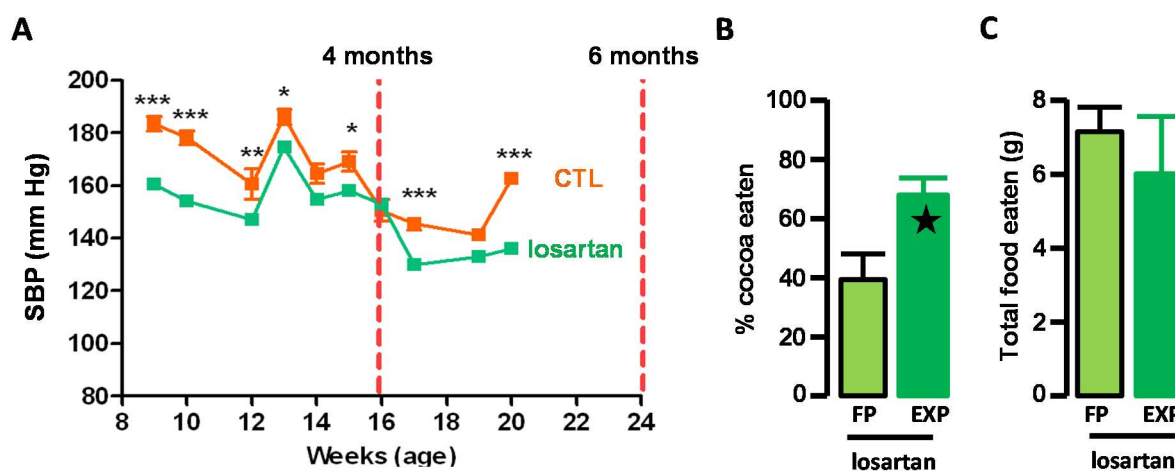


**Figure 104: comparison of odor threshold of detection for cocoa in Sprague Dawley (SD) and SHR strains.** (two-way ANOVA, interaction  $F_{(3,29)} = 0.455$ ,  $p = 0.71$ ; % of cocoa:  $F_{(3,29)} = 2.866$ ,  $p = 0.053$ , SD vs SHR:  $F_{(1,29)} = 0.547$ ,  $p = 0.46$ ). Columns expressed means  $\pm$  sem.

These results suggest that the odour discrimination threshold, the genetic background and the age of SHRs are not responsible for the associative olfactory remote memory deficit.

### 3.2.4. Relation between AHT and memory impairment

The rationale for the use of Losartan was to assess if the general reversion of the BP increase was able to counteract the deleterious effects of hypertension on memory consolidation, strengthening the idea that sustained AHT is involved in the observed cognitive deficits of SHRs and likely interferes with systems-level memory consolidation. The treatment consisted of administrating this drug with a posology of 13-18 mg/kg/day (in tap water) in SHRs from the age of 2 months to the sacrifice of the rats (6/7 months old), in order to avoid the development of hypertension (Demirci et al., 2005; He et al., 2014). The blood pressure was controlled to adjust the concentration of the drug to assure an efficient treatment and to stabilize the blood pressure (Fig. 105A). Finally, the remote memory retrieval was tested in 6 months SHRs treated with Losartan. The experimental group ate more cocoa than the food preference group (Fig. 105B), without any modification of the total food eaten (Fig. 105C). This result suggests that the Losartan treatment rescues the blood pressure increase and restores the remote memory in 6 month-old SHRs.

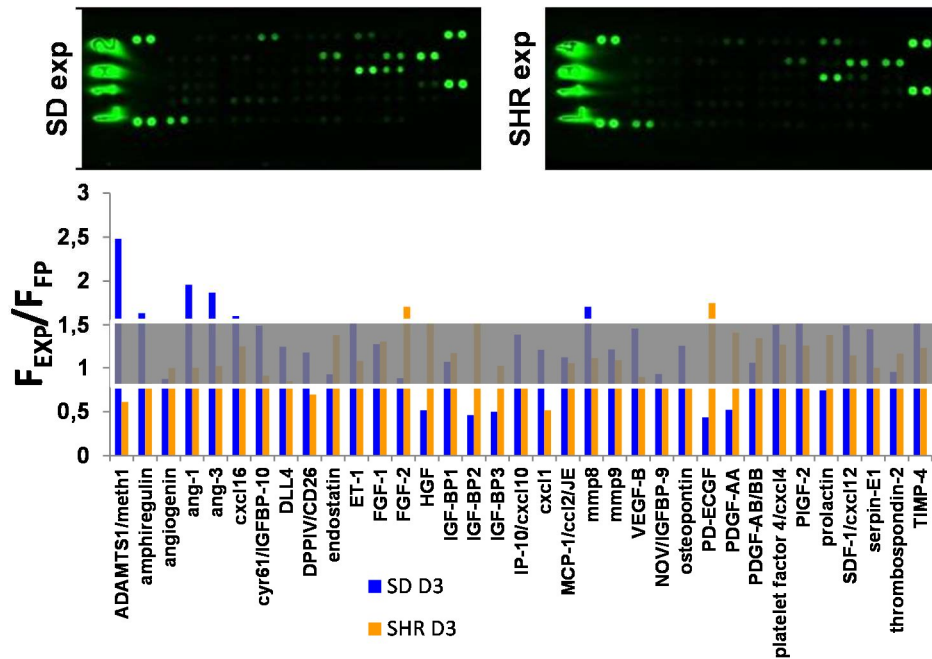


**Figure 105: Effects of Losartan treatment (13-18 mg/Kg/day, from 2 to 6 months of aging) on blood pressure and remote memory.** A- Systolic blood pressure (SBP), measured by the tail cuff method in SHR, without treatment (CTL) and in SHR treated with losartan. B- Percentage of cocoa eaten during retention test by the rats exposed to losartan (t-test,  $t_6 = 2.737$ ,  $p = 0.034$ , ★). C- Total food eaten during the test (t-test,  $t_6 = 0.686$ ,  $p = 0.518$ ). Columns expressed means  $\pm$  sem.

Altogether, these results suggested that the hypertensive phenotype is responsible for the remote memory impairment.

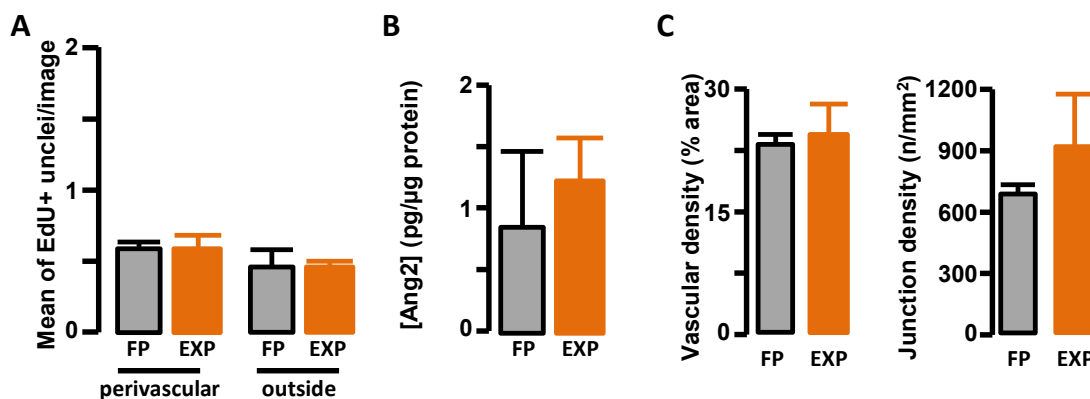
### 3.2.5. Correlation between angiogenic impairment and remote memory deficit

As performed on SD rats (Fig. 86), we investigated the angiogenesis profile and outcome during the early phase of memory consolidation. We evaluated the angiogenesis pathway 3 days after the encoding with the proteome profiler dedicated to angiogenesis and we compared the result obtained in SHRs to SDs ones. We decided to express the results showing the increase of proteins induced by the formation of associative olfactory memory formation, thus making the ratio of the fluorescence found in experimental rats on the food preference one. As illustrated in Fig. 106, the angiogenesis pathways are globally activated in both SHRs and SD rats but with several differences.



**Figure 106: analysis of angiogenic pathways with proteome profiler.** On the top typical images of proteome profiler membrane (both membranes were simultaneously imaged with Odyssey-imager and contrasted for the illustration) on the bottom, fluorescence analysis of each dot is reported as the ratio of the fluorescence of experimental group on the food preference group. All membranes were treated together. F: fluorescence.

Moreover, the memory induced vascular cell proliferation (protocol described in Fig. 90), revealed by the co-localization of EdU labelling and anti-collagen-IV antibody (Fig. 107A), as well as the Ang-2 increase measured by ELISA (Fig. 107B), detected in SDs, were absent in this strain (protocol described in Fig. 88), Accordingly, the vascular and junction densities were similar in both experimental and food preference groups (Fig. 107C).

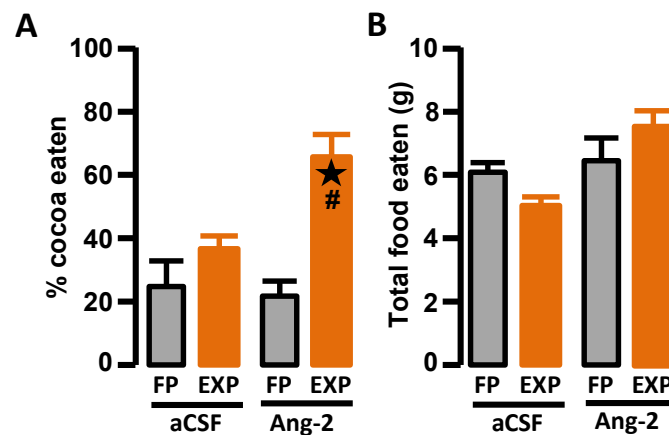


**Figure 107: angiogenesis in SHR at D3** A- proliferative cells in ACC labelled with EdU: number of EdU positive nuclei pairs as measured in SD rats in the perivascular area (t-test,  $t_9 = 0.093$ ,  $p = 0.93$ ) and outside the vessel periphery (t-test,  $t_9 = 0.093$ ,  $p = 0.93$ ). B- Ang-2 concentration measured by ELISA (t-test,  $t_{15} = 1.878$ ,  $p = 0.08$ ). C- The immunohistofluorescence labelling of Collagen-IV analysed with Angiotool was performed on rat sacrificed 3 days after interaction, vascular density expressed as the area of the collagen-IV on the total area of the image, in ACC (t-test,  $t_9 = 0.684$ ,  $p = 0.64$ ) and vascular branching expressed as the number of junctions per  $\text{mm}^2$  in ACC (t-test,  $t_9 = 1.033$ ,  $p = 0.33$ ). Columns expressed means  $\pm$  sem.

These results suggested that the angiogenesis process induced by the encoding of the associative olfactory memory was impaired in SHRs.

### 3.2.6. Injection of Ang-2 restores the memory consolidation impairment in 6 month-old SHR

The key point of this work was the use of an invasive approach to test if the memory consolidation impairment measured in SHR could be restored by acting on the angiogenesis pathway. Thus, SHR rats were injected with Ang-2 in ACC during the early phase of memory consolidation, following the protocol used for SDs (Fig. 95) and the remote memory performance was tested 30 days after the social interaction. aCSF-injected animals showed the same behavioural performance as non-injected SHR, revealing a remote memory impairment. The Ang-2-injected experimental rats ate more cocoa than Ang-2-injected food preference rats and aCSF experimental rats (Fig. 108A), displaying a rescued remote memory performance. The total food eaten was not affected, showing that motivation does not affect the memory performance (Fig. 108B).

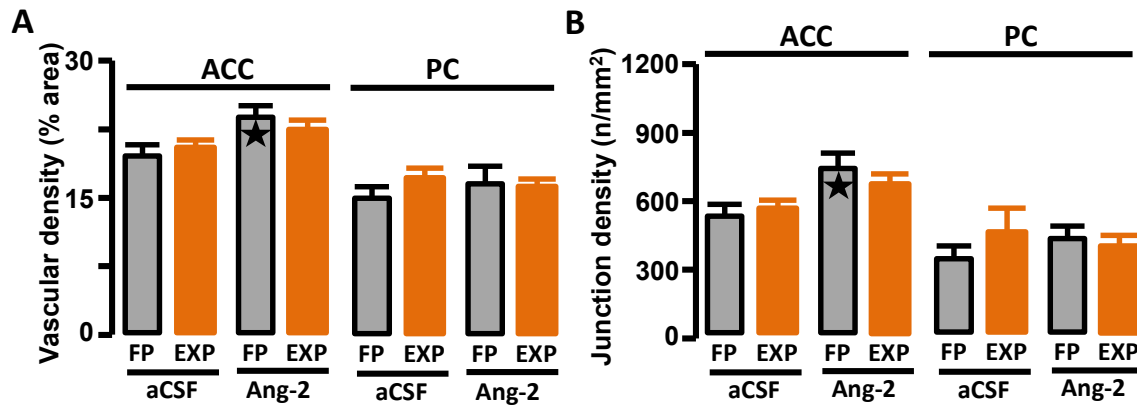


**Figure 108: remote memory evaluated with STFP task on SHR 6 months old treated with Ang2 or aCSF** **A-** % cocoa eaten (two-way ANOVA, interaction  $F_{(1,26)} = 6.961$ ,  $p = 0.013$ ; treatment:  $F_{(1, 26)} = 4.556$ ,  $p = 0.042$ ; STFP groups:  $F_{(1, 26)} = 17.35$ ,  $p = 0.0003$ ; post-hoc Tukey Ang-2 FP vs EXP:  $p = 0.0002$ , ★; EXP aCSF vs Ang-2:  $p = 0.001$ , # ). **B-** Total food eaten (two-way ANOVA, interaction  $F_{(1,26)} = 0.0059$ ,  $p = 0.93$ ; treatment:  $F_{(1,26)} = 4.661$ ,  $p = 0.022$ ; STFP groups:  $F_{(1,26)} = 0.0452$ ,  $p = 0.83$ ). Columns expressed means  $\pm$  sem.

**This result demonstrated that the memory impairment in 6 month-old SHR is restored by injection of Ang-2 in ACC during the early phase of memory consolidation, suggesting that the permissive mechanism of angiogenesis in memory consolidation could be affected in this model.**

Finally we investigated the effect of Ang-2 injections on the vascular density 30 days after the encoding: brain slices were labelled with the anti-collagen-IV antibody and analysed with Angiotools software. The vascular density and the number of branching were calculated in ACC and PC. No differences concerning vascular density and number of branching were found between the groups in PC. Surprisingly, in ACC, Ang-2 injections increased the vascular density and branching in the food preference group (Fig. 109).





**Figure 109: vascular density and branching in ACC and PC from rat injected with Ang-2 or aCSF.** The immunohistofluorescence labelling of Collagen-IV analysed with Angiotool was performed on rat killed 30 days after interaction and just after the retrieval. **A-** vascular density expressed as the area of the collagen-IV on the total area of the image, in ACC (two-way ANOVA, interaction  $F_{(1,25)} = 1.242$ ,  $p = 0.27$ ; treatment:  $F_{(1, 25)} = 10.51$ ,  $p = 0.0034$ ; STFP groups:  $F_{(1, 26)} = 0.3738$ ,  $p = 0.55$ ; post-hoc Tukey FP Ang-2 vs aCSF:  $p = 0.0406$ , ★); in PC (two-way ANOVA, interaction  $F_{(1,25)} = 2.046$ ,  $p = 0.16$ ; treatment:  $F_{(1, 25)} = 0.1927$ ,  $p = 0.66$ ; STFP groups:  $F_{(1, 26)} = 0.8203$ ,  $p = 0.37$ ). **B-** Vascular branching expressed as the number of junction per mm<sup>2</sup> in ACC (two-way ANOVA, interaction  $F_{(1,25)} = 1.155$ ,  $p = 0.29$ ; treatment:  $F_{(1, 25)} = 10.58$ ,  $p = 0.0033$ ; STFP groups:  $F_{(1, 26)} = 0.2820$ ,  $p = 0.60$ ; post-hoc Tukey FP Ang-2 vs aCSF:  $p = 0.0423$ , ★). ); in PC (two-way ANOVA, interaction  $F_{(1,25)} = 1.981$ ,  $p = 0.17$ ; treatment:  $F_{(1, 25)} = 1.16$ ,  $p = 0.29$ ; STFP groups:  $F_{(1, 26)} = 0.1307$ ,  $p = 0.72$ ). Columns expressed means  $\pm$  sem

**This result showed that the Ang-2 injections increased basal vascular network in SHR, and that this vascular enhancement lasted for at least 30 days after the encoding. This suggests that the ability of SHR to create new vessels is maintained. Despite non-significant, a trend towards an increase was detected for vascular density and number of branching points in Ang-2 injected experimental SHR, suggesting a potential beneficial effect on vascular network**

### 3.3. Discussion

We used the SHR model as a complementary approach to further explore the functional contribution of cortical vascular networks to the formation of remote memory. Our goal was to determine whether the early cortical angiogenic mechanism triggered upon memory encoding in physiological conditions (adult Sprague Dawley rats) could be altered in a pathological condition, namely hypertension. In recent years, several studies have pointed to the existence of a potential link between the disruption of the vascular system and the onset or the acceleration of cognitive pathologies (Carnevale et al., 2012; Carnevale et al., 2016; Faraco and Iadecola, 2013; Iadecola, 2004, 2013). For instance, hypertension has been proposed as one of the most important risk factors able to induce and/or increase memory impairments associated with vascular dementia or Alzheimer's disease (Carnevale et al., 2016), although the underlying mechanism remains to be explored. We took advantage of our behavioural findings in SHR revealing the deleterious effects of sustained high blood pressure on the organization of recent and remote memory, with a selective impact on the formation of remote memories indicative of a hypertension-induced disruption of the memory consolidation process.

Moreover, since SHR develops spontaneous AHT over time (Meneses et al., 2011a; Tayebati et al., 2012), this pathological animal model appeared as particularly suitable for a longitudinal study aimed at examining the impact of vascular impairment on retrieval of recent and remote memory. Different behavioural paradigms have shown that SHR exhibit a potential impairment in learning and



memory (reviewed in (Meneses et al., 2011a; Sagvolden, 2000), but an important bias in the interpretation of these results is introduced by the behavioural phenotype of these animals, in particular their hyperactivity that can influence the majority of memory tasks based on exploratory behaviours such as for instance maze exploration (Sontag et al., 2013). To circumvent this issue, we chose to use the STFP paradigm that takes place in the animal's home cage and in which the expression of memory performance does not rely on exploration of the animal's environment.

## **Memory profile and AHT**

Because hypertension develops progressively, SHR rats were tested at the age of 4 (beginning of stable hypertension) and 6 months (established level of hypertension). The recent and remote memory performances in 4 months-old SHR rats were not impaired. Similar results were obtained with two olfactory flavour pairs (cumin/thyme and cocoa/cinnamon) that permit to minimize a potential bias in the odour salience of a given pair or in the taste preference of SHR rats. In 6 and 7 months old SHR rats, recent memory was not affected whereas remote memory was selectively impaired. Only a few studies have examined memory performance in SHR rats during the course of the pathology. It was shown that 3-month-old SHR rats learned faster than Sprague Dawley rats in an 8-arm radial maze task (Wyss et al., 1992) while both 6-month-old rats of these two strains have similar spatial memory performance in the Morris Water Maze task (Diana et al., 1994). Memory impairments are only apparent in 12-month-old SHR rats in both spatial tasks (Diana et al., 1994; Wyss et al., 1992). However, these studies could not segregate the effects of aging from the chronicity of hypertension. In our study, 6 month-old SHR rats are not considered as aged rats. Moreover, remote memory retrieval is not affected in SHR rats before 7 months of age as it is also the case in spatial memory (Diana et al., 1994; Wyss et al., 1992), therefore excluding aging as a contributing factor weakening the strength of the encoded memory trace and potentially causing a faster decay. In other words, the selective impairment in remote memory formation and retrieval without alteration of recent memory in the same animals suggests successful encoding but an inability to adequately stabilize and/or retrieve remotely acquired information. Taken together, our findings identify SHR rats as a valuable animal model of memory consolidation deficit.

