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Nicolas Blin

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THÈSE PRÉSENTÉE
POUR OBTENIR LE GRADE DE

**DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX**

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ
SPÉCIALITÉ : NEUROSCIENCES

Nicolas BLIN

**ROLE OF ADULT-BORN DENTATE NEURONS
IN THE RESILIENCE TO COGNITIVE AGING**

**RÔLE DES NÉO-NEURONES DU GYRUS DENTÉ DANS LA RÉSILIENCE AU
VIEILLISSEMENT COGNITIF**

Sous la direction de : Dr. Djoher Nora ABROUS

Soutenue le 14/12/2023

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Blin, N., Rupprecht, L., Lie D.C., Farrugia, F., Charrier, V., Abrous, D.N. Successful cognitive aging relies on healthy adult-born hippocampal neurons. Symposium Neurocentre Magendie – 2022 – Bordeaux, France.

Blin, N., Rupprecht, L., Lie D.C., Farrugia, F., Montaron, MF., Charrier, V., Abrous, D.N. Responsiveness and health of dentate neurons generated throughout adult life is associated with resilience to cognitive aging. European Brain & Behavior Society (EBBS) – 2021 – Lausanne, Switzerland.

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ABSTRACT

The progressive decline of memory associated with aging affects a significant portion of the population but varies in speed and degrees among individuals. Some individuals preserve good memory abilities with age and are considered resilient, while others experience a significant loss in their abilities and are considered vulnerable. The hippocampus, a crucial brain structure for memory processes, undergoes numerous changes during aging, partially explaining the decline in memory abilities with age. Nevertheless, the dentate gyrus of the hippocampus maintains a form of plasticity throughout life consisting in the creation of new dentate granule neurons. This phenomenon plays a predominant role in learning and memory, especially in spatial learning, and represents a factor of resilience against cognitive decline. Due to this continuous production of new neurons, the dentate gyrus is composed of heterogeneous populations of granule neurons, each adopting specific morphologies depending on the period of life during which they are produced. Measuring spatial memory in the watermaze as an indicator of cognitive abilities, we first causally identified using optogenetics which ontogenic wave of dentate granule neurons is involved in learning in young animals. We highlighted the predominant role of neurons generated in adulthood (or adult-born neurons), as well as the transient role of neurons generated during adolescence, as long as adult-born neurons are not mature enough to assume these functions. In the context of aging and inter-individual differences, we demonstrated using Zif268 as a proxy for neural activity that the maintenance of good memory abilities at old age is related to the ability of adult-born neurons (and them only) to be recruited during learning. This ability is associated with the preservation of the mitochondrial homeostasis and glutamatergic synaptic inputs to adult-born neurons. Specifically, our results suggest that the preservation of inputs from mossy cells and neurons in the supramammillary nucleus is a key factor in preserving learning abilities. Finally, we were able to demonstrate that the stimulation of adult-born neurons by optogenetic is sufficient to improve memory retention at old age. In conclusion, this thesis project has highlighted the essential role played by adult-born neurons in maintaining memory functions with age.

Key words: Cognitive aging, inter-individual differences, memory, hippocampus, neurogenesis.

RÉSUMÉ

Le déclin progressif de la mémoire associé au vieillissement concerne une part importante de la population mais est néanmoins variable et se développe à des vitesses et degrés différents entre individus. Certains individus parviennent à préserver de bonnes capacités de mémoire et sont considérés comme résilients, tandis que d'autres subissent une nette détérioration de leurs capacités et sont considérés comme vulnérables. L'hippocampe, une structure cérébrale essentielle pour les processus de mémoire, subit de nombreuses altérations au cours du vieillissement, ce qui explique en partie le déclin des capacités mnésiques avec l'âge. Néanmoins, le gyrus denté de l'hippocampe préserve une forme de plasticité cérébrale tout au long de la vie ; la création de néo-neurones granulaires. Ce processus de plasticité joue un rôle important dans l'apprentissage spatial, et constitue un facteur de résilience face au déclin cognitif. En raison de cette production continue de neurones, le gyrus denté est composé de populations hétérogènes de neurones granulaires, chacune adoptant des morphologies particulières selon son origine temporelle. En mesurant la mémoire spatiale dans le labyrinthe aquatique de Morris comme indicateur des capacités cognitives des animaux, nous avons dans un premier temps identifié de manière causale la vague ontogénique de neurones granulaires impliquée dans l'apprentissage spatial chez de jeunes animaux. Nous avons mis en évidence le rôle prépondérant des neurones générés à l'âge adulte (ou néo-neurones) dans l'apprentissage spatial, ainsi qu'un rôle transitoire des neurones générés lors de l'adolescence tant que les néo-neurones ne sont pas suffisamment matures et à même d'assumer ces fonctions. Dans le contexte du vieillissement et des différences inter-individuelles, nous avons démontré en utilisant Zif268 comme proxy de l'activité neuronale que le maintien de bonnes capacités mnésiques au vieil âge est lié à la capacité des néo-neurones (et eux seuls) à être recrutés lors d'un apprentissage. Le maintien de ce recrutement est possible grâce à la préservation de l'homéostasie métabolique mitochondriale et des afférences synaptiques glutamatergiques des néo-neurones. Plus précisément, nos résultats suggèrent que la préservation des afférences synaptiques en provenance des cellules moussues et des neurones du noyau supramammillaire est un facteur clé dans la préservation des capacités d'apprentissage. Enfin, nous avons montré que la stimulation de ces nouveaux neurones par optogénétique est suffisante pour

augmenter la rétention mnésique chez les animaux. Il a été possible d'améliorer les capacités de rétention en les stimulant artificiellement par optogénétique. En conclusion, ce projet de thèse a permis de mettre en évidence le rôle essentiel joué par les néo-neurones dans le maintien des fonctions mnésiques avec l'âge.

Mots-clés: Vieillissement cognitif, différences inter-individuelles, mémoire, hippocampe, neurogénèse.

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ABBREVIATIONS

Adol-DGNs	Adolescence-born dentate granule neurons
Adu-DGNs	Adult-born dentate granule neurons
AHPs	Afterhyperpolarizations
Ara-C	Cytosine-D-arabinofuranoside
BLBP	Brain lipid binding protein
BrdU	Bromodeoxyuridine
CA	Cornu Ammonis
CldU	Chlorodeoxyuridine
DCX	Doublecortin
Dev-DGNs	Developmentally-born dentate granule neurons
DG	Dentate gyrus
DGNs	Dentate granule neurons
EC	Entorhinal cortex
Emb-DGNs	Embryonically-born dentate granule neurons
FGF-2	Fibroblast growth factor 2
GAD67	Glutamic acid decarboxylase-67
GCL	Granule cell layer
GVC	Ganciclovir
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
HDACs	Histones deacetylases
HICAP	Hilar commissural-associated pathway related
HIPP	Hilar perforant path-associated
HPA	Hypothalamo-pituitary-adrenal
IdU	Iododeoxyuridine
IEGs	Immediate early genes
IFN-γ	Interferon-gamma
IGF-1	Insulin-like growth factor 1
IML	Inner molecular layer
LEC	Lateral entorhinal cortex
LTD	Long-term depression
LTP	Long-term potentiation
MAM	Methylazoxymethanol acetate
MEC	Medial entorhinal cortex
mEPSCs	Miniature excitatory post-synaptic currents
MFBs	Mossy fiber boutons
mIPSCs	Miniature inhibitory post synaptic currents
ML	Molecular layer
MML	Middle molecular layer
MOPP	Molecular layer perforant path-associated
Neo-DGNs	Neonatally-born dentate granule neurons
NSCs	Neural stem cells
NPY	Neuropeptide Y
OML	Outer molecular layer
PSA-NCAM	Polysialylated-neural cell adhesion molecule
S100β	S100 calcium-binding protein β
SGZ	Sub-granular zone
SOM	Somatostatin
SuM	Supramamillary area
TGF-α	Transforming growth factor α
TGF-β	Transforming growth factor β
TMZ	Temozolomide
VEGF	Vascular endothelial growth factor

CHAPTER I: INTRODUCTION

FOREWORD

The work presented in this thesis has for objective to better understand the neurobiology of cognitive aging focusing on adult hippocampal neurogenesis. Instead of focusing on the effect of aging *per se* based on the differences between young and old individuals, our research centered on exploring the variability within the aging process, using spatial learning as an indicator of memory abilities. Specifically, we investigated why some individuals age successfully while others do not. In this context, we aimed at elucidating the role of one among many potential contributors to this variability: adult neurogenesis in the dentate gyrus.

However, before addressing the role of adult neurogenesis in the context of aging, several preliminary steps were needed. Currently, one prevailing working hypothesis in the neurogenesis field stipulates that once adult-born dentate granule neurons (adu-DGNs) reach maturity, they become indistinguishable from the rest of granule neurons born during the development (dev-DGNs). According to this hypothesis, adu-DGNs and dev-DGNs should therefore play a similar role in spatial memory and in aging. Another prevailing hypothesis in the field suggests that adu-DGNs are functionally relevant only when they are in their immature state, and that they are later removed from the network or that they become functionally irrelevant. This hypothesis predicts that inter-individual variabilities in cognitive aging would only depend on immature adu-DGNs.

In the first chapter of this work, we aimed at clarifying whether adu-DGNs and dev-DGNs populations were indeed indistinguishable and both needed for spatial learning in young adults. We also aimed at investigating whether adu-DGNs are involved in learning when immature or mature (Masachs et al., 2021). As a result of these experiments, we showed a specific involvement of mature adu-DGNs in spatial learning. This preliminary step of identification done, we then focused on cognitive aging. In our experiments, we therefore adopted a unique approach relying on the study of long mature adu-DGNs in the inter-individual differences in spatial memory. The results are presented in the second chapter (Montaron et al., 2020) and third chapter (Blin et al., *in submission*), in which we monitored the aging of mature adu-DGNs over 1 to 2 years.

I. COGNITIVE AGING

A. An emerging major player in future health

The world population has significantly increased over the past decades. Demographic predictions continue to forecast a clear rise in the years to come (Figure 1A). This increase is predominantly due to population aging, with life expectancy having markedly risen, especially due to new medicines and therapeutic approaches. The age category of 65+ years is predicted to be one of the age groups that will undergo one of the most significant growth in the next decades, with nearly double numbers. The 65+ population is predicted to outnumber the number of people aged under 24 years-old, marking a milestone in demographics (Figure 1B).

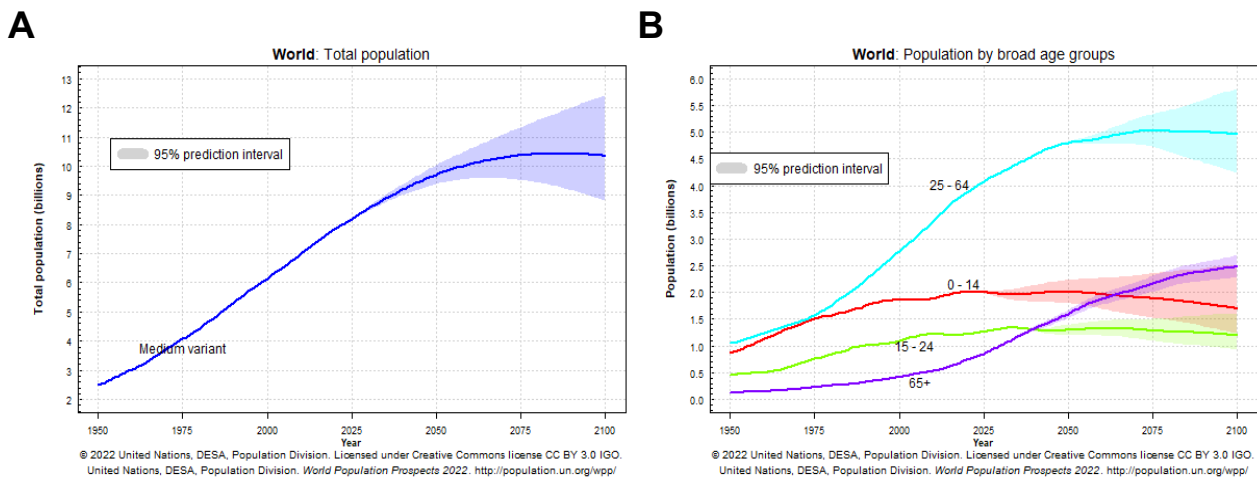


Figure 1: World population Trends and Projections: Overall Growth (1950-2100) for the total population and by broad age groups. (A) World total population evolution and projection (in blue). (B) Population evolution and projection by age groups, with 0-14 in red, 15-24 in green, 25-64 in blue and 65+ in purple. From United Nations World Population prospects 2022 <https://population.un.org>

Unfortunately, this augmentation is accompanied by an increase in age-related disorders. Therefore, the increase in demographics is going to become a new “epidemic” situation with the older population at high-risk for disorders. These disorders cover many conditions, such as physical pain, vision and hearing loss, cardiovascular diseases, diabetes, osteoarthritis, cancer and decline in memory capacities. Altogether, these disorders are a threat for the quality of life and affect the elderly population independence. The development of novel therapeutics and diagnostic methods to help the aging populations therefore seems of essential importance in research today. Aging itself is not considered as a disease, but a natural biological process.

However, given such implications for the future of the population, it has received more and more attention, including the brain and its evolution with aging, for which the maintenance of mental capacities has become a top-tier goal. The work presented here will only focus on cognitive aging and will set aside degenerative-related pathologies. Indeed, age-related cognitive impairments can be divided into two main categories: pathological aging, most often characterized by degenerative diseases leading for example to Parkinson's disease or Alzheimer's disease to cite the most well-known, and non-pathological aging or "normative" aging, which will be referred to as cognitive aging.

Among all the brain functions impacted by aging, cognition is the most affected. Cognition refers to the mental ability to assimilate and process received information through experiences, thoughts and intelligence, and subsequently convert it into knowledge. Cognition allows multiple functions, such as learning, memory, attention, reasoning, judgment or decision making. These functions allow for an individual to adapt its behavior to environmental demands and stresses. Cognitive aging is characterized by a multitude of alterations, such as impaired processing speed, altered language, impaired visuospatial and executive functions, but also impaired memory abilities (Harada et al., 2013). The work presented here will focus on hippocampal-dependent memory and its decline associated with aging.

B. Memory in cognitive aging

1. The different types of memory

Memory refers to the process through which information is acquired, stored or forgotten, and retrieved. It involves the dynamic interactions of various brain regions and neural networks that enable the encoding (acquisition of the experienced information), consolidation (long-term storage) and retrieval (restitution of the memory) of experiences. Eventually, non-necessary information will be forgotten and erased from memory. As first hypothesized in 1972 by Endel Tulving (Tulving, 1972), memory is numerous and complex as represented in [figure 2](#). It allows to address variable situations encountered and to face challenges, and thus represents an adaptive mechanism needed for the survival of the individual but also of the species.

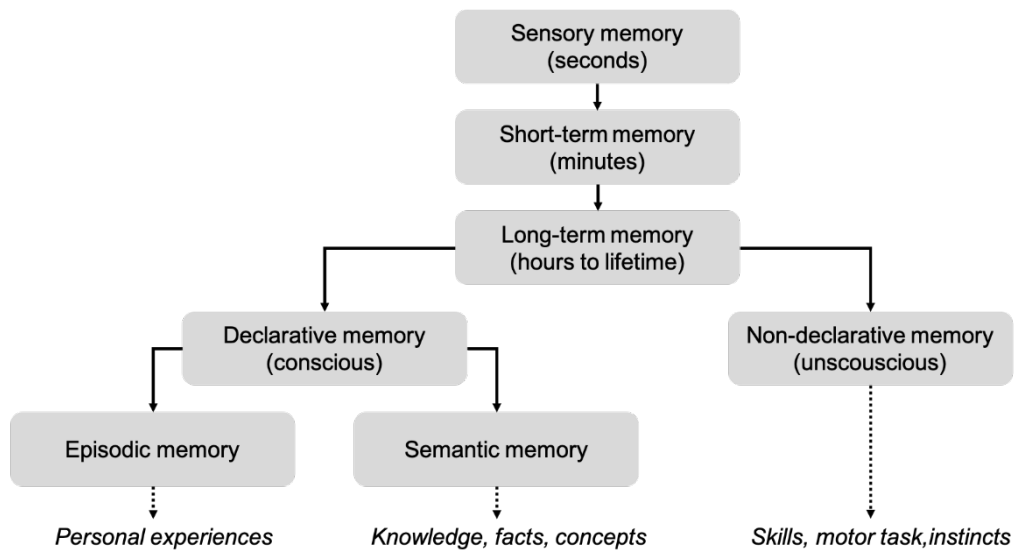


Figure 2: Classification of the different types of memory. Sensory memory encodes and stores information for a few seconds. Short-term memory stores information for several minutes, and if necessary, the information is encoded into long-term memory, divided into declarative memory and non-declarative memory. Non-declarative memory is unconscious and implicit. Declarative memory is composed of episodic memory (personal experiences) and semantic memory (general knowledge of facts and concepts).

The formation of a memory starts with the sensory memory, which encodes rapidly (in a matter of seconds) the visual, auditory and tactile information in relation with the emotional and internal state of the individual at the moment of encoding. The sensory memory is then transferred into short-term memory if selected to last. Short-term memory (or working memory) refers to the information that can be briefly stored for a few minutes. If needed, this information is stored for a longer period (from hours to a lifetime) in what is termed the long-term memory (Cowan, 2008).

Long-term memory can be further sub-divided into two main parts: declarative and non-declarative memory. Briefly, the non-declarative memory, refers to the unconscious storage and retrieval of skills, motor tasks and instincts. On the other hand, the declarative memory refers to the encoded information that can be consciously retrieved and used. It can be sub-divided into the semantic and episodic memory. The semantic memory refers to the storage of general knowledge, facts and concepts without contextual or personal experiences associated to it. On the contrary, episodic memory refers to the storage and retrieval of personal experiences and events that can be associated to specific time and place (spatio-temporal context) in at what, where and when context, linked to the emotional and internal state at the moment of encoding (Eichenbaum, 1997). Memories have to be encoded as a permanent trace, which is referred to as

the “engram” in the brain network, and then have to be maintained and consolidated in time, and finally to be retrieved (or erased) when needed. For the work presented in this thesis, we will focus on the hippocampal formation and its associated memory functions, a limbic structure essential for episodic memory and spatial memory (Nadel et al., 2000; Rosenbaum et al., 2000).

2. Episodic memory

Episodic memory is a conscious form of long-term memory that refers to the ability to store and retrieve specific personal events and experiences, often referred to as autobiographical. It allows individuals to remember not only the factual details of an event but also the context, emotions, and personal significance associated with it. Episodic memory is associated with temporal information (when the event happened), spatial context information (where the event took place) and internal state. According to this, one facet of episodic memory is to form distinct representations of similar and overlapping events, to avoid interferences. This is made possible by the formation of a relation between the specific associative and non-associative information of this event that are linked in a unique representation. These properties support inferential reasoning (Zeithamova et al., 2012), a process by which existing memories are retrieved and recombined to cope with novel situations. It requires the flexible use of learned information and depends both on the ability to form distinct memory representations that share overlapping elements during encoding (“pattern separation” or memory discrimination) and on the ability to retrieve them from partial inputs (pattern completion).

Episodic memory is very sensitive to age-related alterations (Churchill et al., 2003; Nilsson, 2003). Crucial features of episodic memory are altered with age; such as the decreased abilities to perceive contextual details, the increase in interferences between similar features and the reduced response to new stimuli, which attenuates the ability to discriminate events (Leal and Yassa, 2015). It has been hypothesized that during aging there is even a shift from pattern separation toward pattern completion. Therefore, memory processes would tend to rely on already stored information rather than encoding new ones to better discriminate (Vieweg et al., 2015), which eventually would lead to less flexible adaptations at old age.

The evaluation of episodic memory abilities in animals represents a challenge, as it is not possible to directly ask rodents about their subjective experiences. Such experiences typically involve training rodents to remember specific details about an event, such as the location and identity of objects, and then testing their ability to recall these details at a later time. The contextual fear conditioning, an associative learning and memory task, has often been used to

study episodic-like memory in animals. In this task, animals are conditioned to associate a neutral stimulus with a fearful or aversive stimulus (a shock) in a specific context, leading to the development of a conditioned fear response to the previously neutral stimulus. By being re-exposed to the context without aversive stimulus, it is then possible to assess the contextual memory abilities of animals by measuring their freezing behavior. Trace fear conditioning is a variant of fear learning that requires the association of a cue and shock across an empty trace interval of several seconds. During aging, associative memory deficits based on contextual-fear conditioning have been described in human (Battaglia et al., 2018) and in rodents (Villarreal et al., 2004; Kaczorowski and Disterhoft, 2009; Lux et al., 2016).

As mentioned, episodic memory allows memory discrimination, in order to assess such abilities in animals, the fear conditioning task can be adapted with the re-exposition of animals to the conditioning context (still without aversive stimulus) but either to a very similar context, which allows to assess complex memory discrimination, or to a dissimilar context, which allows to assess simple memory discrimination. While simple memory discrimination would be rapidly achieved by animals with high memory discrimination abilities, their complex discrimination abilities can be assessed over several days, in which animals will progressively exhibit less freezing behavior in the similar context than in the conditioning context, indicative of their learning to differentiate. Spatial memory discrimination is also often assessed using the radial arm maze. In this task, animals are habituated to find a reward in one of the arms (sample phase) while all other arms are closed. Then, animals are tested for their ability to select, from a choice of two arms, the arm location that had not been presented in a previous sample phase and is now rewarded over several trials. The distance of separation between the two open arms (previously rewarded during sample phase and rewarded during test) can vary, the higher the separation the simpler the memory discrimination task will be. A decline in memory discrimination abilities in aged animals has been reported using fear-based contextual discrimination or object location discrimination (Wu et al., 2015; Cès et al., 2018).

Although trace eye blink conditioning is not typically considered to be an example of episodic-like memory, it has been used in the context of inter-individual differences especially by Disterhoft's lab. This task is a type of classical conditioning that involves pairing a neutral stimulus, such as a tone, with an unconditioned stimulus, such as an air puff to the eye. Over time, the neutral stimulus becomes associated with the unconditioned stimulus and elicits a conditioned response, such as an eye blink.

3. Spatial memory

Spatial memory is generally considered as part (or a least complementary) of episodic memory as it refers to the ability to remember and associate spatial information between different objects or locations in an environment. It involves understanding how different elements are arranged in relation to each other in order to navigate through space, via the establishment of a cognitive map (O'Keefe and Nadel, 1978). In order to study spatial memory, animal models are vastly used. Indeed, the limitation of animal models to study memory in general resides in the inability for animals to describe what they experience during a test. This makes the investigation for age-related impairments in memory challenging.

Two types of strategies can illustrate spatial navigation; the egocentric and allocentric strategies. The egocentric strategy corresponds to a self-centered representation of an individual into space, where the subject position is used as reference. This type of strategy is non-dependent of the hippocampus and allows very little flexibility and adaptation (Chersi and Burgess, 2015). The allocentric strategy relies on the use of spatial cues in the environment as references in navigation and uses them in order to reach the goal. In that case, the subject creates a spatial map of the environment and can flexibly use it to reach the goal. This strategy is dependent of the hippocampus (Nadel, 1991) and considered more adaptive as it allows for greater flexibility and generalization to new situations. In rodents, spatial learning is mostly studied using the Morris watermaze (Morris, 1981), a task where animals have to locate a hidden platform in a pool of opaque water over several trainings. Egocentric strategy can be studied in several ways: the cue-based strategy in which the platform is made visible with a cue in the pool and the animals associate this visual cue with its goal. In that case, no matter the departure point in the pool, the animals can reach the platform thanks to the cue. A second egocentric strategy relies on the path-integration strategy, in which the platform is hidden but the departure point remains constant. In that case, animals learn a combination of movements to reach their goal and do not use cues to form a spatial map. For the allocentric learning, the platform is hidden underneath the surface of the water and remains at a constant location, and the departure point pseudo-randomly varies, forcing animals to create a spatial map.

Allocentric spatial memory (and not egocentric) in rodents is impaired with age, similarly to what is observed in humans (Lester et al., 2017). Deficits are already observable at middle-age in rodents (Gage et al., 1984a; Rapp et al., 1987; Gallagher et al., 1993; Robitsek et al., 2008). Rodents serve as a good model to study the aging of spatial memory abilities, as several studies showed age-related impairments in several spatial memory task, such as the Morris watermaze (Gallagher

et al., 1993), the water radial-arm maze (Bimonte et al., 2003), the radial-arm maze (8 or 12 arms) (Ingram et al., 1981; Ikegami, 1994), the Y maze (Weiss et al., 1998; Temido-Ferreira et al., 2020) or the Barnes maze (Barrett et al., 2009). A correct spatial navigation is a fundamental element of spatial learning and the preservation of the brain structures assuring this function seems therefore essential in the context of cognitive aging. Given the role of the hippocampus in allocentric learning, it has been proposed to be responsible for the loss of spatial memory abilities with age.

C. Inter-individual differences in cognitive aging

A very central point of the work presented in this thesis which has not been introduced yet is that cognitive aging is not a uniform process in the aging population. Indeed, the more the population, the more individuals will differ from each other in terms of ability. Therefore, age itself is not a good predictor of cognitive aging. On the contrary, it is characterized by a high variability across the elderly population. While some individuals maintain high memory abilities with minimal age-related decline, achieving what is termed successful cognitive aging, others experience varying degrees of memory impairments, corresponding to unsuccessful cognitive aging (Nyberg et al., 2020). The first group of individuals will be referred to as resilient individuals, whereas the latter will be mentioned as vulnerable individuals. This concept and evolution of cognitive performances are illustrated in **figure 3**.

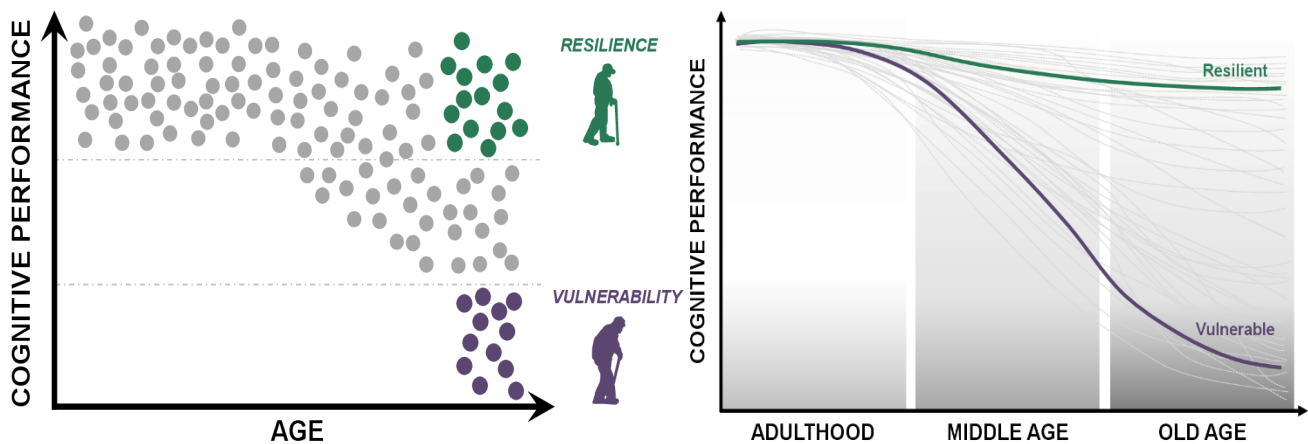


Figure 3: Illustration of inter-individual differences in cognitive performances with age. Illustration of the different cognitive performance levels of individuals (represented by dots) at different ages on the left. Illustration of the different patterns of evolution during aging of cognitive aging of individuals (represented by lines) on the right. Resilient individuals are depicted in green, vulnerable individuals are depicted in purple.

The term “resilience” implies that the brain employs mechanisms to avoid memory loss. Three complementary hypotheses are mentioned in the literature today to support this concept; brain reserve, brain maintenance and brain compensation. Brain reserve refers to the fact that an individual is equipped with increased neural resources prior to age-related decreased abilities, a reserve that has been built over the lifetime. The brain maintenance concept relies on the “active” preservation of these neural resources during age-related alterations. Finally, brain compensation relies on the use of these neural resources to better cope with the demand or the attacks linked to aging (Nyberg et al., 2012; Cabeza et al., 2018; Stern et al., 2023). In rodents, a significant variability in memory abilities among aging individuals can also be observed, especially in tasks measuring spatial memory abilities (Rapp and Amaral, 1992; Gallagher et al., 1993), making it a good model to study inter-individual differences.

The inter-individual variability is believed to originate from hippocampal alterations. Indeed, the impairments observed due to aging are similar to those observed after hippocampal lesions (Morris et al., 1982; Eichenbaum et al., 1990; Moser et al., 1995; Redish and Touretzky, 1998; Stoelzel et al., 2002), genetic manipulations (Tsien et al., 1996; Nakazawa et al., 2003) and have been associated with defects in hippocampal circuitry and plasticity (Markowska et al., 1989; Gallagher et al., 1990; Rapp and Amaral, 1992; Rowe et al., 2007). It could then be hypothesized that the resilient individuals manage to prevent the alteration of their hippocampal formation while the vulnerable do not, leading to the behavioral outcome observed.

In this thesis, we will set aside the study of aging *per se* and therefore the observations made on differences between young and old animals (resilient and vulnerable pooled together). We will focus on the differences observed between resilient and vulnerable animals. Indeed, taking the old population as a whole and comparing it to a young population can either conceal the resilience-associated mechanisms put in place or lead to misinterpretations of the age-related effects observed. For example, in the case that resilient animals adopt a mechanism that vulnerable do not, pooling these individuals together can hide this mechanism when compared to young individuals alone. On the other hand, differences observed between young and old populations are frequently automatically attributed to age-related impairments, indicative of unsuccessful aging. However, an alternative interpretation could imply that the observed disparities in the old population underlie a mechanism linked to successful aging, put in place by resilient individuals. Conversely, in that case vulnerable individuals would show an inability to adapt and to adopt this mechanism, and would remain similar to the young population.

In summary, the first chapter of introduction introduces the concept of memory and its age-related impairments during aging. It highlights that cognitive aging is a highly variable process at the population level as not all individuals experience the same reduction in their memory abilities. Therefore, understanding which brain structures are affected during aging and lead to these inter-individual variabilities seems of high importance. As mentioned, learning and memory are fundamentally linked to the hippocampal formation, which will therefore be our focus for the rest of this introduction.