As to the hypertensive profile of our SHR rats, our results are in agreement with previous findings (Tayebati et al., 2012) indicating that BP levels are constant between 4 to 6 months in these rats. This suggests that the observed vascular damage are predominantly related to the chronicity of the pathology rather than the level of BP. Concurring with this assumption, the hypertension-induced memory impairments were always detectable a few months after the start of hypertension in mice (Carnevale et al., 2012). Surprisingly, recent memory impairment, measured with the STFP task can be revealed in Dahl-salt hypertensive rats, although the hypertension status of these rats was not examined (Ruiz-Opazo et al., 2004). This suggests that the model of hypertension (genetic bases and duration of hypertension) can impact the pattern of observed memory impairments.

Ongoing experiments aim at quantifying the spine density (synaptophysin labelling) and neuronal activation (c-fos labelling) in SHR rats in order to correlate, according to our hypothesis, the lack of memory consolidation to the absence of neuronal/synaptic changes as described previously in hypertensive mice (Dai et al., 2016).

## **Control strain and exclusion of potential bias in the memory performance interpretation**

Our results confirm the higher locomotor activity of SHR rats compared to WKYs (Berton et al., 1997; Langen and Dost, 2011; Pardey et al., 2009) but also underline a similar emotional reactivity and locomotion between SDs and SHR rats. The results on anxiety obtained in the open field task are in

accordance with those acquired with the elevated plus maze, showing that SHR are less anxious (Berton et al., 1997). Moreover, the locomotor and mood behaviours are slightly modified by aging as also described (Meneses et al., 2011a; Zhang-James et al., 2013). In conclusion, SDs could be a better control to study what happens in SHR in terms of memory, considering that the memory is based on a single social interaction. Anyway, since SHR strain was used as a model of ADHD (Meneses et al., 2011a; Sagvolden, 2000), it is important to control that the strength of the memory trace encoded by the animals is similar, excluding that this aspect is responsible for the faster decay.

In fact, the memory retrieval can be linked to the strength of encoded memory trace as well as its consolidation (Lechner et al., 1999). In the STFP task, memory performance is dependent on the interaction between the demonstrator and the observer rats (Galef and Whiskin, 1998) and the ability to discriminate, upon retrieval, the odours presented. There are no established relationships between olfactory impairment and hypertension in humans (Landis et al., 2004) and the question has not been fully investigated in animal model of hypertension, except in terms of innate preference to different odours as measured in the STFP task (Ruiz-Opazo et al., 2004). Our findings show a similar olfactory sensitivity (odour detection threshold) in both SHR and SD strains. Finally, the analysis of the number and duration of nose contacts during the social interaction phase of the STFP task used as an index of social interaction is still ongoing, nevertheless, previous studies indicated that despite SHRs explore more, they are not more aggressive in presence of intruder, as this is the case during interaction (Berton et al., 1997). Altogether, the results suggested that SDs and SHRs have comparable behaviours. Then, the differences detected between SHRs and SDs in STFP task should be interpreted as a memory consolidation specific effect.

To explain the memory deficits observed in 6month-old SHRs, several studies suggested a reduction of neuronal density in HPC (Bendel and Eilam, 1992; Johansson, 1986; Ritter and Dinh, 1986; Sabbatini et al., 2002; Sabbatini et al., 2001; Tajima et al., 1993) and glucose consumption (Johansson, 1986; Wei et al., 1992). In our study, the learning and the recent memory are not impaired despite of the engagement of HPC. Anyway, in light of these results, we can assume that this neuron density reduction and decrease glucose consumption can affect remote memory retrieval by decreasing the HPC-ACC dialogue and thus the robustness of memory trace. Despite that, the Ang-2 injection probably compensates the neuronal loss via the increase of vascular network efficiency responsible for the metabolic intakes.

### **Memory restoration with antihypertensive drug (Losartan)**

The chronic treatment with Losartan (20 weeks; 13-18 mg/kg/day in drinking water), designed to decrease the hypertensive phenotype in SHRs, reduced their BP and rescued their remote memory deficit. The effects of antihypertensive drugs on memory performance have been previously reported (Meneses et al., 1997; Wyss et al., 1992). Moreover, the Losartan treatment did not affect food intake, neither during the habituation phase nor during the retrieval test. Thus, the STFP task was not affected by known side effects of Losartan such as the decrease of food intake via a decrease of appetite and chemosensory perception (Doty et al., 2003) or the impairment of taste without modification in odour detection (Schlienger et al., 1996; Srinivasan et al., 2003).

The choice of this particular antihypertensive molecule was implemented to limit, as much as possible, the typical side effects of antihypertensive treatments on brain functions. Even if AT1 angiotensin-II receptors have been described in the brain, their direct implication in memory processes has not been confirmed (Wright et al., 2013). Moreover the crossing of the blood-brain barrier by our Losartan preparation is minimal (as underlined in the information note of the medicinal preparation that we have used), thus minimizing any central effects in our study. Other antihypertensive drugs could

directly bind on neurons and modify their excitability such as calcium blockers that can decrease memory impairment in mouse models of Alzheimer's disease (Jansone et al., 2015; Jansone et al., 2016) but also affect odour preference memory via the decrease of calcium entry into pyriform cortical neurons (Mukherjee and Yuan, 2016). In Alzheimer mice, Losartan ameliorates the acquisition and recall of spatial memory independently of the level of BP and can rescue the neurovascular and neurometabolic coupling and the cerebrovascular dilatory capacity (Ongali et al., 2014), suggesting that several signalling pathways including those which regulate angiotensin-II could be implicated in the improvement of memory processes. Interestingly, angiotensin-II can modulate angiogenesis depending on the model analysed, the structure explored and the array of receptors targeted (Benndorf et al., 2003; Clapp et al., 2009; Ribatti et al., 2007; Walther et al., 2003). Recently, Cablesartan, a pharmacological compound from the Losartan's family was described to induce a prolonged pro-angiogenic effect on brain vessels after ischemia (Soliman et al., 2014). Moreover, preservation of cognitive functions induced by neurovascular protective drugs such as cilostazol whose effects are related to the restoration of angiogenesis and pericyte proliferation in hypertension-induced stroke models has been reported (Omote et al., 2014). Based on these studies, and in light of our current results obtained in physiological conditions with Sprague Dawley rats (remote memory improvement or impairment induced by modulation of Ang-2 expression) and in SHR (Ang-2 induced rescue of remote memory deficit), we anticipate that the Losartan-induced beneficial effects on remote memory may be mediated, at least in part, via an angiogenic mechanism. To explore this angiogenesis hypothesis, we propose to analyse the modifications of the vascular architecture in Losartan-treated SHRs.

### **Angiogenic impairment in SHRs: correlative approach**

As mentioned in the introduction, opposite results concerning the angiogenic status of SHRs have been found: 1/ While new vessels can be produced in fibrin implants (Hudlett et al., 2005), angiogenesis was decreased in SHRs implanted with subcutaneous sponges containing pro-angiogenic molecules (Wang et al., 2004) 2/ the mesenteric microvasculature network in SHRs is basally impaired (Murfee and Schmid-Schonbein, 2008), 3/ Angiogenic processes induced following ischemia are impaired in SHRs muscles (Lu et al., 2016), and are differently activated by exercise depending on muscle types (Fernandes et al., 2012). However, all these findings do not provide insights into the possibility of a physiological angiogenesis induced by neuronal activity, despite they give us the idea about the link between AHT and angiogenesis impairment.

The global angiogenic proteome profiler assay we have used suggests that memory consolidation triggers angiogenesis in both SHRs and Sprague Dawley rats, albeit with a different temporal profile. Moreover, we found that Ang-2 expression was not increased in experimental SHRs at day 3 post social interaction. Furthermore, we assessed the lack of memory-associated vascular increase and vascular proliferation, 3 days after the encoding of associative olfactory memory. These results suggest that 1/ the detected memory deficit can be linked to an impairment of vascular plasticity during the early phase of memory consolidation, similarly to what happen in SDs; 2/ angiogenic processes in SHRs are modified or delayed in response to a physiological stimulation such as an increase of neuronal energy demand (Lu et al., 2016; Wang et al., 2004) and 3/ the induction of Ang-2 production is impaired in SHRs, as shown when shear stress or reactive oxygen species are increased (Korff et al., 2012).

Thus, a perspective experiment can be performed to understand how the recruitment of the Ang-2 pathway is either impaired or temporally delayed in SHRs. Whatever the case, the SHR angiogenic impairment we have observed appears to be sufficient to block the formation and expression of remote memory.

## **The angiogenic impairment is responsible for the memory deficit of SHRs**

The invasive approach, consisting in injecting Ang-2 in the ACC during the early phase of memory consolidation restored the remote memory performance of SHRs, thus pointing to the crucial role of cortical angiogenesis in the rescue of the remote memory deficit. The prediction of the ongoing quantification of neuronal activity and spine density is that the remote memory rescue should be accompanied by the increase of neuronal plasticity.

The quantification of vascular density and number of branching 30 days after the interaction reveals an unexpected result. Ang-2 multiple injections were able to significantly increase the complexity of the vascular network in ACC of food preference rats, whereas only a non-significant trend towards an increase was detected for vascular density and number of branching points in Ang-2 injected experimental SHRs. This result suggests that the Ang-2 pathway is basally affected in SHRs but the comparison between the aCSF-injected SHRs and Sprague Dawley rats was not able to reveal a vascular rarefaction, contrasting with what was observed in the brainstem of 3 month-old SHRs (Sokolova et al., 1985). However, our result is in accordance with the study by Werber and colleagues (1990) (Werber et al., 1990) in which no cerebral arteriolar decrease was observed in SHRs. Two hypotheses could explain this result. Firstly, SHRs can overexpress Tie-2 receptors (Wang et al., 2002). In this case, the vascular increase mediated by the injection of Ang-2 would be amplified. Nevertheless, this would not explain the lack of increase of Ang-2 in experimental rats. Secondly, the Ang-2 partial agonist nature on Tie-2 could act on the stabilization of the vascular network. This hypothesis is more difficult to test because the context dependent agonist nature of Ang-2 is not fully understood (Fagiani and Christofori, 2013), and we cannot exclude that others angiogenic molecules are implicated, as revealed by our results obtained with the proteome profiler array.

Taken together these results show that early cortical angiogenesis is a crucial mechanism which drives the formation and stabilization of remote memories. These exciting results prompted us to extend our analysis to the effects of gravity modification on the cerebral microvasculature.

## **CHAPTER 4: Effects of hypergravity on memory consolidation.**

## **Chapter 4: Effects of hypergravity on memory consolidation.**

### **4.1. Introduction**

In the previous chapter, we showed that hypertension can impair the consolidation of the associative olfactory memory. Among the modifications of gravity observed during spaceflights or hypergravity obtained by centrifugation, several studies indicated impairment of spatial memory (Bojados and Jamon, 2014; Gueguinou et al., 2012; Mandillo et al., 2003; Mitani et al., 2004; Qiong et al., 2016) but these works did not segregate the effects on learning and on consolidation. Moreover, there are no data on other forms of memory. Finally, in rats exposed to simulated microgravity (hindlimb suspended or unloaded rats) hypertension-like effects have been observed concerning vascular reactivity of cerebral arteries (Dabertrand et al., 2012; Xie et al., 2005). Thus, we proposed to study the effect of hypergravity on the associative olfactory memory, trying to segregate the putative effect induced by hypergravity on consolidation or encoding

The protocol was based on a group of 12 rats (Sprague Dawley) exposed to centrifugation during 21 or 60 days (2g group), whereas another group of 12 rats (1g group) were placed in normogravity condition in the centrifuge room, to mimic environment parameters. This centrifugation protocol induced a continuous hypergravity (2g). All rats were weighed before their installation in the centrifuge and control devices (Fig.71, right) to follow the weight as an index of good health and as the principal metabolic parameter.

### **4.2. Protocols and Results**

#### **4.2.1. Effect of 21 days of hypergravity on memory consolidation and remote memory retrieval**

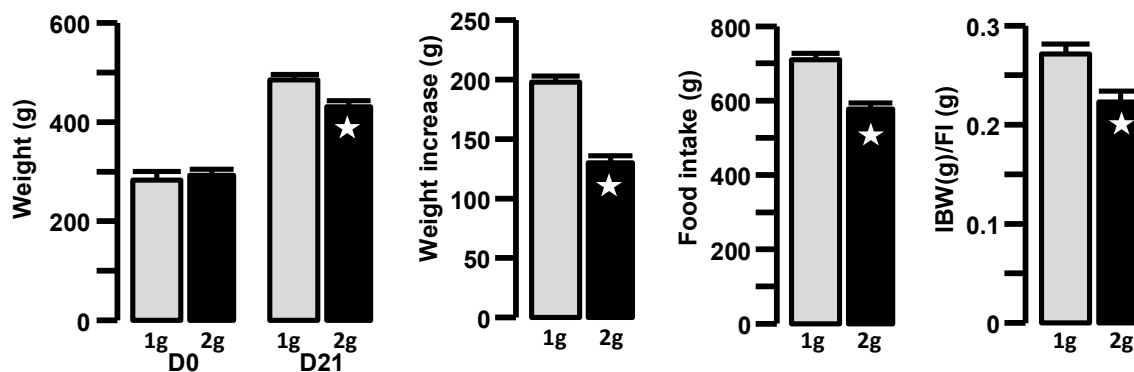
The first protocol was designed as illustrated in Fig. 110 and was aimed to detect the impact of gravity alterations on remote memory consolidation. Briefly, the observer rats interacted with demonstrators fed with cumin or with control food in the centrifuge and after 20 hours the centrifuge was launched and rats were centrifuged during 21 days. The retrieval test was performed 30 days after interaction. This behavioural experiment was performed 3 times (2015-2016), whereas the assays measuring plasma concentrations of corticosterone, circulating IgG and nitrite/nitrate balance were performed only with the two last experiments.



**Figure 110: protocol for centrifugation between interaction and retrieval test.** The observer rats were placed in the centrifuge for habituation, interacted with demonstrator in the centrifuge and 20 h after interaction the centrifuge is launched. After 21 days of centrifugation the rats were food restricted during 3 days and their remote memory was tested.

The weights measured after the end of the centrifuge were significantly increased in both conditions. Despite this, the weight growth was different between 1g and 2g: the increase of body weight was

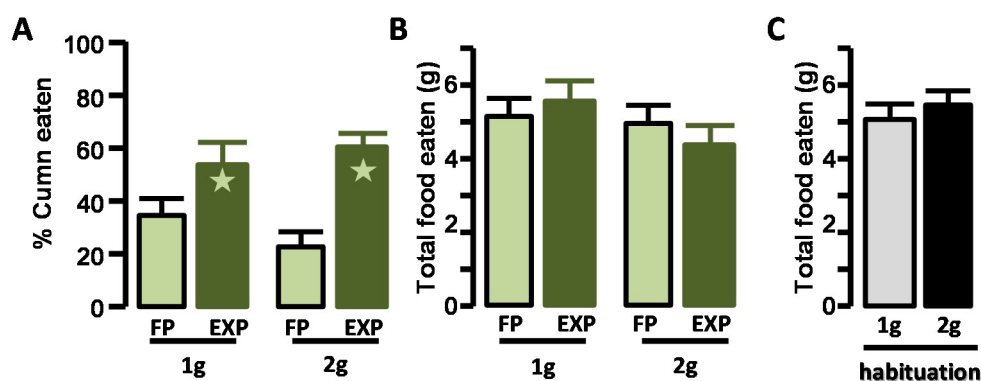
significantly decreased by the exposure to hypergravity as well as the food intake. Finally, the ratio of the increase of body weight on food intake was also sharply decreased (Fig. 111).



**Figure 111: effects of hypergravity on the weight of rats exposed to 2G during 21 days.** From the left to the right, the body weights were measured before centrifugation (D0) (t-test,  $t_{46} = 1.321$ ,  $p = 0.19$ ) and 2h after the stop of the centrifuge (D21) (t-test,  $t_{46} = 5.874$ ,  $p < 0.0001$ ) in rats exposed to 2G (2g) and maintained in normogravity (1g), the increase of body weight were calculate individually (t-test,  $t_{46} = 9.332$ ,  $p < 0.0001$ ) as food intake (t-test,  $t_{46} = 9.711$ ,  $p < 0.0001$ ) and the ratio of the increase of body weight on food intake (IBW/FI) (t-test,  $t_{46} = 5.198$ ,  $p < 0.0001$ ). Columns expressed means  $\pm$  sem.

This result indicated that the rats in the centrifuge were not able to increase their body weight as rats placed in normogravity suggesting an altered metabolism/catabolism and a deficit in the food intake.

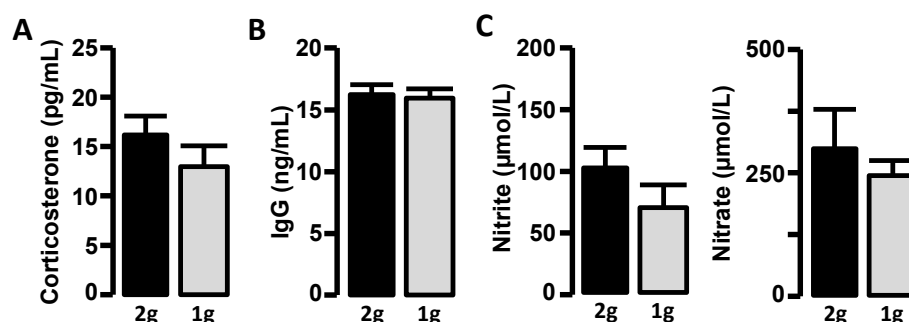
The remote memory performance was evaluated in each condition (1g vs 2g) and in both observer groups as the percentage of cumin eaten (Fig. 112A). The two-way ANOVA analysis indicated that experimental group ate more cumin than the food preference group; this effect was similar in both gravity conditions. Likewise, there was no difference in the total food eaten during the retrieval test (Fig. 112B), and the values were not different from the total food eaten measured during the habituation phase (Fig. 112C).



**Figure 112: Hypergravity (21 days, 2g) effects on remote memory tested with social transmission of food preference.** **A-** Percentage of cocoa eaten during retention test by the rats exposed to normogravity (1g,  $n = 17$ ) and hypergravity (2g,  $n = 19$ ) (two-way ANOVA, interaction  $F_{(1,65)} = 1.917$ ;  $p = 0.17$ ; gravity:  $F_{(1,65)} = 0.272$ ;  $p = 0.603$ ; STFP groups:  $F_{(1,65)} = 25.67$ ;  $p < 0.0001$ ; post-hoc Tukey 1g-FP vs 1g-EXP  $p = 0.052$ ; 2g-FP vs 2g-EXP  $p = 0.0001$ ,\*). **B-** Total food eaten during the test (two-way ANOVA, interaction  $F_{(1,65)} = 0.935$ ;  $p = 0.34$ ; 1g vs 2g:  $F_{(1,65)} = 1.606$ ;  $p = 0.20$ ; FP vs EXP:  $F_{(1,65)} = 0.096$ ;  $p = 0.76$ ). **C-** Total food eaten during the habituation period, before hypergravity exposure (t-test,  $t_{32} = 0.479$ ,  $p = 0.634$ ). Columns expressed means  $\pm$  sem.

These data suggest that there was no remote retrieval impairment of the information encoded before hypergravity exposure, suggesting that hypergravity was not able to impair the consolidation of associative olfactory memory.

Finally, the plasma concentrations of corticosterone, IgG and nitrite/nitrate balance were evaluated with different assays. None of these parameters was affected by the hypergravity exposure (Fig. 113).

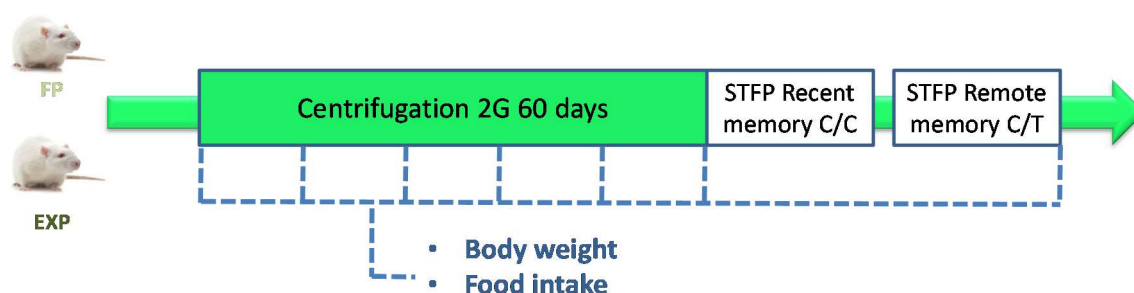


**Figure 113: Effects of hypergravity (2G, 21 days) on plasma concentration of corticosterone (A), IgG (B) and Nitrite and nitrate (C).** Measured individually by ELISA and colorimetric assays after the retrieval test in rats exposed to hypergravity (2g) or maintained in normogravity (1g). **A-** Plasma corticosterone (t-test,  $t_{39} = 1,528$ ,  $p=0.211$ ). **B-** Plasma IgG (t-test,  $t_{30} = 1.528$ ,  $p = 0.137$ ). **C-** Plasma nitrite (t-test,  $t_{30} = 9.711$ ,  $p < 0.137$ ) and endogenous nitrate (t-test,  $t_{30} = 0.886$ ,  $p = 0.382$ ). Columns expressed means  $\pm$  sem.

This result suggested that the rats, after 21 days in the centrifuge and 3 days after the stop of centrifuge are not stressed, moreover the blood NO level is not affected.

#### 4.2.2. Effect of 60 days of hypergravity on recent and remote memory

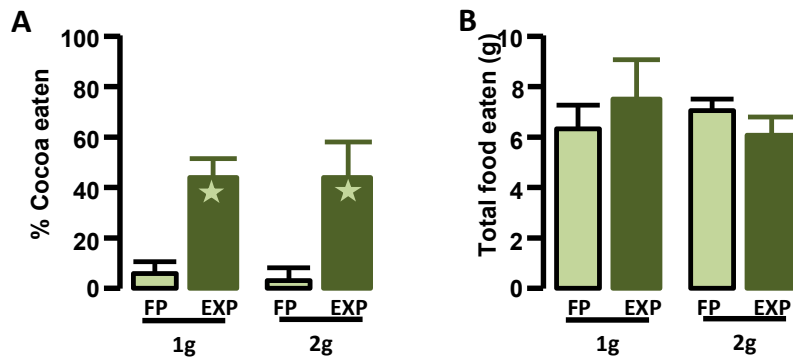
A second set of experiments was performed to evaluate if, as hypertension, a longer exposure to hypergravity during 2 months could affect associative olfactory memory (Fig. 114). Since centrifugation was performed before the encoding, a possible gravity effect can be ascribed both to encoding and to consolidation deficit.



**Figure 114: protocol for centrifugation before STFP task.** The observer rats were centrifuged for 60 Days before STFP paradigm; the rats were food restricted during 3 days and their recent and remote memories were tested.

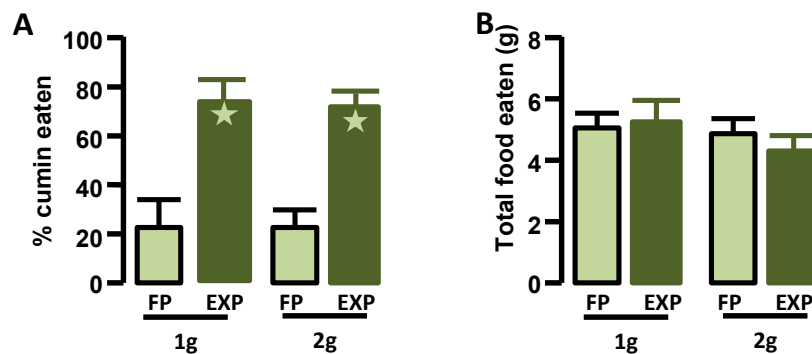
In this experiment performed 3 days after the stop of the centrifuge, the 1g and 2g observer rats, interacting with demonstrators fed with cocoa or normal food, were tested 1 day after to evaluate the recent memory (Fig. 115). Both groups showed an intact recent memory (fig. 115A), independently of the gravity level; moreover, the quantity of food eaten was similar in all groups, showing no difference in motivation of food intake (Fig. 115B).





**Figure 115: Hypergravity (60 days, 2g) effects on recent memory tested with social transmission of food preference.** **A-** Percentage of cocoa eaten during retention test by the rats exposed to normogravity (1g,) and hypergravity (2g,) (two-way ANOVA, interaction  $F_{(1,19)} = 0.023$ ,  $p = 0.88$ ; 1g vs 2g:  $F_{(1,19)} = 0.041$ ,  $p = 0.841$ ; FP vs EXP:  $F_{(1,19)} = 18.29$ ,  $p = 0.0004$ ; post-hoc Tukey 1g/FP vs 1g/EXP  $p = 0.034$ , ★; 2g/FP vs 2g/EXP  $p = 0.030$ , ★). **B-** Total food eaten during the test (two-way ANOVA, interaction  $F_{(1,19)} = 0.828$ ,  $p = 0.37$ ; 1g vs 2g:  $F_{(1,19)} = 0.159$ ,  $p = 0.69$ ; FP vs EXP:  $F_{(1,19)} = 0.004$ ,  $p = 0.94$ ). Columns expressed means  $\pm$  sem.

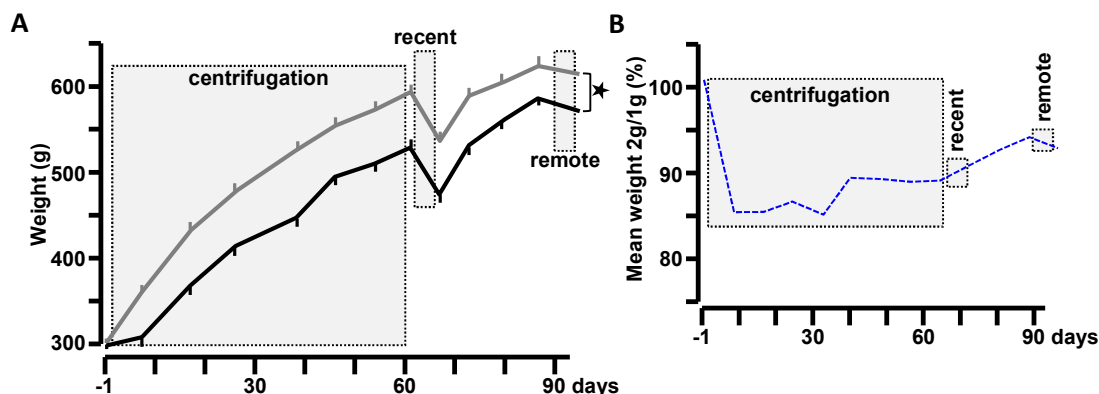
After this test, the observer rats interacted with a different demonstrator fed with cumin to test remote memory 30 days after interaction (Fig. 116). Both 1 g and 2g groups showed an intact remote memory (fig. 116A), independently by the gravity level; food consumption during the retrieval test was similar across groups (Fig. 116B).



**Figure 116: Hypergravity (60 days, 2g) effects on remote memory tested with social transmission of food preference** **A-** Percentage of cumin eaten during retention test by the rats exposed to normogravity (1g) and hypergravity (2g) (two-way ANOVA, interaction  $F_{(1,13)} = 0.0067$ ,  $p = 0.935$  gravity:  $F_{(1,13)} = 0.050$ ,  $p = 0.826$ ; STFP groups:  $F_{(1,13)} = 25.42$ ,  $p = 0.0002$ ; post-hoc Tukey 1g-FP vs 1gEXP  $p = 0.021$ , ★; 2g-FP vs 2g-EXP  $p = 0.012$ , ★). **B-** Total food eaten during the test (two-way ANOVA, interaction  $F_{(1,13)} = 0.055$ ,  $p = 0.81$ ; gravity:  $F_{(1,13)} = 1.450$ ,  $p = 0.250$ ; STFP groups:  $F_{(1,13)} = 0.090$ ,  $p = 0.768$ ). Columns expressed means  $\pm$  sem.

**These results showed that neither the recent nor the remote memories were significantly affected by the exposure to hypergravity (2G during 60 days).**

In this experiment, the weight of the rats was constantly measured (during the change of the litter). After 1 week the weight of the centrifuged rats (2g) was different from the control rats (1g) as illustrated in Fig. 117A. This difference slightly decreased over time, but it persisted during the centrifugation period and during the month between the stop of the centrifuge and the retrieval test of remote memory (Fig. 117B).



**Figure 117: body weight and food intake measured in rats exposed to 60 days to 2g. A-** Mean of body weight. **B-** Ratio of the mean body weight of rats exposed to hypergravity (2g) on rats exposed to normogravity (1g) (n = 12 for both groups). Dots expressed means  $\pm$  sem.

According to the results obtained after 21 days of centrifugation, these results suggest that centrifugation affect the body weight of the rats.

### 4.3. Discussion

Our results show that exposure to hypergravity (2g) during 21-60 days could not affect the associative olfactory memory. In these experiments, animals were exposed to continuous centrifugation to study the hypergravity as a new living condition.

These sets of experiments indicate that hypergravity affects neither the remote retrieval of the associative olfactory memory when the learning phase (interaction) has been performed just before the exposure to hypergravity, nor the recent and remote retrievals when the interaction and retrieval followed the hypergravity exposure. There results contrast with those obtained on the remote spatial memory. In fact, spatial memory retrieval could be impaired or reduced by continuous exposure to hypergravity (Bojados and Jamon, 2014; Mandillo et al., 2003; Mitani et al., 2004) as well as to simulated microgravity (Cassady et al., 2016; Qiong et al., 2016), suggesting that the change of the gravity level is sufficient to alter spatial memory. These effects were always concomitant with vestibular dysfunctions and alteration of emotional status (Bojados and Jamon, 2014; Gueguinou et al., 2012). In the STFP, the locomotor and vestibular components have probably limited effect because they are poorly engaged in social interaction and odour detection, whereas the emotional status could affect social interaction more (Cordero and Sandi, 2007; van der Kooij and Sandi, 2012). It has been shown that the increase of corticosterone level associated to stress-related responses is related to the level of hypergravity (Bojados and Jamon, 2014; Petrak et al., 2008), Nevertheless, in our previous study on mice exposed to hypergravity, we have shown that hypergravity level can affect the expression of many genes and several of them are implicated in neurogenesis and angiogenesis in hippocampus (Pulga, Porte, Morel, submitted manuscript in annexes). But, in the present study 3 days after chronic exposure to 2G, the plasma concentration of corticosterone was not increased, suggesting that the centrifuged animals were not more stressed than controls.

Taken together, both studies allow to hypothesize that the modulation of gene expression in hippocampus is not sufficient to impair the encoding and the consolidation of the olfactory associative memory. It could be interesting to assess the ACC vascular plasticity, as in SHRs, since this region seems to be more sensitive to vessels alterations. As a matter of fact, we found a region-specific persistence of hypoxia in this region after encoding, accompanied by a memory-induced vascular

sprouting and Ang-2 overexpression. This region was shown to be sensitive to vascular alterations as observed in SHRs and it can be potentially affected by adaptation induced by hypergravity, compared to other regions such as HPC.

Hypergravity has been proposed as a countermeasure against microgravity since its beneficial effects in reducing skeletal muscle and bones loss induced by microgravity (Bojados and Jamon, 2014; Gnyubkin et al., 2015), suggesting that hypergravity (2G but not more) could be considered as a continuous exercise which is reported to enhance the memory performance or to decrease the memory impairment induced by aging or Alzheimer's disease, as reviewed recently (Duzel et al., 2016; Herring et al., 2016; Paillard, 2015). Exercise is also known to increase angiogenesis (Fernandes et al., 2012; Roque et al., 2013) and reduce hypertension deleterious effects (Lin et al., 2015; Park et al., 2012; Shi et al., 2016). Thus, these studies allow us to assume that hypergravity can have beneficial effects on memory. Moreover, in our study, we noticed a decrease of weight suggesting a modification in the metabolism. The observed effect can be compared to those observed in caloric restriction diet and antioxidant treatment. As reviewed, these treatments are proposed to reverse or decrease memory impairment observed in Alzheimer's disease models (Perluigi et al., 2014). Moreover, hypergravity can reverse the effects of a sedentary lifestyle, known to have similar effects of microgravity both characterized by ectopic fat storage (Bergouignan et al., 2011).

All of these data allow us to issue the following hypothesis: hypergravity, similarly to physical exercise or enriched environment, could restore the memory impairment induced by hypertension.

## **GENERAL DISCUSSION AND PERSPECTIVES**

## **General discussion**

According to the standard model of systems-level memory consolidation, the formation of memory traces requires a transitory hippocampal-cortical interaction allowing the progressive remodelling of cortical neuronal networks involved in remote memory storage (Squire and Alvarez, 1995). According to this model, the HPC acts as an anatomical index allowing the integration of the different features of the memory trace encoded by distributed cortical network in order to create a coherent memory trace. Moreover, cortical neurons must undergo an *early* “tagging process”, upon encoding, to ensure the progressive hippocampal-driven rewiring of cortical networks that support remote memory storage (Lesburgueres et al., 2011). To pinpoint accurately the time-course of the hippocampal-cortical dialogue during the course of systems-level memory consolidation, we selected the social transmission of food preference (STFP). Its associative nature requires the HPC and specific cortical regions such as the orbitofrontal (OFC) and anterior cingulate (ACC) cortices, which are both involved in processing olfactory associative information (Frankland and Bontempi, 2005; Lesburgueres et al., 2011). In this task, the progressive increase of cortical neuronal activity is accompanied by an increase of dendritic spines detectable as early as one day following social interaction (Lesburgueres et al., 2011) which may reflect the neuronal allocation of specific cortical neurons to a given memory trace that will subsequently undergo consolidation (Rogerson et al., 2014; Silva et al., 2009).

Thus, neuronal allocation and cortical tagging processes involve neuronal activity and require energy. Of course, this neuronal request is translated into the activation of neurovascular coupling, but the induced metabolic request is sustained and needs more than localized and transitory vasodilation and increased of cerebral blood flow in response to action potentials. Our hypothesis is that rapid changes in the architecture of vascular networks, namely vascular rewiring in the form of an angiogenic process, is needed to support the quick activation of neuronal networks in cortical areas required for the consolidation of the memory trace. This angiogenic process would allow the responding to a higher metabolic request of the neurons implicated in the creation of new synapses and in the reinforcement of the pre-existing ones (weight and wiring plasticity). In other words, angiogenesis would provide to the neurons the proper nutritional environment required to support their recruitment during memory processes..

**Thus, during the course of this PhD, we have investigated the following questions:**

- **Is an angiogenic process initiated during memory consolidation?**
- **What is the temporal signature of this angiogenic process? It is time-limited or sustained during the entire course of the memory consolidation process?**
- **What are the functional properties of this angiogenic process? Is it specifically required for memory consolidation or just a consequence of neuronal activity?**

### **A physiological angiogenic process is activated during systems consolidation**

Preliminary investigations performed in the team (Anaïs Giacinti, Ph.D. 2014) suggested a modification of both the reactivity and architecture of cerebral vessels during the formation of associative olfactory memory after social interaction. In particular, it was shown that encoding of an associative olfactory memory was accompanied by a transitory increase in vessel density and number of vessel branching points in the anterior cingulate cortex (ACC), suggesting the triggering of an angiogenic process. Our results extend these findings by revealing early transitory changes of vascular networks in the ACC, supporting the early phase of memory consolidation, including a time-dependent and region-specific increase of hypoxia, followed by a region-specific increase in

Angiopoietin-2 levels (key and early regulator of angiogenesis) which selectively act on vascular network and the creation of newly-born endothelial cells. Overall, we provide evidence for the existence of time-dependent changes in the microvascular architecture at the cortical level. These angiogenesis-related processes are concomitant to the establishment of the hippocampal-cortical dialogue required for the memory consolidation process.

However, it is important to point out that this cortical angiogenic mechanism differs from the neurovascular coupling by its duration and probably its activation. In fact, the neurovascular coupling is immediately activated by the action potentials generated by the neurons. Moreover, the neurovascular coupling principally regulates the vasodilation of the capillary network around the activated neurons (Attwell et al., 2010; Cauli and Hamel, 2010; Filosa et al., 2016).

**Overall, our results suggest that the temporal signature of the cortical angiogenic process is cocommittant to that of the neuronal allocation process, making it relevant to provide the optimal environment for specific neurons to be tagged and to support the progressive stabilization and embedding of distinct memory traces within cortical neuronal networks.**

### **A physiological permissive role of vascular networks during systems consolidation**

Is the cortical angiogenic process a permissive mechanism allowing the creation of dendritic spines and neuronal architecture modifications that are essential for remote memory formation?

Our data showed that the manipulation of the transitory cortical angiogenesis achieved by selectively blocking or stimulating the Ang-2 signalling pathway impaired or improved remote memory retrieval, respectively. Moreover, exogenous Ang-2 administrations in SHR, which exhibit a lack of Ang-2 production when cognitively challenged and are unable to adequately stabilize and/or retrieve remotely acquired information, was efficient in rescuing the observed memory deficit. **Thus, these results underline that this early angiogenesis can assist the initial maturation of neuronal cortical networks, anticipating the stabilization of the memory trace, and conditioning the following corticalization of the memory trace and its retrieval.**

Several studies have revealed that cognitive impairments were associated to vascular dysfunctions in both humans and rodent models (Carnevale et al., 2012; Carnevale et al., 2016; Faraco and Iadecola, 2013; Iadecola, 2004, 2013; Meneses et al., 2011a; Tayebati et al., 2012). The originality of our study lies in showing that hypertension affects selectively remote memory while sparing recent memory. Moreover, this memory profile is associated to the progression of the pathology and probably related to an angiogenesis deficit. Interestingly, angiogenic pathways are altered during hypertension. The first hypothesis is that at the beginning of hypertension, the increase of cerebral blood pressure results in an excess of brain perfusion and thus leads to over-oxygenation. This prolonged hyperoxia induced the downregulation of VEGF and other proangiogenic factors (Adair and Montani, 2010). Conversely, others studies showed an increased expression of angiogenic growth factors in hypertensive patients (Filiz et al., 2015; Sane et al., 2004) in response to 1/ endothelial damage (Felmeden et al., 2003), 2/ excessive cyclical mechanical stretch caused by hypertension (Chang et al., 2003; Serne et al., 2001; Zheng et al., 2001), 3/ smooth muscle cells hypoxia during arterial remodelling as a compensatory mechanism (Kuwahara et al., 2002), and 4/ other factors such as ET-1 and Angiotensin II known to induce VEGF production (Chua et al., 1998; Williams et al., 1995). But if these factors are released, why are they not promoting angiogenesis?