II. THE HIPPOCAMPAL FORMATION

A. Anatomical organization

The hippocampal region is a subcortical region of the brain which belongs to the limbic system. It is composed of two main areas: the hippocampal formation (or hippocampus) and the para-hippocampal areas. The hippocampus has a C-shaped morphology, consistently found across all mammals. It extends along a dorsal (septal) to ventral (temporal) orientation in rodents, equivalent to a posterior to anterior orientation in humans. The dorsal part plays a crucial role in memory capacities, and especially spatial memory (Moser et al., 1993). The ventral part is more engaged in the regulation of emotional responses (Gray, 1982; Papez, 1995; Bannerman et al., 2004). The hippocampus itself can be subdivided into three main regions: the dentate gyrus (DG), the Cornu Ammonis (CA), with its subfields named CA3, CA2 and CA1, and the subiculum (Witter et al., 2000). The hippocampus is mostly composed of two principal types of cells; the granule neurons of the DG and the pyramidal cells of the CA and subiculum (Amaral and Witter, 1989).

The connectivity between the sub-regions of the hippocampus is generally considered as unidirectional; the granule neurons of the DG project via their axons, called mossy fibers, to the pyramidal cells of the CA3; which in turns project to the CA1 via the Schaffer collaterals. Then, the pyramidal cells of the CA1 project to the subiculum. Finally, the pyramidal cells in the subiculum project out of the hippocampus mostly to the entorhinal cortex (EC) (Köhler, 1985) but also other sub-cortical areas such as the thalamus or hypothalamus (Swanson and Cowan, 1975; O'Mara et al., 2001; Kim and Spruston, 2012). This anatomical organization is represented in [figure 4](#).

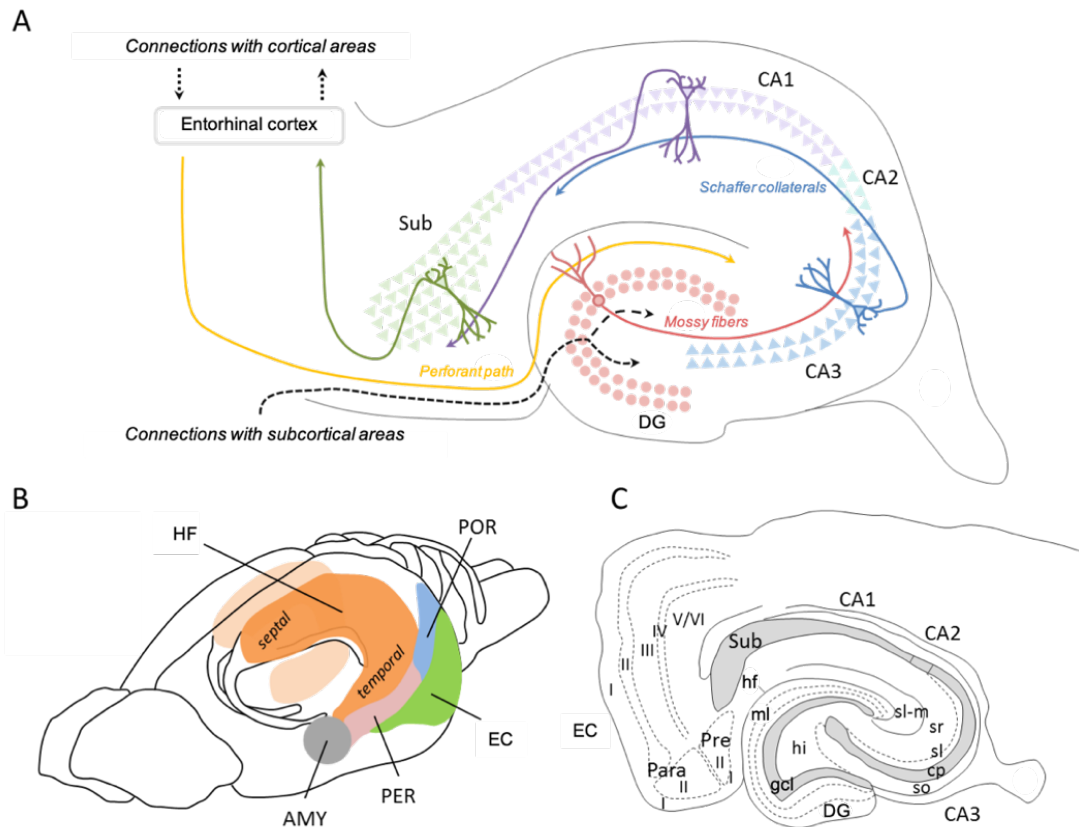


Figure 4: The hippocampal formation architecture and its relative position in the rodent brain. (A) Representation of the connectivity in and out of the hippocampus and the intra-hippocampal tri-synaptic circuit. As depicted, the entorhinal cortex projection to the granule neurons of the dentate gyrus (DG), called perforant path, the mossy fibers from the granule neurons connecting the pyramidal cells of the CA3 sub-field and the Schaffer collaterals from the pyramidal cells of the CA3 connecting the CA1 sub-field. Finally, the connections from the CA1 to the subiculum (Sub) and then out of the hippocampus back to the entorhinal cortex. (B) Position of the hippocampal formation (HF) in the rat brain. The HF is indicated in orange, surrounded by the close structures, the amygdala (AMY), the perirhinal cortex (PER), the post-rhinal cortex (POR) and the entorhinal cortex (EC). (C) Transversal schematic representation of the hippocampus and its different regions and sub-layers. Roman numerals represent cortex layers. *cp*: cortical plate; *DG*: dentate gyrus; *EC*: entorhinal cortex; *gcl*: granule cell layer; *hf*: hippocampal formation; *hi*: hilus; *ml*: molecular layer; *Para*: parasubiculum; *Pre*: presubiculum; *sl*: stratum lucidum; *sl-m*: stratum lacunosum moleculare; *so*: stratum oriens; *sr*: stratum lucidum; *sub*: subiculum. Adapted from (Amaral and Lavenex, 2006; Furtak et al., 2007).

It has now been demonstrated that the connectivity of the hippocampus is more complex with connections between all regions, with the CA3, CA1 and subiculum back-projecting in the hippocampus itself but also with the DG making direct connections with the CA1. In addition, a proximodistal organization of the projections within the trisynaptic network adds to the complexity of this structure and of the memory processing linked to this organization (Igarashi et al., 2014; Nakamura et al., 2013; Beer et al., 2018; Flasbeck et al., 2018).

The hippocampus is also composed of many other cells playing essential roles in its functions. These cells are composed of inhibitory interneurons (GABAergic) and excitatory glutamatergic mossy cells (see part III.B. for additional details) (Amaral, 1978; Freund and Buzsáki, 1996). The para-hippocampal areas are composed of the perirhinal, postrhinal, and entorhinal cortices, as they account for the large majority of the cortical inputs and outputs of the hippocampus (Burwell, 2000).

B. Implication of the hippocampal formation in the inter-individual variability during aging

A large literature has focused in understanding the brain and neuronal basis of inter-individual variabilities in learning and memory in the old population. The data discussed here will focus on the hippocampal implication and what has been shown in the rodent literature. The findings found for each sub-regions of the hippocampal region supporting resilience or vulnerability to cognitive aging are summarized in [table 1](#).

1. Network and cellular activity

The first report about the effect of aging on the rodent brain activity has been made with the use of autoradiographic [14C]-2-deoxy-D-glucose (Gage et al., 1984b). This method allows to measure glucose utilization in brain areas and therefore their metabolic activity. The extent of learning abilities impairments measured in the watermaze was shown to be related to the extent of reduction in glucose utilization in old animals. This was shown in a few specific regions of the limbic system such as the DG, the CA1 and the CA3 sub-fields (along with the medial septum, diagonal band area and prefrontal cortex). These results were the first hints towards a variable activity of brain areas according to the memory abilities.

There are very few investigations made in the context of aging about the network activity at a cellular resolution. Cellular activity can be investigated using the expression of immediate early-genes (IEGs). IEGs are a group of genes that are rapidly and transiently activated in response to various stimuli, including neuronal activity. These genes play a crucial role in the early phases of cellular responses to stimuli, and therefore often serve as proxy for cellular activity (Minatohara et al., 2015). The most commonly used are *Arc*, *cfos* and *Zif268* (or *Egr1*). Not much investigations were made regarding the activity of the DG between resilient and vulnerable animals, if not that no major changes in the total expression of *Zif268* in granule neurons could be shown with aging (Desjardins et al., 1997) or at resting state and after novelty exposition (Gheidi et al., 2013). This

gap in the knowledge of the DG in the context of inter-individual differences calls for more investigations to better understand the ins and outs of the DG activity in successful and unsuccessful aging.

Concerning the CA3, reduced learning abilities at old age were shown to be positively correlated with the hyperactivity of the CA3 using *cfos* in old animals cognitively-assessed in the watermaze (Haberman et al., 2017) and *Arc* in animals cognitively-assessed in the T-maze (Tomás Pereira et al., 2015) or during an object discrimination task (Maurer et al., 2017). In this last study, a hyperactivity of the CA3 afferent neurons from the lateral entorhinal cortex could be also noted, suggesting a possible link between the hyperactivity of both regions. More information about the rest of the para-hippocampal areas is still lacking in the literature. Concerning the CA1, the opposite could be observed using *Arc* in old animals cognitively-assessed in a contextual fear conditioning task, with a hypoactivity of the CA1 related to reduced learning abilities (Lux et al., 2016; Atucha et al., 2021). Of note, in these last two studies, a normalized activity could be observed in the CA3 between resilient and vulnerable animals, in contradiction with the previously mentioned observations in this region. Finally, transcriptomic analysis in the CA1 showed lower gene expression of *cfos* in vulnerable animals (Smith et al., 2020), further corroborating the hypoactivity of the CA1 in this cognitive population.

Such results indicate that the functional integrity of both the CA1 and CA3 might be required to maintain optimal cognitive functions over time. It overall seems that vulnerability is linked to CA3 hyperactivity and CA1 hypoactivity. Nevertheless, both sub-regions are positioned downstream in terms of their connectivity relative to the DG. It could then be hypothesized that the modified activity of the CA areas results from DG alterations at the entry of the hippocampus. This highlights again the need to better understand the DG activity between resilient and vulnerable animals.

2. Number of neurons and synapses

A straightforward solution to explain the decline in memory abilities among vulnerable individuals would be the reduction in the number of neurons in the hippocampal formation, as observed in several forms of degenerative disorders. However, resilient and vulnerable animals were shown to display similar number of neurons in the granule cell layer and the hilus of the DG, in the CA3 and the CA1 and in para-hippocampal regions (medial and lateral entorhinal cortices, perirhinal and postrhinal cortices) (Rapp and Gallagher, 1996; Rapp et al., 2002; Drapeau et al.,

2003; Spiegel et al., 2013). Therefore, while neurons do not die, one could suppose that they suffer from progressive disconnection from the rest of the network, rendering them inefficient.

The number of synapses in the DG of resilient and vulnerable animals has been investigated. Two types of synapses were studied, the axospinous (when the axon projects to the spines) and the axodendritic (when the axon projects to the shaft of the dendrite). While the first one is considered as excitatory, as spines mostly receive glutamatergic inputs, the second one is considered as inhibitory, as GABAergic interneurons project at the level of the dendritic shaft. Resilient and vulnerable animals aged of 26 months exhibited a similar number of axodendritic synapses in the DG, suggesting no differences in the total inhibition that granule neurons receive. On the contrary, the number of axo-spinous synapses was higher in resilient animals, suggesting that the preservation of excitatory inputs is related to better learning. Going even further, the axo-spinous synapses were investigated according to their status as non-perforated or perforated, which corresponds to having a continuous or discontinuous post-synaptic density (PSD). A reduction in the number of perforated (and not non-perforated) synapses has been reported in the DG of vulnerable animals (Geinisman et al., 1986). Perforated synapses are believed to be representative of a structural modification resulting in a sustained augmentation of synaptic efficacy (Siekevitz, 1985).

The DG presynaptic connectivity was also shown to be altered in vulnerable animals using an immunohistochemical labeling for the presynaptic vesicle marker synaptophysin (Smith et al., 2000). Of note, the decreased number of synapses and presynaptic marker were shown to be specific of the medial molecular layer in both studies, which indicates a preservation of specific inputs (see part III.C. for details and significance). This sub-layer had already been identified in the context of aging as it suffers from reduced number of axo-spinous synapses in old animals compared to young ones (Geinisman et al., 1992).

While these results are mostly believed to concern glutamatergic inputs, granule neurons also receive cholinergic inputs, which can project to the spine even if their exact projection site on the dendrite remains unclear. In the context of aging, the degree of spatial navigational impairments has been shown to be correlated with morphological changes in the medial septum and the diagonal band of Broca, both major forebrain cholinergic cell groups (Fischer et al., 1989). These alterations appear progressively (Fischer et al., 1992) and are associated with acetylcholine release (Ikegami, 1994). However, the role of acetylcholine in cognitive decline has been challenged by the discovery that deafferentation of septohippocampal cholinergic inputs in young animals does not produce memory loss (Brayda-Bruno et al., 2013; Parent and Baxter, 2004). It

has been proposed that acetylcholine *per se* is not essential for memory, but that the connectivity changes due to cholinergic neurons atrophy might predispose the emergence of subsequent memory loss.

Concerning the inhibitory circuits of the DG, a variety of interneurons were investigated using the glutamic acid decarboxylase-67 (GAD67), somatostatin and neuropeptide Y markers. While no differences between resilient and vulnerable animals were shown in the number of inhibitory neurons in the granule cell layer and molecular layer of the DG, a specific down-regulation of GAD67 and somatostatin (but not neuropeptide Y) could be detected in the hilus (Spiegel et al., 2013; Koh et al., 2014). These results highlight a specific vulnerability of the hilar interneurons, which could specifically participate in the decrease of learning abilities with age.

In the CA3, the balance of axospinous synaptic weights was shown to be dysregulated in vulnerable animals compared to resilient animals, where the DG-CA3 connections are replaced by auto-associational synapses CA3-CA3 (Buss et al., 2021). This shift suggests a reduction in the quantity of DG-CA3 mossy fibers synapses which would result in a reduced regulatory influence exerted by the DG onto CA3 and more auto-activation of the CA3. This finding potentially aligns with the previously mentioned hyperactivity of the CA3 in vulnerable animals. The levels of synaptophysin labelling in the CA3 of vulnerable animals were also shown to be decreased, indicating a reduced presynaptic innervation (Smith et al., 2000). About inhibitory circuits, no differences in the number of interneurons expressing GAD67 or somatostatin were detected between resilient and vulnerable animals (Spiegel et al., 2013).

In the CA1, the total number of axospinous synapses was shown to be similar between resilient and vulnerable animals (Geinisman et al., 2004; Buss et al., 2021). Yet, this lack of synaptic loss was found to be related with a decrease in the size of the post-synaptic density at the level of perforated synapses in vulnerable rats (Nicholson et al., 2004), suggesting that hippocampal perforated synapses become less efficient in vulnerable animals with age, and could therefore explain the CA1 hypoactivity observed. About inhibitory circuits, no differences in the number of interneurons expressing GAD67 could be detected between resilient and vulnerable animals (Spiegel et al., 2013).

Concerning para-hippocampal areas, the synaptophysin levels were shown to be higher in the lateral entorhinal cortex of resilient animals (and not the medial entorhinal cortex) (see part III.C. for details), suggesting a preserved presynaptic connectivity of the lateral entorhinal cortex in resilient animals. In addition, the levels of Reelin expression, a marker indicative of synaptic plasticity and phosphorylation in the entorhinal cortex neurons, were shown to be specifically

reduced in the lateral entorhinal cortex of vulnerable animals, indicating reduced synaptic plasticity and higher levels phosphorylated tau, which could result in a reduced integrity of the perforant path to the DG (Stranahan et al., 2011).

3. Excitability

The alterations enumerated above could lead to neuronal dysfunction in the different sub-regions of the hippocampus. Aged individuals with cognitive impairments could therefore display abnormalities in their neuronal excitability, which is most often indicative of an imbalance in the excitatory/inhibitory balance. To check for such alterations, electrophysiological recordings were most often performed *ex vivo* on slices. In the DG, the preservation of spatial memory abilities in the watermaze was shown to be correlated with higher amplitudes of miniature excitatory post-synaptic currents (mEPSCs) and miniature inhibitory post synaptic currents (mIPSCs) in granule neurons (Tran et al., 2018). This indicates that resilient animals have stronger synaptic connections than vulnerable ones, which corroborates the observations made on synaptic connectivity before. The only information documented on the aging CA3 excitability concerns an age-related effect for which there is an increase in the firing rate of CA3 pyramidal neurons in old individuals compared to young ones (Oh et al., 2016). The excitability of CA3 pyramidal cells between resilient and vulnerable animals has not been addressed in the literature. For the CA1, no differences between resilient and vulnerable animals were found in the amplitude of mEPSCs and mIPSCs; however the average value of tonic inhibition strongly correlated with the memory abilities, suggesting a reduced somatic excitability of CA1 pyramidal cells. This is believed to enhance the signal to noise ratio and improve memory encoding (Tran et al., 2018). However, it does not align with the hypoactivity of the CA1 observed in vulnerable animals. Conversely, afterhyperpolarizations (AHPs) in the CA1 of vulnerable animals (tested in both the watermaze and contextual fear conditioning) were shown to be larger compared to resilient animals (Tombaugh et al., 2005; Kaczorowski and Disterhoft, 2009). The AHPs correspond to a period of hyperpolarization that occurs after a series of action potentials and relates to a decrease in the neuronal excitability. Larger AHPs in vulnerable animals suggest that this hyperpolarization is longer than usual and could limit the recruitment of these neurons into the neural circuitry, and would therefore align with the decreased activity of the CA1 in vulnerable animals in that case.

Concerning para-hippocampal areas, an increase in persistent firing probability (the ability to continue to fire action potential after the termination of a triggering stimulus) in the lateral entorhinal cortex was shown to be related to preserved temporal associative memory abilities

measured in the trace eye blink conditioning task (Lin et al., 2020), suggesting better synaptic plasticity. Interestingly, a drastic reduction in the plastic properties of entorhinal-DG synapses in middle-aged animals (8-10 month-old) was reported, placing limits in the communication of information between the hippocampus and the rest of the brain (Amani et al., 2021). A reduced recruitment of feedback inhibition in vulnerable rats in response to lateral entorhinal cortex activation has also been described (Tran et al., 2019). These results fit with the alterations described for the hilar interneurons (that are providing feedback inhibition onto granule neurons) which are associated with impaired performance in aging (Spiegel et al., 2013). This alteration of feedback inhibition contrasts with the absence of correlation between measures of excitatory synaptic strength of the LEC inputs and behavioral performance in the aged rats (Tran et al., 2019).

Studies on synaptic plasticity in resilient and vulnerable individuals are scarce. In respect to electrophysiological measures of hippocampal synaptic plasticity such as kindling (long-lasting alteration in neuronal excitability brought about by repeated, low level electrical stimulation) or long-term potentiation (LTP), only aged rats with poor memory exhibit a significant decline in synaptic plasticity efficacy in the dentate gyrus (Barnes, 1979; deToledo-Morrell et al., 1988). The status of LTP and long-term depression (LTD) has been examined in the associative/commissural pathway (A/C → CA3), which provides the majority of excitatory inputs to CA3 pyramidal neurons. Whereas A/C → CA3 LTP is minimally affected, two forms of LTD, involving NMDA receptors and metabotropic glutamate receptors (mGluR), are both greatly reduced in vulnerable rats (Yang et al., 2013). These findings indicate that the integrity of mGluR-LTD at A/C → CA3 inputs may play a crucial role in maintaining the spatial memory performance of CA3 circuitry in aging. More recently, aging has been associated with a shift in the form of plasticity induced by a weak stimulus (low-frequency stimulus elicited LTP instead of LTD), as a consequence of increased NMDAR activation and Ca^{2+} influx, in CA1 synapses. This plasticity profile correlates significantly with the behavioral memory index in aged rats, whereby a higher LTD-to-LTP shift corresponds to a worse spatial memory (measured in a Y-maze) (Temido-Ferreira et al., 2020).

The hippocampus is composed of cells that respond specifically to the current location of an individual in the environment, called place cells (O'Keefe and Dostrovsky, 1971; Park et al., 2011). This has been one of the most important neurobiological support to point out the hippocampus as the locus of an internal map of the spatial environment. In terms of excitability, place cells were shown to fire only when an individual occupies a particular location in the environment, referred to as their "place field". This firing specificity enables to encode different spatial information and to create a spatial map of the surrounding environment, as each place cell will have its own place

field which allows to represent distinct areas (Moser et al., 2008). The degree of firing variability of place cells, referred to as overdispersion, describes to which extent the firing rate of a place cell varies from its expected average firing rate. Overdispersion is a common feature of place cells and is important for encoding spatial information by allowing more flexibility, as a higher overdispersion allows for a more accurate representation of different locations, allowing to better nuance the cognitive map of the environment. In the context of aging, the place cells in the CA1 of old vulnerable animals were shown to have a lower degree of overdispersion compared to resilient ones (Hok et al., 2012). This finding has been confirmed in the context of the watermaze, in which place cells of the CA3 and CA1 exhibited a higher “rigidity” in vulnerable animals (Wilson et al., 2003). Therefore, the “rigidity” of place cells in vulnerable animals could represent one for the element for reduced spatial learning abilities, as vulnerable animals would have troubles in elaborating an accurate and more nuanced cognitive map. Whether these findings also extend to the place cells of the DG remains unknown and calls for more investigation in this region.

4. Gene expression

While anatomical structures and neuronal excitability have provided crucial insights, it has become increasingly evident that genetic and epigenetic factors also play pivotal roles in supporting cognitive functions. Transcriptomic analysis of the DG and the CA3 revealed that poorer learning performances in animals subjected to a spatial discrimination task were related to the decreased expression of transthyretin, a key gene for the maintenance of cognitive processes (Brouillette and Quirion, 2008; Masser et al., 2014). Vulnerable animals were also shown to display decreased gene expression of genes involved in synaptic transmission and neurogenesis (Haberman et al., 2011; Ianov et al., 2017; Smith et al., 2020). In particular, genes related to synaptic plasticity and excitatory neurotransmission in the CA3 were upregulated in vulnerable animals compared to resilient ones, which aligns with the hyperactivity of the CA3 (Haberman et al., 2011). In the CA1, impaired cognition was shown to be associated with altered expression of synaptic genes and genes involved in Ca²⁺ regulation (Ianov et al., 2017). Nothing is known about the para-hippocampal areas genetic expression regarding resilience and vulnerability to cognitive aging. Data collected on genetic studies largely vary between one-another study. While all of them detected an age-related effect in gene expression, most of them only found a cognitive-related effect only in one or two of the three sub-hippocampal regions, and these regions were not always the same according to the study. Such differences highlight the complexity of hippocampal aging but also reflect the importance for methodological comparison

and uniformity across studies. First, the behavioral assessment of cognitive abilities differs between the studies, a lack of gene up or down-regulation expression in one sub-regions could be due to the fact that the assessed cognitive process does not depend on this sub-region. The time for hippocampal dissection after training also seems to be one factor for variability across studies (varies from hours to weeks) as well as gene-expression analysis (microarray or RNAsequencing).

While alterations on the genome sequence have direct and evident effect on gene expression, it can also be modified through epigenetic modifications. A large range of modifications exist that can either promote or restrain gene expression; and the epigenetic mechanisms altered during aging include DNA methylation, histone modifications, nucleosome remodeling, and microRNA-mediated gene regulation (Harman and Martín, 2020).

Investigation about the epigenetic profile of the hippocampus between resilient and vulnerable animals are sparse in the literature, and even fewer looked at the sub-regions of the hippocampus separately. In terms of acetylation, while no differences in histones deacetylases (HDACs) protein levels could be detected in basal conditions according to the cognitive status, resilient animals displayed a decreased HDACs protein levels in the DG and CA1 (and not the CA3) following learning, an adaptation that vulnerable animals did not display (Castellano et al., 2012). These results suggest that successful learning supposedly induces a remodeling of proteins regulating acetylation and therefore a possible increase in acetylation. However, the total levels of acetylation (both basal and post-training) were similar between resilient and vulnerable animals (Castellano et al., 2012) and increasing hippocampal acetylation levels failed to rescue memory performances in vulnerable animals (Castellano et al., 2014). Therefore, it seems safe to presume that the observed adapted level of HDACs following learning in resilient animals does not result in increased acetylation and that the biological value for such adaptation in resilient animals remains unknown.

In terms of DNA methylation, a general hypomethylation has been observed in the old hippocampus (Chiavellini et al., 2022) which could be linked to the decrease expression of the DNA methyltransferase Dnmt3a2, a DNA methyltransferase mediating DNA methylation. Indeed, rescue of Dnmt3a2 levels in the CA1 restored memory functions measured in a contextual trace fear and novel object recognition task (Oliveira et al., 2012a). Yet, the underlying mechanism of resilience seems more subtle as the hypomethylation of genes coding for transcriptional factors was shown to be correlated with higher learning abilities (Chiavellini et al., 2022). Thus, successful aging probably does not depend on a complete and general hypo/hypermethylation or acetylation of the whole genome. The maintenance of learning abilities could therefore rely on a more

complex association of both, which would therefore explain the variable results found in the literature.

	Cortex	DG	CA3	CA1	References
Network activity		Res>Vul	Res>Vul	Res>Vul	Gage et al., 1984b
Cellular activity	LEC: Res<Vul (Arc)	Res = Vul (Zif268)	Res<Vul (cfos & Arc)	Res>Vul (Arc)	Maurer et al., 2017; Haberman et al., 2017; Tomás Pereira et al., 2015; Lux et al., 2016; Atucha et al., 2021; Gheidi et al., 2013
Number of neurons	Res=Vul	GCL: Res=Vul Hilus: Res=Vul	Res=Vul	Res=Vul	Drapeau et al., 2003; Spiegel et al., 2013; Rapp et al., 2022; Rapp and Gallagher 1996
Number of synapses	Septal nuclei: neuronal atrophy (cholinergic)	Axodendritic: Res=Vul Axospinous: Res>Vul	Axospinous: Res>Vul	Axospinous: Res=Vul PSD size: Res>Vul	Geinisman et al., 1986, 2004; Fischer et al., 1989; Buss et al., 2021; Nicholson et al., 2004
Pre-synaptic vesicles (synaptophysin)	LEC: Res>Vul MEC: Res=Vul	Res>Vul	Res>Vul	Res>Vul	Stranahan et al., 2011; Smith et al., 2000
Interneuron markers		GCL&ML GAD67: Res=Vul Hilus GAD67: Res>Vul SOM: Res>Vul NPY: Res=Vul	GAD67: Res=Vul SOM: Res=Vul	GAD67: Res=Vul	Spiegel et al., 2013; Koh et al., 2014
Excitability	LEC persistent firing Res>Vul	mEPSC & mIPSC Res>Vul		mEPSC & mIPSC Res=Vul	Tran et al., 2018
Synaptic plasticity	LEC to DG feedback inhibition recruited by LEC activation: Res>Vul	Kindling & LTP: Res>Vul	LTP: Res=Vul LTD: Res>Vul	LTP -> LTD in Vul	Barnes, 1979; deToledo-Morrell et al., 1988; Yang et al., 2013; Tran et al., 2019; Temido-Ferreira et al., 2020
Place cells overdispersion			Res>Vul	Res>Vul	Hok et al., 2012; Wilson et al., 2003
Gene expression		Transthyretin Res>Vul Synaptic function Res>Vul Neurogenesis Res>Vul	Transthyretin Res>Vu Synaptic transmission Res>Vul Synaptic plasticity Res<Vul Excitatory transmission Res<Vul	Neuronal activity (cfos) Res>Vul Synaptic genes Res>Vul Ca2+ regulation Res>Vul	Haberman et al., 2011; Masser et al., 2014; Ianov et al., 2017; Smith et al., 2020

Table 1: Implication of the hippocampal formation in the inter-individual variability observed during cognitive aging. *GAD67: glutamic acid decarboxylase-67; GCL: granule cell layer; HDACs: histones deacetylases; LEC: lateral entorhinal cortex; MEC: medial entorhinal cortex; mEPSCs: miniature excitatory post-synaptic currents; mIPSCs: miniature inhibitory post synaptic currents; ML: molecular layer; NPY: neuropeptide Y; Res: resilient; SOM: somatostatin; Vul: vulnerable.*

In summary, this second chapter of introduction highlights the implication of the hippocampal region in the inter-individual variability for age-related impairments of memory abilities. Particularly, it emphasizes that each sub-region of the hippocampus experiences different age-related impairments and that resilience/vulnerability is not a uniform process. Particularly, information about the DG remains limited and some gaps still call for more investigations. In the work presented in this thesis, we will focus on the DG because i) it represents the entry point of the hippocampus, therefore alterations in this sub-region could have repercussions downstream and relate to the alterations described in the CA3 and CA1. This implies that the rescue of impairments in the DG might have a cascade effect of recovery on the try-synaptic circuit. ii) The DG keeps a peculiar form of plasticity during the entire life of an individual; called adult neurogenesis, which supports learning and memory and therefore makes it a good candidate to support resilience to cognitive aging.

III. THE DENTATE GYRUS OF THE HIPPOCAMPAL FORMATION

A. Anatomical organization

The DG is composed of three different layers; the sub-granular zone (SGZ), the granule cell layer (GCL) and the molecular layer (ML). The SGZ, GCL and ML form a V-shaped structure that surrounds the last part of the DG called the hilus. The GCL can be sub-divided into two blades, the supra and infrapyramidal blades. The region of the V where the two blades meet is called the crest. Finally, the ML can be further sub-divided into three sub-layers; the inner molecular layer (IML), the middle molecular layer (MML) and the outer molecular layer (OML). The anatomical organization of the DG is shown in [figure 5](#).