The proposed mechanism is an endothelial resistance to angiogenic factors in hypertensive patient (Sane et al., 2004) as well as in SHR (Yang et al., 2002). Our result showed a similar basal

level of Ang-2 concentration in SD and SHRs as well as a similar basal vascular density. Moreover, our results provide additional insights into the hypertension modulation of angiogenesis: the angiogenesis activation by a physiological challenge, as it is the case for memory-induced neuronal activity, is impaired. A similar finding supports the dissociation between basal and activated states which impact memory: the loss of cholinergic activation and associated memory deficit in aged mice has been shown to be related to a dissociation between a comparable functional basal neuronal activity and an incapacity to adequately respond to cognitive stimuli (Lebrun et al., 1990).

**Importantly, interfering with the transitory angiogenesis process was sufficient to affect remote memory consolidation, suggesting that angiogenesis can be one of the *condiciones sine quibus non* of the expression of remote memory, together with neuronal plasticity.**

Despite the crucial importance of the cortical angiogenesis process in the stabilization and retrieval of remote memories, we can be surprised by its transitory profile. In fact, notwithstanding the possible contribution of angiogenesis in neuronal allocation, we do not know whether these newly born vessels are maintained or have regressed.

### **Much Ado about Nothing? Meaning of transitory angiogenesis**

Preliminary data describing the vascular plasticity time window together with the absence of vascular network enhancement 30 days after encoding (Anais Giacinti Ph.D thesis) suggest that the increase in vascular density, induced by the formation of the associative olfactory memory is transitory and that the vascular network regresses rapidly. But the detected vascular density decrease does not reveal whether the potential regression concerns the newly-born vessels or the existing (older) ones.

Two destinies can be predicted for the newly-formed vascular network: 1/ the new vessels achieve the maximal utility in the early phase of memory consolidation, expressing their functionality as a support of neuronal and synaptic allocation. After this period, their presence is no longer functionally advantageous, and this leads to vascular regression. 2/ the functional advantage of the new vessels, such as a closer position to tagged neuronal network during encoding or more efficient response to neuronal request, ensures their survival, while the existing older vascular network becomes less important, potentially less perfused, thus useless to maintain, and for this reason is intended to regress.

The kinetics of vascular remodelling can be rapid as observed in models of altered gravity (Wang et al., 2013b) but the creation and regression of a new vessel can take days as shown in model of hypoxia-induced cerebral angiogenesis (Harb et al., 2013; Pichiule and LaManna, 2002). We have acquired preliminary data describing the vascular plasticity time window, suggesting that the vascular increase, induced by the formation of the associative olfactory memory, is transitory and the vascular network regresses rapidly, after 3 days following encoding. This detected decrease in vascular density does not reveal whether the potential regression concerns the new vessels or the existing (older) ones.

**Thus, our results suggest that the angiogenic process can participate to the allocation of neuronal networks, potentially increasing the nutritional exchanges and thus the neuron efficiency, as it is the case for astrocytes which participate to the maintenance of the synaptic transmission.**

## Lessons from hypergravity-induced vascular adaptation

Neuronal and vascular plasticity share common molecular pathways (Madri, 2009; Palmer et al., 2000; Yamashima et al., 2004). Thus, several studies assumed that a global non-region specific vascular plasticity precedes and/or follows an increase of neuronal activity as shown by an increase in angiogenesis following exposure to environmental enrichment and physical activity (Black et al., 1987; Black et al., 1991; Isaacs et al., 1992; Palmer et al., 2000; Wallace et al., 2011). In the memory field, all previous studies have tested memory performance after the event supposed to increase the angiogenesis. Our study show for the first time the functionality of angiogenesis during post-encoding phases. But is it possible to detect this physiological memory-induced angiogenesis in a model in which the vascular network is globally increased before the task due to environmental enrichment and physical activity?

Part of the answer to this question can be derived from the effects of hypergravity.

Since changes in gravity modify vascular reactivity (Colleran et al., 2008; Dabertrand et al., 2012; Looft-Wilson and Gisolfi, 2000) in order to induce a vascular redistribution of body fluids (De Santo et al., 2001; Grigoriev and Egorov, 1996; Norsk, 1992, 1997) and can alter the level of angiogenesis measured in vitro (Dimmeler et al., 1999; Hood et al., 1998; Ku et al., 1993; Laham et al., 2003), we raised the hypothesis that hypergravity could interfere with the memory consolidation process, as observed in SHR. Our findings reveal that this is not the case. Rats that submitted to hypergravity did not show any memory deficit, even in experimental conditions in which the centrifugation period was increased. This may be due to the insufficient level of hypergravity we have used, since it can induce increases of bone and muscle mass but is without deleterious effects on metabolism, immunity and mood. Thus, it could be considered as a form of sustained physical exercise. Physical activity has been reported to induce angiogenesis (Fernandes et al., 2012; Roque et al., 2013), to reduce the deleterious effects of hypertension (Lin et al., 2015; Park et al., 2012; Shi et al., 2016) and to improve memory performance (Duzel et al., 2016; Herring et al., 2016; Kerr et al., 2010; Paillard, 2015). Thus, we can assume that the beneficial effects of hypergravity, due to the increase of physical activity, counterbalance its potential deleterious effects related to vascular dysfunctions. So, to complete our study, we have planned to test whether exposure to hypergravity can rescue the memory deficit detected during hypertension in SHR. Moreover, this experiment could increase the knowledge about the impaired mechanism affecting angiogenesis in SHR.

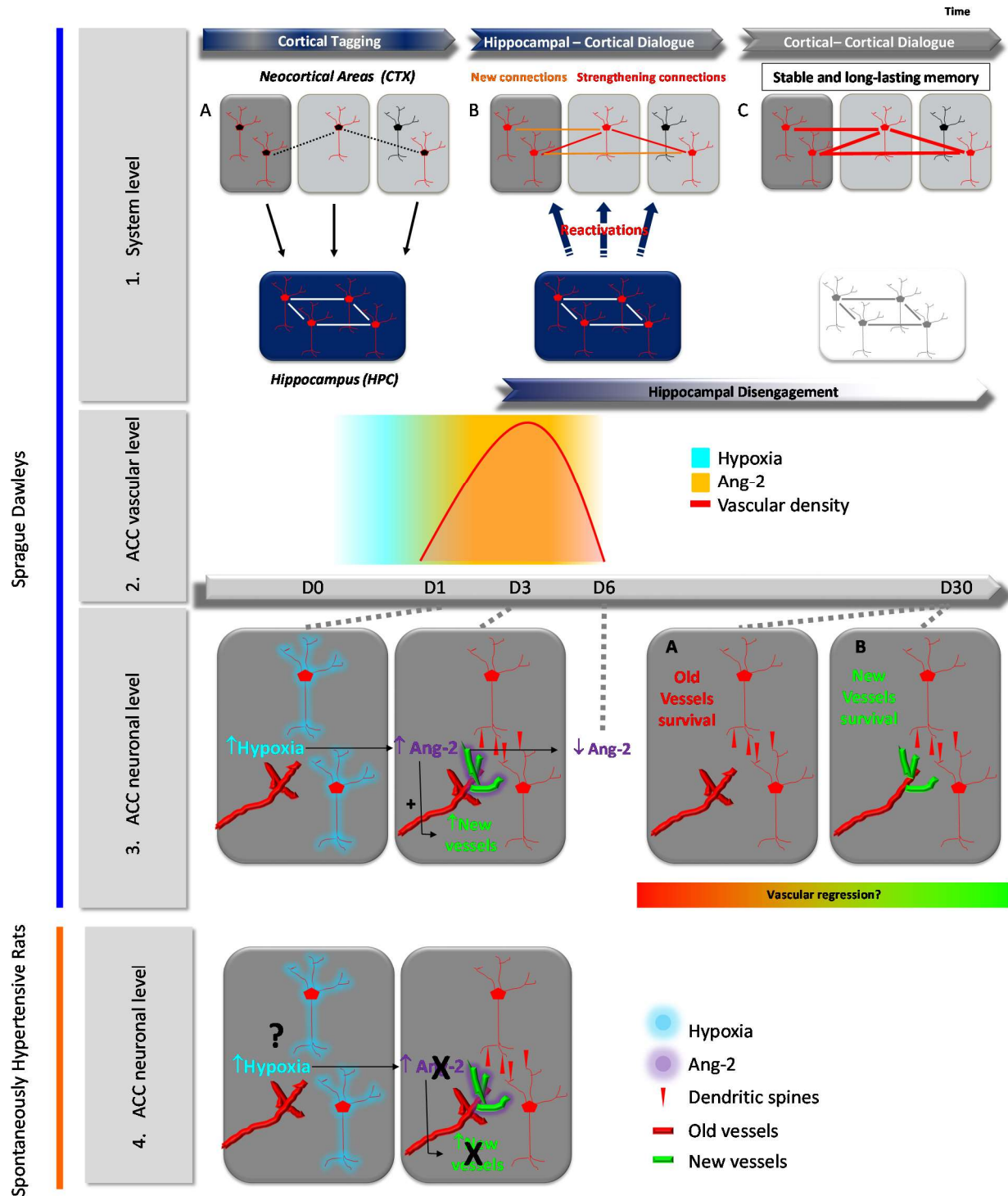
## Conclusions and perspectives

During this thesis, we provided correlative evidence suggesting that remote memory formation induced a rapid and sustained hypoxic signal in the ACC, which in turn, triggered a time-limited angiogenic mechanism (increased vascular density, cellular proliferation and Ang-2 expression) during the early phase of the memory consolidation process. This led us to propose that this transitory mechanism may as a **functional and preparatory process** allowing the progressive stabilization of the memory trace.

Importantly, we provided causal evidence to support the functional validity of this angiogenic mechanism. We showed that impairment of this mechanism both during hypertension (lack of increase of vascular density, cellular proliferation and memory-induced Ang-2 production) or achieved by pharmacological Ang-2 inhibition during the early phase of the memory consolidation process (ASON against Ang-2) was sufficient to interfere with the formation and/or retrieval of remote memory.



Conversely, intracerebral delivery of exogenous Ang-2 during the early phase of the memory consolidation process enhanced performance upon remote memory retrieval in both physiological or pathological (hypertension) conditions. These findings highlight not only the functionality of angiogenesis during the early phase of consolidation of an associative olfactory memory, but also its permissive role allowing the progressive establishment of the memory trace at the cortical level. Based on our main findings, we propose a schematic model of vascular and neuronal interactions during the course of the memory consolidation process (Figure 100).



**Figure 9300: A putative model of neuronal and vascular interactions during the course of memory consolidation.** 1. System level. A) Information is initially encoded within primary and associative neocortical sites (CTX, black neurons with red contour line). The hippocampus integrates information arising from these distributed cortical sites that represents the various sensory-motor features of a whole experience and then fuses these features into a coherent memory trace by means of an topographical index which contains the cortical

addresses of the memory trace. At this stage, hippocampal-cortical neurons are tagged to enable an adequate hippocampal-cortical dialogue. B) Successive cortical-hippocampal reactivations induce the progressive reinforcement of pre-existing connections (red connection lines) together with the creation of new connections (orange connection lines), the final outcome being the stabilization of the cortical memory trace within tagged neurons. C) Over days to weeks, as the cortical memory mature (thick red connection lines), the role of the hippocampus gradually diminishes, presumably leaving cortical areas to become capable of ensuring permanent memory storage and retrieval independently. Adapted from (Frankland and Bontempi, 2005; Lesburgueres et al., 2011). **2. Vascular level.** Memory-induced neuronal activation induces a rapid and sustained hypoxic signal in the cortex, which in turn triggers an Ang-2-dependent angiogenic mechanism during the early phase of the memory consolidation process, the final outcome being a transient increase of vascular density in the cortex. **3. Neuronal level:** Memory storage in the cortex is accompanied by a progressive increase of dendritic spine density (Frankland et al., 2004; Lesburgueres et al., 2011; Maviel et al., 2004; Restivo et al., 2009; Vetere et al., 2011). Although transitory, the angiogenic mechanism is necessary and permissive, in that it enables structural plasticity to occur within neuronal networks, a prerequisite for the formation of enduring memories. **4. Dysfunctional vessels.** SHR are unable to form and/or retrieve remote memory, a profile associated with an impaired angiogenic. Ang-2 intracortical injections (data not depicted) were successful in improving remote memory retrieval in SD rats (physiological conditions) or rescuing the memory deficits of SHR.

These exciting results open new questions regarding the understanding of the early cortical angiogenic mechanism:

- Why is the permissive action of angiogenesis rapid and transitory?

The unexpected very transitory nature of the cortical angiogenic process induced by encoding of associative olfactory memory points to its functional relevance predominantly during the early phase of the memory consolidation process. Thus the increased density of the vascular network may reflect the quick neuronal activation and plasticity required, as early as encoding, for the cortical allocation of the neuronal networks to the memory trace which subsequently undergoes stabilization. This possibility is further reinforced by the observation of the temporal overlapping of the angiogenic process with the progressive surface redistribution of cortical NMDA receptors (Benjamin Bessieres, Ph.D. 2016) involved in the induction and regulation of synaptic plasticity (Lau and Zukin, 2007; Mayford et al., 2012) and by the fact that early angiogenic stimulation can improve remote memory performance. Future studies will be required to provide mechanistic insights into how the Ang-2-induced remote memory amelioration is achieved. In light of our current findings, we can propose two hypotheses: 1/ Ang-2 administration can maximize the non-specific energy delivery to the neuronal networks supporting the processing of the memory trace via an increased density of the cortical vascular network, thereby generating an advantageous environmental substrate for neuronal activity. 2/ Ang-2 administration may prolonged the time window of action of the cortical angiogenic process, therefore further supporting weight and wiring plasticity within neuronal networks actively involved in the stabilization of the memory trace. If this hypothesis is correct, injections of Ang-2 should induce a longer time window of vascular remodelling and secondly that the progressive surface redistribution of cortical NMDA receptors is temporally increased.

- Is the angiogenic response to memory consolidation an information-specific mechanism or does it takes place only once in order to ensure the optimization of the vascular network to respond adequately to the metabolic request of neurons? Is the angiogenic process triggered each time that new information is encoded?

Despite some common characteristics between neuronal and vascular networks (shared developmental features, specific guidance cues and cellular and molecular signalling events, neurovascular coupling (Tam and Watts, 2010)), the dynamics of these two components are likely to be different. For instance, while the early cortical tagging process revealed by Lesburgueres and colleagues (Lesburgueres et al., 2011) is information specific, it is difficult to conceive a similar property for the cortical angiogenic process, which can be expected not to be information-specific. Accordingly, the use of a dedicated

protocol in which animals encode successively two associative olfactory information and the angiogenic process triggered by the second information is selectively blocked should not impair remote memory retrieval of both information, the sparing of the second information being attributable to the cortical angiogenic process initiated by the first information and still beneficial for the second information.

Finally, the interactions between microgravity and memory functions should be examined further together with its potential effects on vascular/angiogenic processes. The possibility of human life exploring Mars does no longer belong to science fiction movies but may well become a reality in the near future. NASA, ESA and all spatial agencies are focusing their efforts on studying the effects of long-term periods of space exposure on the human physiology. Converging evidence indicate that spatial perception and memory functions of astronauts can be altered by extended period of time in space. Maintenance of their optimal cognitive performance is therefore essential for decision making in the event of problem solving during a mission. A better understanding of memory and vascular disturbances related to microgravity changes and the development of pertinent countermeasures will be key to limit their deleterious consequences on crews during spaceflights. Moreover, the use of hypergravity - as a countermeasure to microgravity to reduce bone and muscular loss in astronauts - can also open the perspective to use it in the rescue of memory impairment due to hypertension.

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## ANNEXES

## **Publications and communications:**

### **Communications:**

- **Dynamics of vascular changes in spontaneously hypertensive rats during memory consolidation.** Pulga A., Bontempi B., Morel JL. 14<sup>th</sup> Scientific Day of the Graduate School of Life Sciences and Health of Bordeaux, Arcachon, France. April 9, 2014
- **Comparison of effects due to corticosterone injection and hypergravity on mouse hippocampal transcriptome.** Alice Pulga, Yves Porte, Jean-Luc Morel 9th FENS Forum of Neuroscience Milan, Italy. July 5-9, 2014
- **Dynamics of vascular changes in spontaneously hypertensive rats during memory consolidation.** Pulga A., Costet A., Giacinti A., Hambucken A., Biendon N., Macrez N., Bontempi B., Morel JL. 15th Scientific Day of the Graduate School of Life Sciences and Health of Bordeaux, Arcachon, France. April 16, 2015
- **Dynamics of vascular changes during memory consolidation in hypertensive rats.** Equipe Bontempi. HCERES IMN Bordeaux, France. February 11-12, 2015
- **Modelization of hypertension-induced memory deficit in rat.** Annabelle Costet\*, Alice Pulga\*, Jean-Luc Morel. EAVPT 2015, Nantes, France. July 19-22, 2015
- **Memory consolidation and cerebral angiogenesis are impaired in a rat model of hypertension.** Pulga A., Hambucken A., Biendon N., Costet A., Giacinti A., Macrez N., Bontempi B., Morel JL. 16<sup>th</sup> Scientific Day of the Graduate School of Life Sciences and Health of Bordeaux, Arcachon, France. April 6, 2016
- **Cerebrovascular modifications induced by hypergravity: effects on blood brain barrier.** Pulga A., Vanden Bossche A., Vico L., Morel JL. Federation of European Physiological Societies and the French Physiological Society, Paris, France. 29th June-1st July 2016
- **Deleterious effects of hypertension on the organization of recent and remote memories in the spontaneously hypertensive rat.** Pulga A., Hambucken A., Costet A., Morel J.L.\*, Bontempi B.\* Society for Neuroscience 2016 Annual Meeting, San Diego, California. Nov 12–16, 2016

### **Publications:**

- **Changes in C57BL6 mouse hippocampal transcriptome induced by hypergravity mimic acute corticosterone-induced stress.** Alice Pulga, Yves Porte, Jean-Luc MOREL. Submitted to Journal: Frontiers in Molecular Neuroscience

# Changes in C57BL6 mouse hippocampal transcriptome induced by hypergravity mimic acute corticosterone-induced stress.

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**Running title:** Hypergravity effects on hippocampal transcriptome

**Key words:** Aging, chronic stress, acute stress, gravity, hippocampus, transcriptome, Illumina

## Abstract

Centrifugation is a widely used procedure to study the impact of altered gravity on Earth, as observed during spaceflights, allowing us to understand how a long-term physical constraint can condition the mammalian physiology. It is known that mice, placed in classical cages and bred during 21 days in a centrifuge at 3G gravity level, undergo physiological adaptations due to hypergravity and/or stress. Indeed, an increase of corticosterone levels has been previously measured in the plasma of 3G-exposed mice. Corticosterone is known to modify neuronal activity during memory processes. Although measurements of learning and memory cannot be performed in the centrifuge, literature largely described a large panel of proteins (channels, second messengers, transcription factors, structural proteins) which expressions are modified during memory processing. Thus, we used the Illumina technology to compare the whole hippocampal transcriptome of three groups of C57Bl6/J mice, in order to gain insights into the effects of hypergravity on cerebral functions. Namely, a group of 21 days 3G-centrifuged mice was compared to (1) a group subjected to an acute corticosterone injection, (2) a group receiving a transdermal chronic administration of corticosterone during 21 days and (3) aged mice because aging could be characterized by a decrease of hippocampus functions. Our results suggest that hypergravity stress induced by corticosterone administration and aging modulate the expression of genes in the hippocampus. However, the modulations of the transcriptome observed in these conditions are not identical. Hypergravity affects per se the hippocampus transcriptome and probably modifies its activity. Hypergravity induced changes in hippocampal transcriptome were more similar to acute injection than chronic diffusion of corticosterone or aging.