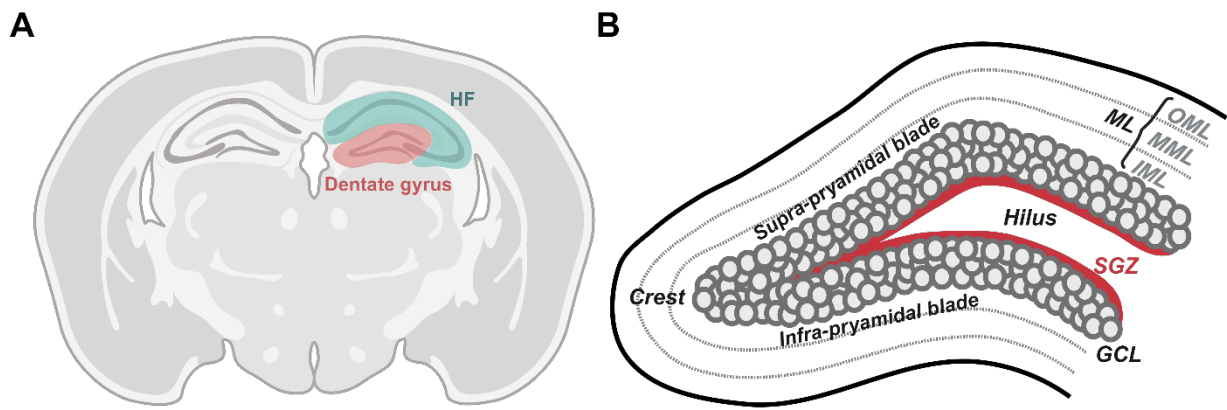


Figure 5: Schematic representation of the architectural organization of the dentate gyrus. (A) Schematic representation of the position of the hippocampal formation (HF – in green) and dentate gyrus (DG – in red) in a coronal view of the rodent brain. (B) Schematic representation of the DG organization showing the different layers, the hilus, the crest and the two blades of the granule cell layer. The sub-granular zone is indicated in red. *GCL: granule cell layer; HF: hippocampal formation; ML: molecular layer; IML; inner molecular layer; MML: middle molecular layer; OML: outer molecular layer; SGZ: sub-granular zone.*

B. Neurons of the dentate gyrus

1. Granule neurons

The principal neurons of the DG are the granule neurons that are glutamatergic excitatory neurons in nature. They are closely juxtaposed to each other, and their cell bodies constitute the GCL. Their total count is estimated at approximately 1 demi million per DG in mice and 1 million per DG in rats, although this number varies based on the mice or rat strain (Boss et al., 1985; Amrein et al., 2004). They have an elliptical cell body with a width of approximately 10 μm and a height of 18 μm in rats (Claiborne et al., 1990). The dendritic arborization of the granule neurons develops in the ML, where the inputs are received. The dendritic tree resembles a cone, with branches extending toward the superficial portion of the ML. They generally cross the entirety of the ML with the majority of their distal ends terminating at the end of the ML (Claiborne et al., 1990).

The axons of the granule neurons, called mossy fibers (MFs), cross through the hilus to contact the pyramidal cells of the CA3. The connection sites between granule cells and pyramidal cells and the end of the mossy fibers axons are named mossy fiber boutons (MFBs) (Amaral and Dent, 1981; Chicurel and Harris, 1992; Rollenhagen et al., 2007). The MFBs connect with the pyramidal cells in the *stratum lucidum* of the CA3. The MFBs are large contact sites able to make

several synapses on pyramidal cells. Additionally, smaller MFBs (3-6 μ m of diameter) are distributed all along the axon allowing one granule cells to make connection with several other pyramidal cells. A single presynaptic extension of a mossy fiber can make approximately 40 synaptic contacts with a single dendrite of a CA3 pyramidal neuron (Gonzales et al., 2001). Each granule cell is likely to influence 14 to 28 pyramidal neurons, whereas it is estimated that each pyramidal neuron is contacted by 50 granule cells (Acsády et al., 1998). Granule neurons innervate differently the CA3 according to their position in the DG, the ones in the upper-blade of the DG contact distal parts of the CA3 while the ones in the lower-blade contact the proximal part of the CA3 (Witter, 2007). While crossing through the hilus, mossy fibers also develop collaterals that will contact interneurons and mossy cells in the hilus with *en passant* synapses (Claiborne et al., 1986).

2. Mossy cells

Mossy cells are a type of excitatory glutamatergic neuron found in the hilus of the DG. These cells have a distinctive morphology with an ovoid/fusiform-shaped large cell body and several thick primary dendrites (Amaral, 1978). The dendrites of mossy cells are covered with large spines called thorny excrescences, where they receive in the major part *en-passant* synapses from the mossy fibers of the granule neurons. They send their axons in the IML where they innervate the proximal dendrites of granule neurons in the DG (Buckmaster et al., 1996), allowing a direct reciprocal excitation. However, they also have a dual function as they contact many interneurons in the DG, which upon activation from mossy cells will in turn inhibit granule neurons (Scharfman, 2016). Mossy cells are more active than granule neurons and exhibit a spatially organized firing during navigation with multiple space fields, suggesting a potential role of mossy cells in spatial memory (Danielson et al., 2017; Senzai and Buzsáki, 2017). Besides, their alteration in Alzheimer-like models lead to spatial cognitive deficits (S. Li et al., 2022) and mossy cells were shown to be essential for memory retrieval (Li et al., 2021). Thus, recent research pointed out mossy cells as a key component in the DG for learning and memory. However, their role in age-related memory impairments and inter-individual variability remains unknown.

3. Interneurons

There is a huge variety of GABAergic interneurons in the DG depending upon the evaluation criteria (Houser, 2007). However, their exact individual functions are unknown, probably due to the lack of molecular tools to specifically target one or another.

In the granule cell layer. The chandelier cells are located within or immediately adjacent to the GCL, their main distinguishing feature is the termination of their axon that extensively

branches and contacts the axon of granule neurons. Chandelier cells are parvalbumin positive (Arellano et al., 2004). The basket cells are located in the GCL close to the hilar border, they have a large cell body and long atypical dendrites, giving them a basket-like shape. Their axons remain in the GCL where they contact granule neurons or other basket cells directly on the soma. Basket cells are parvalbumin positive.

In the molecular layer. The molecular layer perforant path-associated (MOPP) cells have their cell bodies in the ML and thus likely receive direct excitation from the same inputs that the granule neurons. Their axons innervate the MML and OML and participate in the feed-back inhibition of granule neurons (Li et al., 2013). No specific molecular marker is attributed to MOPP cells.

In the hilus. The hilar perforant path-associated (HIPPA) and hilar commissural-associated pathway related (HICAP) cells are interneurons found in the hilus of the DG. They are hardly morphologically distinguishable with similar cell bodies. The difference between them resides in their axonal projections, the HIPPA cells innervate the MML and OML while the HICAP cells innervate the IML. This suggests that they inhibit different excitatory information received by the granule neurons according to the part of the ML which received different excitatory inputs (see part III.C. for details). They are both somatostatin positive.

C. Inputs to the granule neurons of the dentate gyrus

Inputs to the DG come from the entire brain. Some are intra-hippocampal, others are long-range. They fall under a variety of neurotransmission; as already mentioned the DG receives glutamatergic and GABAergic inputs, but also cholinergic and monoaminergic to name a few. This variety of inputs allows for the complexity of DG encoding and response to environment demands. Inputs in the DG are received on the ML, which as mentioned can be further subdivided into the IML, MML and OML. This sub-division is due to the fact that each sub-layer receives different and specific inputs, as illustrated in [figure 6](#).

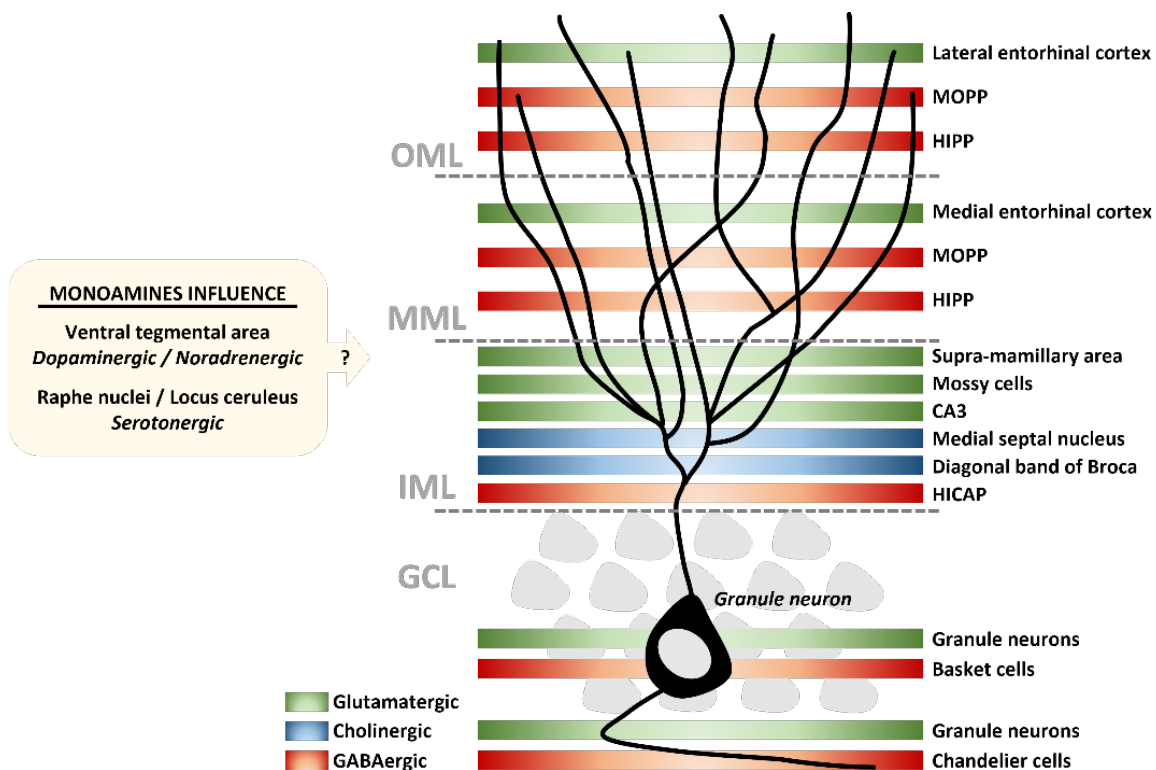


Figure 6: Schematic representation of the innervation of granule neurons in the dentate gyrus. GCL: granule cell layer; IML: inner molecular layer; MML: middle molecular layer; OML: outer molecular layer; MOPP: molecular layer perforant path-associated cells; HIPP: hilar perforant path-associated cells; HICAP: hilar commissural-associated pathway related cells.

1. Intra-hippocampal inputs

Interneurons. As mentioned before, the OML and MML of the DG receive inputs from MOPP and HIPP interneurons. The IML is innervated by HICAP interneurons. The soma of granule neurons is innervated by basket cells and their axon by chandelier cells. This difference in innervation by interneurons suggest that they can each inhibit the received information on the dendrites, the neuronal excitability at the level of the soma and the output information at the axon of granule neurons. Not much is known about the evolution of these inputs during aging.

Mossy cells. The mossy cells found in the hilus innervate the IML of the DG. Not much is known about the evolution of these inputs during aging.

CA3 back projections. The hippocampus is typically described by the tri-synaptic circuit. However, intrinsic connectivity within the DG is more complex with retrograde connections. A major one concerns the CA3-DG back projections to the DG, which are mostly described as indirect inhibitory projections as they contact and activate interneurons in the hilus, which in turn will inhibit granule neurons (Scharfman, 2007). However, staining of pyramidal cells and projections suggested a direct innervation of granule neurons in the IML (Cutsuridis et al., 2010), a finding validated by monosynaptic tracing of granule neurons inputs that revealed a direct excitatory

connection with CA3 pyramidal cells (Vivar et al., 2012), suggesting a dual inhibitory/excitatory function of these back projections. Not much is known about the evolution of these back projections during aging.

CA1 and subiculum. Retrograde tracing studies have shown back projections from the CA1 to granule neurons as well. Similarly, they were the first to describe direct back projections from the subiculum to granule neurons (Deshpande et al., 2013; Bergami et al., 2015). As no indication in the literature about the site of projection of their axons in the DG, these projections were not included in **figure 6**. Again, nothing is known about the evolution of these inputs during aging.

Granule cell layer. While direct connections between granule neurons are not believed to exist under normal conditions, (Vivar et al., 2012) demonstrated their existence via monosynaptic retrograde tracing. These connections were suggested as mechanisms by which granule neurons modulate the activity of other granule neurons (via excitation or inhibition) differently according to the variety of incoming synaptic information (Luna et al., 2019).

2. Extra-hippocampal inputs

Entorhinal cortex. The DG receives its primary inputs predominantly from the entorhinal cortex (EC) through the perforant path. The direct projections from the EC to granule neurons are glutamatergic. However, some long-range GABAergic projections to the hippocampus originating from the EC have been reported (Germroth et al., 1989). Yet, they were shown to be indirect and not directly innervating granule neurons but rather interneurons (Melzer et al., 2012). The perforant path axons mainly originate from the layer II of the EC and can be differentiated into two distinct bundles: the medial perforant path from the medial entorhinal cortex (MEC) and the lateral perforant path from the lateral entorhinal cortex (LEC). These projections from these two sub-parts of the EC are segregated and innervate respectively the MML and OML. Such differences imply different functions for both. The MEC is believed to convey spatial information as it contains the grid-cells and exhibits spatial firing properties, contributing to the encoding and representation of spatial information (Diehl et al., 2017). The LEC serves for the representation of non-spatial information, often referred to as "what" elements. It is related to the processing of objects (Deshmukh and Knierim, 2011). As mentioned, during aging, perforant path projections to the DG tend to decrease in the vulnerable population and support the decrease of learning abilities. As mentioned before, some observation suggest that these projections degenerate with age in the vulnerable population, and could underlie cognitive decline.

Supra-mammillary area of the hypothalamus. The second major long-range inputs to granule neurons come from neurons in the supra-mammillary area of the hypothalamus (SuM). Projections from the SuM innervate the IML of the DG (Amaral et al., 2007) and participate in the theta frequencies of the DG (Pan and McNaughton, 1997; Ruan et al., 2011) and spatial memory retrieval (Aranda et al., 2008; Li et al., 2020), a role that could be link to modulation of neurogenesis in the DG by SuM inputs (Li et al., 2022). It seems important to mention that these inputs have the peculiarity to co-release both glutamate and GABA (Pedersen et al., 2017; Hashimoto et al., 2018). Not much is known about the evolution of these inputs during aging.

Septal nuclei. The DG receives a relatively limited number of inputs from subcortical structures. Among these inputs, the most robust originates from the septal nuclei. These septal projections come from neurons located within the medial septal nucleus and the nucleus of the diagonal band of Broca and innervate mostly the IML (but the rest of the ML and the hilus are also innervated). Of note, while the majority of these projections are cholinergic (50 to 75%); some cells from the septal nuclei projecting to the DG are also GABAergic, but mostly innervate interneurons such as basket cells. Therefore, granule neurons only receive cholinergic inputs from the septal nuclei (Amaral et al., 2007) which is believe to modulate their excitability. As mentioned earlier, aging is associated with morphological changes in the medial septum (Fischer et al., 1992) and also by a decline of presynaptic cholinergic activity (Springer et al., 1987) and decreased acetylcholine release (Ikegami, 1994). However, these were shown to be age-dependent and not cognitive-dependent between resilient and vulnerable animals (Bañuelos et al., 2013).

Of note, granule neurons are also influenced by dopaminergic, noradrenergic and serotonergic inputs, which project from the ventral tegmental area, raphe nuclei and locus ceruleus respectively to interneurons in the hilus and the molecular layer, which in turn connect with granule neurons (Amaral et al., 2007). However, some evidences indicate population of granule neurons expressing dopamine and serotonin receptors (Gangarossa et al., 2012; Tanaka et al., 2012), suggesting a direct influence even though direct inputs from dopamine and serotonergic regions were not observed in retrograde tracing studies. No conclusive data are available to our knowledge in terms of their evolution during aging.

In summary, this third chapter of introduction highlighted the cell variety within the DG; allowing to balance excitation and inhibition. With a focus on the granule neurons, the main excitatory cells of the DG, the diversity of their inputs has been emphasized. Each single one of these inputs are essential for the function of the granule neurons and the DG as a whole, and their maintenance throughout aging seems therefore as an essential feature for the preservation of learning and memory abilities. Granule neurons are unique within the brain, because while the majority is produced during embryogenesis and the post-natal period for rodents, some granule neurons keep being added to the network until old age, a unique phenomenon called adult neurogenesis. We will therefore focus on neurogenesis and this variety of granule neurons.

IV. NEUROGENESIS IN THE DENTATE GYRUS

A. Morphogenesis of the dentate gyrus

The DG is the last structure of the hippocampal formation to develop during the course of embryonic and post-natal development. The granule neurons composing the DG originate from four distinct stages: late embryonic, early postnatal, adolescent and adult stages. The germinal zone responsible for generating granule neurons at each of these stages is different. Granule neurons are derived, respectively, from precursor cells in the dentate neuroepithelium (primary matrix) bordering the lateral ventricles and adjacent to the fimbria, from precursors located in the hilar region (secondary matrix), and finally from precursors situated in the DG and sub-granular zone (tertiary matrix) during adulthood (Schlessinger et al., 1975).

The proliferation process begins during embryogenesis during which stem cells detach from the ventricular zone and migrate tangentially towards the crest of the DG (E18-E20), where they form the secondary matrix. Subsequently, the cells migrate into the DG between postnatal day 1 and 2 (P1-P2) and settle into layers. The prominent tertiary matrix, which is most pronounced between P3 and P10, starts to regress in the hilar region between P20 and P30. In rodents, the majority of granule neurons are formed during the first three weeks of post-natal period, with a boost of proliferation in rats around P5-P7 and in mice around P1-P2 (Nicola et al., 2015). During adulthood, a discrete proliferative zone persists only in the SGZ of the adult DG in mammalian

species, as demonstrated in rodents (Cameron et al., 1993), adult primates (Gould et al., 1999b) and humans (Eriksson et al., 1998).

B. Development and integration of adult-born dentate granule neurons

1. Neuronal stem cells in the adult dentate gyrus

Neural stem cells (NSCs) are a type of cells with the ability to self-renew over an extended period and generate the three main cell types of the central nervous system, namely neurons, astrocytes, and oligodendrocytes. The identity of NSCs in the adult brain has been a subject of debate for long. The most widely accepted model suggests that a special category of astrocytes with radial glial properties constitutes the NSCs in the SGZ of the DG (Alvarez-Buylla and Lim, 2004; Garcia et al., 2004). These NSCs are referred to as “type I” and are predominantly quiescent. Their soma is found in the SGZ and they have a primary radial process that crosses the GCL. They can be identified through the expression of various markers such as GFAP (glial fibrillary acidic protein), GLAST (glutamate aspartate transporter) and BLBP (Brain lipid binding protein). However, they differ from astrocytes in their expression of nestin, Sox2 and Ascl1, all markers for neuronal stem cells and progenitors, and the absence of expression of mature astrocyte markers such as S100 β (S100 calcium-binding protein β) (Filippov et al., 2003). Of note, these "radial glia-like cells" are the most described type I NSCs in the literature, but other type I NSCs have also been observed with short horizontal extensions that run along the boundary between the GCL and the hilus (Lugert et al., 2010; Suh et al., 2007) (**Figure 7**).

2. Neuronal lineage and differentiation

In the adult SGZ, when type I NSCs exit their quiescent state and begin to proliferate they give rise through asymmetric division to non-radial precursor cells, referred to as “type II”, which then have the potential to differentiate into neurons and astrocytes (Encinas et al., 2011). Two subgroups of type II cells have been identified: type II-a, which consistently expresses the markers nestin, Sox2 and Ascl1, and type II-b, which stops expressing nestin and Sox2 but begins to express specific markers indicative of neuronal commitment, such as T-box brain protein 2 and Neurogenin 2 (Kempermann et al., 2004; Suh et al., 2007; Dhaliwal and Lagace, 2011).

The type II-b cells then give rise to cells known as neuroblasts that migrate a short distance within the GCL and undergo morphological modifications underlying the start of their functional differentiation. These lineage-committed neuronal cells are destined to give rise to newborn

granule neurons and express markers of immature granule neurons such as doublecortin (DCX) and PSA-NCAM (polysialylated-neural cell adhesion molecule). They even start to express the neuronal marker Prox1, which will be continuously expressed until full maturation (Kempermann et al., 2004; Lavado et al., 2010).

As the neuroblasts migrate through the GCL, the expression of DCX decreases, and markers of late neuronal maturation begin to appear, such as calbindin (Kempermann et al., 2004). The newly generated neurons become functionally and structurally mature glutamatergic granule neurons in approximately 7 to 8 weeks in mice (van Praag et al., 2002; Zhao et al., 2006) (Figure 7).

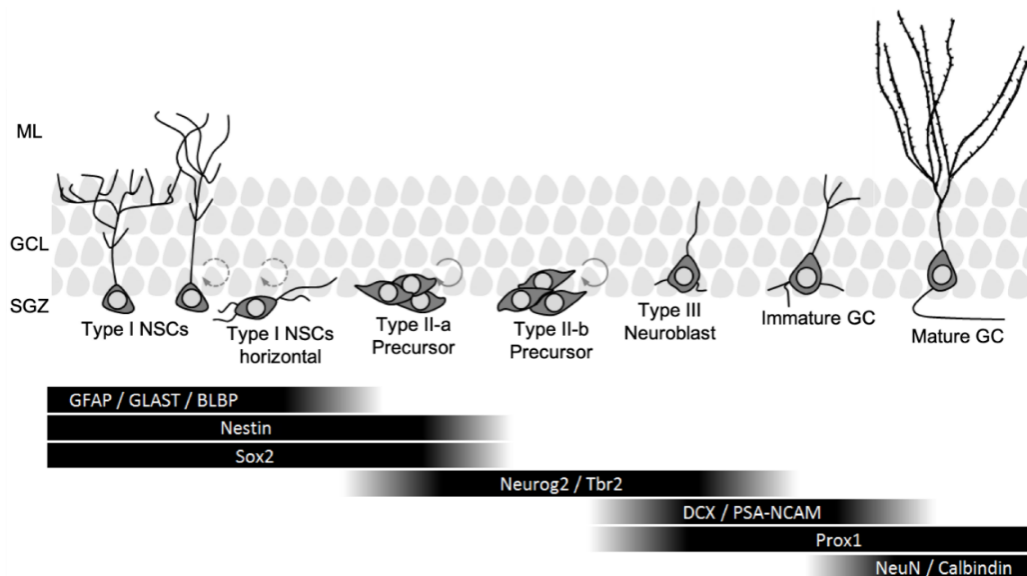


Figure 7: Development of newborn granule neurons in the adult dentate gyrus. *BLBP: Brain Lipid Binding Protein; DCX: doublecortin; GCL: granule cell layer; GFAP: glial fibrillary acidic protein; GLAST: glutamate aspartate transporter; ML: molecular layer; Neurog2: Neurogenin 2; NSCs: neuronal stem cells; PSA-NCAM: polysialylated-neural cell adhesion molecule; SGZ: sub-granular zone; Tbr2: T-box brain protein 2.*

3. Selection and morphological maturation

The maturation of adult neurons is a slow process that spans over several weeks from the birth of the neurons (Zhao et al., 2006). The evolution of the morphology (dendritic arborization mostly) and the progressive establishment of the connectivity has been well documented. During the first stages of neuronal maturation, during the transition from amplifying progenitor to post-mitotic neurons, there is a notable peak of neuronal death observed in the SGZ. This reflects the critical initial phase of survival that newly generated neurons face during their maturation (Sierra et al., 2010). Over 50% of the newly formed cells eventually undergo cell death (Gould et al., 1999a). Between the first and second post-mitotic week, newly formed neurons that survived

undergo considerable axonal elongation and branch toward the CA3 region (Markakis and Gage, 1999; Hastings and Gould, 1999; Hastings et al., 2002). Simultaneously, a thick process, distinct from the newly formed axon, redirects its growth toward the ML, where it gives rise to the first apical dendrite. New neurons primarily develop their dendritic tree during the first three weeks in a phase of active growth, followed by a period of pruning of surplus dendrites. It is during these initial three weeks that dendrites reach the outermost layer of the ML (Sun et al., 2013; Gonçalves et al., 2016). It is around this time (16-18 days after birth) that the first spines on the dendrites start to appear (Zhao et al., 2006). At the level of their MFBs corresponding to the granule cells synaptic outputs, most of their growth undergoes between the 2 to 7 weeks of age, during which the MFBs size nearly doubles, with a peculiar peak of growth between the 4th and 7th. However, it is important to mention that they keep growing consequently (by 20%) up to the 24th week of age (Cole et al., 2020).

4. Functional integration: network analysis

One of the final stages in the development of new neurons in the DG is their functional integration into the pre-existing network circuits. This stage represents a significant difference from embryonic neurogenesis as unlike developmentally-born dentate granule neurons (dev-DGNs), adult-born dentate granule neurons (adu-DGNs) develop within an already mature and functional network.

The conventional view stipulates that before the emergence of the first synapses, adu-DGNs are only exposed to ambient GABA during their first two weeks of life (Ge et al., 2006), but other evidences indicate the NSCs are also influenced by glutamate (Deisseroth et al., 2004). Up to two weeks of age, GABA exerts a depolarizing action capable of triggering action potentials. Indeed, like newborn neurons during embryogenesis, newly generated adu-DGNs have a high intracellular concentration of chloride. Upon GABA stimulation, the membrane is therefore depolarized. This GABAergic excitation has been shown to promote neuronal differentiation (Tozuka et al., 2005) when adu-DGNs start to project their first outputs to the CA3 pyramidal cells (Markakis and Gage, 1999; Hastings and Gould, 1999; Hastings et al., 2002). Besides, this GABA-dependent depolarization has been shown to be essential for the latter development of GABA and glutamatergic synapses (Tozuka et al., 2005; Espósito et al., 2005; Ge et al., 2006).

The first glutamatergic projections appear with the first spines on the dendrites of newborn cells at around the second week of age. However, the synapses on adu-DGNs are still silent at that time as they only have NMDA receptors and lack AMPA receptors (Deisseroth et al., 2004; Tashiro

et al., 2006). Retrograde tracing revealed a strong but transient innervation from mature granule neurons to newborn adu-DGNs around 3 weeks after birth. At this time, glutamatergic innervation from other regions is sparse with a majority from the mossy cells (Vivar et al., 2012) that provide the first glutamatergic synapse to adu-DGNs (Chancey et al., 2014). Therefore, the transient innervation from mature granule neurons and the innervation from mossy cells seems predominant and could represent a critical glutamatergic input for the survival of new cells. Following the third week after birth, the innervation from mature granule neurons collapses in aid of the other glutamatergic inputs (mostly entorhinal cortex). Of note, these 3 week-old immature adu-DGNs also receive strong distal cholinergic connections from the medial septum and the diagonal band of Broca (Vivar et al., 2012; Deshpande et al., 2013) that may also be involved in the integration of new immature neurons. It is important to mention that this is the conventional way of the progressive connectivity of newborn adu-DGNs. Recent evidences however noticed that one-week old adu-DGNs already receive GABAergic and cholinergic inputs as well as glutamatergic innervation (from mossy cells, mature dev-DGNs and pyramidal cells) (Sah et al., 2017).

From the third-fourth and sixth week following birth, adu-DGNs are highly plastic, hyper excitable, and more prone to develop LTP within the DG in response to a stimulation of the medial perforant path (Ge et al., 2007; Schmidt-Hieber et al., 2004). This period from 4 to 6 weeks of neuronal age corresponds to what has been called the “critical period” of immature adu-DGNs. Upon the 8th week of age, it is generally considered that adu-DGNs have finished their maturation in mice as they exhibit functional properties similar to other mature granule neurons (Laplagne et al., 2006). While this critical period of increased synaptic plasticity has been well characterized by performing LTP on adu-DGNs via the medial perforant pathway, some differences were shown more recently by inducing LTP via the lateral perforant pathway. Such activation did not elicit LTP in adu-DGNs during the traditional critical period at immature stages (4-6 weeks) and beyond (>8 weeks). They even often displayed LTD after theta burst stimulation. However, over the course of 3–4 months of age, adu-DGN displayed enhanced synaptic plasticity, suggesting a wider critical period (Vyleta and Snyder, 2021).

In addition, it was found that adult neurogenesis is actually a key process able to influence the plastic properties of the DG by facilitating the expression of LTP (Snyder et al., 2001; Massa et al., 2011) and LTD (Massa et al., 2011): reducing adult neurogenesis dramatically impairs synaptic transmission and both LTP and LTD at medial perforant path synapses. In addition, different time courses for LTP and LTD recovery was observed after adult neurogenesis rescue suggesting that

newborn neurons that integrated into the DG network are first contacted by synapses that can be strengthened only through LTP. This situation changes later as those synapses acquire the ability to display bidirectional plasticity (Massa et al., 2011).

5. Maturation of granule neurons depends on multiple conditions

It seems important to point out that the literature regarding neuronal maturity raises an interesting and complex question: when can we precisely consider neurons to have reached maturity? Should maturity be considered as reached when the morphology is stable, or rather when functions are undertaken? Besides, the function attributed to a neuron might actually change according to its immaturity or maturity state, which points out for a better understanding of maturity-function of granule neurons. Furthermore, it appears that neurons have different maturation periods according to their ontogenic origin, the latter they are born the longer it will take for them to fully mature. Indeed, even though few studies have addressed this question, it appears that dev-DGNs fully mature within 6 weeks against a minimum 8 weeks for adu-DGNs in mice (Overstreet Wadiche et al., 2005; Zhao et al., 2006; Cahill et al., 2017; Kerloch et al., 2019) and even longer when neurons are produced at middle-age (Trincherro et al., 2017). Even further complexifying the issue, neuronal maturation appears to differ across species with indications that it occurs more rapidly in rats compared to mice, for which adu-DGNs would reach maturity at 6 weeks of age (Snyder et al., 2009). To add complexity, the switch between these immaturity/maturity does not seem to be uniform within the same ontogenic population: neurons from the same ontogenic population do not necessarily change behavior at the same time, and neurons born at different times may behave similarly (Brunner et al., 2014). Finally, genetic (Kempermann and Gage, 2002; Zocher et al., 2021) and environmental factors (Kempermann et al., 1997; Tashiro et al., 2007; Alvarez et al., 2016) can also influence the maturation process, by either facilitating or impeding it. Altogether, maturation depends on the ontogenic origin, the species, the individual neuron itself and the genetic and environmental influences.