## Introduction

Stress is known to modulate memory by acting on the hippocampus. As reviewed recently (Kim et al., 2015; Pearson-Leary et al., 2015), norepinephrine and glucocorticoids act on memory via modulations of neuronal functions, neurogenesis and glial cells. The effects of glucocorticoids depend on their concentration, synthesis and turn-over. Moreover, as suggested by the high density of glucocorticoids receptors, the hippocampus, together with the amygdala, represents one of the main targets of glucocorticoids (Reul and de Kloet, 1985). Indeed, infusions of corticosterone (or its analogues) in the hippocampus affect memory performance in different paradigms (Micheau et al., 1984; Roozendaal and McGaugh, 1997). They appear to be necessary for memory consolidation, yet while acute post-training injection of low doses enhance performance in aversive and spatial tasks, higher doses or chronic treatment with low doses impair memory and hippocampal functions (Marks et al., 2015; McGaugh and Roozendaal, 2002). However, a very high dose of corticosterone is able to mimic the memory impairment observed in the post-traumatic stress disorder (PTSD) (Kaouane et al., 2012), which is characterized by hypermnnesia for the core traumatic event associated with a memory deficit for peritraumatic contextual cues.

Besides the typical events leading to stress, an increase of corticosterone levels in plasma samples has been observed in rats (Petrak et al., 2008) and mice (Gueguinou et al., 2012) exposed to conditions of altered gravity.

Life is conditioned by the gravity vector, from conception to adult stages. Adult mammals, during their whole life, need to integrate this force to coordinate the communication between organs and maintain their physiology in a balanced steady-state. The alteration of gravity is a model used to shed light on fundamental processes implicated in environmental adaptation influencing the genomic expression, and it may be helpful to understand how organisms can evolve and adapt to their life on Earth. However, during space flights, astronauts undergo gravity modifications. The most remarkable effects induced by microgravity observed after spaceflights in both humans and rodents are bone decalcification, decrease of musculature and cardiovascular deconditioning, but they also show spatial disorientation, depressive-like and cognitive disorders (Porte and Morel, 2012). In space, astronauts as animal models are exposed to several risk factors, such as secondary radiations (due to high energy protons from solar radiation), modification of light-dark cycle, confinement and modification of gravity level. In order to isolate the impact of gravity and confinement from the radiation exposures, we have used a model consisting of the confinement of mice in a centrifuge, which creates a modification in gravity level.

Using the same protocol, a behaviour analysis using a Morris water maze 15 days after exposure to hypergravity (21 days, 3G) suggested that the memory processes could be affected (Bojados and Jamon, 2014). In the same conditions, corticosterone blood levels also revealed that the stress level in these mice was increased in the hours following centrifugation (Gueguinou et al., 2012). The increase of corticosterone levels observed in this study could be due to (1) a long lasting stress induced by the hypergravity and/or (2) an acute stress due to the centrifuge brake.

Since gravity changes induce modifications of genome expression in several tissues (Morel et al., 2013), we hypothesized that hypergravity as well as stress (induced by acute or chronic administration of corticosterone) could affect the genome expression in the hippocampus, with putative effects on memory. To assess this paradigm, we have performed a transcriptomic analysis of the hippocampus of mice which underwent either centrifugation or chronic and acute corticosterone administration.

Finally, since deleterious effects induced by gravity modifications have been presented as an acceleration of aging (Vernikos and Schneider, 2010), we compared the hypergravity situation with aging effects on the hippocampus transcriptome (Vernikos and Schneider, 2010); moreover, aging is also associated with an increase of plasma corticosterone levels and modifications in hippocampus-dependent processes (Garrido et al., 2012a; Garrido et al., 2012b; Lo et al., 2000).

## Material and Methods



### ***Animals, centrifugation and corticosterone treatments***

Eight week-old C57BL/6J male mice were purchased from Charles River (Les Oncins, 69210 Saint Germain sur l'Arbesle, France) and housed in standard cages (4 mice per cages, 36 × 20 × 14 cm) under standard conditions (22°C, 55% humidity, 12/12h light–dark cycle) with free access to standard food and water. Mice were habituated to animal room for two weeks prior to beginning testing.

Mice were divided into different groups described as follows: 24 mice (6 cages) were placed in the centrifuge at 3G for 21 days (3G group = hypergravity); 24 mice were placed in normogravity (1G group = confinement) in the centrifuge room and confined in the same gondola as used in the centrifuge. The cages were supplied with enough food and water to allow an uninterrupted 21 days centrifugation/confinement period. This part of the experiment was performed in the animal facility containing the centrifuge (Hôpital de la Timone; Marseille, France; Marc Jamon was responsible for centrifugation).

A group of 10 mice were implanted subcutaneously with Matrix-Driven Delivery (MDD) pellets (Innovative Research of America, Sarasota, FL, USA) containing 10 mg of corticosterone, allowing the constant and continuous delivery of corticosterone during 21 days (CC group). Likewise, a control group of 10 mice were implanted with the placebo pellets during 21 days (PL group), in order to rule out a putative confounding effect of implantation and surgery in the CC group. One mouse treated with placebo died before the end of the protocol. Furthermore, 10 mice (13 weeks old, aged-matched with CC and PL groups on the day of euthanasia) received a single intraperitoneal injection of corticosterone (in 2-hydroxypropyl- $\beta$ -cyclodextrin complex; 1.5 mg/Kg; in a volume of 0.1 ml/10 g bodyweight; group AC), one hour before euthanasia as described previously (Kaouane et al., 2012). Finally, a group of 15 mice of 22 month (AGE group) was also included in the study to compare the effect of aging to hypergravity and corticosterone-induced stress.

CC, PL, AC and AGE mice were housed in the animal facility of our laboratory, in Bordeaux, France. All animal groups are summarized in Table 1.

The project was validated by the French Ministry of Research in accordance with the European Community and French guiding principles. The principal investigator is authorized by French authorities to perform animal experiments (n° C33-01-029).

Mice were euthanized in the same time window (9-11 AM) by a lethal dose of pentobarbital, brains were extracted from the skull and blood was collected. Hippocampi were dissected and placed in 2 mL tubes containing 1 mL of tri-reagent (TR-118, MRC, Cincinnati, OH) and 10-12 ceramic bead (SiLibeads, ZS, 2-2.2 mm diameter, Labomat Essor, Saint-Denis France) and frozen in dry ice. Samples of group 1G and 3G were transported to Bordeaux in dry ice. All samples were prepared for transcriptomic analysis as described below.

### ***Preparation of RNA samples for transcriptomic assays***

After disruption and homogenization of all samples with minilys (Precellys, distributed by Ozyme France, Montigny le Bretonneux) in tri-reagent (Molecular Research Center, Inc., Cincinnati, USA), isolation of the total RNA from each hippocampus was performed following the supplier procedures. RNA integrity and purity were verified by using RNA HighSens Analysis Kit (Experion, BioRad, Marne-la-Coquette, France) and the concentration of RNA was measured with spectrophotometry (NanoDrop Technologies, Wilmington, DE) for each hippocampus. We have determined that the total RNA in one hippocampus was not sufficient to be sequenced by Illumina. Thus, the RNAs from 3-8 mice were pooled in order to generate 3 samples for each experimental condition (supplementary figure 1). Samples were sent to GATC, the company which verified the quality of the samples and performed the transcriptomic analysis using Genome Sequencer Illumina HiSeq2000\* (sequence mode single read 1 x 50bp), and sequences were mapped. The quantities of sequences obtained in each sample were sufficient to perform a statistical analysis (supplemental figure 2).

### ***Corticosterone assays***

Blood was drawn just after the cervical dislocation. After 10 min, samples were centrifuged during 15 min at 1000 g and supernatants were collected and stored at -20°C before assays. The quantification of endogenous mouse corticosterone in blood samples was performed using EIA kit DetectX

Corticosterone (Arbor Assays, Michigan distributed by Euromedex) following the instructions of the supplier: samples and standards solutions were diluted (1:100) in the buffer and placed in the plate in duplicates. The reaction buffer containing anti-corticosterone antibody was added and after 2 h of incubation the chemoluminescent substrate was added. The chemoluminescence was measured with the Optima apparatus (BMG Labtech, Champigny sur Marne, France).

### ***Statistical analysis***

The first steps of transcriptomic data analysis were performed by GATC. To validate the statistical analysis, p-values were corrected using the Benjamini and Hochberg procedure controlling false discovery rate (FDR). Supplementary statistical analyses were performed with Graphpad prism software (Graphpad software Inc, La Jolla, CA). Data are expressed as means  $\pm$  S.E.M.; *n* represents the number of tested animals. The samples were compared pair by pair with t-test and with one-way ANOVA. The *P* values  $< 0.05$  were considered as significant and indicated by ★ in figures.

The list of proteins was analyzed using Database for Annotation, Visualization, and Integrated Discovery (DAVID; <http://www.david.niaid.nih.gov>), in order to identify the associated pathways and function of the different genes modified in the experimental conditions. The query was made as proposed: *sp-pir-keywords* for functional categories and *biocarta*, *KEGG pathway* and *panther pathway* for signaling pathways.

## **Results**

### ***Corticosterone levels***

As described previously in mice centrifuged in the same device (Gueguinou et al., 2012), we measured an increase of the plasma level of corticosterone during the two hours following a period of 21 days in the centrifuge at 3G ( $59.4 \pm 8$  ng/mL versus  $113 \pm 11.6$  ng/mL for 1G and 3G groups, respectively; *n* = 24 for each group; *p* = 0.0004). We also noticed that in the 3G group the variability of individual measurements was increased (Figure 1A). We therefore decided to complete the statistical analysis by the Thompson-test to determine the outliers' points. The result was not modified ( $52.5 \pm 7$  ng/mL, *n* = 22 versus  $107 \pm 10$  ng/mL, *n* = 23 for 1G and 3G groups, respectively; *p* = 0.0001).

To compare the effects of hypergravity on the transcriptome to those observed in stress conditions, we treated mice chronically with corticosterone pellets. The levels of corticosterone in blood of animals treated with chronic corticosterone and placebo were evaluated ( $33.6 \pm 7$  ng/mL, *n* = 9 versus  $51.3 \pm 8.2$  ng/mL, *n* = 10 for PL and CC groups, respectively; *p* = 0.118), and the *p* value was ameliorated by the use of the Thompson-test ( $28.4 \pm 5.3$  ng/mL, *n* = 8 versus  $51.3 \pm 8.2$  ng/mL, *n* = 10 for PL and CC groups, respectively; *p* = 0.042), as reported in Figure 1B.

The acute intraperitoneal injection of corticosterone induced an increase of blood corticosterone concentration close to 10-fold more than in the 3G group ( $1201.8 \pm 232.7$  ng/mL, *n* = 10; one-way ANOVA, *p* = 0.001 in comparison with all other groups).

Finally, in aged mice corticosterone levels were not significantly affected in comparison with the placebo group used as control younger mice ( $33.6 \pm 7$  ng/mL, *n* = 9 versus  $52.7 \pm 17.1$  ng/mL, *n* = 15; *p* = 0.193. After the Thompson test, values were  $28.4 \pm 5.3$  ng/mL, *n* = 8 versus  $38.0 \pm 9.3$  ng/mL, *n* = 14; *p* = 0.383; for PL and AGE groups, respectively).

### ***Transcriptomic analysis***

The Illumina analyses revealed the presence of 33842 different sequences in the transcriptome of the hippocampus. The statistical comparison showed that the expressions of 82 transcripts were affected by hypergravity compared to normogravity, corresponding to 77 identified genes, while 5 sequences

were not associated to known genes. In the other conditions, some genes were associated with several sequences; we have mentioned them in Table 2 as repeated sequences. After excluding sequences not associated with gene names and repeated sequences, the expressions of 110 transcripts were modified by acute injection of corticosterone compared to placebo, 102 by chronic administration of corticosterone compared to placebo, and finally 3308 by aging compared to young mice placed in normogravity (Table 2).

The global modifications of transcriptome produced by the different experimental conditions were compared and summarized in a Venn diagram in Figure 2. The profile of the effect of hypergravity was qualitatively and quantitatively more similar to the effect of the acute injection of corticosterone than to the long lasting delivery of corticosterone.

To map the putative effects of hypergravity, we have used the DAVID database. Seventy six genes were analyzed and dispatched in 37 groups. Figure 3 summarized how the genes could be grouped according to their known functions and cellular localization. The detailed DAVID queries are described in supplementary file (DAVID-queries-PPM.xls).

The statistical comparisons between control and experimental groups were grouped in supplementary file (EXPRESSION-PPM.xls). In details, the increases of expression levels of 8 transcripts and the decreases of the expression of 2 transcripts were specifically affected by hypergravity only (Table 3). The Table 4 summarized the comparison between the modification of transcriptome induced by hypergravity and the other conditions (AC, CC and AGE). Hypergravity and acute injection of corticosterone similarly affected the expression of 49 transcripts (but for 26 of them, a marked difference is noticeable between PL and 1G groups), and 3 transcripts were altered in opposite ways when compared respectively to normogravity and PL controls (Table 5).

The chronic administration of corticosterone and hypergravity affected the expression of 4 transcripts similarly (but for 2 of them, a marked difference is noticeable between PL and 1G groups) and showed opposite effects on the expression of 2 transcripts compared respectively to normogravity and PL controls (details in supplementary file EXPRESSION-PPM.xls).

Hypergravity and aging together affected the expression of 45 transcripts, of which 13 were similarly modified and 32 were affected contrariwise compared to adult mice in normogravity (details in supplementary file EXPRESSION-PPM.xls). Hypergravity and the acute injection of corticosterone resulted to be the closest experimental conditions in modulating the hippocampal transcriptome. Table 6 lists the 47 transcripts affected by acute injection of corticosterone but not by hypergravity (but for 15 of them, a marked difference is noticeable between PL and 1G groups).

To compare the putative effects of 3G and other experimental conditions, we have crossed the results of DAVID queries to generate Venn diagrams. The effects are not stackable in any functional category proposed by DAVID. As illustrated in Figure 4, some categories are affected in all conditions (in A-C) or only 3 (D-E) or 2 (F) of them. As expected, the aging group is the most affected by the number of gene and the number of function (supplementary file: DAVID-queries-PPM)

## Discussion

We have confirmed that 1) C57BL6 mice exposed for 21 days to hypergravity showed an increase of their corticosterone plasma levels, 2) the previously reported variability of the individual adaptation to gravity modifications was indeed present (Beraneck et al., 2012; Gnyubkin et al., 2015).

It is also interesting to compare our results with those obtained in other species, devices and protocols. In rats, the level of corticosterone could increase during hypergravity exposure and after the stop of centrifugation (Petrak et al., 2008), but also increased or decreased depending on the hypergravity protocol and age of the animals, as shown respectively in (Abe et al., 2013; Casey et al., 2012). Thus, the design of the hypergravity protocol is crucial. For example, a regular “stop and go” during the centrifugation can lead to a habituation that can reduce the stress effects.

Taken together, all these studies indicate that the design of the centrifugation protocol and the animal model could influence the stress produced by hypergravity. It would be very interesting to test

how centrifugation protocols could influence physiological parameters as plasma concentration of several hormones on different species (mouse and rat) to determine general rules in biological adaptation to gravity levels. Finally, these experiments could also reinforce the theory of the gravity continuum proposed and discussed in the biology and gravity fields (Plaut et al., 2003; VanLoon, 2016). Probably, as suggested by these studies, the effect of hypergravity close to 2G is the opposite of microgravity, while the effects induced by 3G acceleration could be different, without following a linear trend. The continuum of gravity should be established independently for each physiological function or tissues.

The absence of handling during the 21 days of centrifugation obliged us to limit the manipulation of mice in the other groups as well; thus the wide range in and relatively high corticosterone levels in 1G controls may reflect a release of corticosterone in response to the stress of handling (Pitman et al., 1988). In this work, we decided to consider the PL group as a control group for AC, despite the fact that more adequate controls would have been acutely injected with saline; however, the animal sacrifice was performed one hour after the acute injection, time by which its effects on corticosterone levels were unneglectable (Freund et al., 1988). Noteworthy that all sacrifices were performed in the same time window, not only to limit the bias due to the circadian rhythm of corticosterone synthesis, but also to decrease misinterpretation linked to circadian rhythm of protein synthesis and gene regulation implicated in memory processes (Eckel-Mahan, 2012).

The chronic treatment with corticosterone increased the level of plasma corticosterone ( $p = 0.042$ , after the Thompson-test revealing one outlier for 19 mice in both groups), yet in a lesser extent than expected. As reported recently, in order to reach a 1.8-2 fold increase of the blood corticosterone concentration in mice after 21 days, a dose of 40mg/Kg/day would be required (Kolinko et al., 2015; Weng et al., 2016). In our experiment, the designed pellet delivery was close to 20 mg/Kg/day and the increase of the measured plasma corticosterone concentration was 1.5 fold compared to PL group. Since drug delivery problems can be excluded, this level of blood corticosterone can be due to different turn-over mechanisms.

Interestingly, by comparison with placebo-treated animals, we also observed a slight increase of the corticosterone concentration in group 1G ( $p = 0.052$ , t-test's comparison) close to the ones observed in aging and chronically-treated mice. This result suggests that containment alone is sufficient to induce a rise in corticosterone levels. Future experiments should clarify if this increase can impact the memory performance by itself (Salehi et al., 2010).

Before our study, several studies have reported that aging as chronic stress affect similarly hippocampus function as learning and memory (Bonhomme et al., 2014; Tronche et al., 2010; Wang et al., 2016a), then we have hypothesized that the modifications of hippocampus transcriptome induced by hypergravity should be similar to those observed in aged mice and/or mice treated with corticosterone.

The hippocampus transcriptome is modified in each experimental condition. Our results show that there is no overlap between experimental conditions but 61 %, 38 % and 5 % of transcripts modified by hypergravity were respectively modified by AC, aging and CC in the same way; whereas 14%, 4%, and less than 3% of transcripts modified by hypergravity were respectively modified by aging, AC and CC in the opposite way.

A global analysis of the transcriptome can give a snapshot of the hippocampus status, thereby revealing possible modifications of its functions induced by hypergravity, corticosterone (acute i.p. and long lasting s.c. delivery) and aging. It appears from our result that a very large part of the hypergravity effects (summarized in Figure 2) is reproducible by an acute injection of corticosterone used to replicate the symptomatology of PTSD (Kaouane et al., 2012). The most probable event comparable to acute stress in our protocol is the stop of the centrifuge. Consequently, we suggest that the stop of the centrifuge is perceived by animals as an acute stress that could induce cognitive

disorders close to PTSD. Further experiments should be performed to analyze the memory performance once normogravity is reestablished, using behavioral experiments, like fear conditioning for example. Moreover, in order to decrease the effect of rapid gravity modifications as well as to better compare the effects of the gravity in rodents to those seen in humans, animals could be trained to several acceleration training phases or modulation of velocity in both “lift-off” and “landing” phases, etc... Indeed, studies on humans differ largely from the ones using rodents: first, astronauts undergo sustained training before being submitted to space flight; second and most importantly, since humans beings are conscious, they can prepare themselves to the changes of gravity during the mission (i.e. since they know when and why the gravity changes, thus the situation is less stressful).

Surprisingly, the long lasting subcutaneous diffusion of corticosterone used to mimic a chronic stress is the most different situation in comparison with hypergravity, and in another hand there only 14 gene affected by both acute and chronic corticosterone treatments. These treatments were used to mimic stress effects and several studies reported the effects of the acute and chronic stresses modified differently the hippocampus transcriptome (Li et al., 2013; Stankiewicz et al., 2015). Furthermore, the nature of the stressor is also crucial (Li et al., 2013; Porter et al., 2012; Stankiewicz et al., 2015; Suri et al., 2014). Similarly to stress, modifications of living conditions, as well as long-term mild or intense exercise, can modulate the hippocampus transcriptome (Inoue et al., 2015). In our case, the effect of the stop of the centrifuge creating a higher increase of plasma corticosterone than those observed in chronic corticosterone treatments and reveals a similar modification of the hippocampus transcriptome as observed after an acute injection of corticosterone.