In this context, the literature has examined the functions of immature and mature granule neurons in various ways with different time windows for maturity, often yielding conflicting conclusions due to the diverse timeframes used to define maturity. Here, by separating neurons born during embryogenesis (emb-DGNs), neo-natal period (neo-DGNs) from birth to weaning at P20-21, adolescence (adol-DGNs) after weaning to P63 (Spear, 2000) and adulthood (adu-DGNs), we will address the differences between granule neurons according to both their ontogenic origin and their maturity, in order to better understand their functional implication in memory. Given

the results exposed before, all dev-DGNs (emb/neo/adol-DGNs) will be considered as mature once 6 week-old in mice and rats. Adu-DGNs born during young adulthood will be considered as mature once 8 week-old in mice and once 6 week-old in rats. For neurons born later at middle-age or old-age, the exact age at which maturity is reached remains unknown but will be considered as more than 8 week-old. This working hypothesis is illustrated in **figure 8**. We realize that it might be questioned again in the future, especially for the maturity of adu-DGNs at 8 week-old, as recent *in vivo* analysis revealed that 9-12 weeks adu-DGNs still exhibit different properties than mature granule neurons (McHugh et al., 2022).

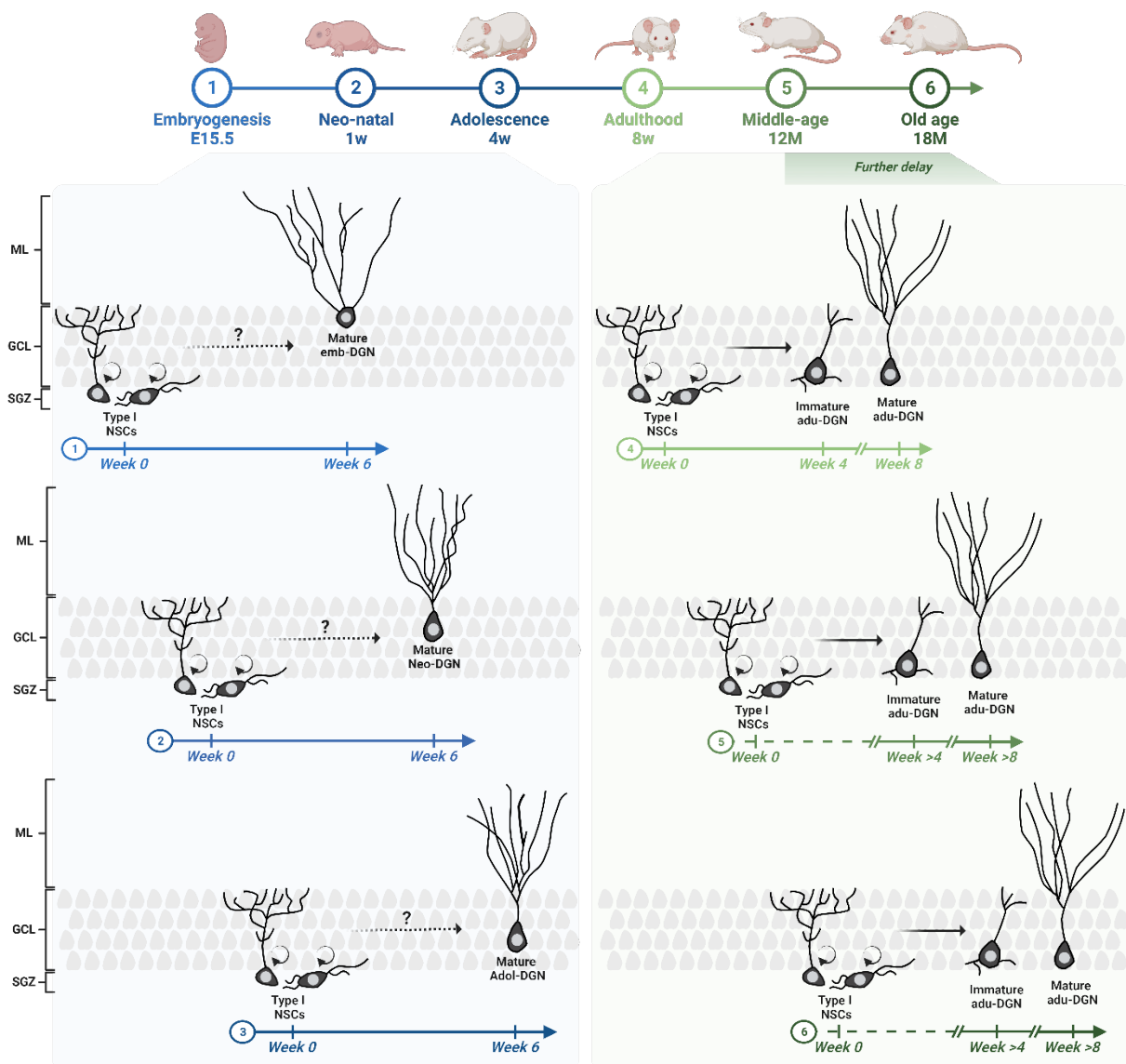


Figure 8: Different maturation periods of granule neurons of the dentate gyrus according to their ontogenic origin. *adu-DGNs: adult-born dentate granule neurons; adol-DGN: adolescence-born dentate granule neuron; emb-DGNs; embryogenesis-born dentate granule neurons; NSCs: neuronal stem cells; neo-DGNs: neonatally-born dentate granule neuron.*

C. Developmentally-born and adult-born dentate granule neurons: similar or different?

As introduced in the foreword, one of the dogma of the adult neurogenesis field is that once they completed their maturation, adu-DGNs resemble and become indistinguishable from dev-DGNs and even eventually retire from the network (Alme et al., 2010; Gu et al., 2012). This is exemplified in the different papers of the literature by the distinction between immature neurons (considered as adu-DGNs) and mature neurons (that include mature adu-DGNs and dev-DGNs). However, this is not exactly correct as we will develop in this section.

1. *Morphological differences*

Adu-DGNs follow similar developmental steps than dev-DGNs and exhibit the same basic morphological properties: their dendrites develop throughout the ML, their soma is found in the GCL and they extend an axon through the hilus toward the CA3 (Llorens-Martín et al., 2016). This seems to be supported by the fact that single-cell RNA-sequencing analysis confirmed that regardless of their ontogenic origin, neurons go through similar molecular identity from neuroblasts to immature neurons (Hochgerner et al., 2018). However, the morphological similarities stop at this basic observation. Indeed, Kerloch and colleagues have conducted a more in-depth study on the morphology of mature granule neurons according to their ontogenic origin either born during embryogenesis (born E14.5, aged 11w), neo-natal period (born P0, aged 12w) and adolescence (born P21, aged 15w) or during adulthood (born 12w, aged 12w) in mice. First, they confirmed that the DG develops in an outside-in layering pattern (Schlessinger et al., 1975), and therefore that the cell body of granule neurons born last will be situated in the inner part of the GCL close to the hilus. The key point in this study is not so much the difference between dev-DGNs and mature adu-DGNs globally, but rather the distinction within the subgroups of DGNs in comparison to the rest. In terms of broad morphology, emb-DGNs were shown to have the shortest dendritic length while neo-DGNs were shown to have the longest, with adol-DGNs and adu-DGNs in between. For more subtle morphological features, the emb-DGNs population (born E14.5, aged 11w) was shown to exhibit differences from neo-DGNs (born P0, aged 12w), adol-DGNs (born P21, aged 15w) and adu-DGNs (born 12w, aged 12w), which were shown to be more similar to each other. Emb-DGNs have multiple primary dendrites, while the other DGNs have only one. Additionally, emb-DGNs show greater complexity in their proximal dendrites compared to the other DGNs, but less complexity in their distal dendrites. Finally, emb-DGNs also display a

wider branching angle of their dendrites compared to the others (Kerloch et al., 2019). This suggests differences in the strength and integration of synaptic inputs from different pathways between the populations.

In another morphological study made in rats, the morphological differences between neo-DGNs (born at P8) and adu-DGNs were also reported (Cole et al., 2020). Spine densities in adu-DGNs reach and surpass that of neo-DGNs. Interestingly, the variations in spine (mushroom type) densities across the IML, MML, and OML were also examined. While mature neo-DGNs (born at P1, aged 16w) and immature adu-DGNs (born 9 to 14w, aged 2 to 7w) exhibit lower mushroom density in the IML compared to the MML and OML, mature adu-DGNs (born 8w, aged 24w) demonstrate similar densities in all three layers. This indicates that mature adu-DGNs adopt a different connectivity pattern compared to neo-DGNs. Neo-DGNs seem to be more influenced by the EC projecting to the MML and OML than by the projections into the IML, namely from mossy cells and the SuM. On the contrary, adu-DGNs are equally connected in all three layers, suggesting an equal influence of the EC, mossy cells and SuM. Moreover, this study also examined MFBs between the ontogenic populations. The MFBs of immature adu-DGNs (born 9 to 14w, aged 2 to 7w) were shown to progressively increase in size with neuronal maturation, until reaching a wider size in mature adu-DGNs (born 8w, aged 24w) compared to mature neo-DGNs (born at P1, aged 16w). Preliminary data obtained in the laboratory in which the MFBs of emb, neo, ado, and adu-DGNs were investigated indicate that MFBs of DGNs are bigger the later they are born (Mortessagne, P., Pacary, E., *unpublished data*).

In terms of connectivity, a study comparing emb-DGNs (born E15.5, aged 13w) and adu-DGNs (born 6w, aged 6w) indicated that adu-DGNs display a higher degree of global connectivity ratio compared to their embryonic counterparts, for both intra-hippocampal and long range projections (Sun et al., 2021).

		Animals	Animal age Labelling	Animal age Experience	Neuron age Experience	Observation	Conclusion	References																																																																																															
Position GCL	Emb/Neo	Mice	E14.5/P0/P21	11w/12w/15w	12w	Outside-in layering pattern	Emb>Neo>mAdu	Schlessinger et al., 1975 Kerloch et al., 2019																																																																																															
	mAdu		12w	24w	12w				Dendritic length	Emb/Neo	Mice	E14.5/P0/P21	11w/12w/15w	12w	Neo-DGNs have the longest dendrites, emb-DGNs have the shortest	Emb<Adu<Neo	Kerloch et al., 2019	mAdu	12w	24w	12w	Primary dendrite	Emb/Neo	Mice	E14.5/P0/P21	11w/12w/15w	12w	Emb-DGNs have more primary dendrites than neo-DGNs and adu-DGNs	Emb>Neo=mAdu	Kerloch et al., 2019	mAdu	12w	24w	12w	Proximal dendrites complexity	Emb/Neo mAdu	Mice	E14.5/P0/P21 12w	11w/12w/15w 24w	12w 12w	Emb-DGNs have more complex proximal dendrites	Emb>Neo=mAdu	Kerloch et al., 2019	Distal dendrites complexity	Emb/Neo mAdu	Mice	E14.5/P0/P21 12w	11w/12w/15w 24w	12w 12w	Emb-DGNs have less complex distal dendrites	Emb<Neo=mAdu	Kerloch et al., 2019	Branching angle	Emb/Neo mAdu	Mice	E14.5/P0/P21 12w	11w/12w/15w 24w	12w 12w	Emb-DGNs have wider branching angle	Emb>Neo=mAdu	Kerloch et al., 2019	MFBS	Neo	Rats	P1	16w	16w	iAdu MFBS increase in size during maturation until reaching wider size than neo-DGNs	Neo<mAdu	Cole et al., 2020	iAdu	14w/12w/9w	16w	2w/4w/7w	mAdu	8w	32w	24w	Mushroom density	Neo	Rats	P1	16w	16w	IML<MML=OML IML<MML=OML IML=MML=OML	Neo=iAdu>mAdu	Cole et al., 2020	iAdu	14w/12w/9w	16w	2w/4w/7w	mAdu	8w	32w	24w	Connectivity ratio	Emb	Mice	E15.5	12w	13w	iAdu have higher connectivity ratio compared to emb-DGNs	Emb<iAdu
Dendritic length	Emb/Neo	Mice	E14.5/P0/P21	11w/12w/15w	12w	Neo-DGNs have the longest dendrites, emb-DGNs have the shortest	Emb<Adu<Neo	Kerloch et al., 2019																																																																																															
	mAdu		12w	24w	12w				Primary dendrite	Emb/Neo	Mice	E14.5/P0/P21	11w/12w/15w	12w	Emb-DGNs have more primary dendrites than neo-DGNs and adu-DGNs	Emb>Neo=mAdu	Kerloch et al., 2019	mAdu	12w	24w	12w	Proximal dendrites complexity	Emb/Neo mAdu	Mice	E14.5/P0/P21 12w	11w/12w/15w 24w	12w 12w	Emb-DGNs have more complex proximal dendrites	Emb>Neo=mAdu	Kerloch et al., 2019	Distal dendrites complexity	Emb/Neo mAdu	Mice	E14.5/P0/P21 12w	11w/12w/15w 24w	12w 12w	Emb-DGNs have less complex distal dendrites	Emb<Neo=mAdu	Kerloch et al., 2019	Branching angle	Emb/Neo mAdu	Mice	E14.5/P0/P21 12w	11w/12w/15w 24w	12w 12w	Emb-DGNs have wider branching angle	Emb>Neo=mAdu	Kerloch et al., 2019	MFBS	Neo	Rats	P1	16w	16w	iAdu MFBS increase in size during maturation until reaching wider size than neo-DGNs	Neo<mAdu	Cole et al., 2020	iAdu	14w/12w/9w	16w	2w/4w/7w		mAdu		8w	32w	24w				Mushroom density	Neo	Rats	P1	16w	16w	IML<MML=OML IML<MML=OML IML=MML=OML	Neo=iAdu>mAdu		Cole et al., 2020		iAdu	14w/12w/9w	16w				2w/4w/7w	mAdu	8w	32w	24w	Connectivity ratio	Emb	Mice	E15.5	12w	13w	iAdu have higher connectivity ratio compared to emb-DGNs	Emb<iAdu	Sun et al., 2021	iAdu	6w
Primary dendrite	Emb/Neo	Mice	E14.5/P0/P21	11w/12w/15w	12w	Emb-DGNs have more primary dendrites than neo-DGNs and adu-DGNs	Emb>Neo=mAdu	Kerloch et al., 2019																																																																																															
	mAdu		12w	24w	12w				Proximal dendrites complexity	Emb/Neo mAdu	Mice	E14.5/P0/P21 12w	11w/12w/15w 24w	12w 12w	Emb-DGNs have more complex proximal dendrites	Emb>Neo=mAdu	Kerloch et al., 2019	Distal dendrites complexity	Emb/Neo mAdu	Mice	E14.5/P0/P21 12w	11w/12w/15w 24w	12w 12w	Emb-DGNs have less complex distal dendrites	Emb<Neo=mAdu	Kerloch et al., 2019	Branching angle	Emb/Neo mAdu	Mice	E14.5/P0/P21 12w	11w/12w/15w 24w	12w 12w	Emb-DGNs have wider branching angle	Emb>Neo=mAdu	Kerloch et al., 2019	MFBS	Neo	Rats	P1	16w	16w	iAdu MFBS increase in size during maturation until reaching wider size than neo-DGNs	Neo<mAdu	Cole et al., 2020	iAdu	14w/12w/9w	16w	2w/4w/7w		mAdu		8w	32w	24w				Mushroom density	Neo	Rats	P1	16w	16w	IML<MML=OML IML<MML=OML IML=MML=OML	Neo=iAdu>mAdu	Cole et al., 2020	iAdu	14w/12w/9w	16w	2w/4w/7w		mAdu		8w	32w	24w			Connectivity ratio		Emb	Mice	E15.5	12w	13w	iAdu have higher connectivity ratio compared to emb-DGNs	Emb<iAdu	Sun et al., 2021	iAdu	6w	12w	6w											
Proximal dendrites complexity	Emb/Neo mAdu	Mice	E14.5/P0/P21 12w	11w/12w/15w 24w	12w 12w	Emb-DGNs have more complex proximal dendrites	Emb>Neo=mAdu	Kerloch et al., 2019																																																																																															
Distal dendrites complexity	Emb/Neo mAdu	Mice	E14.5/P0/P21 12w	11w/12w/15w 24w	12w 12w	Emb-DGNs have less complex distal dendrites	Emb<Neo=mAdu	Kerloch et al., 2019																																																																																															
Branching angle	Emb/Neo mAdu	Mice	E14.5/P0/P21 12w	11w/12w/15w 24w	12w 12w	Emb-DGNs have wider branching angle	Emb>Neo=mAdu	Kerloch et al., 2019																																																																																															
MFBS	Neo	Rats	P1	16w	16w	iAdu MFBS increase in size during maturation until reaching wider size than neo-DGNs	Neo<mAdu	Cole et al., 2020																																																																																															
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Mushroom density	Neo	Rats	P1	16w	16w	IML<MML=OML IML<MML=OML IML=MML=OML	Neo=iAdu>mAdu	Cole et al., 2020																																																																																															
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Connectivity ratio	Emb	Mice	E15.5	12w	13w	iAdu have higher connectivity ratio compared to emb-DGNs	Emb<iAdu	Sun et al., 2021																																																																																															
	iAdu		6w	12w	6w																																																																																																		

Table 2: Morphological evidences supporting differences between different ontogenic dentate granule neurons populations. *Dev: developmentally-born dentate granule neurons; emb; embryonically-born dentate granule neurons; GCL: granule cell layer; iAdu: immature adult-born dentate granule neurons; IML: inner molecular layer; mAdu: mature adult-born dentate granule neurons; MFBS: mossy fibers boutons; mGCs: mature granule neurons; MML: middle molecular layer; neo: neonatally-born dentate granule neuron; OML: outer molecular layer.*

2. Electrophysiological differences

As mentioned before, the fact that adu-DGNs are distinct from dev-DGNs when immature has been well documented in the literature, but supposedly only during their traditional critical period. At this time they display higher plasticity, enhanced somatic (Schmidt-Hieber et al., 2004; Espósito et al., 2005; Ge et al., 2007) and output terminals excitability (Vyleta and Snyder, 2023). However, once fully mature, adu-DGNs are believed to share highly similar electrophysiological properties with neo-DGNs born at P7 (Laplagne et al., 2006). Yet, the “adu-DGNs” population was labelled at 6 weeks of age in mice, an age that corresponds to the adolescent period. Therefore, it can only be concluded that mature adol-DGNs (born 6w, aged 14w) share similar electrophysiological properties with mature neo-DGNs (born P7, aged 19w) (Laplagne et al., 2006). A more recent study reported that emb-DGNs (born E12.5, aged 6 to 25w) exhibited early firing and lower excitability compared to mature granule neurons (Save et al., 2019), a fact undetected before as dev-DGNs were mostly studied as neo-DGNs. While this study did not specifically compare them to other dev-DGNs or adu-DGNs, it does suggest distinct electrophysiological

properties according to the ontogenic origin. Nevertheless, it could be argued that even if granule neurons do reach similarity in their electrophysiological characteristics when mature, it does not necessarily indicate identical functions, but simply implies that they reached functional maturity and are able to undertake their roles.

	Animals	Animal age Labelling	Animal age Experience	Neuron age Experience	Observation	Conclusion	References
Synaptic plasticity & somatic excitability	iAdu mGCs	Rats	-	2-3 M	PSA-NCAM+ PSA-NCAM-	Higher plasticity & excitability in iAdu	iAdu>mGCs Schmidt-Hieber et al., 2004
Synaptic plasticity & somatic excitability	Neo Adol	Mice	P7 6w	20w 20w	19w 14w	Similar plasticity & excitability	Neo=Adol Laplagne et al., 2006
Excitability	Emb mGCs	Mice	E12.5 ?	5w-24w 5w-24w	High variability	Emb-DGNs have early firing but lower excitability	Emb<mGCs Save et al., 2019
Somatic & terminal excitability	Neo iAdu mAdu	Mice	P0-P5 6-8w 6-8w	12-29w 10-12w 12-28w	12-29w 4-6w >8w	Enhanced excitability of soma and MFBs	iAdu>Neo=mAdu Vyleta and Snyder, 2023

Table 3: Electrophysiological evidences supporting differences between different ontogenic dentate granule neurons populations. *Emb*; embryonically-born dentate granule neurons; *iAdu*: immature adult-born dentate granule neurons; *mAdu*: mature adult-born dentate granule neurons; *mGCs*: mature granule neurons; *neo*: neonatally-born dentate granule neuron

In summary, this fourth chapter of introduction detailed the generation and integration process of newborn adu-DGNs. It highlighted the diversity in the maturation processes between dentate granule neurons according to their ontogenic origin. The morphological and electrophysiological differences between granule neurons have been presented according to their ontogenic origin and maturity state. This diversity calls for possible different functional involvement between the populations of granule neurons.

In the next section we therefore focused on understanding the reciprocal relationship between neurogenesis and learning and memory, but also by taking into account both the ontogenic origin of the neurons and their maturity state.

V. DENTATE GRANULE NEURONS AND MEMORY

A. Background

Before the investigations on adult neurogenesis, the DG was a largely “ignored” region. Most of the “old” studies were based on the use of colchicine, an alkaloid that binds tubulin and blocks mitosis and axoplasmic transport, and preferentially destroys granule neurons (Goldschmidt and Steward, 1980, 1982). These lesions resulted in the impairment of spatial reference memory (allocentric memory) measured in the watermaze and radial maze (Xavier and Costa, 2009). Both acquisition (lesion before training) and retention (lesion after training) were impaired indicating that the DG is necessary for both. The role of the DG in generating associative memories was first reported using a contextual fear conditioning task. A deficit in forming context-specific fear memory was observed in DG-lesioned animals (Lee and Kesner, 2004; Hernández-Rabaza et al., 2008). In addition to its role in the formation of spatial and associative memories, the DG was described as essential to detect novel spatial arrangements relative to previously experienced ones (Gilbert et al., 2001; Lee et al., 2005). The ability of DG-lesioned animals to detect novel spatial arrangements was found to depend upon the proximity of the locations. When the spatial cues were distal (i.e. high distance between the two objects), with less overlap resulting in less similarity, DG-lesioned animals were not impaired. In contrast, when spatial locations were proximal (i.e. short distance between the two objects), a condition increasing overlap and spatial similarity, DG-lesioned animals were impaired (Gilbert et al., 2001). This ability of the DG to discriminate metric relationships between objects (Goodrich-Hunsaker et al., 2008) refers to memory discrimination (“pattern separation”) already mentioned at the beginning of this introduction.

Altogether, these data indicate that the DG is required for the different phases of spatial and associative learning: encoding, retention/consolidation and retrieval, although its role in memory storage remains to be clearly defined. It is also essential for detecting novel spatial arrangements, and for establishing positional relationships among multiple independent environmental cues thus allowing the flexible use of acquired information, and finally it is involved in memory discrimination. Given the heterogeneity of the DG neuronal population, one could

wonder whether all these functions are equally assumed by all populations or if these functions are divided among them.

B. Influence of spatial learning on neurogenesis

Given our interest in spatial learning abilities at old age, it seems important to address the fact that spatial learning itself impacts the neurogenesis process at different steps. In 1999, Elisabeth Gould demonstrated for the first time in rats that spatial learning increases the survival of neurons generated one week before the learning process (Gould et al., 1999a). It is important to note that this effect is hippocampal-dependent, because it was absent in animals subjected to egocentric cue-based learning, which is hippocampal-independent, or to yoked animals (i.e. animals submitted to learning without a platform) (Gould et al., 1999a). This effect has since been confirmed, with additional details in rats in the laboratory (but not in mice) (Trincherro et al., 2015). Spatial learning in the watermaze can be sub-divided into two phases; the early phase, during which spatial performances improve rapidly, and the late phase, during which performances stabilize. It was confirmed that adu-DGNs generated just before the learning process (1 week) undergo an increased survival. Interestingly; the adu-DGNs born during the early phase of learning were shown to die when animals become experts (late phase/end of learning) (Döbrössy et al., 2003). This decline in neurogenesis was shown to be necessary for the stabilization of the memory as it negatively correlates with the performances. This decline was later shown to result from an apoptotic elimination of the newly born cells during the early phase (Dupret et al., 2007). Consequently, blocking apoptosis by pharmacological approach has been shown to block cell death, to prevent the increase in survival of neurons born just before learning and to impair learning (Dupret et al., 2007). This selection made by spatial learning could be due to the fact that non-selected adu-DGNs need to "make space" for the selected adu-DGNs. Following this balance of survival/death induction during the early phase of learning, the late phase of learning was shown to promote the proliferation of new adu-DGNs, which then survive for up to five weeks after animals have acquired the task and differentiate into neurons (Döbrössy et al., 2003). Interestingly, blocking cell death of neurons born during the early phase of learning also blocked this rebound in cell proliferation, indicating that all these process are intermingled and homeostatically regulated (Dupret et al., 2007).

In terms of maturation, spatial learning has been shown to promote the dendritic development and to increase the number of spines of adu-DGNs generated one week before the start of learning (therefore the ones selected by spatial learning for survival) as well as the ones

whom survived the elimination wave. Interestingly, blocking cell death also blocks the dendritic remodeling indicating competitive events between cohorts of new cells. This homeostatic regulation of cell number and dendritic development is also regulated by NMDA receptors, highlighting the role of glutamate in shaping new network. Learning-induced changes in dendritic arborization is long-lasting and endures for weeks (Tronel et al., 2010) and is not limited to immature adu-DGNS: even adu-DGNs aged of 4 months, thus long mature and integrated into the network, displayed increased dendritic length (and number of spines) following learning (Lemaire et al., 2012). Of note, only adu-DGNs were shown to reshape in response to learning, which was not the case for dev-DGNs (Tronel et al., 2010). These observation challenge: i) the notion that adu-DGNs are malleable only during their traditional critical period but rather indicate that they remain so for at least 4 months after complete maturation and ii) that adu-DGNs and dev-DGNs are similar once maturity is reached, as dev-DGNs are not influenced by spatial learning, except if one considers that they are still not mature at 4 months of age.

C. Role of dentate neurons in memory

As mentioned earlier, the DG is highly heterogenic as it is constituted by neurons having different ontogenic origin, which actually display highly different morphological properties and potential different connectivity. We propose that such differences based on their ontogenic origin underlie different functional implications. This hypothesis derived from a pioneer work of the laboratory showing that neo-DGNs are recruited when animals have to navigate into two very different contexts whereas adu-DGNs are recruited by spatial navigation (Tronel et al., 2015b). The following section will focus on reviewing the literature in order to highlight the concept that both the ontogenic origin and maturity state adu-DGNs are important variables to take into consideration when addressing the role of adu-DGNs.

Different tools have been used to study the role of adult neurogenesis: i) irradiation that leads to a permanent depletion of stem cells (Wojtowicz, 2006), ii) pharmacological tools such as cytosine-beta-D-arabino-furanoside (Ara-C) (Doetsch et al., 1999), methylazoxymethanol acetate (MAM) (Shors et al., 2002), or temolozomide (TMZ) (Garthe et al., 2009), iii) transgenic mice or rats leading to decreased cell proliferation (Deng et al., 2010), or increase cell survival (Sahay et al., 2011) or accelerate cell maturation (Farioli-Vecchioli et al., 2008), iv) and more recently optogenetic (Gu et al., 2012) and pharmacogenetics (Alvarez et al., 2016) tools to silence or stimulate the activity of adu-DGNs.

1. *The dentate gyrus sparse coding*

Before delving into the memory functions of granule neurons, it seems important to mention and investigate their basic physiological control of the DG. Indeed, one particularity of the DG is its sparse and low activity compared to other brain regions. Adult neurogenesis is believed to participate in this sparse coding, due to the high excitability of immature adu-DGNs during their critical period. The first work suggesting this idea relied on neurogenesis ablation (by irradiation and transgenic approaches) that led to the increase of gamma bursts amplitude in the DG (Lacefield et al., 2012). It seems worthy to mention that ablation started at 6 weeks of age in this study, therefore resulting in the ablation of adol-DGNs as well as adu-DGNs, suggesting a possible shared-function of the two ontogenic populations. In the same line of thoughts, irradiation-mediated ablation of immature and mature adu-DGNs (born 8w, aged 0-14w) led to increased DG excitability (Ikrar et al., 2013). Conversely, increasing the number of immature and mature adu-DGNs (born 8w, aged 0-8w) was shown to decrease the DG excitability (Ikrar et al., 2013). Similar effects were reported when only the population of immature adu-DGNs (born 8w, aged 6-7w) has been increased (Anacker et al., 2018). Later on, less invasive techniques showed that the optogenetic stimulation of immature adu-DGNs (born 8w, aged 7w) resulted in the inhibition of surrounding mature granule neurons. This inhibition has been shown to be indirect via the activation of interneurons which then inhibit mature granule neurons in return (Drew et al., 2016). On the contrary, the chemogenetic silencing of immature adu-DGNs (born 8w, aged 6-7w) was shown to increase the activity of mature granule neurons. Recently, a bigger picture study established that immature adu-DGNs (born 4-6 months, aged 4-7w) indeed impose the sparsity coding of the DG but also to the CA3 and CA1, when mature adu-DGNs (born 4-6 months, aged 9-12w) did not (McHugh et al., 2022). Particularly, these mature adu-DGNs were rather shown to code for the precision of memory. This study was the first to really address the function of mature adu-DGNs and to show that they were specifically not involved in the sparse coding of the DG (even though they still keep functional roles in the network).

Before that, conflicting results stipulated that the sparse coding of the DG could also be assumed by mature adu-DGNs (Temprana et al., 2015). However, the studied granule neurons were generated in 5 to 7 week-old mice, thus adolescent mice. These results actually also points out toward a possible role for adol-DGNs in the DG inhibition as mentioned before (Lacefield et al., 2012). In addition, chemogenetic silencing of immature adol-DGNs in the context of spatial learning (born 6w, aged 4w) also resulted in the increased activity of mature neo-DGNs (born P6, aged 10w) (Ash et al., 2023).

	Animal	Manipulation	Animal age manipulation	Animal age Experience	Neuron age Experience	Observation	Conclusion	References
Adol & iAdu & mAdu	Mice	Irradiations	6w	12-16w	0-10w	Adol, iAdu and mAdu ablation leads to gamma bursts in the DG	iAdu & mAdu & Adol	Lacefield et al., 2012
		Transgenic (GFAP-TK) (reduced neurogenesis by GCV injection)	6-8w	16-20w	0-12w			
iAdu & mAdu	Mice	Irradiations	8-9w	22-23w	0-14w	iAdu & mAdu ablation increases DG excitability	iAdu & mAdu	Ikhar et al., 2013
iAdu & mAdu		Transgenic Nestin-Bax (blocking of apoptosis)	8w	16w	0-8w	iAdu & mAdu increased number decreases photostimulation evoked DG excitability	iAdu & mAdu	
Adol	Mice	Optogenetic stimulation & inhibition	5-7w	9-14w	4-7w	Adol activate feedback inhibition (via interneurons) in the DG	Adol	Temprana et al., 2015
iAdu mGCs	Mice	Optogenetic stimulation	8w	15w	7w	iAdu stimulation resulted in mGCs inhibition	iAdu	Drew et al., 2016
iAdu mGCs	Mice	Chemogenetic silencing	8w	14-15w	6-7w	iAdu inhibition increases mGCs activity	iAdu	Anacker et al., 2018
iAdu	Mice	Transgenic Nestin-Bax (blocking of apoptosis)	8w	14-15w	7w	iAdu increased number decreases DG excitability	iAdu	Anacker et al., 2018
iAdu mAdu	Mice	Optogenetic stimulation	16-24w	16-24w	4-7w	iAdu impose the sparsity of the DG and not mAdu-DGNs	iAdu	McHugh et al., 2022
Adol (immature)	Rats	Chemogenetic silencing	6w	10w	4w	Immature adol inhibition increases the activity of neo-DGNs	Adol	Ash et al., 2023

Table 4: Implication of the different ontogenic populations of dentate granule neurons in the dentate gyrus sparse activity. *Adol: adolescence-born dentate granule neurons; DG: dentate gyrus; GCV: ganciclovir; iAdu: immature adult-born dentate granule neurons; mAdu: mature adult-born dentate granule neurons; mGCs: mature granule neurons; neo: neonatally-born dentate granule neuron.*

2. Associative memory

As discussed before, the DG has been shown to be involved in associative memory, often via the study of contextual-fear memory. Concerning adult neurogenesis, irradiation-mediated ablation of a mixed population of immature and mature adu-DGNs (born 12-25w, aged 0-12w) (given the period before testing) (Saxe et al., 2006) or (born 9-11w, aged 0-9w) (Hernández-Rabaza et al., 2008) was shown to impair contextual-fear memory. Similar observations were made following irradiation-mediated ablation of only immature adu-DGNs (born 16w, aged 0-4w or 0-5w) (Winocur et al., 2006; Wojtowicz et al., 2008). Later on, this irradiation-mediated effect was shown to be specific of the ablation of immature adu-DGNs aged 4 to 6 weeks, that is during their traditional critical period (Denny et al., 2012). Genetic ablation or impairment of immature adu-DGNs (born 12-20w, aged 0-6w) was shown to result in similar outcomes (Saxe et al., 2006), and that for immature adu-DGNs aged either 0 to 3 weeks or 4 to 7 weeks (Farioli-Vecchioli et al., 2008; Mateus-Pinheiro et al., 2021). Recent studies with more specific manipulation of neurogenesis then confirmed by optogenetic silencing of immature adu-DGNs (born 8w, aged 6w) that they were needed for contextual-fear memory (Danielson et al., 2016). This was confirmed by a similar optogenetic silencing study of immature adu-DGNs (and a portion of adol-DGNs given

the age of mice) (born 7-10w, aged 6w) (Huckleberry et al., 2018). Similarly, chemogenetic stimulation (during memory test) of immature adu-DGNs (born 8w, aged 1w during conditioning and 6w during test) was shown to improve contextual-fear memory (Lods et al., 2022). While these studies point out toward an involvement of immature adu-DGNs in associative memory, other studies found no effects: pharmacological ablation of immature adu-DGNs (born adulthood, aged 2w) with MAM had no effect on contextual-trace fear memory (Shors et al., 2002) nor did genetic ablation of immature adu-DGNs (born 8w, aged 0-6w) (Groves et al., 2013) or immature adu-DGNs (born 8w, aged 0-4w) reduced proliferation (Zhang et al., 2008). Such differences could be due to the extent of adult neurogenesis ablation or to the level of maturity of adu-DGNs. Additionally, some studies have found an implication of adol-DGNs in contextual-fear memory (Farioli-Vecchioli et al., 2008; Arruda-Carvalho et al., 2011). When a more mature population of adu-DGNs has been targeted (born 8w aged 0-9), their ablation did not impair the ability of the mice to associate a shock with a context in a fear contextual protocol (Dupret et al., 2008; Tronel et al., 2012). Therefore, there are conflicting results about the involvement of DGNs in contextual-fear memory. While we have argued that such discrepancies could rely either depending on the ontogenic origin, the maturity or the extent of neurogenesis impairments, it can also depend on the procedure (single/multiple trial, pre-exposure) followed to induce fear memory during conditioning (Drew et al., 2010).

The only study in which the role of adol-DGNs has been (unintentionally) addressed shown by optogenetic inhibition of adol-DGNs (born 5-6w, aged 4w) an impairment in contextual-fear memory (Gu et al., 2012).

	Animal	Behavioral test	Manipulation	Animal age manipulation	Animal age Experience	Neuron age Experience	Observation	Conclusion	References
iAdu	Rats	Fear conditioning	Pharmacological ablation (MAM)	Adult (no age)	Adult (3w after arrival)	2w	iAdu ablation decreases trace fear conditioning but not contextual-fear memory	Not iAdu	Shors et al., 2002
iAdu	Rats	Fear conditioning	Irradiations	16w	20w	0-4	iAdu ablation impairs contextual-fear memory	iAdu	Winocur et al., 2006
iAdu & mAdu			Irradiations	12-25w	24-37w	0-12w	iAdu & mAdu ablation impairs contextual-fear conditioning	iAdu & mAdu	
iAdu	Mice	Fear conditioning	Transgenic (GFAP-TK) (reduced neurogenesis by GCV injection)	12-20w	18-26w	0-6w	iAdu reduced numbers impairs contextual-fear conditioning	iAdu	Saxe et al., 2006
iAdu & mAdu	Mice	Fear conditioning	Transgenic Nestin-Bax (ablation by apoptosis)	8w	14-16w	0-8w	iAdu & mAdu ablation has no effect on contextual-fear conditioning	Not iAdu & mAdu	Dupret et al., 2008
iAdu	Mice	Fear conditioning	Transgenic Tlx (reduced proliferation)	8w	12w	0-4w	iAdu reduced proliferation has no effect on contextual-fear memory	Not iAdu	Zhang et al., 2008
Adol	Mice	Fear conditioning	Transgenic Nestin-PC3 (accelerated maturation)	P30 (4w)	P95 (14w)	0-10w	Impaired contextual-fear memory	Adol and iAdu	Farioli-Vecchioli et al., 2008
iAdu	Mice	Fear conditioning		P138 (19w)	P160 (22w)	0-3w			
iAdu	Rats	Fear conditioning	Irradiations	16w	21w	0-5w	iAdu ablation impairs contextual-fear memory	iAdu	Wojtowicz et al., 2008
iAdu & mAdu	Rats	Fear conditioning	Irradiations	9-11w	18-20w	0-9w	iAdu & mAdu ablation impairs contextual-fear memory	iAdu & mAdu	Hernández-Rabaza et al., 2009
Adol	Mice	Fear conditioning	Transgenic Nestin-DTR (cell death by diptheria toxin injection)	4w	11w	7w	Adol ablation post-training impairs contextual-fear memory	Adol but post-training	Arruda-Carvalho et al., 2011
iAdu				9w	11w	0-2w	iAdu ablation (0-2w) has no effect on contextual-fear conditioning		
iAdu	Mice	Fear conditioning	Irradiations	9w	13w	0-4w	iAdu ablation (0-4w) has no effect on contextual-fear conditioning	iAdu in a specific window of 4-6w	Denny et al., 2012
iAdu				9w	15w	0-6w	iAdu ablation (0-6w) decreases contextual-fear conditioning		
Adol	Mice	Fear conditioning	Optogenetic silencing	5-6w	10w	4w	Adol silencing impairs contextual-fear memory	Adol	Gu et al., 2012
iAdu	Rats	Fear conditioning	Transgenic (GFAP-TK) (reduced neurogenesis by GCV injection)	8w	14w	0-6w	iAdu reduced number has no effect on contextual-fear conditioning	Not iAdu	Groves et al., 2013
iAdu	Mice	Fear conditioning	Optogenetic silencing	8w	14w	6w	iAdu silencing impairs contextual-fear memory	iAdu	Danielson et al., 2016
Adol & iAdu	Mice	Fear conditioning	Optogenetic silencing	7-10w	13-16w	6w	Adol & iAdu silencing impairs contextual-fear memory	Adol & iAdu	Huckleberry et al., 2018
iAdu	Rats	Fear conditioning	Transgenic (GFAP-TK) (reduced neurogenesis by GCV injection)	12w	19w	4-7w	iAdu reduced number decreases contextual-fear conditioning	iAdu	Mateus-Pinheiro et al., 2021
iAdu	Rats	Fear conditioning	Chemogenetic stimulation	8w	9w (conditioning) & 14w (test)	1w (conditioning) & 6w (test)	iAdu stimulation during test improves contextual-fear memory retention	iAdu	Lods et al., 2022

Table 5: Implication of the different ontogenic populations of dentate granule neurons in associative memory. *Adol*: adolescence-born dentate granule neurons; *GCV*: ganciclovir; *iAdu*: immature adult-born dentate granule neurons; *mAdu*: mature adult-born dentate granule neurons; *MAM*: methylazoxymethanol acetate.

3. Memory discrimination

As discussed before, memory discrimination (“pattern separation”) can be measured using different tasks: either using the contextual-fear conditioning task or the radial arm maze. The ability for complex memory discrimination has been supposed to rely on adu-DGNs, given the role of the DG in associative memory and the complexity of this function. Indeed, mice with ablated immature and mature adu-DGNs (born 8w, aged 0-9w) displayed impaired complex spatial discrimination abilities measured in the radial maze and in a touchscreen test (Clelland et al., 2009). In the contextual-fear conditioning, ablating a mixed population of immature and mature adu-DGNs (born 8w, aged 0-9w) did not impaired the ability to distinguished two similar contexts.

However, when animals were over-trained for one week and the cells became older (born 8w, aged 0-10w), then deficits emerged suggesting that the inability to distinguish between two similar contexts were linked to mature adu-DGNs (Tronel et al., 2012). Similarly, impairing mature adu-DGNs synaptic plasticity (born 8-10w, aged 8w) (Kheirbek et al., 2012) or ablating by irradiation immature adu-DGNs (born 9-12w, aged 0-6w) (Nakashiba et al., 2012) delays complex contextual-fear discrimination. Conversely, genetically increasing the number of mature adu-DGNs (born 6-10w, aged 0-8w) was shown to improve contextual-fear discrimination (Sahay et al., 2011). Interestingly, a study latter addressed the involvement of the same adu-DGNs population both when immature and mature in a touchscreen discrimination task. Silencing a “younger” mixed population of adu-DGNs (born 6-10w, aged 5-10w) led to impaired discrimination abilities, while it did not when “older” of a few weeks (born 6-10w, aged 14-18w) (Zhuo et al., 2016). More specific studies showed that immature adu-DGNs (born 8w, aged 6w) optogenetically silenced during exposure to a similar context than the conditioning one impaired discrimination abilities (Danielson et al., 2016). Surprisingly, optogenetic silencing of immature adu-DGNs (and a portion of adol-DGNs given the age of mice) (born 7-10w, aged 6w) resulted in improved discrimination abilities, which might be due to silencing performed during test session and not during context discrimination training (Huckleberry et al., 2018).

Importantly, the irradiation-mediated ablation of immature and mature adu-DGNs did not impair the ability to spatially discriminate highly separated arms in the radial arm maze (Clelland et al., 2009) nor did the impairment of adu-DGNs plasticity affect the ability to discriminate two dissimilar contexts (Kheirbek et al., 2012), indicating no role for adu-DGNs in simpler memory discrimination task. Indeed, it has been uncovered using IEGs that simple memory discrimination is not dependent of adu-DGNs but rather of neo-DGNs (born P7, aged 28w) (Tronel et al., 2015b). Besides, it was shown that old granule neurons (which corresponds to dev-DGNs) do not contribute to the discrimination between similar environments (Nakashiba et al., 2012). However, they noticed a trend for their involvement in discriminating relatively distinct contexts. In addition, they showed that old granule neurons were also responsible for pattern completion using the watermaze with “degraded” spatial cues.

These observations align with the notion of different functions according to the ontogenic origin. Dev-DGNs assume simple memory discrimination while adu-DGNs more complex one. Conflicting results about immature/mature adu-DGNs appear, which could suggest a possible shared functions.

	Animal	Behavioral test	Manipulation	Animal age manipulation	Animal age Experience	Neuron age Experience	Observation	Conclusion	References
iAdu & mAdu	Mice	Radial maze / Touch-screen test	Irradiations	8w	17w	0-9w	iAdu & mAdu ablation impairs spatial pattern separation	iAdu & mAdu	Clelland et al., 2009
mAdu	Mice	Fear conditioning	Transgenic Nestin-Bax (blocking of apoptosis)	6-10w	14-18w	0-8w	mAdu increased number accelerates contextual-fear discrimination	mAdu	Sahay et al., 2011
mAdu	Mice	Fear conditioning	Transgenic Nestin-NR2B (impaired synaptic plasticity)	8-10w	16-18w	8w	mAdu impaired synaptic plasticity delays contextual-fear discrimination	mAdu	Kheirbek et al., 2012
iAdu & mAdu	Mice	Fear conditioning	Transgenic Nestin-Bax (blocking of apoptosis)	8w	17w	0-9w	iAdu & mAdu ablation impairs contextual-fear discrimination 10w post-training after longer maturation	mAdu	Tronel et al., 2012
iAdu	Mice	Fear conditioning	Irradiation	9-12w	15-18w	0-6w	iAdu ablation delays contextual-fear discrimination	iAdu	Nakashiba et al., 2012
Neo mAdu	Rats	Watermaze / Drymaze	IEGs based study	P7	28w	28w	Neo (and not mAdu) are activated in dissimilar contexts	Neo, simple memory discrimination	Tronel et al., 2015b
iAdu mAdu	Mice	Location discrimination task	Optogenetic silencing	7-8w	12-18w 21-26w	5-10w 14-18w	iAdu (not mAdu) silencing impairs spatial pattern separation	iAdu	Zhuo et al., 2016
iAdu	Mice	Fear conditioning	Optogenetic silencing	8w	14w	6w	iAdu silencing impairs contextual-fear discrimination	iAdu	Danielson et al., 2016
Adol & iAdu	Mice	Fear conditioning	Optogenetic silencing	7-10w	13-16w	6w	Adol & iAdu silencing improves contextual-fear discrimination	Adol & iAdu	Huckleberry et al., 2018

Table 6: Implication of the different ontogenic populations of dentate granule neurons in memory discrimination. *Dev: developmentally-born dentate granule neurons; iAdu: immature adult-born dentate granule neurons; mAdu: mature adult-born dentate granule neurons; neo: neonatally-born dentate granule neuron.*

4. Spatial learning and memory

Spatial learning allows to navigate through space via the formation of a cognitive map of the environment. This has long been linked to the DG and hypothesized as relying on adult neurogenesis. However, the literature has addressed the involvement of adult neurogenesis in many different ways and several opposite conclusions were drawn. Given the multitude of studies addressing this subject, and the equal number of conclusions, we will first address them according to their methodological approach to manipulate neurons before trying to draw a global conclusion.

Using irradiation, ablation of immature and mature adu-DGNs (given the extent of time before spatial learning) (born 12-25w, aged 0-12w) did not impair spatial learning or memory retention in the watermaze (Saxe et al., 2006). Similarly, only immature adu-DGNs (born 16w, aged 0-5) ablation by irradiation did not impact spatial learning abilities of rats (Wojtowicz et al., 2008). Similar conclusions were reached when animals were raised in an enriched environment with ablation of immature and mature adu-DGNs (born 10w, aged 0-16) (Meshi et al., 2006). Pharmacological ablation of immature adu-DGNs (born during adulthood, aged 1w or 2w) with MAM or Ara-C had no effect on spatial learning abilities (Shors et al., 2002; Lemaire et al., 2012). Similarly, pharmacological reduction of immature adu-DGNs production (born 8w or 44w, aged 0-4w) using TMZ did not induce any deficits in spatial memory. Interestingly, such deficits appeared

only when the treatment was carried out during adolescence reducing the number of immature adol-DGNs (born 4w, aged 0-4w) on spatial memory (but not learning) (Martinez-Canabal et al., 2013a). Nevertheless, concerning adu-DGNs, when the delay between the pharmacological ablation by Ara-C and behavioral testing has been extended, resulting in ablation of mature adu-DGNs aged of 2 to 4 months, deficits in spatial learning emerged, suggesting a role of mature adu-DGNs in spatial learning (Lemaire et al., 2012).

Later studies started to use transgenic mice to manipulate adult neurogenesis, which are more specific. By doing so, the alteration of immature adu-DGNs (born 19w, aged 0-3w) due to their precipitated maturation (Farioli-Vecchioli et al., 2008) or the ablation (Mateus-Pinheiro et al., 2021) of immature adu-DGNs (born 15 or 19w, aged 1-3w or 4-7w) resulted in decreased spatial learning performances. The reduced proliferation of immature adu-DGNs (born 8w, aged 0-4w) was shown to impair short-term memory retention (but not long-term) (Zhang et al., 2008). Similarly, genetic reduction of the number of a mixed population of immature and mature adu-DGNs (born 8w, aged 0-8w) resulted in impaired spatial learning and memory retention, and that only when the allocentric strategy is used and not the egocentric one (Dupret et al., 2008). Another transgenic approach showed that ablation of immature adu-DGNs (born 8w, aged 2-3w) had no effect on spatial learning but did disturb memory retention. Particularly, they also showed that mature adu-DGNs (born 8w, aged 9-11w) ablation had no effect on both learning and memory retrieval (Deng et al., 2009). Later on, the increased number of immature adu-DGNs (born 8w, aged 1 or 6w) after enriched environment resulted in enhanced spatial memory only when adu-DGNs reached the age of 6 week-old (Stone et al., 2011a). Similarly, transgenic reduction in the number of immature adu-DGNs (born 7-10w, aged 0-2w) resulted in decreased spatial avoidance in an active place avoidance task (Vukovic et al., 2013). Therefore, these transgenic approaches seem to tend toward an involvement in spatial learning and/or memory of immature and mature adu-DGNs. However, one study showed that the reduction in the number of immature adu-DGNs (born 8w, aged 0-6w) had no effect on spatial learning (Groves et al., 2013).

Even though transgenic approaches did bring more insights about adu-DGNs involvement in spatial learning, there are still some conflicting results. More recent techniques now allow to manipulate the activity of a neuron without disturbing the “normal” physiological network by addition or removal of neurons, which could represent a bias. Some studies have used optogenetic silencing or chemogenetic silencing/stimulation to better look at the role of adu-DGNs in spatial learning and memory. By doing so, it was shown in rats that adu-DGNs immature (born 8w, aged 1w) during learning (without manipulation during this learning phase) are not needed for memory

retrieval performed 2 days later as their chemogenetic silencing during the memory test had no effect on memory performances. However, adu-DGNs mature (born 8w, aged 6w) during learning (without manipulation during this learning phase) are needed for memory retrieval performed 2 days later as their silencing during the test resulted in impaired memory retrieval performances (Lods et al., 2021). While these results point out for a major role played by mature adu-DGNs in spatial memory, it seems worth noting that the adu-DGNs immature during learning were actually shown to be needed for long-term memory persistence several weeks later and therefore probably keep a “trace” of the learning experience *per se* (Lods et al., 2021). In the same line of idea, it was shown that adu-DGNs either immature (born 8w, aged 1w) or mature (born 8w, aged 6w) at the time of learning (without manipulation during this learning phase) and chemogenetically reactivated during memory retrieval 4 weeks later resulted in enhanced memory retrieval abilities (Lods et al., 2022). We can note here that the period before testing is longer than in the previous experiment with chemogenetic silencing, suggesting that the adu-DGNs immature during learning need to progress through maturation before being able to play an active role in memory retrieval. Nevertheless, this confirms that even though they are immature during learning, they are probable re-shaped by learning and keep a “trace” of this experience as mentioned. Of importance, when neo-DGNs (born P3, aged 8w) at the time of learning are reactivated 4w later during retrieval, it has no effect on memory performances, indicating no role for neo-DGNs in the spatial task, confirming previous data of the lab (Lods et al., 2022).

Therefore, manipulation of adu-DGNs in the context of spatial learning has produced several different conclusions. We can notice that ablation-mediated approaches often brought negative results, which could be due to the impairment of the whole network. On the contrary, more specific methodologies seem more consistent for a functional implication of adu-DGNs.

One other possibility to study the neuronal involvement in spatial learning without manipulating at all the network relies on the use of immediate early genes (IEGs), which are used as proxy to study neuronal activity. A first study reported that adu-DGNs (born at least 8w) were indeed preferentially recruited by spatial learning once they reach the age of 6 week-old compared to the rest of the granule neurons (Kee et al., 2007). However, in a subsequent study, a functional equivalence between adu-DGNs (born 8w, aged 7.5w) and neo-DGNs (born P7, aged 7.5w) (Stone et al., 2011b). A third study pointed out the specific recruitment of adu-DGNs (born 8w) and not neo-DGNs (born P7) by spatial learning (Tronel et al., 2015b). This divergence could be due to the fact that the first study assessed IEGs after a memory test while the second one

assessed it after learning only. It could also be due to the stage of maturity of adu-DGNs, while in the first paper the recruitment of dev-DGNs is compared to immature adu-DGNs, in the second one it is compared to mature adu-DGNs.

Concerning the implication of adol-DGNs, it was revealed by optical controlling a time-dependent role for adol-DGNs: silencing a cohort of ~4 week-old cells during a probe test, but not 2 or 8-week old cells, substantially disrupt memory retrieval (Gu et al., 2012), suggesting their involvement during a temporal window around 1 month-old. Of note, it was concluded that from this study that adu-DGNs retire from the network when mature. However, this conclusion was misleading as the cells were generated in adolescent mice (born at 5-6w). Note that inhibition of adol-DGNs during learning did not influence the performances. Two other studies showed decreased spatial learning abilities following their accelerated maturation by genetic manipulation (born 4w, aged 0-10w) (Farioli-Vecchioli et al., 2008) or by their pharmacological ablation (born 4w, aged 0-4w) with TMZ (Martinez-Canabal et al., 2013a). Similarly, irradiation-mediated ablation of adol-DGNs when supposedly still at their immature age (born 5w, aged 0-4w) had no impact on spatial learning but did impair memory retention once they would have supposedly reach their maturity (born 5w, aged 6-9w) (Snyder et al., 2005). However, these findings should be taken carefully given that the period between irradiation and learning encompasses the beginning of adulthood, some adu-DGNs were also ablated in this study, which could explain the results especially at the latter time point (more adu-DGNs concerned). Indeed, chemogenetic inhibition of immature adol-DGNs (born 6w, aged 4w) had no effect on spatial learning performances in rats (Ash et al., 2023). Once again, it seems of high important to emphasize the importance of the age of animals when granule neurons are manipulated. In these studies, animals were either 5 or 6 week-old at the time of manipulation, which corresponds to the adolescence period, and not adulthood as stipulated. Therefore, conflicting results also exist about adol-DGNs. No investigations were made about adol-DGNs using specific targeted approaches or using IEGs and therefore seems lacking considering these conflicting results.

	Animal	Behavioral test	Manipulation	Animal age manipulation	Animal age Experience	Neuron age Experience	Observation	Conclusion	References
iAdu	Rats	Watermaze	Pharmacological ablation (MAM)	Adult (no age)	Adult (3w after arrival)	2w	iAdu ablation has no effect in the spatial learning phase	Not iAdu, for spatial learning	Shors et al., 2002
Adol & iAdu	Rats	Watermaze	Irradiations	5w	9w 11-14w	0-4w 0-9w	No effect on spatial learning performances Decreased memory retrieval performances	Adol, not for spatial learning but for memory retention	Snyder et al., 2005
iAdu & mAdu	Mice	Watermaze	Irradiations	12-25w	24-37w	0-12w	iAdu & mAdu ablation has no effect on spatial learning or memory retention	Not iAdu & mAdu, for spatial learning and memory	Saxe et al., 2006
iAdu & mAdu	Mice	Watermaze	Irradiations	10w	26w	0-16w	iAdu & mAdu ablation has no effect on spatial learning	Not iAdu & mAdu, for spatial learning and memory	Meshi et al., 2006
iAdu	Mice	Watermaze	IEGs based study	8w	9, 10, 12, 14 or 16w	1, 2, 4, 6, or 8w	iAdu are recruited by spatial learning preferentially once they reach 6 week-old	iAdu	Kee et al., 2007
iAdu	Mice	Watermaze	Transgenic Tlx (reduced proliferation)	8w	12w	0-4w	iAdu reduced proliferation impairs short-term memory retention (not long-term)	iAdu, spatial memory	Zhang et al., 2008
iAdu	Rats	Watermaze	Irradiations	16w	21w	0-5w	iAdu ablation has no effect on spatial learning abilities	Not iAdu, spatial learning	Wojtowicz et al., 2008
Adol iAdu	Mice	Watermaze / Radial maze	Transgenic Nestin-PC3 (premature maturation)	4w 19w	P95 (14w) P160 (22w)	0-10w 0-3w	Impaired learning (WM and radial) and reversal (WM) Impaired learning (WM and radial)	Adol & iAdu	Farioli-Vecchioli et al., 2008
iAdu & mAdu	Mice	Watermaze	Transgenic Nestin-Bax (ablation by apoptosis)	8w	14-16w	0-8w	No deficits in egocentric learning but impaired allocentric learning and memory. Impaired flexibility with novel departure point	iAdu & mAdu, for spatial learning and memory	Dupret et al., 2008
iAdu mAdu	Mice	Watermaze	Transgenic (Nestin-TK) (reduced neurogenesis by GCV injection)	8w	11w 19w	2-3w 9-11w	iAdu reduced number does not affect learning but impairs memory retention No effect on spatial learning nor memory retention	iAdu, not learning but memory mAdu, not learning nor memory	Deng et al., 2009
iAdu iAdu	Mice	Watermaze	Enhanced neurogenesis by entorhinal cortex stimulation	8w	9w 14w	1w 6w	iAdu increased number enhances spatial memory once they reach 6 week-old	iAdu, spatial memory	Stone et al., 2011a
Emb Neo iAdu	Mice	Watermaze	IEGs based study	E18 P7 (1w) 8w	6,5w 8,5w 15,5w	7,5w 7,5w 7,5w	Functional equivalence between all temporally born granule cells in spatial memory	Emb, PN and iAdu	Stone et al., 2011b
iAdu mAdu	Rats	Watermaze	Pharmacological ablation (AraC)	8w	9w 16w or 24w	1w 8w or 16w	iAdu ablation has no effect on spatial learning abilities mAdu ablation impairs spatial learning abilities	mAdu	Lemaire et al., 2012
Adol	Mice	Watermaze	Optogenetic silencing	5-6w	8w or 10w or 14w	2w or 4w or 8w	Adol silencing impairs memory retrieval only when aged 4 week-old	Adol	Gu et al., 2012
iAdu	Mice	Active place avoidance	Transgenic DCX-DTR (ablation by diphtheria toxin of DCX+ cells)	7-10w	9-12w	0-2w	iAdu ablation decreases spatial learning for place avoidance task but not the memory trace later on	iAdu, for spatial learning but not memory	Vukovic et al., 2013
iAdu	Rats	Watermaze / Radial maze	Transgenic (GFAP-TK) (reduced neurogenesis by GCV injection)	8w	14w	0-6w	iAdu reduced number has no effect on spatial learning	Not iAdu, for spatial learning	Groves et al., 2013
Adol iAdu	Mice	Watermaze	Pharmacological ablation (TMZ)	4w 8w 44w	8w 12w 48w	0-4w 0-4w 0-4w	Adol (and not iAdu) reduced numbers impairs spatial memory retention (but not learning)	Adol, for spatial memory	Martinez-Canabal et al., 2013
Neo mAdu	Rats	Watermaze	IEGs based study	P7 8w	8 or 16w or 24w	8 or 16w 16w	mAdu (and not neo) are necessary for spatial learning	mAdu, for spatial learning	Tronel et al., 2015b
iAdu	Rats	Watermaze	Transgenic (GFAP-TK) (reduced neurogenesis by GCV injection)	12w	15 or 19w	1-3w or 4-7w	iAdu reduced number has no effect on spatial learning	Not iAdu, for spatial learning	Mateus-Pinheiro et al., 2021
iAdu mAdu	Rats	Watermaze	Chemogenetic silencing	8w	9w 14w	1w 6w	Silencing iAdu has no effect on short-term memory retrieval Silencing mAdu impairs spatial memory retrieval	mAdu, for spatial memory	Lods et al., 2021
Neo iAdu mAdu	Rats	Watermaze	Chemogenetic stimulation	P3 8w 8w	8w (learning) & 13w (test) 9w (learning) & 14w (test) 14w (learning) & 19w (test)	8w (learning) & 13w (test) 1w (learning) & 6w (test) 6w (learning) & 11w (test)	Neo stimulation during retrieval has no effect iAdu & mAdu non-stimulated during learning but stimulated during memory test enhance memory retrieval performances	iAdu & mAdu, for long-term spatial memory	Lods et al., 2022
Adol	Rats	Watermaze	Chemogenetic silencing	6w	10w	4w	Adol inhibition has no effect on spatial learning performances	Not Adol, for spatial learning	Ash et al, 2023

Table 7: Implication of the different ontogenic populations of dentate granule neurons in spatial learning and memory. Adol: adolescence-born dentate granule neurons; DCX: doublecortin; Emb; embryonically-born dentate granule neurons; GCV: ganciclovir; iAdu: immature adult-born dentate granule neurons; IEGs: immediate early genes; mAdu: mature adult-born dentate granule neurons; MAM: methylazoxymethanol acetate; neo: neonatally-born dentate granule neuron; TMZ: temozolomide.