Aging is characterized by a substantial modification of the hippocampus transcriptome (close to 10% of the sequences are affected). This result is probably due to an important dispersion of individual physiological status/history but also to the absence of selection of the mice based on learning and memory performances, for example. The number of gene affected by aging depends also on the age of the mice (Stilling et al., 2014). Similarly, the lists of genes affected by both aging and corticosterone treatments contain only 55-57 genes, largely different from the hypergravity. These observations underscore that aging as other experimental conditions integrate multiple signals and their complex marks; a transduction pathway can be affected in one or more different steps. Ultimately, it is possible to implicate several identical pathways affected in all conditions as revealed by the results of DAVID queries.

Before, the measured effect should be analyzed remembering that the hippocampus contains several cell types as neurons, glial cells but also endothelial cells and pericytes constituting blood vessels. Likewise, the comparison between our results and those described recently (Stankiewicz et al., 2015) suggests that the choroid plexus could be affected by hypergravity. In fact, *Ttr*, *Igf2*, *Igfbp2*, *Prlr*, *Enpp2*, *Sostdc1*, *1500015O10RIK(Eerg4)*, *Kl*, *Clic6*, *Kcne2*, *F5*, *Slc4a5*, and *Aqp1* have been described as more highly expressed in the choroid plexus as in the brain parenchyma (Stankiewicz et al., 2015). Our results show that the transcription levels of these genes were affected by hypergravity. We cannot totally exclude a bias due to the dissection step. Nevertheless, the effect of gravity modifications on the choroid plexus should be more investigated, as blood and cerebrospinal fluid pressures are both sensitive to hypergravity (Iwasaki et al., 2012).

Globally, the query of DAVID database indicated that genes affected by hypergravity encode for proteins implicated in several functions. Probably, the most important common effects of hypergravity and acute injection of corticosterone concerned signaling pathways affecting nucleus functions. Moreover, hypergravity significantly affects cellular interactions as modulation of tight junctions. However, the cellular metabolism does not seem to be disturbed, as observed in other studies concerning different organs in a pregnant rat model (Casey et al., 2015; Casey et al., 2012). The effects of hypergravity on hippocampal transcripts are not solely overlapped to acute stress and 10 transcripts were *per se* modified by hypergravity only. The transcriptomic approach could be used to determine targets potentially affected by environmental modifications (Huttenrauch et al., 2016).

Hypergravity modulate the expression of genes implicated in neuronal differentiation and migration via *Plagl1* expression (Adnani et al., 2015) and neurite outgrowth and vascular remodeling via *Smad5* (Hegarty et al., 2013; Mathieu et al., 2008). The hypergravity can alter learning and memory via the modulation of *ppp1r1b*, *Dnajb1*, *Rbp1* and *Hspa5* genes encoding proteins involved in the regulation of molecular pathways of memory and linked to neurodegenerative disorders (Heyser et al., 2013; Ignacak et al., 2009; Leil et al., 2003; Seo et al., 2014; Udan-Johns et al., 2014; Witt, 2013). The role of *CTRP5* encoded by *C1qtnf5-Mfrp* gene is not described in the brain but it is associated with macular degeneration (Hayward et al., 2003) and lipid oxidation (Yang and Lee, 2014). If the functions of *Igfn1* and *Krt18* are unknown in neurons, their function in smooth muscle cells (Baker et al., 2010) and implication in intracerebral arteriovenous malformations (Sasahara et al., 2007) indicate that hypergravity could act on pericytes to modify the cerebrovascular function and hippocampus perfusion. Similarly, *Sdf2l1*, an endoplasmic reticulum stress-inducible gene (Fukuda et al., 2001) is implicated in the folding of proteins (Tiwari et al., 2013) but the function of the encoded proteins in brain cells (neurons, glia and vessels) remains largely unknown.

In conclusion, even if it is not completely possible to segregate the effects of stress and hypergravity in this model, the transcriptomic analysis indicated that the expression of genes encoding proteins implicated in excitability, cellular interactions, migration, protein synthesis and cell death, was modified after 3G exposure. An effect on memory thus cannot be excluded since memory requires a coordinated regulation of many known and unknown proteins (Jarome and Helmstetter, 2014). It is also known that stress affects deeply the cerebrovascular network (Longden et al., 2014; Scheuer et al., 2007). Therefore, a modulation of the vascular network, due to hypergravity, should not be totally excluded and could act on brain functions especially in animal models without training as in astronauts (cognitively, emotionally and physically). Considering that 10 genes were modified specifically after 3G exposure, it appears that hypergravity alone modulates the hippocampal activity. We can thus suggest that both the neurogenesis and angiogenesis would be impaired (decrease of both *plagl1* and *smad5*), whereas neuroprotection against hypoxia effects would be ameliorated (increase of *rbp1* and *dnajb1*). Moreover, the increases of expression of factors regulating protein folding (*sdf2l1*, *hspa5*) and NMDA-dependent signals (*ppp1r1b*) most probably affect the neuronal architecture. Finally, our results could also be compared to other studies using the same approach to understand how the hippocampus transcriptome was adapted to environmental conditions (Huttenrauch, 2016).

### Legends of figures:

**Figure 1: Blood corticosterone concentrations.** (A) in 1G- (circle) and 3G- (square) exposed animals; each dot represents the mean of the duplicate measured for each animal. (B) Mean of corticosterone concentration measured in placebo and chronic corticosterone treatment. The results were statistically compared with t-test; ★,  $p < 0.05$ . (C) Mean of corticosterone concentration measured in placebo, 1G and aged mice groups.

**Figure 2: Venn diagram comparing the effects of all experimental conditions on hippocampus transcriptome.** The diagram was produced by the software available on <http://bioinformatics.psb.ugent.be> using the lists of genes statistically affected in each experimental condition. The number of genes affected by the experimental conditions is indicated in each colored subset.

**Figure 3: Putative functions and localizations of proteins encoded by genes affected by 3G.** The list of genes modified by hypergravity was analyzed using DAVID database and genes listed in DAVID were further grouped according to the localization (purple subsets) and the functions (blue subsets) of their encoding proteins. In green, genes not identified by DAVID were groups in “not determined” subsets; genes identified in other lists were indicated in “other function subsets” with the function indicated in parenthesis. Genes’ names in red indicate those only affected by 3G.

**Figure 4: Venn diagrams comparing the queries of DAVID database.** The lists of genes modified by all experimental conditions were analyzed using DAVID database and Venn diagrams were created considering 6 categories of genes encoding: (A) signal peptide, (B) secreted proteins, (C) proteins of the extracellular matrix, (D) proteins implicated in calcium-dependent mechanisms, (E) proteins implicated in Wnt pathway and (F) proteins implicated in ion transport.

### Conflict of interest:

The authors have not conflict of interest concerning this study. The authors thank the CNES and ANR for granting the project.

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### Authors and contributors:

The project was initiated by YP continued by AP under supervision of JLM. YP and JLM prepared samples; AP and JLM analyzed the data and wrote the manuscript. All authors approved the manuscript.

**Table 1:** Design of experimental groups

Group	3G	1G	AC	CC	PL	AGE
n	24	24	10	10	10	15
Age	10 weeks at the beginning of the protocol					80 weeks
Gravity level	3 x G	Normogravity (1G)				
Treatment	Centrifuge	None	Corticosterone Intraperitoneal	Corticosterone Subcutaneous 21days	Placebo	None

**Table 2:** Number of transcripts affected by experimental conditions

Group comparison	3G vs 1G	AC vs PL	CC vs PL	1G vs AGE
Sequences	33842	33842	33842	33842
Fail	1061	994	943	892
Lowdata	93	93	93	93
No test	4049	1122	1891	1959
Seq analyzed	28639	31633	30915	30898
<b>P &lt; 0.05</b>	<b>82</b>	<b>131</b>	<b>108</b>	<b>4095</b>
Not associated with genes	<b>5</b>	<b>20</b>	<b>6</b>	<b>592</b>
Repeated sequences	<b>0</b>	<b>1</b>	<b>0</b>	<b>195</b>
<b>Identified sequences</b>	<b>77</b>	<b>110</b>	<b>102</b>	<b>3308</b>

**Table 3:** Transcript expression levels affected only by 3G compared to normogravity (1G) (decreased in italic; all others increased). The values are expressed as Fragments Per Kilobasepair per Million (FPKM) of the gene in sample with respective p-value and q-value, which is the FDR-adjusted p-value.

Gene	Locus	Group 1G	Group 3G	log2(fold change)	p value	q value
<i>Plagl1</i>	<i>10:12810656-12851500</i>	<i>6.098</i>	<i>4.172</i>	<i>-0.547476</i>	<i>1.274E-04</i>	<i>0.046170</i>
Igfn1	1:137878484-137880871	0.241	0.961	1.997340	1.137E-04	0.043405
Hspa5	2:34627514-34655580	45.661	61.084	0.419833	3.614E-07	0.000259
Dnajb1	8:86132079-86135915	19.787	26.955	0.446044	1.794E-06	0.001048
C1qtnf5,Mfrp	9:43909713-43917327	5.721	11.043	0.948863	1.253E-06	0.000816
Rbp1	9:98325199-98347038	12.090	18.123	0.584057	4.914E-05	0.021004
Ppp1r1b	11:98209550-98219164	15.030	19.941	0.407903	5.064E-05	0.021328
Krt18	15:101861304-101862451	0.507	3.744	2.884170	6.800E-05	0.027046
Sdf2l1	16:17130229-17132410	10.438	17.327	0.731153	1.432E-07	0.000108



**Table 4:** Effect of hypergravity on gene expression. In red box increase of expression, in green box decrease of expression and the grey box indicated that the gene expression is not modified. The values log2(fold-change) between 3G and 1G (Group 3G); AC and 1G (Group AC); CC and 1G (Group CC) AGE and 1G (Group AGE).

Gene	Group 3G- log2(fold_change)	Group AC- log2(fold_change)	Group CC- log2(fold_change)	Group AGE- log2(fold_change)
Sulf1	1.30335	2.54783		-0.826247
F5	1.93139	3.95502		2.32089
1500015O10Rik	1.75095	2.99507		0.834877
Igfbp2	0.880923	1.56046		0.600096
Kcnj13	1.97071	4.47569		3.10135
Ankrd57	0.594451	0.867332		
Egr2	1.21264			3.06703
Dcn	-0.573591			2.21481
Ace	1.41923	2.55624	0.632951	
Sostdc1	1.40975	2.78276		0.981031
Gm11274	-0.974941			2.23011
Tgfb1	0.866265	1.06921		
Otx2	1.49754	3.16522		-1.31388
Cab39l	0.466337	0.803683		
Nr4a1	0.475274			1.73113
Enpp2	1.4905	2.51697		
Arc	0.634743			2.49228
Cldn1	1.38973	1.75401		
Col8a1	1.5564	3.57162		
Vgll3	-1.19884	2.09066	1.56852	1.75075
Kcne2	2.07954	4.69944		2.9754
Clic6	1.68149	3.52024		
Cdkn1a	0.733637	0.939498		
Sik1	0.557851	1.03223		-0.519891
Hspa1b	0.849894		0.567793	1.65455
Capn11	-1.65456			3.03854
Ezr	0.388112			0.974911
Ttr	2.18462	4.38164	0.964938	2.88912
Egr1	0.566316			1.7932
Lbp	1.37942	1.97479		
Wfdc2	2.03256	3.83212		
Col9a3	1.23352	1.64091		
Lcn2	1.72437	-2.70037	1.6198	2.3442
Abca4	1.51953	2.96413		
Col8a2	1.24666	2.33567		
Pla2g5	1.75196	2.39452		
Trpv4	1.7617	2.94136		
Kl	1.23118	2.05559		3.02789
Msx1	0.940395			1.4076
Steap1	1.83045	3.40711		
Rbm47	1.65002	2.80976		
Tmem72	1.8963	5.47826		
A2m	0.701867	1.41704		-1.87054
Slco1c1	0.355327			0.628088

Slc13a4	0.764586	1.97186		1.66465
Pdia4	0.462279			0.574577
Pon3	0.938093	1.95674		
Aqp1	2.3098	4.09336		2.03643
Slc4a5	2.30275	4.71954		1.95522
Egr4	0.538811			2.43387
Folr1	1.99678	3.64892		1.95393
Cdr2	0.604981	1.10548		-1.43491
Igf2	0.706057	1.52015		1.61939
Fosb	0.818794			1.39926
Spint2	0.626409	1.05814		
Abhd2	0.319449	0.599141		0.509787
Fzd4	0.542651	0.784285		
Cdh3	2.78157	4.28946		
Vat1l	0.649073	1.36456		-2.60184
Junb	0.471423			2.56019
Cpne2	-0.38987	0.661188	0.378233	0.472237
Ccdc135	1.10931	2.51124		
Slc37a2	0.917782	1.25844		
Cgnl1	0.752135	1.08226		
Cldn2	2.13111	4.68814		2.30732
Gpr101	-0.598134			-0.677419

**Table 5:** Transcript expression levels affected by both 3G and AC compared to 1G and placebo, respectively. In bold: when groups 1G and PL presented values different of more than 2 fold, i.e. confinement effect; in italic: when the effects between 1G and 3G and between PL and AC were in opposite ways; for all other sequences, there was no difference between 1G and PL, and both comparisons showed similar increases. The values are expressed as Fragments Per Kilobasepair per Million (FPKM) of the gene in sample.

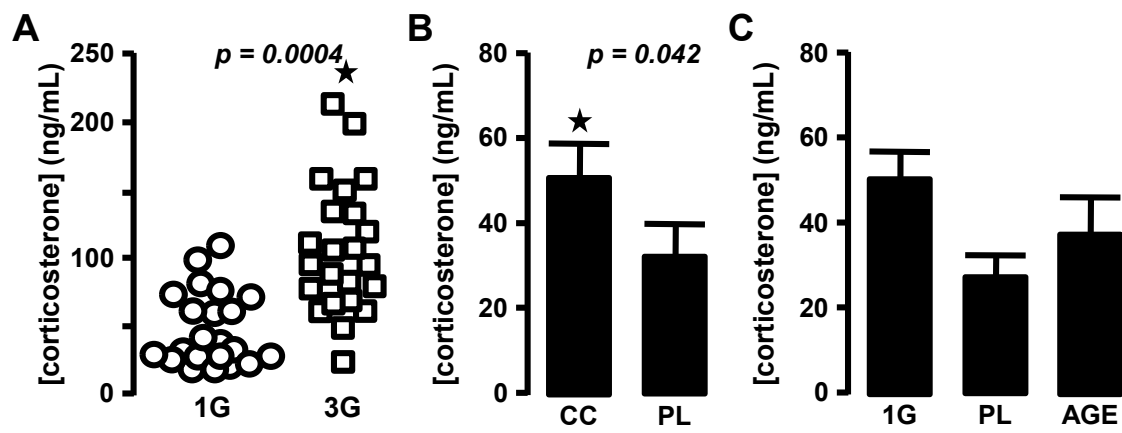
Gene	Locus	Group 1G	Group 3G	Group PL	Group AC
Sulfl	1:12682400-12851259	1.98	4.888	1.024	5.989
Igfbp2	1:72871044-72899041	15.406	28.372	9.198	27.13
Col9a3	2:180332941-180356965	5.792	13.619	3.828	11.937
Lbp	2:158132322-158158118	2.452	6.38	1.687	6.633
Pla2g5	4:138355177-138375157	0.345	1.163	0.202	1.064
Kl	5:151755320-151796908	5.741	13.477	3.428	14.25
A2m	6:121586106-121629223	0.896	1.457	0.697	1.862
Pon3	6:5167314-5206224	0.808	1.549	0.49	1.901
Cdr2	7:128100553-128125695	3.29	5.005	2.772	5.964
Igf2	7:149836670-149846925	18.885	30.807	10.796	30.966
Spint2	7:30041283-30067035	7.703	11.891	5.302	11.039
Abhd2	7:86418109-86510391	13.81	17.232	12.624	19.122
Fzd4	7:96552850-96561625	1.873	2.729	1.836	3.162
Vat1l	8:116729446-116897994	6.811	10.681	5.001	12.877
Slc37a2	9:37035195-37063010	0.624	1.178	0.495	1.183
Cgnl1	9:71474312-71619366	2.871	4.835	2.367	5.012
Ankrd57	10:58684611-58689177	2.013	3.04	1.622	2.959
Tgfb1	13:56710929-56740717	1.199	2.185	1.124	2.359
Cab39l	14:60059798-60167840	7.41	10.238	6.317	11.027
Enpp2	15:54670216-54785330	93.005	261.333	51.59	295.292
Cldn1	16:26356751-26371926	0.692	1.814	0.714	2.409
Cdkn1a	17:29230693-29237671	3.861	6.42	4.491	8.613
Sik1	17:31981268-31992685	2.661	3.917	2.023	4.138
<b>F5</b>	<b>1:166081948-166150560</b>	<b>1.042</b>	<b>3.977</b>	<b>0.3036</b>	<b>4.709</b>
<b>Kcnj13</b>	<b>1:89223550-89347501</b>	<b>0.765</b>	<b>2.999</b>	<b>0.1747</b>	<b>3.887</b>
<b>1500015O10Rik</b>	<b>1:43787414-43799794</b>	<b>4.944</b>	<b>16.642</b>	<b>1.926</b>	<b>15.353</b>
<b>Wfdc2</b>	<b>2:164387945-164393983</b>	<b>0.706</b>	<b>2.887</b>	<b>0.196</b>	<b>2.797</b>
<b>Abca4</b>	<b>3:121746980-121882965</b>	<b>1.112</b>	<b>3.188</b>	<b>0.442</b>	<b>3.451</b>
<b>Col8a2</b>	<b>4:125973864-125991692</b>	<b>0.988</b>	<b>2.345</b>	<b>0.488</b>	<b>2.464</b>
<b>Trpv4</b>	<b>5:115072163-115108406</b>	<b>1.419</b>	<b>4.812</b>	<b>0.533</b>	<b>4.097</b>
<b>Steap1</b>	<b>5:5736328-5749282</b>	<b>0.791</b>	<b>2.814</b>	<b>0.266</b>	<b>2.823</b>
<b>Rbm47</b>	<b>5:66407880-66437455</b>	<b>0.263</b>	<b>0.826</b>	<b>0.128</b>	<b>0.9</b>
-	<b>6:116629116-116635214</b>	<b>0.527</b>	<b>2.175</b>	<b>0.037</b>	<b>2.371</b>
<b>Tmem72</b>	<b>6:116641552-116666943</b>	<b>0.338</b>	<b>1.257</b>	<b>0.074</b>	<b>3.298</b>
<b>Slc13a4</b>	<b>6:35217813-35258151</b>	<b>2.567</b>	<b>4.361</b>	<b>1.262</b>	<b>4.949</b>
<b>Aqp1</b>	<b>6:55286159-55298535</b>	<b>0.941</b>	<b>4.664</b>	<b>0.274</b>	<b>4.67</b>
<b>Slc4a5</b>	<b>6:83187361-83254934</b>	<b>0.649</b>	<b>3.2</b>	<b>0.123</b>	<b>3.233</b>
<b>Folr1</b>	<b>7:108988596-109017870</b>	<b>4.438</b>	<b>17.713</b>	<b>1.327</b>	<b>16.641</b>
<b>Cdh3</b>	<b>8:109079141-109079838</b>	<b>0.131</b>	<b>0.903</b>	<b>0.043</b>	<b>0.844</b>
<b>Ccdc135</b>	<b>8:97579011-97602287</b>	<b>2.471</b>	<b>5.331</b>	<b>1.082</b>	<b>6.166</b>
<b>Ace</b>	<b>11:105829288-105851266</b>	<b>3.804</b>	<b>10.173</b>	<b>1.706</b>	<b>10.035</b>
<b>Sostdc1</b>	<b>12:37040651-37045032</b>	<b>2.486</b>	<b>6.606</b>	<b>0.946</b>	<b>6.513</b>
<b>Otx2</b>	<b>14:49277354-49287141</b>	<b>1.418</b>	<b>4.005</b>	<b>0.483</b>	<b>4.332</b>
-	<b>14:76651174-76652137</b>	<b>5.640</b>	<b>14.079</b>	<b>1.432</b>	<b>10.791</b>