5. Cognitive flexibility

Cognitive flexibility corresponds to the ability to flexibly use and ignore familiar associations when contingencies change, which enables the most appropriate response in face of new challenges. Even though this ability is mostly attributed to the prefrontal cortex, some evidences suggested a role of the DG as well. In the context of spatial navigation, cognitive flexibility can be studied in different ways. First, following allocentric learning (constant platform, variable departure points), the platform position can be moved to another location, a protocol often referred to as reversal learning. Animals with good flexibility will quickly re-learn the novel platform localization. It can also be studied following egocentric learning (constant platform, constant departure point), when the platform is also moved to another localization and the departure point remains the same. Similarly, following egocentric learning, it can be studied with the platform localization remaining constant but a change in the departure point, in that case animals will have to flexibly use the cues to go back to the platform. It was this last form of flexibility test that first identified a role for the hippocampus in cognitive flexibility, as animals with hippocampal lesions were able to undergo egocentric learning and to find the platform (place learning) but were unable to find it from a novel starting point (Eichenbaum et al., 1990). This led to the relational theory of memory proposing that the hippocampus is important for inferential reasoning which depends upon the ability to use learned information in a novel condition. In the same behavioral protocol as described for the previous study, the role of adult neurogenesis in this flexibility was shown by ablation of a mixed population of immature and mature adu-DGNs (born 8w, aged 0-10w) which resulted in a similar outcome with an inability to find the platform from a new departure point (Dupret et al., 2008). In a protocol test of reversal learning (change in platform position after allocentric learning), ablation of immature adu-DGNs (born 12w, aged 1-3w or 4-7w) by genetic approaches also resulted in impaired flexibility (Mateus-Pinheiro et al., 2021). Recently, a multi-modal training in the watermaze including first allocentric learning followed by reversal learning and then by context-discrimination learning showed that immature adu-DGNs (born 16w, aged 6 or 8w) were needed for complex cognitive flexibility (Amelchenko et al., 2023). A similar observation was later made in other behavioral tests, in which transgenic ablation of immature adu-DGNs (born 7-10w, aged 0-2w) resulted in impaired flexibility in a touch-screen discrimination task (Swan et al., 2014). Additionally, irradiation-mediated ablation of immature and mature adu-DGNs (born 10w, aged 0-16/17w) resulted in impaired flexibility in an active place avoidance task (Burghardt et al., 2012; Park et al., 2015). These two studies looked at the behavioral outcome 16 and 17 weeks post-irradiation, indicating that some of the ablated

neurons should be mature at the time of behavior. Therefore, the observed reduced cognitive flexibility could also rely on the decreased number of mature adu-DGNs. Altogether, all studies are consistent in underlying the role of immature adu-DGNs in cognitive flexibility, with a hint for them still undertaking this function once they reach maturity, but further specific investigations are needed to conclude for mature adu-DGNs.

Concerning dev-DGNs, some studies again unintentionally targeted adol-DGNs (born 6-8w, aged 5-7w) and showed that after egocentric learning in the watermaze, their ablation with TMZ resulted in an impaired ability to adapt to a new platform localization (Garthe et al., 2009).

	Animal	Behavioral test	Manipulation	Animal age manipulation	Animal age Experience	Neuron age Experience	Observation	Conclusion	References
iAdu & mAdu	Mice	Watermaze	Transgenic Nestin-Bax (ablation by apoptosis)	8w (beginning of ablation)	14-16w	0-10w	iAdu & mAdu needed for finding unchanged platform from new departure point (training made from constant departure point)	iAdu & mAdu	Dupret et al., 2008
Adol	Mice	Watermaze	Pharmacological ablation (TMZ)	6-8w	14-16w	5-7w	Adol needed for finding new platform localization (training made from constant departure point)	Adol	Garthe et al., 2009
iAdu & mAdu	Mice	Active place avoidance	Irradiations	10w	23-27w	0-17w	iAdu & mAdu needed for shock zone switch avoidance	iAdu & mAdu	Burghardt et al., 2012
iAdu	Mice	Active place avoidance	Transgenic DCX-DTR (ablation by diphtheria toxin of DCX+ cells)	7-10w	9-12w	0-2w	iAdu reduced number impairs avoidance of new shock zone	iAdu	Vukovic et al., 2013
iAdu	Mice	Touch-screen discrimination task	Transgenic (GFAP-TK) (reduced neurogenesis by GCV injection)	10-12w	14-16w	0-4w	iAdu reduced number impairs performances after reversal	iAdu	Swan et al., 2014
iAdu & mAdu	Mice	Active place avoidance	Irradiations	10w	22-26w	0-16w	iAdu & mAdu ablation impairs avoidance of new shock zone	iAdu & mAdu	Park et al., 2015
iAdu	Rats	Watermaze	Transgenic (GFAP-TK) (reduced neurogenesis by GCV injection)	12w	15 or 19w	1-3w or 4-7w	iAdu reduced number impairs finding new platform localization (training made from variable departure points)	iAdu	Mateus-Pinheiro et al., 2021
iAdu	Mice	Watermaze	Irradiations	16w	19, 22 or 24w	3, 6 or 8w	iAdu aged 6w or 8w but not 3w needed for cognitive flexibility	iAdu	Amelchenko et al., 2023

Table 8: Implication of the different ontogenic populations of dentate granule neurons in cognitive flexibility. Adol: adolescence-born dentate granule neurons; DCX: doublecortin; GCV: ganciclovir; iAdu: immature adult-born dentate granule neurons; mAdu: mature adult-born dentate granule neurons; TMZ: temozolomide.

6. Forgetting

Closely linked to the notion of flexibility, the ability to adapt suggest that adult neurogenesis could also support the erasure of previously learned patterns. Established memories can interfere with the encoding of new memories, especially when the new memory conflicts with the already established one. Adult neurogenesis, by facilitating the ongoing elimination of hippocampal associations, may serve to reduce these instances of "proactive interference" and therefore enhance behavioral flexibility (Frankland et al., 2013). The first hints toward this involvement were brought to light using transgenic mice deficient for the enriched environment-mediated increased neurogenesis. In this approach, control mice which underwent fear conditioning and directly after

were submitted to enriched environment for 2 weeks (and so had increased neurogenesis) displayed lower contextual-fear memory than transgenic mice than underwent the same procedure but for which enriched-environment had no effect on adult neurogenesis (Feng et al., 2001). This indicates that preventing the increased number of immature adu-DGNs post-training decreased memory clearance and forgetting. This was later confirmed with another transgenic approach allowing the reduction in the number of immature adu-DGNs (born 8w, aged 2-3w) before training which led to impaired extinction in a fear conditioning paradigm (Deng et al., 2009). It was later showed that increasing the number of immature adu-DGNs (born 8w, aged 0-6w) by physical exercise or pharmacological manipulations after memory encoding induces forgetting of both a contextual fear conditioning memory trace and a spatial memory trace acquired in the watermaze (Akers et al., 2014). Similarly, increasing adu-DGNs numbers by running after learning (born during (no age), aged between 0 and 4w) induced forgetting of a spatial memory trace (Epp et al., 2016) and complex paired associates learning (Epp et al., 2021). These results were reproduced in each memory task (fear conditioning, watermaze and paired-associate learning) in rats in which increasing the number of adu-DGNs by physical exercise (born adulthood (no age), aged 2-3w) promoted forgetting of the memory trace (Scott et al., 2021). Interestingly, the observed forgetting of memories due to increased neurogenesis post-training were shown to be dependent of the delay between training and increased neurogenesis. When physical exercise-mediated increased number of immature adu-DGNs is done directly after learning, then the memory trace is forgotten. However, if there is a delay between learning and physical exercise-mediated increased adult neurogenesis, the memory trace remains intact (Gao et al., 2018). Given that there is an homeostatic regulation of cell and “synapses” number (Dupret et al., 2007), it can be proposed that increasing cell genesis may erase the trace through competitive mechanisms. However, another study performed on rats upon which neurogenesis has been increased post-training in the watermaze (born 6w, aged 0-4w) did not observe forgetting of the spatial memory trace 4 weeks later. It seems worth mentioning that the age of animals make them increased both adol-DGNs and adu-DGNs if we consider rats still adolescent at 6 week-old (Kodali et al., 2016).

Animal	Behavioral test	Manipulation	Animal age manipulation	Animal age Experience	Neuron age Experience	Observation	Conclusion	References	
iAdu	Mice	Fear conditioning	Transgenic (FB-KO) (Deficient in enriched environment induced neurogenesis)	11-14 months	11-14 months (learning) & 13-16 months (test)	0-2w	Prevention of iAdu increased number after training prevents memory clearance and forgetting	iAdu	Feng et al., 2001
iAdu	Mice	Fear conditioning	Transgenic (Nestin-TK) (reduced neurogenesis by GCV injection)	8w	11w	2-3w	iAdu reduced number before training impairs extinction	iAdu	Deng et al., 2009
iAdu	Mice	Fear conditioning / watermaze	Running	8w to 14w	8w (learning) & 14w (test)	0-6w	iAdu increased number after learning induces forgetting of the fear and spatial memory	iAdu	Akers et al., 2014
Adol & iAdu	Rats	Watermaze	Running	6w to 10w	6w (learning) & 10w (test)	0-4w	Adol&iAdu increased number has no effect on the spatial memory trace	Not adol & iAdu	Kodali et al., 2016
iAdu	Mice	Watermaze	Running	8w to 12w	8w (learning) & 12w (test)	0-4w	iAdu increased number after learning induces forgetting of spatial memory	iAdu	Epp et al., 2016
iAdu	Mice	Fear conditioning	Running	8w to 12w 10w to 14w	8w (learning) & 14w (test) 8w (learning) 14w (test)	2-4w 0-3w	iAdu increased number directly after learning induces forgetting iAdu increased number "long" after learning has no effect on memory	iAdu, but produced shortly after training	Gao et al., 2018
iAdu	Mice	Paired-associate learning task (PAL)	Running	?	?	0-4w	iAdu increased number after learning induces forgetting of complex paired associates learning	iAdu	Epp et al., 2021
iAdu	Rats	Fear conditioning / Watermaze / PAL	Running	Adult (no age)	Adult (no age)	2-3w	iAdu increased number after learning promotes forgetting of fear, spatial and paired associate learning	iAdu	Scott et al., 2021

Table 9: Implication of the different ontogenic populations of dentate granule neurons in forgetting. Adol: adolescence-born dentate granule neurons; GCV: ganciclovir; iAdu: immature adult-born dentate granule neurons; mAdu: mature adult-born dentate granule neurons; PAL: paired-associate learning task.

In summary, this comparative study of the literature taking into account the ontogenic origin of granule neurons allowed us to highlight an unclear division of the role of adu-DGNs according to their maturity state, which calls for more investigations and clarification. We also highlighted that a consequent number studies unintentionally investigated populations of granule neurons generated during adolescence rather than during adulthood. This added complexity to the issue of maturation, because dev-DGNs mature more rapidly than adu-DGNs. Nevertheless, by addressing several memory functions attributed to the DG, we were able to show that they are indeed not assumed uniformly by all DGNs. Depending on both their ontogenic origin but also their maturity state, DGNs will undertake different roles.

D. Neurogenesis and memory in the aging dentate gyrus

1. Decreased proliferation

Among the first studies addressing adult neurogenesis in the context of aging, it was evident that even though newborn neurons production continues until old age (up to 27 month-old in rats), the rate of proliferation drastically decreases, with a more than 80% reduction between 6 and 12 month-old (Kuhn et al., 1996). More detailed analysis in a pseudo-longitudinal study revealed that cell proliferation decreases by 80% from adolescence (28 days) to adulthood (3

months), by 70% from adulthood to middle age (12 months) and by 60% from middle age to senescence (22 months) (Lemaire et al., 2000). Two competing hypotheses have been investigated in the past few years to explain this decrease; i) there is a progressive exhaustion of the pool of NSCs with age or ii) the micro-environment of the NSCs changes with age and becomes non-viable for proliferation therefore leading them to remain quiescent. The first hypothesis has been supported by a study showing that the pool of NSCs progressively disappears due to their conversion into astrocytes by asymmetric divisions (Encinas et al., 2011). However, it has also been shown that the total number of Sox2 positive cells (marker for NSCs) is unaltered in aging rats, even though proliferation decreases (Hattiangady and Shetty, 2008). These findings would therefore tend to confirm the second hypothesis, which is that NSCs are present but quiescent. This theory is further supported by the fact that some NSCs acquire the capacity to return to quiescence and exhibit long-term self-renewal (Bonaguidi et al., 2011). Besides, even in the late stages of aging, stimulating experiences such as physical exercise (van Praag et al., 2005) or enriched environment (Kempermann et al., 1998, 2002) promote proliferation, also suggesting that the pool of NSCs is not depleted but just silent.

Recently, both hypotheses were shown not to be necessarily mutually exclusive, as several types of NSCs would exist, some being short-term lived while other long-term lived. The short-term NSCs rapidly divide in the young brain and get depleted in a short time period, while long-term NSCs remain for months in the SGZ but under quiescent state (Ibrayeva et al., 2021). More precisely, at the beginning of adulthood, all proliferating NSCs are rapidly differentiating, resulting in a rapid depletion of their pool. However, around 6 month-old in mice, dividing NSCs return to quiescence in a state in which they are able to self-renew. In that case, the pool of NSCs is less likely to deplete (Harris et al., 2021).

2. Delayed maturation

The first steps of neuronal maturation do not seem to be impacted, indeed, neuronal fate choice, and survival are similar for adu-DGNs born at old age compared to young adulthood (Rao et al., 2005). However, maturation/differentiation is altered as only 50% of the cells express NeuN (Rao et al., 2005; Montaron et al., 2006). In addition, the migration of the newborn neurons is delayed in old animals even though they eventually reach their expected position compared to neurons born at younger age (Heine et al., 2004; Rao et al., 2005; Montaron et al., 2006). Besides, immature adu-DGNs in the aged hippocampus were shown to have a delayed and longer dendritic maturation process compared to the ones born during young adulthood (Rao et al., 2005). First,

the dendritic tree takes longer to develop to full maturity (Trincherro et al., 2017). Second, the development of spines is delayed; neurons born at the end of adulthood (8 month-old) exhibit an absence of spines at 3 week-old when neurons born earlier already exhibit some, suggesting a delayed emergence of glutamatergic innervation. Eventually, they end up with similar density of spines compared to neurons born in young adults (Morgenstern et al., 2008; Trincherro et al., 2017). Third, glutamatergic synaptogenesis was indeed shown to be delayed (starting around 4 weeks of age, against 3 normally), also resulting in a delayed maturation of electrophysiological properties (reached at 10 week-old again 8 weeks for neurons born earlier) (Trincherro et al., 2017). These results suggest a longer critical period than the traditional one for neurons born at the end of adulthood compared to neurons born earlier. This delay in dendritic maturation can be counteracted and accelerated following physical exercise or exposure to enriched environment (Trincherro et al., 2017, 2019). Concerning the efferences, the development of MFBs contacts with pyramidal cells in the CA3 of neurons born at the end of adulthood was shown to follow similar speed compared to neurons born earlier in adulthood (Trincherro et al., 2019).

3. Factors regulating neurogenesis at old age

Given that it is possible to reverse the decreased proliferation with physical exercise or enriched environment for example, it has been suggested that the reduced proliferation and delayed maturation could originate from age-related changes of the NSCs (that we will not review here) (Gillot et al., 2021) or changes in the SGZ micro-environment (or niche). These later changes could either reside in the increased levels of factors negatively impacting neurogenesis or the decreased levels of factors pro-neurogenesis. In the following section, we will only mention the factors that have been shown to impact memory functions in aged individuals (for more information see (Drapeau and Nora Abrous, 2008)). We will not review the environmental factors that influence neurogenesis such as diet (Zainuddin and Thuret, 2012) or the systemic milieu (Murphy and Thuret, 2015).

a. Growth factors

Growth factors and their corresponding receptors are highly present during development but their quantity tends to decrease with increasing age and might therefore represent an explanation for decreased cell proliferation at old age (Drapeau and Abrous, 2008; Kempermann, 2015). Several growth factors have been shown to strongly control proliferation and differentiation in the young adult network, but few investigations were made in the aged DG.

Although some factors decrease with age, the transforming growth factor β (TGF- β) has been identified as a potential pro-aging factor as it has elevated levels in aged mice. Indeed, the over-expression of this factor in old mice was shown to inhibit proliferation (Buckwalter et al., 2006). Conversely, the systemic attenuation of TGF- β (by inhibition of its receptor) was shown to lead to increased neurogenesis in aged mice (Yousef et al., 2015). On the other hand, the growth factor TGF- α levels of expression were shown to decrease with age (at least in the sub-ventricular zone) (Enwere et al., 2004) and its re-exposition could lead to the restoration of cognitive functions. The vascular endothelial growth factor (VEGF) has been shown to have positive effects on cells proliferation (Jin et al., 2002) but its levels decline in the course of aging (Shetty et al., 2005). Interestingly, VEGF exposition has been shown to promote adult neurogenesis in aged mice without depletion of the NSCs pool (Licht et al., 2016). The insulin-like growth factor (IGF-1) was also reported to have decreased levels with age even though it subsist in neurogenic areas (Rotwein et al., 1988; Shetty et al., 2005). Its infusion in aged rats was shown to restore adult neurogenesis (Lichtenwalner et al., 2001), which could therefore also represent a promising approach to restore memory functions. The hippocampal concentration of fibroblast growth factor 2 (FGF-2) decreases between young adulthood and middle-age, but thereafter remains constant (Shetty et al., 2005). Its infusion in aged mice was shown to restore neurogenesis levels (Jin et al., 2003), which also indicates that it could represent a potential factor to restore memory functions at old age.

b. Neurosteroids and corticosteroids

Neurosteroids are a sub-group of steroids synthesized de novo in the brain, and more particularly in the hippocampus (Baulieu and Robel, 1990). Pregnenolone sulfate has been shown to have positive effects on neurogenesis in aged animals (Mayo et al., 2005). Conversely, corticosteroids are one of the most potent negative regulatory factors for adult neurogenesis. They are release in the circulation following the activation of the hypothalamo-pituitary-adrenal (HPA) axis, primarily by stress. In different stress-induced strategies, increased corticosterone levels were shown to results in decreased cell proliferation (Gould et al., 1998, 1997) and cell survival/differentiation (Pham et al., 2003; Thomas et al., 2007). Similarly, age-related decline in neurogenesis has been shown to be associated with increased levels of corticosterone. Indeed, adrenalectomy performed in old rats was shown to increase levels of neurogenesis (Montaron et al., 1999; Cameron and McKay, 1999). This effect depends on corticosterone secretion as it can be prevented by a substitutive treatment with the hormone (Montaron et al., 1999).

c. Inflammatory factors

One hypothesis put forward to explain age-related cognitive decline is related to brain inflammation, a process deleterious for the production of new neurons. The interferon-gamma (IFN- γ) can drive neuroinflammation in the brain. Transgenic middle-aged mice with limited amounts of IFN- γ were shown to have increased levels of proliferation, indicating that IFN- γ exposition has a negative effect. Interestingly, IFN- γ reduced expression in young adult mice was shown to improve cognitive functions, which suggest possible similar effects at old age (Baron et al., 2008). While many investigations were made on the modulation of adult hippocampal neurogenesis by interleukins, nothing was made in the context of aging to our knowledge.

4. Aging, memory and neurogenesis

Considering the various functions attributed to adu-DGNs in cognition listed before, it appears that focusing on adult neurogenesis as a potential treatment strategy for restoring age-related cognitive decline could be a promising approach (Abrous et al., 2005). Several approaches have been explored to investigate the role of adult neurogenesis in cognitive aging, mostly through correlational studies between the manipulation of neurogenesis and its behavioral outcome, they are all summarized in **table 10**.

a. The rate of neurogenesis determines learning and memory abilities: correlation studies

The laboratory was among the first to offer evidences linking the generation of new neurons and memory in aged animals. Indeed, it was shown that memory abilities were positively correlated to the number of proliferating cells, surviving cells, and new neurons, evaluated three weeks after training in aged rats (Drapeau et al., 2003). Therefore, it has been concluded that spatial performances at old age actually predict the level of neurogenesis in individuals. This correlative relation was confirmed in Fisher 344xBrown Norway hybrid (FBNF1) rats (Driscoll et al., 2006) and in monkeys (Aizawa et al., 2009). Reciprocally, spatial learning was also shown to influence the fate of the newly-born cells according to their birth-date and to individual memory abilities (Drapeau et al., 2007). In resilient rats, learning was shown to increase the survival of cells generated at least one week before the learning episode, whereas it decreased the survival of cells produced during the early phase of learning. On the contrary, cell survival was not influenced by learning in vulnerable rats. These data indicate that learning, and not training, decreased the survival of adu-DGNs, and that spatial memory abilities critically depend on the dynamic homeostatic regulation of adu-DGNs survival.

b. Manipulation of neurogenesis: correlational analysis

Experience-induced increase of adult neurogenesis at old age was shown to lead to improved spatial learning abilities and spatial memory, whether it was achieved by exposure to enriched environment (Kempermann et al., 1998) or physical exercise (van Praag et al., 2005; Gibbons et al., 2014). In addition, an improvement of associative memory following physical exercise could be observed in a fear-conditioning task (Gibbons et al., 2014). In another study, even though physical exercise only tended to increase the number of proliferating cells in aged mice, it was shown to complexify the dendritic tree of newly generated neurons an effect associated with improved contextual-fear discrimination abilities (Wu et al., 2015). Nevertheless, enriched environment and physical exercise do not specifically target adult neurogenesis and can have additional effects on different neural or non-neural elements (glial cells, vessels for example) in different brain areas. In fact, the positive effects of enriched environment and physical exercise on spatial learning were previously shown to be independent of neurogenesis (Meshi et al., 2006; Madroñal et al., 2010).

Another way to indirectly increase the number of new neurons was achieved by performing bilateral adrenalectomy. By this approach, the age related-increase in the activity of the HPA axis was blocked and a low level of corticosterone was maintained through synthesis by the adipose tissue and the testicles. When adrenalectomy has been performed on middle-aged rats (10 month-old), a time at which corticosterone levels begin to increase (Landfield et al., 1978), it was shown to improve spatial learning abilities and memory performances in old rats (25 month-old) (Montaron et al., 2006).

Based on the observation made on the regulating effect of the micro-environment on adult neurogenesis, some approaches have adopted therapies based on the re-exposition to circulating factors. By doing so, treatment with the molecule ER272 inducing increased levels of circulating TGF- α in a mice model for accelerated aging (SAMP8) was shown to promote adult neurogenesis and enhance memory retention of a spatial memory task acquired in the watermaze (Gómez-Oliva et al., 2023). Similarly, IGF-I therapy has led to increased neurogenesis in 28 month-old rats which resulted in improved spatial memory accuracy in the Barnes maze (Pardo et al., 2016). Similarly, exposition to the platelet-derived exerkine factor PF4 in 20 month-old mice resulted in increased neurogenesis and improved spatial avoidance in an active-place avoidance test, as well as improved contextual-fear memory after fear conditioning (Leiter et al., 2023). Some other original approaches have drawn inspiration from parabiosis experiments, a surgical procedure which allows to join two organisms first described in 1864 (Bert, 1864) and led to the observation that

blood from young individuals positively impact the behavior of older individuals (Villeda et al., 2014). Building upon this concept, blood transfer from young individuals to aged mice (18 month-old) resulted in increased neurogenesis and improved spatial learning abilities and memory retrieval performances in the radial-arm watermaze, as well as improved contextual-fear memory following fear conditioning (Horowitz et al., 2020).

Genetic approaches have also been used to manipulate the pool of newborn neurons. Still based on the observations made on the systemic milieu in the aging dentate gyrus, genetic overexpression of the circulating factor TGF- β led to a reduction in cell proliferation and altered neuronal morphology of granule neurons in middle-aged animals (from birth) (Martinez-Canabal et al., 2013b). These results are therefore in accordance with the already discussed observed effect in young animals (Yousef et al., 2015). This resulted in impaired spatial learning abilities as well as decreased memory retrieval performances (Martinez-Canabal et al., 2013b). Similarly, the genetic deprivation of another blood factor β 2-microglobulin (B2m) resulted in increased adult neurogenesis from birth to old age. This resulted in improved spatial learning abilities and memory retention in the radial-arm watermaze as well as improved contextual-fear memory following fear conditioning (Smith et al., 2015).

Another original approach relied on the genetic overexpression of the negative regulator of dendritic spines Klf9 in mature granule neurons; which resulted in their decreased spine density and activity. Consequently, it resulted in the enhanced integration of newly born adu-DGNs in middle-age (11 month-old) or old age (16 month-old) mice. Thereafter, before behavior, the Klf9 expression was restored back to normal in mature granule cells, so adult neurogenesis would go back to normal physiological levels as well (McAvoy et al., 2016). Thus, it allowed an indirect manipulation of the pool of immature adu-DGNs in a specific temporal window. By doing so and indirectly increasing the pool of immature adu-DGNs (aged 5-8w), the contextual-fear memory discrimination abilities of both middle-age and old age animals were improved following fear conditioning (McAvoy et al., 2016).

c. Targeting newly generated neurons: direct evidence

Direct evidence of genetically increased adult neurogenesis at old age have further highlighted its importance to support memory abilities at old age. The first evidence was brought by genetic repression of the *Dkk1* gene in NSCs resulting in increased *Wnt* signaling-pathway leading to increased self-renewal and survival of NSCs and consequently increased number of newly generated neurons. When this approach was adopted in aged mice (16 month-old) for 2 months, it resulted in improved long-term retention of spatial memory (with no effect on spatial

learning) (Seib et al., 2013). Another approach based on lentivirus-mediated expression of Tet2 in middle-age mice (6 month-old) for 2 months led to increased neurogenesis via the increased catalyzation of the 5hmc epigenetic marker in NSCs. While this had no effect on spatial working memory in the radial maze, it resulted in increased associative memory following a fear conditioning task (Gontier et al., 2018). Finally, the genetic overexpression of Cdk4/cyclinD1 in NSCs leading to an increase in the number of newborn neurons in old mice (aged 17-18 months) resulted in improved spatial memory retention in the Barnes maze (while having no effect on spatial learning itself) (Berdugo-Vega et al., 2023). Interestingly, increasing the number of immature adu-DGNs in these old animals resulted in poorer cognitive flexibility and rather in increased perseverance for previous escape location which contrast with what has been described in “young” adult rodents (Dupret et al., 2008; Garthe et al., 2009). However, the increased number of immature adu-DGNs had no effect on the context discrimination abilities in old animals following fear conditioning (Berdugo-Vega et al., 2023). On the contrary, when adult neurogenesis was chronically increased for 10 months from young adulthood (4 month-old) to middle-age (up to 14 month-old), this led to improved context discrimination abilities (Berdugo-Vega et al., 2023). This could be due to the fact that this addition of new neurons during 10 months also increased the pool of mature adu-DGNs which helped enhancing memory abilities.

In summary, increasing hippocampal neurogenesis at old age seems to be a good tool to improve memory functions. However, it is important to note that most often only immature adu-DGNs are investigated in these studies. As previously suggested, mature adu-DGNs continue to play vital functions within the neuronal network, and increasing their numbers and/or stimulating them may offer additional resilience against cognitive decline. In fact, the two studies that prolonged the increase in neurogenesis, consequently also expanding the pool of mature adu-DGNs, demonstrated the most extensive range of restored functions (Montaron et al., 2006; Smith et al., 2015; Berdugo-Vega et al., 2020).

Animal	Manipulation	Outcome	Animal age manipulation	Animal age Experience	Neuron age Experience	Behavioral test	Observation	References
Mice	Enriched environment	Increased neurogenesis & survival	18 months	20 months	0-2 months	Water maze	Improved spatial learning abilities	Kempermann et al., 1998
Mice	Physical exercise (running)	Increased neurogenesis	19 months	20 months	0-1 month	Water maze	Improved spatial learning abilities and memory retention	van Praag et al., 2005
Rats	Adrenalectomy	Increased neurogenesis	10 months	25 months	15 months	Water maze	Improved spatial learning abilities and memory retention	Montaron et al., 2006
Mice	Transgenic (Nestin-Dkk1) increased Wnt activity	Increased neurogenesis and dendritic complexity	16 months	18 months	0-2 months	T maze alternation	Improved spatial working memory	Seib et al., 2013
						Radial-arm maze	No effect on spatial learning	
						Active-place avoidance	Improved long-term retention of spatial memory	
Mice	Transgenic (TGF-B) overexpression of TGF-B	Reduced proliferation and altered morphology	Birth	12 months	0-12 months	Watermaze	Impaired spatial learning abilities and memory retrieval performances	Martinez-Canabal et al., 2013b
Mice	Physical exercise (running)	Increased neurogenesis	19 months	20 months	0-1 month	Water maze	Improved spatial learning abilities and memory retention	Gibbons et al., 2014
						Reversal training - new platform location	No effect on cognitive flexibility	
						Fear conditioning	Improved contextual-fear memory	
Mice	Transgenic (B2m) deprived of B2m blood factor	Increased neurogenesis	Birth	17 months	0-17 months	Radial arm water maze	Improved spatial learning abilities and memory retention	Smith et al., 2015
						Fear conditioning	Improved contextual-fear memory	
Mice	Physical exercise (running)	Tend toward increased number of DCX+ cells but increased dendritic complexity	17 months	18 months	0-1 month	Open field & novelty suppressed feeding	Mixed effects in the openfield, no effect in the novelty-suppressed feeding	Wu et al., 2015
						Fear conditioning	No effect on contextual-fear memory acquisition / Improved context discrimination abilities	
Rats	Insulin-like growth factor-I therapy	Increased neurogenesis	28 months	28,5 months	0-2 weeks	Barnes maze	Improved spatial memory accuracy	Pardo et al., 2016
Mice	Transgenic Klf9 repression in mature granule neurons	Indirect enhanced newborn neurons integration	11 months	12.5 months	5-8 weeks	Fear conditioning	Enhanced context discrimination abilities	McAvoy et al., 2016
			16 months	17.5 months	5-8 weeks			
Mice	Lentivirus injection expressing Tet2	Increased neurogenesis	6 months	8 months	7-8 weeks	Radial maze	No effect on spatial working memory	Gontier et al., 2018
						Fear conditioning	Improved contextual-fear memory	
Mice	Transgenic (Cdk4/cyclinD1) increased proliferation	Increased neurogenesis	17 months	18 months	0-1 month	Barnes maze	Similar learning abilities but rescued allocentric strategy. Decreased flexibility, increased perseverance. Increased memory retention.	Berdugo-Vega et al., 2020
						Fear conditioning	No major effect on contextual-fear memory	
			4 months	7 to 14 months	0-10 months	Barnes maze	Enhanced spatial preference	
						Fear conditioning	Improved contextual-fear memory	
Mice	Blood plasma transfer from young to aged	Increased neurogenesis	18 months	19 months	0-1 month	Radial-arm water maze	Improved spatial learning abilities and memory retention	Horowitz et al., 2020
						Fear conditioning	Improved contextual-fear memory	
Mice	Platelet-derived exerkine PF4	Increased neurogenesis	20 months	21 months	0-1 montg	Active-place avoidance	Improved spatial avoidance of shock zone	Leiter et al., 2023
						Fear conditioning	Improved contextual-fear memory	
Mice (SAMP8)	ER272 treatment	Increased neurogenesis	4 months (accelerated aging)	6 months (accelerated aging)	0-2 months	Watermaze	Improved memory retention	Gómez-Oliva et al., 2023

Table 10: Manipulation of adult neurogenesis and its effects on memory during cognitive aging.