<b>Col8a1</b>	<b>16:57624392-57678659</b>	<b>0.553</b>	<b>1.626</b>	<b>0.181</b>	<b>2.149</b>
<b>Kcne2</b>	<b>16:92292609-92298528</b>	<b>1.715</b>	<b>7.248</b>	<b>0.234</b>	<b>6.082</b>
<b>Clic6</b>	<b>16:92498344-92541580</b>	<b>2.629</b>	<b>8.434</b>	<b>0.831</b>	<b>9.539</b>
<b>Ttr</b>	<b>18:20818884-20832827</b>	<b>375.259</b>	<b>1705.95</b>	<b>69.393</b>	<b>1446.51</b>
<b>Cldn2</b>	<b>X:136335278-136345917</b>	<b>0.831</b>	<b>3.641</b>	<b>0.155</b>	<b>4.004</b>
<i>Lcn2</i>	<i>2:32240155-32243278</i>	<i>0.958</i>	<i>3.167</i>	<i>2.081</i>	<i>0.32</i>
<i>Cpne2</i>	<i>8:97057028-97094435</i>	<i>18.178</i>	<i>13.873</i>	<i>9.387</i>	<i>14.844</i>
<i>Vgll3</i>	<i>16:65828218-65866609</i>	<i>1.016</i>	<i>0.442</i>	<i>0.193</i>	<i>0.821</i>

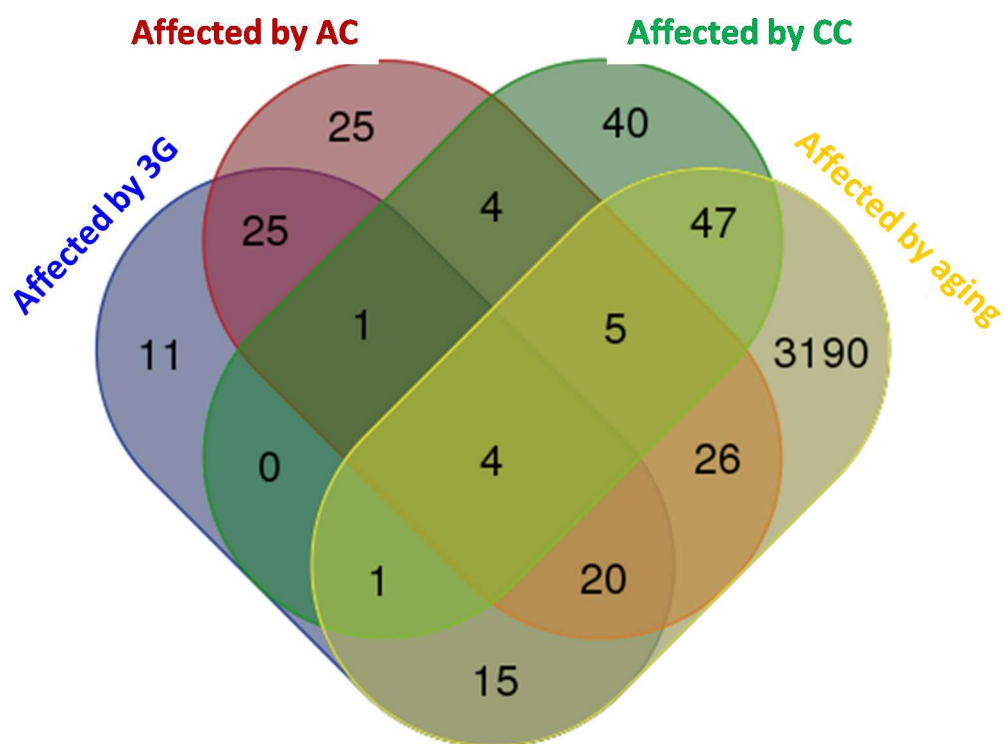
**Table 6:** Transcript expression levels affected by AC but not by 3G and compared to 1G and placebo, respectively (in bold: when groups 1G and PL presented values different of more than 2 fold). The values are expressed as Fragments Per Kilobasepair per Million (FPKM) of the gene in sample.

Gene	Locus	Group PL	Group AC	Group 1G	Group 3G
Prelp	1:135806851-135817995	6.119	10.092	8.991	11.076
Rgs16	1:155587388-155592662	2.468	8.272	2.594	3.453
Plcb4	2:135485156-135840371	4.181	8.883	4.704	4.926
Bmp7	2:172693519-172765977	1.034	2.264	1.743	2.322
Sox18	2:181404542-181406350	2.634	1.088	3.305	3.348
Sgms2	3:131033242-131153082	0.501	1.128	0.690	1.091
Txnip	3:96361859-96365785	6.449	15.570	6.819	9.094
Rims3	4:120527441-120569406	8.236	13.792	8.760	9.828
Id3	4:135699645-135701474	18.669	10.784	18.856	21.987
Alpl	4:137297663-137352249	1.573	3.168	2.559	3.427
Errfi1	4:150229180-150243052	11.745	18.439	12.633	13.599
Hes5	4:154335009-154336494	3.570	1.047	4.734	3.688
Cit	5:116357249-116459005	6.297	11.750	7.865	9.303
AC113316.1	5:147043206-147114511	18.642	291.688	32.813	34.458
Apold1	6:134933638-134936893	0.947	2.796	1.037	1.597
Mdfic	6:15671340-15752163	0.705	2.036	1.041	1.737
Hbb-b1	7:110975050-110982005	355.809	546.092	462.061	501.292
Syt9	7:114514498-114692169	1.253	2.976	1.742	1.506
Zfp36	7:29161807-29164279	1.273	3.202	1.869	2.431
Plekhf1	7:39005604-39012997	1.458	3.225	1.718	1.885
Tnnt1	7:4456181-4466249	0.337	3.947	0.345	0.443
Slc17a6	7:58877006-58926498	3.873	8.414	3.278	3.980
Lars2	9:123276062-123371876	13.944	115.222	14.733	14.913
Amotl1	9:14346410-14447921	2.982	6.149	3.287	3.750
Slc37a2	9:37035195-37063010	0.495	1.183	0.624	1.178
Zic1	9:91252845-91260745	1.643	5.271	2.526	3.483
D10Bwg1379e	10:18299938-18463830	1.924	0.000	2.127	2.047
Perp	10:18564896-18576876	1.122	2.622	1.666	2.276
Sgk1	10:21601862-21719710	17.961	44.267	18.984	25.136
Ddit4	10:59412417-59414734	22.596	35.464	24.492	27.538
Arl4d	11:101526844-101529145	5.293	10.395	6.036	7.725
Adra1b	11:43588132-43649883	0.695	1.875	0.781	0.952
Ramp3	11:6558299-6577482	1.685	6.132	1.948	2.485

Rpl23a	11:77990411-77997079	9.030	2.244	8.150	8.976
Coch	12:52694347-52706789	1.798	4.422	2.338	3.326
Nfkb1a	12:56590395-56593615	8.681	21.278	9.183	11.524
Abhd12b	12:71255075-71285422	0.092	1.096	0.132	0.110
Gadd45g	13:51942029-51943872	11.307	19.046	11.666	14.819
Slitrk6	14:111147307-111154449	0.114	0.589	0.116	0.157
Slc39a4	15:76446259-76447453	0.238	1.038	0.439	0.884
Apol7e	15:77532443-77532675	14.815	0.000	13.428	21.768
Zc3h7a	16:11136694-11176449	18.019	66.577	16.310	16.097
Dusp1	17:26642533-26645639	6.156	10.271	7.292	9.745
AY036118	17:39981973-39985775	78.210	212.687	72.855	73.357
Six3	17:86001750-86025499	0.154	1.102	0.274	0.628
Tcf7l2	19:55816309-56008146	1.157	8.807	1.468	2.675
Htr2c	X:143396975-143631821	6.342	12.437	8.440	9.408
<b>Fmod</b>	<b>1:135933969-135944800</b>	<b>1.600</b>	<b>3.299</b>	<b>3.373</b>	<b>3.279</b>
<b>Ptgds</b>	<b>2:25318620-25321886</b>	<b>0.219</b>	<b>0.935</b>	<b>0.503</b>	<b>0.281</b>
<b>S100a8</b>	<b>3:90473003-90473954</b>	<b>7.764</b>	<b>2.042</b>	<b>1.499</b>	<b>1.563</b>
<b>S100a9</b>	<b>3:90496559-90499221</b>	<b>8.422</b>	<b>2.628</b>	<b>2.099</b>	<b>1.431</b>
<b>Wdr86</b>	<b>5:24216845-24236452</b>	<b>0.240</b>	<b>3.018</b>	<b>0.985</b>	<b>2.856</b>
<b>BC030500</b>	<b>8:61379343-61393163</b>	<b>0.909</b>	<b>0.000</b>	<b>1.982</b>	<b>0.228</b>
<b>Calml4</b>	<b>9:62705887-62723783</b>	<b>1.586</b>	<b>7.113</b>	<b>3.630</b>	<b>8.037</b>
<b>Narg2</b>	<b>9:69245774-69280992</b>	<b>0.091</b>	<b>0.490</b>	<b>0.304</b>	<b>0.482</b>
<b>Pmch</b>	<b>10:87553815-87555214</b>	<b>0.373</b>	<b>2.791</b>	<b>0.155</b>	<b>0.105</b>
<b>Wfikkn2</b>	<b>11:94097301-94104025</b>	<b>0.181</b>	<b>0.651</b>	<b>0.436</b>	<b>0.869</b>
<b>Dio3</b>	<b>12:111517039-111519293</b>	<b>0.689</b>	<b>1.699</b>	<b>2.572</b>	<b>1.545</b>
<b>4930427A07Rik</b>	<b>12:114394889-114403682</b>	<b>1.909</b>	<b>0.244</b>	<b>0.292</b>	<b>0.310</b>
<b>Gm9307</b>	<b>14:103409438-103409867</b>	<b>23.347</b>	<b>5.840</b>	<b>9.150</b>	<b>10.242</b>
<b>Apol7e</b>	<b>15:77532728-77532933</b>	<b>66.098</b>	<b>8.501</b>	<b>5.214</b>	<b>7.550</b>
<b>Glp1r</b>	<b>17:31038889-31077715</b>	<b>0.457</b>	<b>1.020</b>	<b>1.148</b>	<b>1.457</b>



**FIGURE 1**



**FIGURE 2**

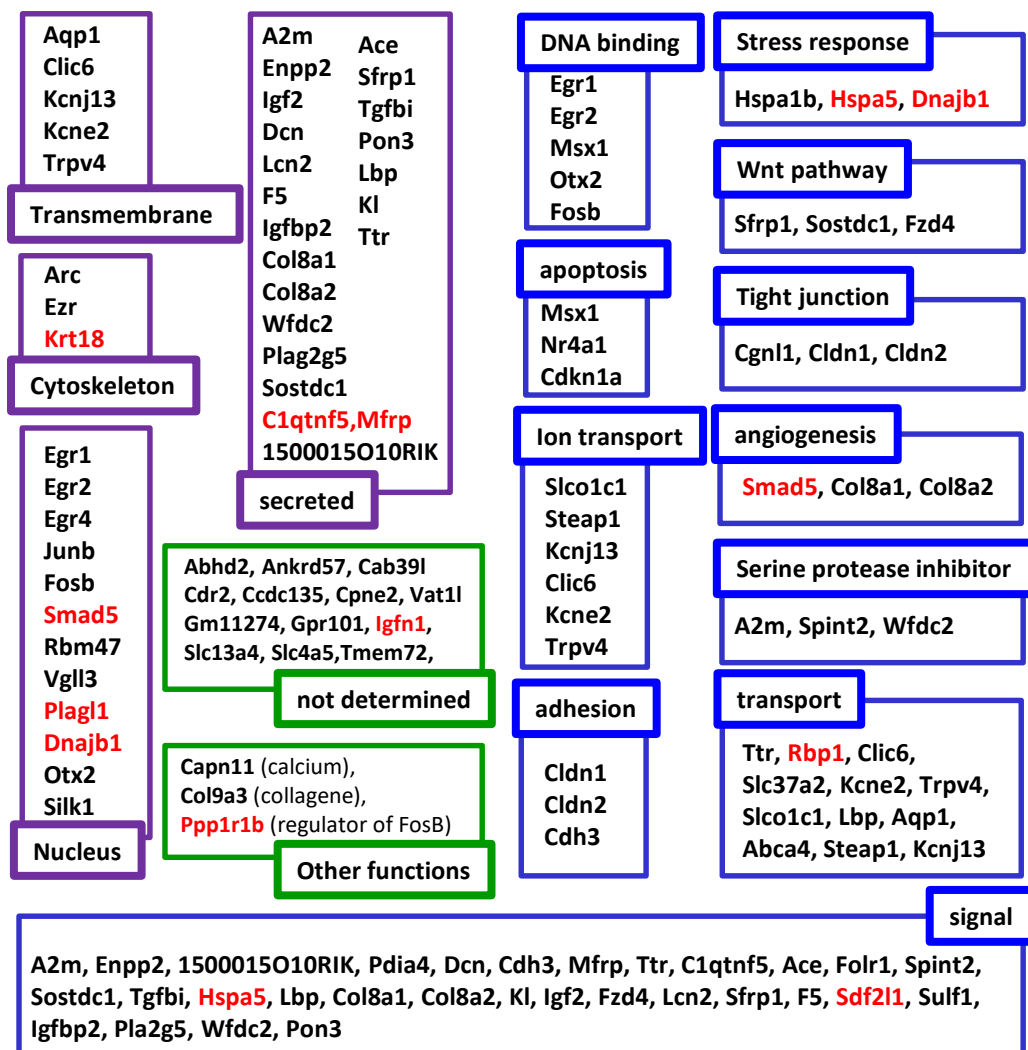
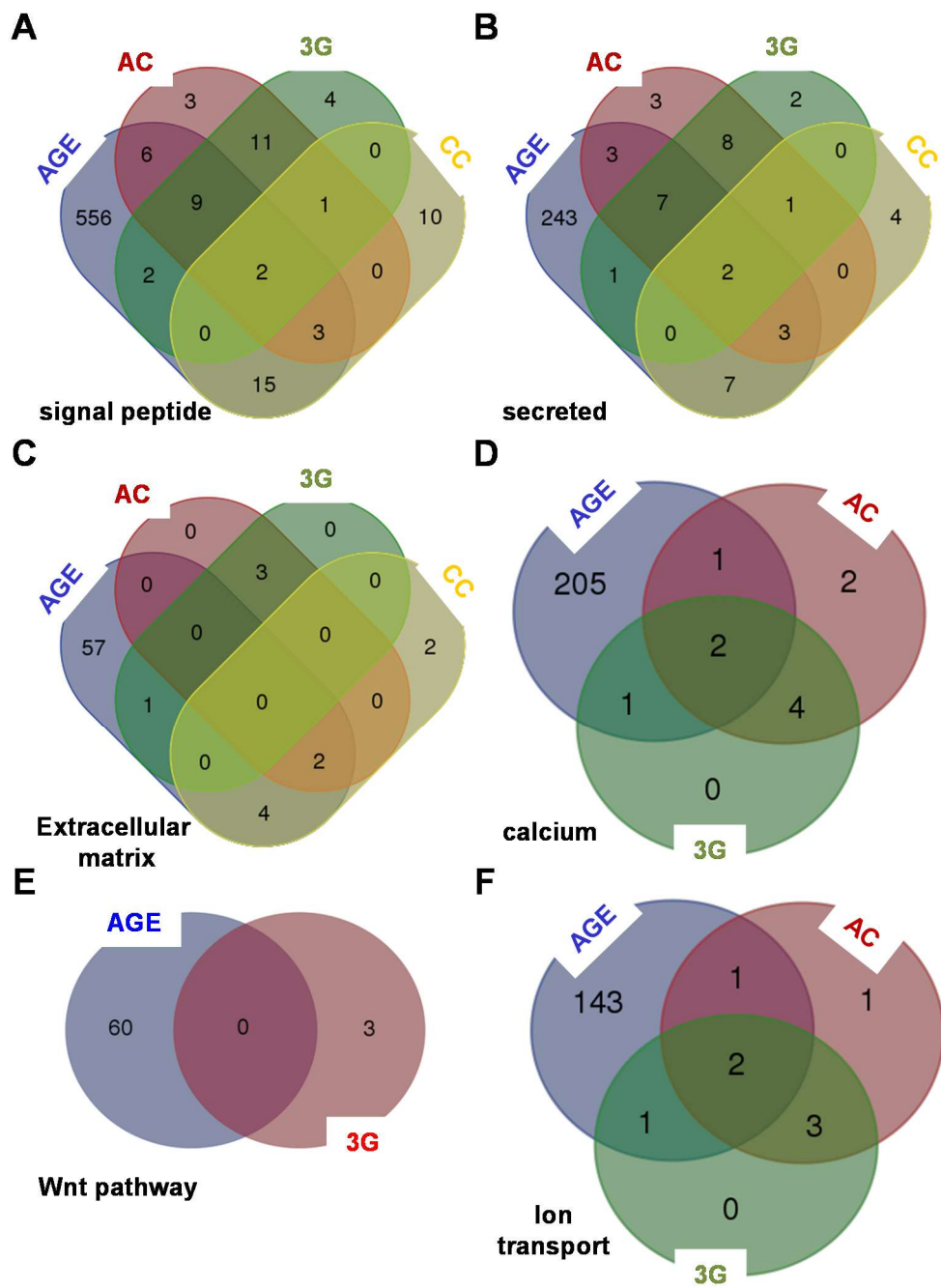


FIGURE 3



**FIGURE 4**



**Supplementary Figure 1:** copy of xls file containing (A) the concentration of RNA from each sample; the color code (B: blue, R red, V green)indicated how the different samples were associated to generate amples for Illumina sequencing and (B) the concentration and RNA mass in each sample sended to GATC for Illumina sequencing.