In summary, this last chapter of introduction highlighted the decrease in neuronal production and delay in newborn neurons maturation in the old DG network compared to the young adult one. It also highlighted the functional relevance of adult neurogenesis in the old DG, with indirect and direct evidences supporting its role as a potential target to rejuvenate memory functions with age. However, there is a major gap in our understanding about the role played by mature adu-DGNs in supporting resilience to cognitive aging, as mostly adult-born neurons born at old age and still immature at the time of behavior were addressed in the literature. Yet, mature adu-DGNs in the old DG are numerous and must also play a role in maintaining memory functions.

VI. OBJECTIVES OF THE THESIS

Taking only the hippocampus and hippocampal-dependent memory into consideration, aging remains a multi-modal and very complex process challenging hippocampal regions in various ways. To understand the mechanisms that underlie resilience to cognitive aging, our focus will be on the DG for several reasons; i) it serves as the primary gate to the hippocampus, making it a key area to target for preventing downstream impairments; ii) it is composed of a highly heterogeneous neuronal population, supporting a wide range of memory functions, from basic to more complex and iii) unlike most of the other brain regions, the DG retains the ability to generate new neurons in adulthood, a unique phenomenon with crucial functions in memory and aging.

To assess the memory abilities of animals, we will use the Morris water maze, a widely used behavioral test to study normal aging in rats, as spatial learning and memory using allocentric strategy are particularly sensitive to the aging process. Additionally, the function of granule neurons within the DG has been predominantly investigated in this type of maze, with their role in other mazes being less known. We used male outbred Sprague-Dawley rats because they are commonly used in aging to study inter-individual differences in spatial memory abilities.

As mentioned, granule neurons within the DG exhibit significant heterogeneity, as they are not all born at the same time of development or adulthood and morphological and functional disparities were shown in the literature. Even though adu-DGNs were previously shown to be recruited during spatial learning and retrieval (Tronel et al., 2015a) while neo-DGNs were not (Tronel et al., 2015b), no causal relationship have been shown until now. Besides, there is to our knowledge a major gap in the understanding of the role played by adol-DGNs in spatial learning.

Indeed, while before adolescence animals rely on the use of proximal cues for navigation, a process independent of the hippocampus (Schenk, 1985; Rudy et al., 1987), animals start to use hippocampal-dependent strategies to navigate around adolescence (Langston et al., 2010). This suggests that some granule neurons have to assume this function during this developmental phase.

Hence, before focusing on aging, the primary objective of this thesis will focus on the identification of the functional role of each ontogenic population of granule neurons in spatial learning in young individuals (first article). The aim will also be to elucidate if there is a difference in their functional recruitment based on the immature or mature state of the neurons. The second objective of this thesis (second article) will reframe the recruitment of each ontogenic wave in the context of aging and inter-individual differences; again, with a particular focus on mature granule neurons. Finally, the third objective (third article) will be to investigate how the identified population of granule neurons crucial for spatial learning maintain their functions throughout aging, ultimately leading to resilience against cognitive decline.

CHAPTER II:

RESULTS

I. FUNCTIONAL RELEVANCE OF EACH ONTOGENIC POPULATION OF GRANULE NEURONS IN SPATIAL LEARNING

A. General presentation

The DG comprises a vast population of granule neurons. Unlike most of the brain, not all granule neurons are generated and integrated into the network during embryogenesis, as the DG is generated during the post-natal period and keeps the ability to produce new neurons during the entire life. Therefore, granule neurons in the DG can be distinguished based on their temporal origin: those generated during embryogenesis, the neo-natal period, adolescence, and adulthood. With a focus on emb-DGNs, neo-DGNs and adu-DGNs, these ontogenic populations were shown to exhibit morphological differences (Kerloch et al., 2019; Cole et al., 2020), suggesting different functional roles. Indeed, it has been hypothesized that each ontogenic population undertakes functions that are most useful to individuals at the developmental stage when they are produced, with the ultimate goal being to adapt as effectively as possible to the challenges that individuals face (Abrous et al., 2022). In this line of thoughts, it has been demonstrated using IEGs as proxy for neuronal activity that adu-DGNs are recruited by spatial learning when emb-DGNs and neo-DGNs are not (Tronel et al., 2015b). These observations align with the mentioned theory, as postnatal animals are unable to use a hippocampal-dependent allocentric strategy during spatial learning. However, this ability starts to be put in place during adolescence (Rudy et al., 1987; Langston et al., 2010), suggesting a possible implication of adol-DGNs. As mentioned before, conflicting results about the involvement of this ontogenic population in spatial learning were found based on their addition or removal from the network (Snyder et al., 2005; Farioli-Vecchioli et al., 2008; Martinez-Canabal et al., 2013a; Ash et al., 2023). Considering the potential influence of such methodological approaches on the network and, consequently, on behavioral outcomes, we addressed the involvement of adol-DGNs in spatial learning by investigating their recruitment using IEGs and subsequently by inhibiting them using optogenetics. This approach allows us to selectively manipulate adol-DGNs without impacting the rest of the network. By applying this approach to each ontogenic population from embryogenesis to adulthood, we aim to causally identify which ontogenic population is functionally involved and relevant to spatial learning.

Article 1:

The temporal origin of dentate granule neurons dictates their role in spatial memory

ARTICLE OPEN



The temporal origin of dentate granule neurons dictates their role in spatial memory

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The dentate gyrus is one of the only brain regions that continues its development after birth in rodents. Adolescence is a very sensitive period during which cognitive competences are programmed. We investigated the role of dentate granule neurons (DGNs) born during adolescence in spatial memory and compared them with those generated earlier in life (in embryos or neonates) or during adulthood by combining functional imaging, retroviral and optogenetic tools to tag and silence DGNs. By imaging DGNs expressing Zif268, a proxy for neuronal activity, we found that neurons generated in adolescent rats (and not embryos or neonates) are transiently involved in spatial memory processing. In contrast, adult-generated DGNs are recruited at a later time point when animals are older. A causal relationship between the temporal origin of DGNs and spatial memory was confirmed by silencing DGNs in behaving animals. Our results demonstrate that the emergence of spatial memory depends on neurons born during adolescence, a function later assumed by neurons generated during adulthood.

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INTRODUCTION

Adolescence is a critical period during which the brain undergoes a series of structural and functional maturation steps allowing the transition from childhood to adulthood [1, 2]. Ranging from approximately postnatal weeks 3–4 to 6–7 in rodents, it has been suggested that this period is a stage in which neural circuitry and synapses are sculpted to support the emergence of social behaviour [2] and the transition from simple to complex forms of learning [3, 4]. This period is also often associated with heightened emotional reactivity, increased impulsivity and vulnerabilities to psychological disorders [1, 2]. Adolescent brain neuronal development in particular has been studied in this context. The maturation of the prefrontal cortex (and its connections) has been linked to pruning, perineuronal net maturation, myelination and neurochemical changes (excitatory/inhibitory balance, endocannabinoid systems, dopaminergic sprouting) to name but a few [5, 6]. In contrast, relatively little is known about the neurobiological developmental changes occurring during adolescence that may be involved in the maturation of spatial memory function.

The dentate gyrus (DG) of the hippocampus is a heterogeneous structure composed of different populations of dentate granule neurons (DGNs), most of which are generated during the early postnatal period [7] but also throughout adult life in a process called adult neurogenesis [8, 9]. In recent years, some investigations have focused on the study of morphological and functional

differences between adult and developmental DGNs showing that these populations present major morphological differences [10, 11], and are recruited in different learning processes [12, 13]. In addition, adult-born DGNs (Adu-DGNs) undergo a process of dendritic plasticity in response to learning [14–16] whereas developmental born neurons seem to be insensitive [14, 15].

However, the specific contribution of DGNs born during adolescence (here called Adol-DGNs) to hippocampal-dependent behaviour is largely unknown. There are a few studies in which Adol-DGNs were indirectly manipulated using either stress [17, 18], running [19], social isolation [20] or genetic mutations [21, 22]. All of these manipulations lead to behavioural consequences on emotion and learning in adult animals, but their indirect nature did not allow a firm causal relationship between Adol-DGNs and behaviour to be established. Thus, new methods that specifically target Adol-DGNs are needed in order to directly assess their involvement in learning and memory, and to establish whether their role differs from those generated earlier (in embryos or neonates) or at adulthood.

In this study we investigated whether the temporal origin of DGNs dictates their role in spatial memory by combining functional imaging, retroviral and optogenetic approaches. Using Zif268 [12] an immediate early gene (IEG), critical for the stabilisation of long-lasting memories [23], we found that DGNs generated in adolescent rats (and not embryos or neonates) are

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transiently involved in memory processing during early adulthood whereas DGNs generated in adulthood are not recruited until a later time point when animals are older. The causal relationship between the temporal origin of DGNs and spatial memory was then demonstrated using retroviral and optogenetic approaches to birth date and silence DGNs during learning [24]. Our results demonstrate for the first time a functional specificity of developmental and adult neurogenesis in hippocampal spatial memory.

MATERIALS AND METHODS

General methods

Animals. A total number of 292 male and 21 pregnant female Sprague-Dawley rats (OFA, Janvier, France) were used to perform these experiments (detailed in Tables 1 and 2). Rats were housed under a 12 h/12 h light/dark cycle (light off at 8 p.m.) with ad libitum access to food and water. Temperature (22 °C) and humidity (60%) were kept constant. Experimental procedures were carried out following Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010. Animal studies were approved by the ethical committee of the University of Bordeaux (CEEA50, Dir 13105, #5306).

Injection of thymidine analogues. BrdU (5-Bromo-2'-deoxyuridine), IdU (5-Iodo-2'-deoxyuridine) and CldU (5-chloro-2'-deoxyuridine) were dissolved in Phosphate Buffer (pH 8.4), 1 N NH₄OH/NaCl and NaCl, respectively. CldU and IdU injections were given with equimolar doses of BrdU (50, 100 and 150 mg/kg, Table 1) [12, 25].

Water maze training. The apparatus consisted of a circular plastic swimming pool (180 cm diameter, 60 cm height) in a 330 cm × 300 cm room that was filled with water (20 ± 1 °C) rendered opaque by the addition of a white cosmetic adjuvant. Before training, the animals were habituated to the pool for 1 min per day for 2 days. During training, the Learning group (L) was composed of animals that were required to locate a submerged platform, which was hidden 1.5 cm under the surface of the water in a fixed location, using the spatial cues available within the room (according to a previously described reference memory protocol [25]). All rats were trained for four trials per day (90 s with an intertrial interval of 30 s and released from three starting points used in a pseudorandom sequence each day). If an animal failed to locate the platform, it was placed on that platform at the end of the trial. The time taken to reach the platform was recorded using a video camera that was secured to the ceiling of the room and connected to a computerised tracking system (Videotrack, Viewpoint). In the first 3 experiments, three control groups were used. The home cage group (HC) consisted of animals that were transferred to the testing room at the same time and with the same procedures as the Learning group but that were not exposed to the water maze. The Swim group (S) was a Yoked control group for the stress and motor activity associated with the water maze training. The Swim group was composed of rats that were placed in the pool without the platform and were paired for the duration of the trial with the Learning animals. The third control group was the Cued group (C) which was composed of rats that were trained to find a visible platform in a fixed location. Animals in this group were tested for four trials per day (90 s with an intertrial interval of 30 s and beginning from three different starting points used in a pseudorandom sequence). Trained animals and different age-matched control groups were perfused transcardially 90 min after the last trial.

Table 1. Summary of the experimental groups and experiments for IEGs experiments.

Experiment	Animal's age at the time of Xdu injection	DGNs' age at the time of WM training	Group size	Xdu (mg/kg)
1. IEGs activation of neurons Adol-1M (Fig. 1A–D)	P28	1 month	Control = 9; Learning = 11; Swim = 9; Cued = 9	IdU: 1 × 150
2. IEGs activation of neurons Adol-2M (Fig. 1E–H)	P28	2 months	Control = 5; Learning = 9; Cued = 5	IdU: 1 × 100
3. IEGs activation of neurons Adol-3M (Fig. 1I–L)	P28	3 months	Control = 5; Learning = 6; Cued = 6	CldU: 1 × 50
4. IEGs activation of neurons Adu-1M (Fig. 2A, B)	2 M	1 month	Control = 3; Learning = 6	BrdU: 3 × 100
5. IEGs activation of neurons Adu-2M (Fig. 2A, B)	2 M	2 months	Control = 3; Learning = 5	BrdU: 3 × 100
6. IEGs activation of neurons Adu-3M (Fig. 2A, B)	2 M	3 months	Control = 5; Learning = 7	BrdU: 3 × 100
7. IEGs activation of neurons generated during embryonic and neonatal periods (Fig. 2C–E)	E18.5 P14	10 weeks 12 weeks	Control = 6; Learning = 13; Cued = 8	CldU: 2 × 50 IdU: 1 × 50

Table 2. Summary of the experimental groups and experiments for optogenetics experiments.

Experiment	Animal's age at the time of Arch injection	DGNs' age at the time of WM training	Group size
1. Optogenetic inhibition of Adol-1M DGNs (Fig. 3A–E)	P28	1 month	Adol-DGNs-Arch-No-Light = 14 Adol-DGNs-Arch-Light = 6 Adol-DGNs-GFP-Light = 12
2. Optogenetic inhibition of Adol-2M DGNs (Fig. 3F–J)	P28	2 months	Adol-DGNs-Arch-No-Light = 13 Adol-DGNs-Arch-Light = 13 Adol-DGNs-GFP-Light = 13
3. Optogenetic inhibition of Adol-4M DGNs (Fig. 3K–O)	P28	4 months	Adol-DGNs-Arch-No-Light = 10 Adol-DGNs-Arch-Light = 8 Adol-DGNs-GFP-Light = 9
4. Optogenetic inhibition of Adu-1M DGNs (Fig. 4A–D)	2 M	1 month	Adu-DGNs-Arch-No-Light = 10 Adu-DGNs-Arch-Light = 11
5. Optogenetic inhibition Adu-6M DGNs (Fig. 4E–H)	2 M	6 months	Adu-DGNs-Arch-No-Light = 11 Adu-DGNs-Arch-Light = 12
6. Optogenetic inhibition of Neo-DGNs (Fig. S4)	P3	2 months	Neo-DGNs Arch-No-Light = 7 Neo-DGNs Arch-Light = 13

Retrovirus production. The murine Moloney leukaemia virus-based retroviral vector CAG-GFP has been described previously [14] and is a gift from Prof. F. H. Gage (Salk Institute, La Jolla, CA). The retrovirus expressing an inhibitory optogene *Archaeorhodopsin-3* (Arch-EGFP) was generated by Dr S. Ge [24]. High titres of retroviruses (between 5×10^8 and 5×10^9) were prepared with a human 293-derived retroviral packaging cell line (293GPG) [26] kindly provided by Dr Dieter Chichung Lie (Friedrich-Alexander Universität Erlangen-Nürnberg, Erlangen, Germany). Virus-containing supernatant was harvested three days after transfection with Lipofectamine 2000 (ThermoFisher). This supernatant was then cleared from cell debris by centrifugation at 3500 rpm for 15 min and filtered through a 0.45 µm filter (Millipore). Viruses were concentrated by two rounds of centrifugation (19,500 rpm 2 h) and resuspended in PBS.

Stereotaxic injections of retrovirus. The retroviral solution was injected bilaterally into the dorsal area of the septal DG. Adult and adolescent rats were anaesthetised in a box using a mix of oxygen and 3% isoflurane and maintained asleep with 2% isoflurane for the duration of surgery in a stereotaxic apparatus. P3 rats were anaesthetised by placing them in ice. Animals also received lidocaine (0.1 ml, sub-cutaneous, Vetoquinol) at the incision site and metacam (1 ml/kg, sub-cutaneously, Boehringer Ingelheim). Glass micropipettes connected to a Hamilton syringe placed into a micro injector (KDS legato 130) directly attached to the stereotaxic apparatus were used. Injections were administered using the following coordinates [27]: For Neo-DGNs groups, [AP]: -1.2 mm; lateral [L]: ±1.35 mm; [P]: -2.6 mm; Adol-DGNs groups, [AP]: -2.8 mm; [L]: ±1.5 mm; [P]: -3.5 mm and for Adu-DGNs groups, [AP]: -3.3 mm; [L]: ±1.6 mm; deepness [P]: -4.2 mm. 1 (Neo-DGNs), 2 (Adol-DGNs) or 3 µl (Adu-DGNs) of viral suspension were injected at a flow rate 0.25 L/min. Cannulas were maintained in position for 3 min after the end of the injections to let the suspension infuse. The skin was then stitched with absorbable sutures and the rat was placed in a recovery chamber (37 °C) until it woke up.

Electrophysiological recording. Animals were deeply anaesthetised (Xylazine 16.7 mg/kg plus Ketamine 167 mg/kg, intraperitoneal, Bayer santé and Merial, Germany, respectively) and sacrificed. Dissected brain was immediately immersed in ice-cold oxygenated cutting solution (in mM: 180 Sucrose, 26 NaHCO₃, 11 Glucose, 2.5 KCl, 1.25 NaH₂PO₄, 12 MgSO₄, 0.2 CaCl₂, saturated with 95% O₂-5% CO₂). 350 µm slices were obtained using a vibratome (VT1200S Leica, Germany) and transferred to a 34 °C bath of oxygenated aCSF (in mM: 123 NaCl, 26 NaHCO₃, 11 Glucose, 2.5 KCl, 1.25 NaH₂PO₄, 1.3 MgSO₄, 2.5 CaCl₂; osmolarity 310 mOsm/l, pH 7.4) for 30 min and then cooled down progressively to room temperature (RT; 23–25 °C) in oxygenated aCSF. After a 45 min recovery period at RT, slices were anchored with platinum wire at the bottom of the recording chamber and continuously bathed in oxygenated aCSF (RT; 2 ml/min) during recording.

Fluorescent DGN cells were identified using fluorescence/infrared light (pE-2 CoolLED excitation system, UK). Neuron action potential firing was monitored in whole-cell current-clamp recording configuration. Patch electrodes were pulled (micropipette puller P-97, Sutter instrument, USA) from borosilicate glass (O.D. 1.5 mm, I.D. 0.86 mm, Sutter Instrument) to a resistance of 2–4 mΩ. The pipette internal solution contained [in mM: 125 potassium gluconate, 5 KCl, 10 HEPES, 0.6 EGTA, 2 MgCl₂, 7 Phosphocreatine, 3 adenosine-5'-triphosphate (magnesium salt), 0.3 guanosine-5'-triphosphate (sodium salt) (pH adjusted to 7.25 with KOH; osmolarity 300 mOsm/l adjusted with d-Mannitol)] with added biocytin 0.4% (liquid junction potential -14.8 mV was corrected on the data and statistics). Electrophysiological data were recorded using a Multiclamp 700B amplifier (Molecular devices, UK), low-pass filtered at 4 kHz and digitised at 10 Hz (current clamp) or 4 Hz (voltage clamp) (Digidata 1440A, Molecular devices, UK). Activation of Arch was obtained after illumination of the recorded area at 570 nm (pE-2 CoolLED excitation system, UK). Signals were analysed offline (Clampfit software, pClamp 10, Molecular devices, UK).

Optic fibre implantation. Home-made optic fibre implants were constructed following a protocol previously described [28]. The implantation step was carried out during a second surgical procedure two weeks before the behaviour, following the same protocol as that described for the retroviral injection. The skull was scratched and three anchor screws were fixed (one in the anterior area and two in the posterior area). The two optic fibres (length: 4.5 mm, diameter: 200 µm, numeric aperture (NA): 0.37) were lowered with a speed of ~2 mm/min upwards to the RV injection site with the following stereotaxic coordinates [27]: Adu-1M group: [AP]: -3.3 mm; [L]: ±1.6 mm; [P]: -4 mm from skull surface; Adu-6M group: [AP]: -3.7 mm; [L]: ±1.6 mm; [P]: -4 mm from skull surface; Adol-1M group: [AP]: -3.2 mm;

[L]: ±1.5 mm; [P]: -3.6 mm from bregma; Adol-2M and Adol-4M groups: [AP]: -3.4 mm; [L]: ±1.5 mm; [P]: -3.8 mm; Neo-DGNs group: [AP]: -3.3 mm; [L]: ±1.6 mm; [P]: -4 mm (see below for the group description). Fibres were stabilised using dental cement. When the cement was dry, scalps were sutured and disinfected with local antiseptic treatment (Betadine). All rats were followed up until the start of the behaviour. During handling, fur was checked and the scar disinfected systematically. Rats were handled every day to habituate them before the behavioural procedure.

Optogenetic manipulation of DGNs during training in the water maze. The water maze (a circular pool with a diameter of 1.80 m and 60 cm deep) was placed in a room (375 cm × 370 cm) equipped with a green laser source (OptoDuet 532.5 nm, 200 mW, Ikecool) that was connected to a rotatory joint to avoid tangling of the patch cords connected to the intra-dentate optic fibres. Light intensity at the optic fibre cable tip was checked every day before the beginning of the session (8–10 mV). Illumination was applied throughout the session at 1 Hz pulses of 750 ms. The same protocol as that described in the section 'Water Maze training' was used in this training. The time taken to reach the platform was recorded using a video camera connected to a computerised tracking system (Polyfiles, Imetric). When animals reached the plateau phase, training was stopped and 72 h later animals were tested in finding the platform. During the probe test, the hidden platform was removed and the time spent in each quadrant was quantified.

Immunohistochemistry. Animals were perfused transcardially with a phosphate-buffered 4% paraformaldehyde solution. After 1 week of fixation, brains were cut using a vibratome as previously described [25]. Free-floating sections (50 µm) were processed using a standard immunohistochemical procedure to visualise the thymidine analogues (BrdU, CldU, IdU) and retrovirus-expressing EGFP in alternating one-in-ten sections using different anti-BrdU antibodies from different vendors (for BrdU: mouse primary at 1/100, Dako; CldU: rat primary at 1/1000, Accurate Chemical; IdU: mouse primary at 1/1000, BD Biosciences) and with a rabbit anti GFP (Millipore, 1/2000). Bound antibodies were visualised with horse anti-mouse (1/200, Abcys for BrdU and IdU), goat anti-rat (1/200, Vector for CldU) and goat anti-rabbit (1/200, Vector for GFP). The number of XdU-immunoreactive (IR) cells in the granule and subgranular layers (GL) of the DG was estimated with systematic random sampling of every tenth section along the septo-temporal axis of the hippocampal formation using a modified version of the optical fractionator method. Indeed, all of the X-IR cells were counted on each section and the resulting numbers were tallied and multiplied by the inverse of the section sampling fraction (1/sf = 10). This counting was performed blind by the person who carried it out.

The activation of embryonic, neonatal, adolescent and adult generated neurons was examined using immunohistochemistry. To visualise the cells that incorporated thymidine analogues, one section out of ten was incubated with BrdU antibodies (BrdU, 1/1000, CldU, 1/500, Accurate Chemical; IdU, 1/500, BD Bioscience). Bound antibodies were visualised with Cy3-goat anti-rat antibodies (1/1000, Jackson for CldU and BrdU) or Cy3-goat anti-mouse antibodies (1/1000, Jackson for IdU). Sections were also incubated with rabbit anti-Zif268 (1/500, Santa Cruz Biotechnology). Bound antibodies were visualised with Alexa488-goat anti-rabbit antibodies (1/1000, Invitrogen). Primary antibodies for CldU (or IdU or BrdU) and IEG (Zif268) were incubated simultaneously at 4 °C for 72 h, and secondary antibodies were incubated simultaneously at RT for 2 h. CldU-IEG and IdU-IEG labelling were not analysed on the same sections, given the cross-reactivity between the secondary (and not primary) antibodies [12]. The sections were then incubated with the two secondary antibodies. The phenotype of the CldU-IR cells was examined by immunofluorescence labelling using NeuN (1:1000; Millipore) revealed with a Cy5-goat anti-mouse (1:1000; Jackson) and the phenotype of IdU-IR cells using Calbindin (Calb, 1:250; Santa Cruz Biotechnologies) revealed with Alexa-Fluor 647 donkey anti-goat (1/1000; Jackson). Double labelling was determined using a SPE confocal system with a plane apochromatic 63X oil lens (numerical aperture 1.4; Leica). The percentage of XdU cells expressing IEG and the number of XdU-cells expressing NeuN or Calb were calculated as follows: $(\text{Nb of XdU}^+/\text{IEG} + \text{cells}) / ((\text{Nb of XdU}^+/\text{IEG} + \text{cells}) + (\text{Nb of XdU}^+/\text{IEG} + \text{cells})) \times 100$. All sections were optically sliced in the Z plane using a 1 µm interval and cells were rotated in orthogonal planes to verify double labelling. In all experiments, a minimum of 200 developmentally generated neurons were analysed per rat.

The morphometric analysis of virus-labelled neurons was performed with an ×100 objective as previously described [10]. Measurements of dendritic parameters as well as Sholl analysis were performed with the

NeuroLucida (software MicroBrightfield, Colechester, VT, USA). Only neurons from the suprapyramidal layer were analysed. The analysis of the dendritic arborisation was performed blind by the person who carried it out. Moreover, only neurons that reached the edge of the molecular layer and had at least two ramification points, were analysed. Data shown is from at least five rats per group with a minimum of four neurons per rat (Supplementary Table 3).

General procedures

Immediate early genes experiments (Table 1). In the first three functional imaging experiments, we ascertained whether neurons born during the adolescent period were activated by spatial learning. Animals were delivered at P21 days old and they were kept in collective cages (2–3 rats per cage). One week later, rats received one injection of IdU or CldU when 28 days-old. One week before the beginning of the water maze training, animals were housed individually. These animals were randomly distributed into 4 (Home Cage, Swim, Cued and Learning) or 3 (Home Cage, Cued and Learning) experimental groups and were trained in the water maze (see above) 1, 2 or 3 months after the XdU injections.

In experiments four, five and six, we examined whether neurons born during adulthood (Adu-DGN) were activated by spatial learning. Animals were delivered at 6 weeks old and received three injections of BrdU when 2 months old. They were housed in collective cages (2–3 rats per cage) until 1 week before the behavioural training when they were transferred to individual cages. These animals were randomly distributed into 2 experimental groups (Home Cage and Learning) and were trained in the water maze one, two or three months after the XdU injections.

In the seventh experiment, we examined whether neurons born during the embryonic period and those born during the second postnatal week (P14) in the same rats were activated by spatial learning. Pregnant female ($n = 12$, 3 months of age on delivery) rats were individually housed in transparent cages and the mothers received two injections of CldU at E18.5 and E19.5. At 14 days-old (P14), males received one injection of IdU. The litters were raised by their biological mothers until weaning (21 days after birth). After weaning, only the male progeny were kept in collective cages (2–3 rats per cage) and transferred to individual cages 1 week before the beginning of the behavioural training. The animals were randomly distributed into three experimental groups (Home Cage, Cued and Learning) and trained in the water maze when they were 3 months-old.

Optogenetic experiments (Table 2). In the first three optogenetic experiments, we wanted to see the effect of inhibiting DGNs born during adolescence on spatial learning (Adol-DGNs). 135 males were delivered at P21 days old and kept in collective cages (2–3 rats per cage). One week later, P28 day-old animals were bilaterally injected with retrovirus (expressing either Arch or GFP) in the DG. Two weeks before the water maze training, optic fibres were implanted bilaterally in the DG and the animals were moved afterwards to individual cages. Rats were trained in the water maze 1 (Adol-1M), 2 (Adol-2M) or 4 months (Adol-4M) after tagging the cells.

Experiments four and five were performed to assess the effect of silencing adult-born DGNs on spatial learning (Adu-DGNs). 60 males were delivered at the age of 6 weeks and held in collective cages (2–3 rats per cage). At 8 weeks old, animals were bilaterally injected with retrovirus (expressing Arch) in the DG, and as in previous experiments, 2 weeks before the water maze training, optic fibres were placed in the DG and rats were transferred to individual cages. Rats were trained 1 (Adu-1M) or 6 months (Adu-6M) after the viral injection.

Finally, for the neonate DGN experiment (Neo-DGNs), 43 male pups from 9 different females were bilaterally injected with retrovirus (expressing Arch). They were raised by their biological mothers until weaning, and they were kept in collective cages (2–3 rats per cage) before optic fibres were implanted 2 weeks before water maze training which was performed 2 months after the retroviral injection. Animals were randomly assigned in the two groups.

At the end of all of these experiments, only animals with GFP-labelled-cells in both hemispheres and the fibre optic implants at the correct site (at the same anteroposterior levels of GFP-DGNs, see Supplementary Fig. 1) were considered for further analysis (Adu-DGN experiments: $n = 44$, Adol-DGN experiments: 64; Neo-DGNs experiment: $n = 20$, for more details see Table 2).

Statistical analysis. All statistical analyses were performed using Statistica software (Statsoft) and results are reported in Supplementary Tables 1–3. One-way ANOVA with between factors ('Group', 'Light') or a within factor ('Quadrant' factor) were used as well as two-way ANOVAs with a between factor ('Group', 'Light') and within factors ('Days', 'Distance to the soma').

ANOVA were followed by Newman-Keuls post-hoc analysis when appropriate. In the case of comparison of two independent groups or comparison to chance levels, Student *t* tests were used. The relationship between memory retention and dendritic morphology was evaluated using a Spearman correlation test. For all tests, significance was set at $p = 0.05$. All data are presented as mean \pm s.e.m.

RESULTS

The temporal origin of DGNs determines their activation during spatial learning

Adol-DGNs born in P28 male rats were labelled using thymidine analogues IdU (5-Iodo-2'-deoxyuridine) or CldU (5-chloro-2'-deoxyuridine) [12] (Table 1) and their recruitment by training in the water maze (WM) was examined by quantifying in IdU or CldU-labelled cells, the expression of Zif268, a proxy for neuronal activity [12, 29]. In the first experiment, rats were trained one month after IdU injection (Fig. 1A,B). IdU-labelled cells differentiated into neurons, as revealed by co-expression with the adult neuronal marker NeuN (% of IdU expressing NeuN, Home Cage: $97.92 \pm 0.66\%$, Learning: $96.57 \pm 0.82\%$; $t_{43} = 1.28$, $p = 0.21$) and were located in the inner granule cell layer. The percentage of activated Adol-DGNs (IdU-Zif268-IR cells) in the Learning group was superior to that measured in the age-matched Home Cage control group (Fig. 1D). Learning to find a visible platform (Cued learning), a hippocampal-independent task, or stress-physical activity (Swim group) did not activate Adol-DGNs (Supplementary Table 1 for statistical analysis) demonstrating that Adol-DGN activation was specific to learning. We then investigated whether the age of these Adol-DGNs at the time of training was critical or whether their contribution was stable over time. We tagged Adol-DGNs with IdU or CldU and trained rats in the WM, two or three months later (Fig. 1E,F,I,J). Under these conditions (2 or 3 month-old neurons), Adol-DGNs were not activated by training (Fig. 1H, L) indicating that Adol-DGNs were activated by learning within a defined temporal window.