**A**

Souris	sample	Concentration ng/ $\mu$ L nanodrop	260/280	260/230	Experion score
1G40	Hippocampe Gauche	390	1,77	2,33	9,4
1G41	Hippocampe Gauche	180	1,82	1,62	9,3
1G42	Hippocampe Gauche	280	1,77	2	9,2
1G43	Hippocampe Gauche	190	1,84	1,22	9,3
1G44	Hippocampe Gauche	290	1,76	2,27	9,2
1G45	Hippocampe Gauche	360	1,77	2,29	9,2
1G46	Hippocampe Gauche	380	1,77	2,28	9,1
1G47	Hippocampe Gauche	300	1,77	2,06	9,2
1G48	Hippocampe Gauche	330	1,84	1,15	9,2
1G49	Hippocampe Gauche	350	1,84	1,09	9,5
1G50	Hippocampe Gauche	250	1,79	0,78	9,4
1G51	Hippocampe Gauche	210	1,79	1,97	9,3
1G52	Hippocampe Gauche	330	1,77	2,26	9,3
1G53	Hippocampe Gauche	200	1,81	1,06	9,4
1G54	Hippocampe Gauche	20	1,64	0,57	8,9
1G55	Hippocampe Gauche	250	1,76	2,02	9,3
1G56	Hippocampe Gauche	170	1,78	1,77	9,3
1G57	Hippocampe Gauche	130	1,87	0,29	9,4
1G58	Hippocampe Gauche	240	1,84	1,25	9,3
1G59	Hippocampe Gauche	220	1,92	2,1	9,2
1G60	Hippocampe Gauche	310	1,78	2,09	9,3
1G61	Hippocampe Gauche	250	1,86	0,61	9,3
1G62	Hippocampe Gauche	280	1,8	1,17	9,2
1G63	Hippocampe Gauche	310	1,78	2,04	9,2
Souris	Echantillon	Concentration ng/ $\mu$ L	260/280	260/230	Qualification experion
1G40	Hippocampe Gauche	330	1,8	1,96	9,2
1G41	Hippocampe Gauche	340	1,79	2,2	9,1
1G42	Hippocampe Gauche	150	1,79	1,51	9,1
1G43	Hippocampe Gauche	250	1,77	2,16	9,1
1G44	Hippocampe Gauche	230	1,78	0,9	9,1
1G45	Hippocampe Gauche	280	1,79	1,05	9,1
1G46	Hippocampe Gauche	220	1,81	1,37	8,3
1G47	Hippocampe Gauche	340	1,92	0,46	8,3
1G48	Hippocampe Gauche	220	1,81	1,21	9,2
1G49	Hippocampe Gauche	310	1,82	0,88	9,3
1G50	Hippocampe Gauche	290	1,81	1,45	9,2
1G51	Hippocampe Gauche	230	1,8	0,75	9,3
1G52	Hippocampe Gauche	180	1,82	0,74	9,2
1G53	Hippocampe Gauche	340	1,73	0,54	9,3
1G54	Hippocampe Gauche	180	1,79	0,63	9,2
1G55	Hippocampe Gauche	300	1,8	0,88	9,1
1G56	Hippocampe Gauche	250	1,81	1,43	9,1
1G57	Hippocampe Gauche	260	1,82	0,71	9,1
1G58	Hippocampe Gauche	260	1,77		9,7
1G59	Hippocampe Gauche	230	1,77	2,2	9,7
1G60	Hippocampe Gauche	240	1,77	1,34	9,4
1G61	Hippocampe Gauche	280	1,79	1,34	9,3
1G62	Hippocampe Gauche	370	1,79	2,03	9,1
1G63	Hippocampe Gauche	250	1,75	2,31	9,3

mouse	sample	Concentration ng/ $\mu$ L	260/280	260/230	Experion
CA1	campe G	275	1,86	0,54	8,7
CA2	campe G	175	1,88	0,65	8,6
CA3	campe G	300	1,75	2,18	9,6
CA4	campe G	300	1,75	2,07	9,6
CA5	campe G	300	1,79	1,84	9,4
CA6	campe G	100	1,85	0,74	9,1
CA7/Ba	campe G	100	1,81	0,71	9,2
CA7/Bb	campe G	50	1,69	0,33	8,5
CA10	campe G	300	1,8	1,24	9,6
CA9	campe G	275	1,7	1,15	9,4

mouse	sample	Concentration ng/ $\mu$ L	260/280	260/230	experion
P1	Hippocampe Gauche	325	1,81	1,5	8,8
P2	Hippocampe Gauche	175	1,92	0,71	8,8
P3	Hippocampe Gauche	250	1,86	1,89	8,8
P4	Hippocampe Gauche	275	1,81	1,13	8,8
P5	Hippocampe Gauche	350	1,6	1,51	8,7
P6	Hippocampe Gauche	275	1,79	1,31	8,7
P7	Hippocampe Gauche	250	1,91	0,81	8,7
P8	Hippocampe Gauche	150	1,91	0,29	8,9
P9	Hippocampe Gauche	200	1,77	1,06	8,5
CC1	Hippocampe Gauche	200	1,74	0,78	9,1
CC2	Hippocampe Gauche	275	1,87	0,38	9,1
CC3	Hippocampe Gauche	375	1,72	0,73	9
CC4	Hippocampe Gauche	250	1,72	1,92	8,9
CC5	Hippocampe Gauche	375	1,83	0,57	8,9
CC6	Hippocampe Gauche	350	1,72	1,96	8,9
CC7	Hippocampe Gauche	225	1,78	0,75	9
CC8	Hippocampe Gauche	700	1,21	0,92	8,9
CC9	Hippocampe Gauche	225	1,82	0,82	9
CC10	Hippocampe Gauche	250	1,81	0,32	8,9

mouse	sample	Concentration ng/ $\mu$ L	260/280	260/230	experion
V1G	ampe G	150	1,7	0,36	9
V2G	ampe G	300	1,69	0,58	9,4
V3G	ampe G	700	1,76	0,81	8,6
V4G	ampe G	450	1,66	0,64	9,3
V5G	ampe G	450	1,72	0,68	8,5
V6G	ampe G	525	1,8	0,94	8,4
V7G	ampe G	475	1,68	0,58	?
V1D	campe	325	1,73	0,93	9,5
V2D	campe	25	1,47	0,14	7,6
V3D	campe	275	1,51	0,31	9,2
V4D	campe	425	1,74	0,83	8,6
V5D	campe	250	1,65	0,7	9,3
V6D	campe	325	1,69	0,52	9,2
V7D	campe	150	1,75	0,26	9

**B**

Echantillon	Groupe	Volume ( $\mu$ L)	Concentration (ng/ $\mu$ L)	Masse ( $\mu$ g)	260/280	260/230
A 1G B	1G	200	275	55	1,87	1,13
A 1G R	1G	200	250	50	1,82	1,01
A 1G V	1G	200	275	55	1,89	1,11
B 3G B	3G	200	225	45	1,86	0,8
B 3G R	3G	200	225	45	1,8	0,47
B 3G V	3G	200	275	55	1,84	0,84
C CA B	Cort Aigu	150	250	37,5	1,85	0,95
C CA R	Cort Aigu	150	225	33,75	1,87	0,7
C CA V	Cort Aigu	150	225	33,75	1,94	0,63
D CC B	Cort Chronique	100	250	25	1,81	0,36
D CC R	Cort Chronique	100	200	20	1,82	1,1
D CC V	Cort Chronique	100	300	30	1,91	0,35
E PL B	Placebo	100	250	25	1,85	1,19
E PL R	Placebo	100	175	17,5	1,85	0,4
E PL V	Placebo	100	225	22,5	1,83	1,42
F VA B	Agé	100	200	20	1,6	0,26
F VA R	Agé	100	325	32,5	1,75	0,5
F VA V	Agé	100	325	32,5	1,71	0,63

**Supplementary Figure 2:** (A) results of sequencing expressing in number of sequenced reads and bases expressed in base and (B) results of mapped reads (copy of GATC reports)

**A**

sample	sequenced reads	sequenced bases
AG1B	39446700	1972335000
AG1R	33624700	1681235000
AG1V	22964500	1148225000
AG3B	34557200	1727860000
AG3R	34119400	1705970000
AG3V	40110200	2005510000
CCAB	41079000	2053950000
CCAR	33144900	1657245000
CCAV	37772000	1888600000
DCCB	35360800	1768040000
DCCR	30866100	1543305000
DCCV	33031500	1651575000
EPLB	32688880	1634444000
EPLR	35147710	1757385500
EPLV	41968300	2098415000
FVAB	40329230	2016461500
FVAR	27225420	1361271000
FVAV	29389890	1469494500

**B**

**NG-6365**

Library	Project	Sample	Total reads	Mapped reads	% Mapped reads
16414	NG-6365	AG1B	39,446,700	37,296,118	94.55%
16415	NG-6365	AG1R	33,624,700	31,901,644	94.88%
16416	NG-6365	AG1V	22,964,500	21,647,527	94.27%
16417	NG-6365	B3GB	34,557,200	32,698,253	94.62%
16418	NG-6365	B3GR	34,119,400	32,344,064	94.80%
16419	NG-6365	B3GV	40,110,200	37,987,368	94.71%
16420	NG-6365	CCAB	41,079,000	38,998,312	94.93%
16421	NG-6365	CCAR	33,144,900	31,259,033	94.31%
16422	NG-6365	CCAV	37,772,000	35,606,665	94.27%
16423	NG-6365	DCCB	35,360,800	33,526,730	94.81%
16424	NG-6365	DCCR	30,866,100	29,176,021	94.52%
16425	NG-6365	DCCV	33,031,500	31,276,350	94.69%
16426	NG-6365	EPLB	28,722,700	26,581,678	92.55%
16427	NG-6365	EPLR	30,811,500	28,639,296	92.95%
16428	NG-6365	EPLV	37,000,800	34,201,239	92.43%
16429	NG-6365	FVAB	35,476,700	32,986,892	92.98%
16430	NG-6365	FVAR	23,844,600	22,088,099	92.63%
16431	NG-6365	FVAV	25,849,800	23,950,410	92.65%

**Reference**

Genome: mm9 release64

Annotation: Mus\_musculus.NCBIM37.64.gtf (ensembl)



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Le cerveau est l'un des organes les plus perfusé du corps, mais a une capacité limitée à stocker l'énergie. Pour cette raison, il dépend fortement du flux sanguin cérébral. Les systèmes neuronaux et vasculaires communiquent étroitement pour coordonner leurs actions. Comme la microcirculation cérébrale est capable d'adapter son activité pour fournir les quantités nécessaires d'énergie et de nutriments imposées par la demande métabolique des réseaux neuronaux, il est raisonnable de penser qu'une réorganisation vasculaire se produit pour permettre l'approvisionnement en énergie adéquat de réseaux neuronaux spécifiques recrutés lors de processus mnésiques.

Ce processus dynamique de plasticité vasculaire est particulièrement mal connu lors des processus cognitifs. C'est pourquoi nous l'avons exploré lors du processus de la consolidation mnésique, qui peut se définir comme le processus par lequel une trace mnésique acquiert stabilité et persistance dans le temps. Dans notre modèle, la consolidation mnésique exige un dialogue temporaire entre l'hippocampe et les régions corticales qui servent de dépôt permanent des souvenirs.

Nous avons choisi un paradigme comportemental particulièrement adapté à l'étude de la consolidation de la mémoire : la tâche de transmission sociale de préférence alimentaire (TSPA). Cette tâche est basée sur des études éthologiques démontrant que les interactions sociales entre des rats vont conduire à l'apparition d'une préférence alimentaire durable pour la nourriture qui a été consommée par les autres congénères, formant une mémoire associative olfactive. Le TSPA permet d'encoder rapidement des informations olfactives lors d'une seule session d'interaction, permettant une phase d'acquisition ponctuelle sans répétition des essais d'apprentissage afin d'offrir un contrôle rigoureux de la cinétique d'induction des mécanismes post-apprentissages impliqués dans la consolidation systémique, et d'induire une mémoire robuste et durable. En plus, sa nature associative nécessite l'hippocampe et des régions corticales spécifiques telles que le cortex orbitofrontal et cingulaire antérieur qui sont impliqués dans le traitement de l'information olfactive associative. Enfin, sa nature non spatiale restreindre l'implication fonctionnelle de l'hippocampe au processus de consolidation.

Dans un précédent ensemble d'études réalisé dans l'équipe de Bruno Bontempi par Anaïs Giacinti pendant son doctorat (Giacinti, 2014), il a été constaté que la réactivité et l'architecture des vaisseaux cérébraux peuvent être modifiées pendant la consolidation de la mémoire (augmentation de la réactivité de l'artère cérébrale postérieure, irrigant l'hippocampe, pendant l'expression de la mémoire récente; augmentation de la réactivité de l'artère cérébrale antérieure, irrigant le cortex, lors de l'expression de la mémoire ancienne). De plus des données préliminaires rapportées dans cette thèse suggèrent une augmentation transitoire de la densité vasculaire dans le cortex cingulaire antérieur (CCA), proposée comme le signe du déclenchement d'un processus angiogénique.

Sur la base de ces résultats prometteurs, nous avons conçu une série d'expériences de suivi spécifiques visant à décrypter ces processus angiogéniques potentiellement déclenchés lors de l'encodage des informations olfactives dans la tâche du TSPA chez les rats Sprague Dawley (SDs). À cette fin, nous avons exploré dans le premier temps les mécanismes putatifs responsables de l'angiogenèse locale dans le CCA. 24h après l'interaction, des cellules de l'ACC, et seulement dans l'ACC, présentent un niveau d'hypoxie détectable par le marqueur hypoxique Pimonidazole. Cette hypoxie persistante de plusieurs cellules, 24 heures après l'encodage pourraient alors déclencher un processus angiogénique afin de réorganiser le réseau vasculaire et le rendre plus performant à proximité des réseaux neuronaux impliqués dans le mécanisme de consolidation.

L'expression des protéines connues pour induire, contrôler et inhiber le processus d'angiogenèse est régulée dans le temps au sein de l'ACC. La littérature sur l'angiogenèse nous apprend que dans les cellules endothéliales l'expression de l'angiopoïétine-2 (Ang-2) est induite par l'hypoxie, et d'autre part la phase angiogénique est corrélée avec une augmentation de l'expression d'Ang-2 et Vascular Endothelial Growth Factor (VEGF), tandis que la phase de maturation des vaisseaux sanguins est associée à une augmentation relative de l'Angiopoïétine-1 (Ang-1) et à une diminution du VEGF. Enfin, à ce jour l'Ang-2 est spécifique de la sphère vasculaire par rapport à d'autres facteurs de croissance, tels que le VEGF, qui agissent et sont exprimés également dans les neurones et les astrocytes dans le cerveau adulte.

Nous avons déterminé que l'Ang-2 est surexprimée précocement au cours de la tâche de TSPA et spécifiquement dans l'ACC, suggérant sa participation à la consolidation de la mémoire associative olfactive. Cette augmentation d'expression de l'Ang-2 induite par la mémoire était absente immédiatement après l'interaction sociale, mais reste significative 3 jours après l'interaction sociale ; dans le même temps l'expression d'Ang-1. Cela suggère que l'Ang-2 peut favoriser une angiogenèse en provoquant la déstabilisation des vaisseaux en inhibant l'action de l'Ang-1. Ce mécanisme est transitoire puisque le taux d'expression d'Ang-2 revient au niveau basal au sixième jour après l'interaction, suggérant que l'angiogenèse est liée à la phase précoce de la consolidation de la mémoire. L'approche corrélative a été poursuivie par la mise en évidence de la présence de cellules en prolifération associées aux capillaires intracérébraux, et que le réseau vasculaire perfusé, révélé par le bleu Evan, était significativement plus important chez les animaux engagés dans le processus de consolidation mnésique. Mais pour affirmer que ce mécanisme pouvait soutenir le dialogue neuronal entre HPC et CCA, nous avons entrepris une approche invasive visant à moduler l'angiogenèse, via une action sur l'Ang-2, au cours de la phase précoce de la consolidation de la mémoire. Nous avons alors émis l'hypothèse que le blocage ou l'amélioration de l'angiogenèse corticale au cours de la phase précoce de consolidation mnésique devrait soit diminuer ou améliorer la formation ultérieure de la mémoire associative olfactive ancienne en influant sur la réorganisation des réseaux neuronaux corticaux soutenant la mémoire à long terme. L'angiogenèse a été inhibée au moyen d'infusions intracorticales d'oligonucléotides antisens dirigés contre Ang-2. Ces infusions post-encodage limitées dans le temps, au sein de la CCA au cours de la phase précoce de consolidation de la mémoire, ont détérioré la récupération de la mémoire ancienne chez les rats testés 30 jours après l'interaction sociale dans le paradigme TSPA, indiquant une incapacité à former correctement et/ou récupérer des informations anciennes acquises dans le temps. Ainsi, même si l'Ang-2 n'est pas le seul acteur impliqué dans la modulation de l'angiogenèse, son inhibition sélective de l'expression était suffisante pour avoir un impact sur la performance mnésique, indiquant la nécessité de ce facteur dans le processus de consolidation mnésique. Vice-versa, l'infusion intracérébrale du peptide Ang-2, au cours de la phase précoce de consolidation de la mémoire, induit une amélioration de la mémoire chez des rats testés 30 jours après l'interaction sociale. Ces résultats ont souligné le rôle de l'Ang-2 comme facteur impliqué dans l'expression de la mémoire à long terme. Ainsi, nous avons pu proposer que l'angiogenèse corticale précoce soit un mécanisme permissif crucial qui soutient l'intégration progressive dans les réseaux neuronaux corticaux.

En parallèle, nous avons étudié la signification fonctionnelle du mécanisme angiogénique corticale identifié dans des conditions physiologiques en examinant les effets délétères potentiels de l'hypertension sur l'organisation de souvenirs récents et anciens en utilisant le modèle de rats hypertendus (SHRs). Après avoir exclu des biais expérimentaux comportementaux qui pouvaient affecter la mesure des performances mnésiques dans le test de TSPA, nous avons pu établir une détérioration sélective de la mémoire à long terme sans atteinte de la mémoire récente, atteinte qui est dépendante de la durée d'hypertension. Ces résultats suggèrent un encodage réussi, mais une incapacité à stabiliser et / ou à récupérer adéquatement des informations acquises à distance. Cet effet semble d'ailleurs bien lié à l'hypertension puisque l'utilisation du Losartan (un antagoniste des récepteurs de l'angiotensine-II, puissant anti-hypertenseur), restaure la consolidation mnésique, tout en régulant la pression artérielle. Ces résultats suggèrent que le phénotype hypertendu serait responsable de l'incapacité des animaux à conserver le souvenir des informations acquises. Dans ce modèle animal, nous avons également mis en évidence l'absence de l'augmentation de l'expression d'Ang-2 au troisième jour suivant l'interaction sociale et l'absence de prolifération cellulaire pericapillaire. Enfin, l'injection d'Ang-2 dans l'ACC chez le SHR restaure la performance de mémoire ancienne confirmant le rôle crucial de ce nouveau mécanisme dans la consolidation de la mémoire ancienne.

L'ensemble de nos résultats suggèrent également un mécanisme permissif de l'angiogenèse corticale stimulée immédiatement après l'encodage dans la consolidation de la mémoire.

Enfin, nous avons étendu notre analyse aux effets de la modification de la gravité sur la microvasculature cérébrale. En effet, les modifications de la gravité modifient d'une part la réactivité vasculaire et d'autre part des travaux suggèrent des atteintes de la mémoire spatiale, nous avons voulu



tester l'hypothèse selon laquelle l'hypergravité pourrait interférer avec le processus de consolidation de la mémoire via la perturbation du système vasculaire dans un test non-spatial comme le test de TSPA. Des rats SDs ont donc subi une exposition chronique à l'hypergravité à 2G et l'impact de ce traitement environnemental sur les processus de codage, de consolidation et de récupération a été évalué en utilisant le paradigme du TSPA. L'hypergravité à 2G appliquée entre l'encodage et la restitution (pendant 21 jours) ou avant l'encodage (pendant 60 jours) n'est pas en mesure de modifier l'organisation de la mémoire. Ces résultats suggèrent que les modifications gravitaires ne sont pas en mesure d'altérer ni la consolidation de la mémoire non-spatiale ni les mécanismes du dialogue hippocampo-cortical.

Dans l'ensemble, notre approche intégrative et transversale nous a permis de fournir de nouvelles connaissances sur la dynamique de la sphère capillaire cérébrale au cours de la consolidation de la mémoire. Nos résultats identifient l'angiogenèse corticale précoce comme un processus neurobiologique crucial pour aider à la formation et la stabilisation de la mémoire ancienne. Ils révèlent l'importance de la plasticité vasculaire dans la modulation des fonctions cognitives et suggèrent que les changements structurels précoces du réseau vasculaire cérébral constituent un mécanisme permissif pour la régulation de la plasticité neuronale au sein des réseaux corticaux impliqués dans la formation progressive et le stockage des souvenirs. Ce mécanisme vasculaire est davantage discuté à la lumière des connaissances existantes sur l'angiogenèse cérébrale et un modèle putatif incorporant l'implication fonctionnelle de la sphère vasculaire cérébrale à la dynamique des interactions neuronales hippocampique-corticales au cours du processus de consolidation de mémoire est proposé.