Then we investigated whether the recruitment of Adol-DGNs was specific to this neuronal population by first focusing on neurons born during adulthood (Adu-DGNs) (Fig. 2A). To this end, 2-month-old rats were injected with BrdU (5-Bromo-2'-deoxyuridine) and submitted to training one month later. We found that in this condition, the percentage of BrdU-Zif268-IR neurons was not influenced by training (Fig. 2B and Supplementary Table 1). In contrast, when the BrdU pulses and training interval were increased to 2 or 3 months, Adu-DGNs were activated by learning (Fig. 2B and Supplementary Table 1). Next, we analysed neurons generated during embryogenesis and during the neonatal period to determine whether this activation was specific to the neurons born during either adolescence or adulthood. Neither 3-month-old DGNs born during either embryogenesis (Emb-DGNs) or the neonatal period (Neo-DGNs) were activated by spatial learning in adult rats (Fig. 2C–E and Supplementary Table 1). Taken together, these data suggest that: i) Adol-DGNs are recruited by learning only when they are one month-old ii) Adu-DGNs (and not neurons born during development) are recruited after the age of 2 months.

Inhibition of Adol- and Adu-DGNs impairs the expression of memory in a time-dependent manner

To confirm the contribution of Adol-DGNs and Adu-DGNs in spatial learning during different time windows, we used an optogenetic approach to silence DGNs using a retrovirus expressing eGFP-tagged *Archaeorhodopsin* (RV-Arch-EGFP), a tool that allows us to inhibit the cells expressing Arch in presence of light [24]. We first validated this tool in the rat DGNs by showing that optical inhibition reversibly silenced RV-Arch-EGFP-expressing neurons in a power-dependent manner as previously reported in mice [24] (Supplementary Fig. 2). Then, we bilaterally injected the RV-Arch-EGFP into the DG of 28 days-old animals, and rats were

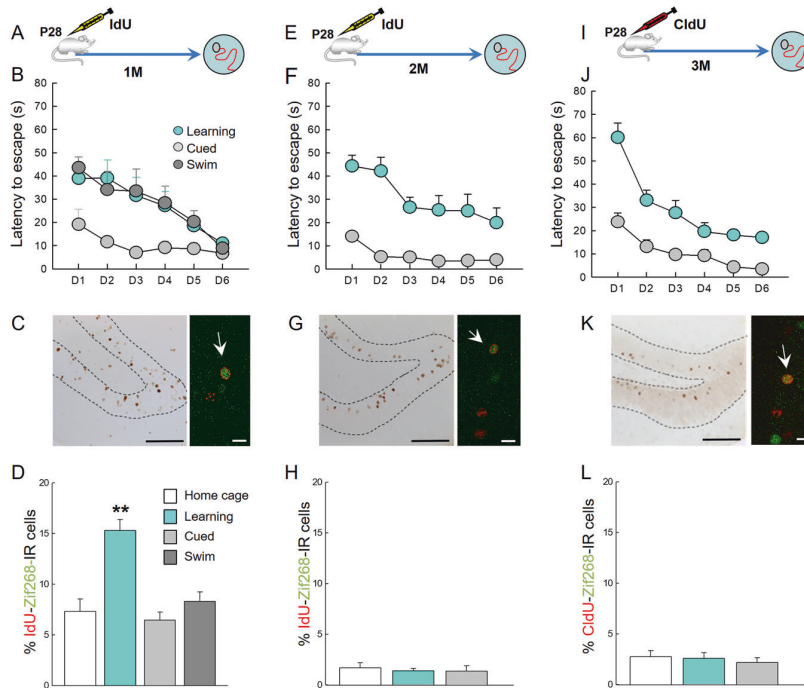


Fig. 1 DGNs born in adolescents are recruited by spatial learning within a defined temporal window. **A, E, I** Experimental designs. **B, F, J** Learning curves. **C, G, K** Left panel: illustration of IdU- or CldU- labelled cells (Bar Scale = 80 μ m). Right panel: confocal example of IdU-Zif-268- or CldU-Zif-268- labelled cells (arrows indicate double labelled cells; Bar Scale = 10 μ m). Spatial learning increased the number of activated Adol-DGNs when the cells were 1 month old (**D**) and not when they were 2 month-old (**H**) or 3 month-old (**L**). **** $p \leq 0.01$** compared to other groups.

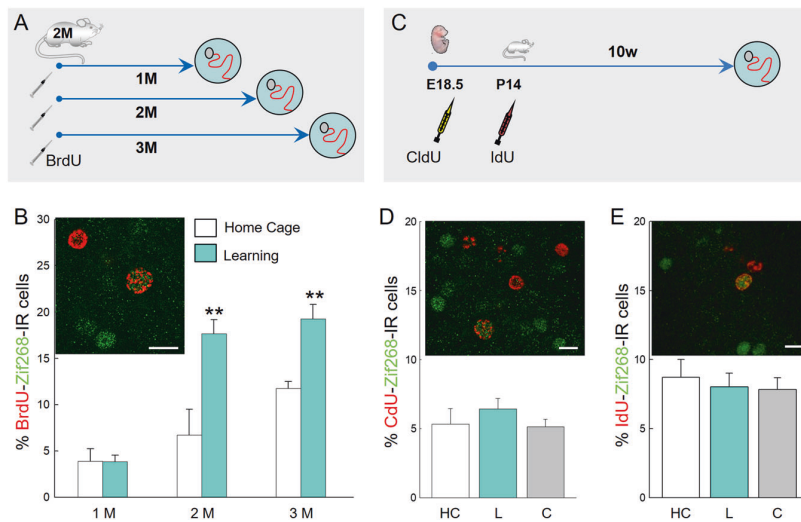


Fig. 2 Mature Adu-DGNs are activated by learning in contrast to DGNs born in embryos or neonates. **A** Experimental design: DGNs born in 2-month-old rats were tagged with BrdU. Rats were tested 1, 2 or 3 months after the injections. **B** Illustration and quantitative analysis of the percentage of BrdU cells expressing Zif-268. **C** Experimental design: DGNs born in embryos were tagged with CldU (injected into the pregnant mothers) and DGNs in the same animals born at P14 were labelled with IdU. Ten weeks after the last injection animals were trained in the water maze. **D** Illustration and quantification of the percent of CldU cells (in red) expressing Zif268 (in green). **E** Illustration and quantitative analysis of the percent of IdU cells expressing Zif268. HC: home Cage; L: Learning, C: Cued. Arrows indicate double labelled cells. **** $p < 0.01$** in comparison to the HC. (Bar Scale = 10 μ m).

trained in the WM 1, 2 or 4 months after tagging the cells. Two weeks before training, optic fibres were implanted bilaterally above the DG and half of the animals were trained with the light on (Adol-DGNs-Arch-Light) and the other half with the light off

(Adol-DGNs-Arch-No-Light). No differences were observed between groups during the learning phase (Supplementary Fig. 3A–C). Seventy-two hours later, memory retention was measured in a probe test. During this test, the platform was removed and the optic

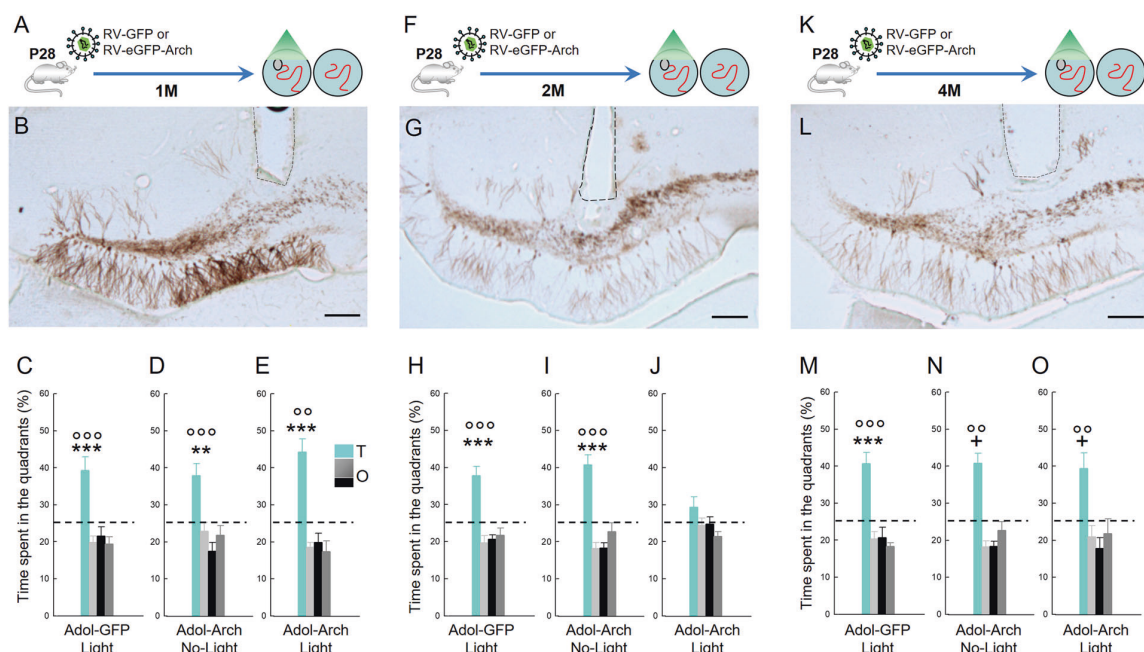


Fig. 3 Optical silencing of Adol-DGNs transiently affects memory retrieval. **A, F, K** Experimental designs. Illustration of Adol-DGNs **B, G, L** infected with RV-GFP or RV-eGFP-Arch; dashed line, position where optic fibres were placed (Bar Scale = 200 μ m). Silencing **C–E** 1-month old Adol-DGNs during training did not impair memory retention, whereas **H–J** silencing 2-month-old Adol-DGNs impaired the ability of the animals to remember the platform location. When 4 months old, silencing Adol-DGNs did not influence memory retention **M–O** at least at $p < 0.05$ and $**p < 0.01$. $***p < 0.001$ compared to the other quadrants. $^{\circ}p \leq 0.01$ and $^{\circ\circ}p \leq 0.001$ compared to chance level (25%). T: target quadrant. O: other quadrants.

fibres connected, but with no light. When there was a lapse of 1 month between retroviral labelling and behavioural testing, both Adol-DGNs-Arch-No-light and Adol-DGNs-Arch-light animals remembered the platform position as they spent more time in the quadrant previously associated with the platform (Target quadrant, T) (Fig. 3D, E and Supplementary Table 2 for complete statistical analysis) compared to the other quadrants (O). In addition, the time spent in the target quadrant differed significantly from the chance level ($>25\%$). In subsequent experiments, rats were trained in the WM 2 or 4 months after tagging the cells. In contrast, retrieval was impaired in the Adol-DGNs-Arch-light group when there was a 2-month delay between retroviral labelling and the behavioural test (Fig. 3I, J and Supplementary Table 2 for complete statistical analysis). Interestingly, no alteration was observed when the delay was increased to 4 months (Fig. 3N, O and Supplementary Table 2 for complete statistical analysis). To confirm that the effect of the light was not responsible for the impairment observed, an extra group of animals was injected bilaterally with RV-GFP without expressing *Archaeorhodopsin* and these animals were tested at the same time as the previous ones. No light effect was found for any analysed time points, either in learning (Supplementary Fig. 3A–C) or in retrieval (Fig. 3C, H, M). After this verification, only the Arch-No-Light group was used as a control in the following experiments.

To ascertain that the contribution of Adol-DGNs was due to the date of birth (Adolescence) and not the age of the population under study (2 months), RV-Arch-EGFP was injected bilaterally into the DG of 3-day-old animals that were trained 2 months later. Both Neo-DGNs-Arch-No-Light and Neo-DGNs-Arch-Light learned and remembered the task (Supplementary Fig. 4 and Supplementary Table 2).

Then we focused our attention on Adu-DGNs by injecting RV-Arch-EGFP bilaterally in 2-month-old animals and testing the impact of silencing 1- and 6-month-old Adu-DGNs. A one month interval was chosen to confirm that the effect observed when manipulating Adol-DGNs (Fig. 3I, J) was specific to this wave of

DGNs and not the age of the rat. The 6-month time point was chosen based on our previous experiments showing that Adu-DGNs are recruited by learning even when these neurons are 6 months old [12]. In both experiments, half of the animals were trained with the light on (Adu-DGNs-Arch-Light) and the other half with the light off (Adu-DGNs-Arch-No-Light). No differences were observed between groups during learning (Supplementary Fig. 3D, E). We then performed the probe test and we observed that in the Adu-1M, both Adu-DGNs-Arch-No-light and Adu-DGNs-Arch-light rats remembered the platform position since they spent more time in it compared to the other quadrants (Fig. 4C, D); in addition the time spent in the target quadrant was significantly different from the chance level ($>25\%$). When a delay between the retroviral labelling and behavioural testing was increased to 6 months (Adu-6M), we observed that Adu-DGNs-Arch-No Light (8-month-old animals) had weaker memories compared with younger control groups from the other experiments (Animals between two and four-months of age, see performance in Fig. 3C, H, M; Fig. 4C; and Supp Fig. 4). Even though, they could still remember the target quadrant where the platform was (Fig. 4G and Supplementary Table 2 for complete statistical analysis). In contrast, Adu-DGNs-Arch-Light animals were unable to remember the platform position as revealed by the time spent in the target quadrant which was no different to that spent in the other quadrants or to the chance level (Fig. 4G, H and Supplementary Table 2 for complete statistical analysis). These data indicate that, as animals become older, memory retention depends on mature adult-born neurons.

Different morphological features of dendritic arborisation among DGN populations

Finally, the morphology of the dendritic arbour of DGNs born at different ontogenetic stages and the effect of light on their organisation were analysed. Clearly, Neo-DGNs exhibited specific

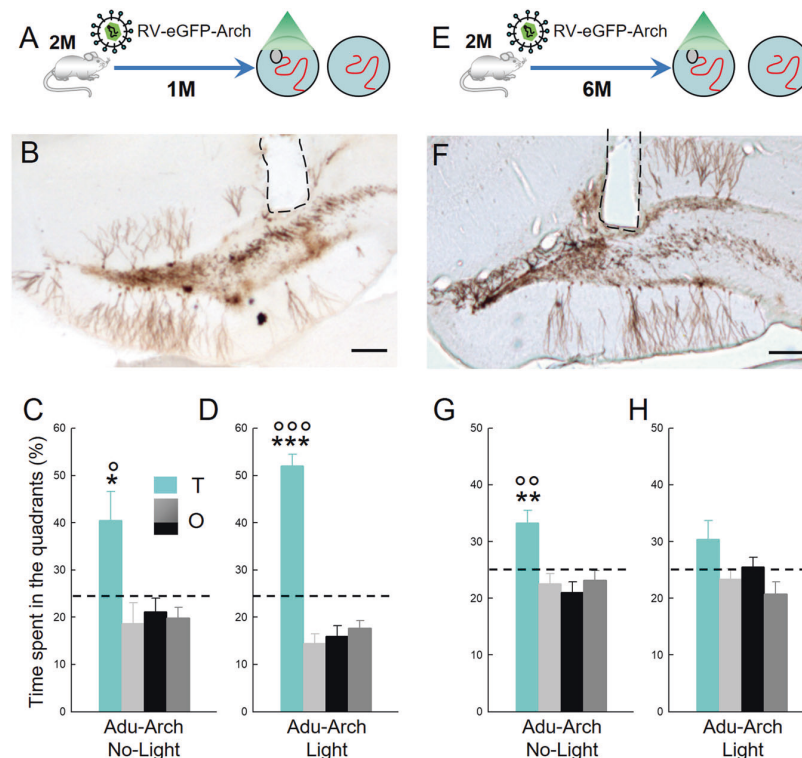


Fig. 4 Optical inhibition of mature Adu-DGNs affects memory retrieval. **A, E** Experimental designs. Illustration of Adu-DGNs **B, F** infected with RV-Arch-EGFP; dashed line, position where the optic fibres were placed (Bar Scale = 200 μ m). Silencing **C, D** 1-month-old Adu-DGNs during training did not impair memory retention, whereas **G, H** silencing 6-month-old Adu-DGNs impaired the ability of the animals to remember the platform location. + at least at $p < 0.05$ compared to other quadrants. ** $p \leq 0.01$ *** $p \leq 0.001$ compared to other quadrants. °° $p \leq 0.01$ and °°° $p \leq 0.001$ compared to chance level (25%). T: target quadrant. O: other quadrants.

features compared to the other cohorts such as multiple primary dendrites, with a wider branching angle and a more complex organisation of the proximal part of the dendritic arbour (Fig. 5A and Supplementary Fig. 5A–C). When dendritic arbours of Adol-DGNs (Adol-1M, Adol-2M and Adol-4M) were compared (Supplementary Fig. 5B–D), no major significant difference could be detected across ages suggesting that their development is completed within a few weeks. However, we did find a difference in the immature Adu-DGNs neurons (Adu-1M) compared with mature ones (Adu-6M), with the latter group presenting an increased number of branches in higher order branches (Fig. 5A and Supplementary Fig. 5D), a feature that was also different from mature Adol-DGNs, indicating a distinct pattern of arborisation in mature Adu-DGNs. Optogenetic inhibition had a different effect on dendritic length depending on the temporal origin and age of the DGNs (Supplementary Table 3). It did not influence the dendritic organisation of Neo-DGNs (Fig. 5B and Supplementary Fig. 5E), which is consistent with the lack of any effect on memory. Similarly, all ages of Adol-DGNs and 1-month-old Adu-DGNs dendritic lengths were not influenced by optogenetic inhibition (Fig. 5B and Supplementary Fig. 5F–I). In contrast, the complexity of the dendritic arbour of mature Adu-DGNs was decreased by the inhibition (Fig. 5B–D), suggesting that these changes are responsible for impairment in retention. To lend weight to this hypothesis, the dendritic length was correlated to the time spent (Fig. 5E) in the area previously containing the platform. We found a positive correlation between memory retention and the dendritic length: memory retention was high in rats with longer dendrites (No Light) whereas memory retention was poor in rats with shorter dendrites.

DISCUSSION

In the present study, we have shown that populations of DGNs of different temporal origin are sequentially involved in spatial memory and also present distinct morphological characteristics. Indeed, Adol-DGNs are involved in memory processing within a particular time window, a function taken up by Adu-DGNs at a later time point when animals become adult. In addition, Adol- and Adu-DGNs exhibit different morphologies and morphological plasticity.

Memory development is not a singular process and learning capacity emerges sequentially from the simple to the complex. The ability to learn spatial location develops later through adolescence into early adulthood. Four- to five-week-old animals use proximal cues to navigate (an independent-hippocampal strategy) and fail to search in the correct quadrant during probe trials [3, 4]. In contrast, 6 week-old animals use a hippocampal-dependent strategy to navigate thought space that requires the establishment of a cognitive map and flexible use of the learned information (spatial relational memory). It has been suggested that the maturation of spatial processing results from the maturation of the hippocampal formation and, in particular, from its spatially responsive cells [30]. Our results suggest that the emergence of navigation through space depends upon the recruitment of DGNs generated during adolescence.

Importantly and surprisingly, this role seems to be transitory since Adol-DGNs are no longer needed (for spatial memory) as animals become adult. Although this transient role was observed by the imaging and silencing of Adol-DGN, a temporal shift was observed between activated (one-month-old) and functionally

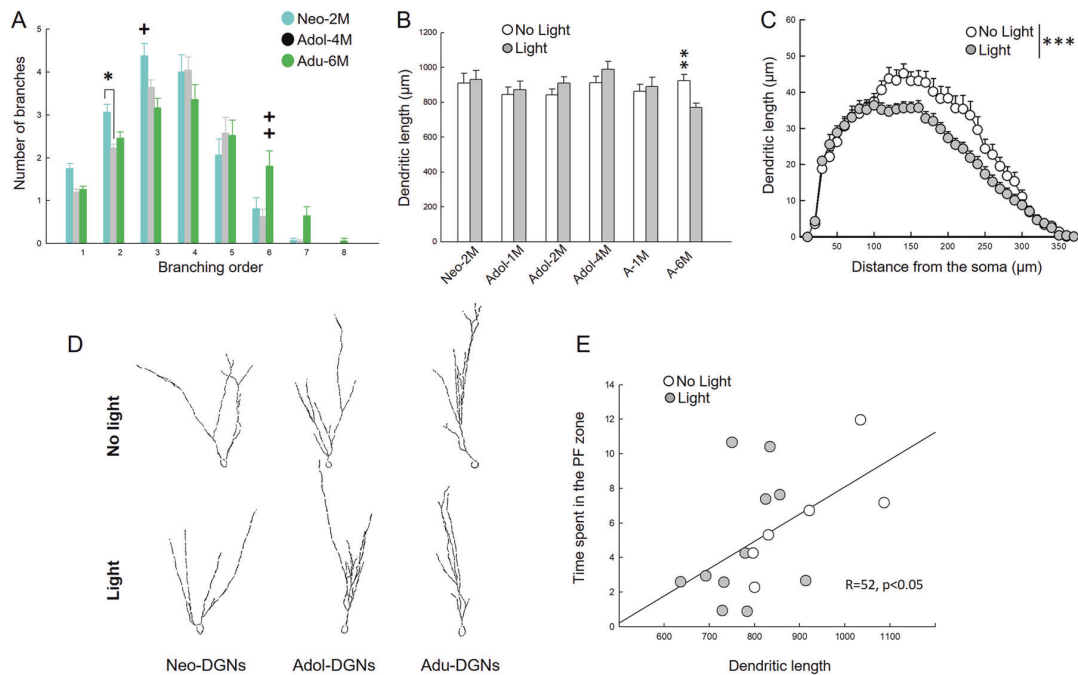


Fig. 5 Different populations of DGNs show distinct features in branching arborisation and they are distinctly modified by the light. A Dendritic branching shows diverse profiles of different mature DGN populations (interaction group \times order: $F_{14,735} = 3.630$, $p < 0.0001$). Neo-DGNs show an increase in low order dendrites, while the Adu-DGNs show an increase in higher order dendrites. When the effect of light on the dendritic arbour was examined, a reduction in the total length was only observed in Adu-DGNs, an effect (B) associated with a decrease in dendritic complexity (C) (See also, Supplementary Table 3). D Representative tracings of the DGN populations analysed. Correlation between dendritic length and the time spent in the PF zone (E) during the probe test of the Adu-DGNs 6 M (ddl = 17, $r = 52$, $p < 0.05$). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, + at least at $p < 0.05$ ++ at least at $p \leq 0.01$.

recruited (two-month-old) Adol-DGNs. To reconcile these conflicting results, we propose that this shift is due to delayed development and integration of Adol-DGNs in optogenetic experiments. Indeed, in this study animals underwent two surgeries (viral injection, optic fibre implantation) and inflammation induced by the cannula [31] and/or sedation [32, 33] are known to delay neuronal development and functional integration.

We previously reported that Neo-DGNs are not recruited when animals are learning to navigate in space either using a water maze or a dry water maze [12]. Here we confirm that Neo-DGNs do not play a role in spatial navigation when animals have reached adulthood because they are not recruited by spatial learning and silencing them during training does not impair memory retrieval. More recently, using functional imaging, we have shown that Neo-DGNs are not involved in remote retrieval-induced memory reconsolidation evaluated in the water maze [12, 13]. Neo-DGNs seem to play a role in more simple functions such as discriminating between very different contexts [12].

As expected, mature Adu-DGNs were important for memory expression, a result consistent with a previous study showing that post-training ablation of 2-month-old Adu-DGNs degrades previously acquired memories [34]. Unsurprisingly, immature Adu-DGNs were not critical for memory expression, most probably because they are less spatially tuned as shown by *in vivo* calcium imaging [35]. However, by combining retroviral and chemogenetic approaches we have recently discovered that at the time of spatial learning (in the water maze) immature Adu-DGNs are necessary for the process of remote retrieval-induced reconsolidation [12, 13]. We speculate that immature Adu-DGNs are primed during learning and that, once mature, they influence the neural representation of the learned information (for discussion see [12, 13]).

Given the sequential involvement of Adol- and Adu-DGNs in memory processing, we propose that the adolescent wave of DGN neurons might be involved in setting up the function (organisational value) whereas adult neurogenesis may have an adaptive value. In support of this framework, Adol-DGNs (P27-35) have been shown to be important for the maturation of social behaviour [36] whereas Adu-DGNs are essential for social memory maintenance [37]. In addition, we have recently observed that the suppression of *Rnd2*, a small Rho GTPase, in DGNs born in neonates (P1) has no effect on anxiety-like behaviour whereas its deletion in Adu-DGNs exacerbates anxiety-like behaviours [38]. Based on this data we suggest that neurogenesis is a “Baldwin mechanism” that allows “ontogenetic adaptation” to a changing environment [39]. Given this framework, deleterious life events (such as chronic stress, drugs and a high-fat diet) during perinatal life and adolescence may have different outcomes and are likely to induce a vulnerable state that conditions the occurrence of disorders later in life [40–47].

In the search for the mechanisms that might explain this different role in spatial memory, we focused on the dendritic structure of DGNs because the shape of a neuron’s dendritic tree influences how synaptic information is received and integrated, which can have functional implications [48]. In the DG, it has been shown that dendritic architectures are predictive of DGN activity during spatial exploration. In particular, in rats, DGNs with high-order dendritic branching patterns are preferentially activated during spatial exploration [49].

We first identified morphological features differentiating the three mature populations: (i) Neo-DGNs have more primary dendrites, a broader branching angle and more ramifications proximal to the soma compared to the two other populations,

(ii) Adol- and Adu-DGNs are indistinguishable for most of the parameters except that mature Adu-DGNs have an increased number of high order dendrites compare to the Adol-DGNs and immature Adu-DGNs. Similarly in mice, we and others found that the dendritic organisation and the synaptic input of the DGNs depend upon the ontogenetic stage of the animals [10, 50]. There are, however, some differences between species since in mice: (i) mature Emb-DGNs and not Neo-DGNs exhibit more primary dendrites, (ii) mature Neo-DGNs exhibit a longer dendritic length compared to Adol- and Adu-DGNs [10]. Not only were differences in the dendritic arborisation studied, it was also shown that Adu-DGNs and Neo-DGNs (P6 in rats) exhibit different critical periods for cell death. Whereas Adu-DGNs die within a few days after their birth [25], Neo-DGNs exhibit a delayed critical period for survival as they die between 2 and 6 months of age [11]. Furthermore, Emb-DGNs and Adol-DGNs exhibit a distinct survival dynamic from that described for Neo-DGNs [11, 51] illustrating a heterogeneity of maturation processes within cohorts of developmentally generated DGNs. Future studies will be of interest to determine whether their temporal origin determines their cellular physiology as described for early embryonic DGNs [52] and for GABAergic neurons in Ammon's Horn [53].

Moreover, we did not observe any time-dependent evolution in the dendritic complexity of Adol-DGNs indicating that maturity is reached within four weeks. We have previously shown in mice that the dendritic arbour of Emb-DGNs and Neo-DGNs reaches maturity within 6 weeks [10]. This suggests that developmentally born neurons mature faster than Adu-DGNs which keep on growing for at least 3 months (Table S2 in [15]), and show an extended period of morphological [54, 55] and physiological [56] maturation. This heterogeneity in the rate of maturation of DGNs could be due to intrinsic differences but also to differences in their local environment [55, 57].

When we compared the effect of DGN inhibition, we found differential reactivity in mature DGNs born in the neonates, adolescent and adult animals. Developmentally-born neurons were not sensitive to light inhibition. In contrast, optogenetic inhibition of Adu-DGNs led to a reduction in dendritic complexity suggesting that these changes were responsible for impairment in memory expression. Even though we were expecting to see anatomical differences in Adol-2M DGNs that could explain the impairment in retrieval, we did not find any effect of the inhibition on the dendritic arbour. As stated previously, insults inflicted during adolescence and adulthood have different outcomes, pointing to a different level of sensitivity in these populations [14, 15]. The maturation of different neurotransmitters and neuromodulators and their signalling cascades in the hippocampus has not yet been achieved at adolescence, and it has been suggested that the maturation of glutamate receptors plays a key role in the "switch" from juvenile- to adult-like functioning [58]. For example, changes in the subunit composition of AMPA receptors have been described around P21–30 [59, 60] or it may be that mGluR5 is necessary for proper neurogenesis at P7 and P28, but is no longer necessary at later time points [61]. For this reason, we speculate that, since glutamate receptors change from birth to adulthood, they can regulate the development, reactivity and function of DGNs differently according to their temporal origin.

In conclusion, we have provided the first evidence of a causal relationship between the temporal origin of DGNs and their role in spatial memory that can provide novel insights with a view to disentangling the ways in which the heterogeneity of populations in the DG may contribute to differences in physiological and pathological states of hippocampal function.

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AUTHOR CONTRIBUTIONS

NM, VC and DNA designed the experiments and wrote the manuscript. NM, VC, ST and NB performed IEGs (development and adolescence) and optogenetic experiments. VL, MFM performed IEG experiments (adult). WM performed electrophysiological recordings. FF produced the retrovirus. SG provided the construct for the RV-Arch-EGFP. CH advised as optogenetic expert. CH, DC, DNA, GM and VDG were part of the Optopath consortium. NM, VC, NB, ST, DC, VDG, GM, CH and DNA revised and approved the manuscript. DNA provided funding and designed the project.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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SUPPLEMENTARY INFORMATION

Experiments	Delay	Nb of XdU-IR cells	Comparison of the number of XdU-IR cells (Group effect)	% of XdU-Zif-268IR cells	Comparison of activated DGNs-IR cells (Group effect)
Adol-DGNs	1 M	Control : 18322 ± 617 Learning : 20351 ± 1384 Cued : 23040 ± 1489 Swim : 22904 ± 1688	$F_{3,37}=2.61$; $p=0.07$	Control : 7.33 ± 1.2 Learning : 15.30 ± 1.09 Cued : 8.29 ± 0.95 Swim : 6.45 ± 0.80	$F_{3,37}=16.67$; $p=0.001$
Adol-DGNs	2 M	Control : 18948 ± 2848 Learning : 15980 ± 1257 Cued : 16140 ± 940	$F_{2,18}=0.85$; $p=0.45$	Control : 1.70 ± 0.50 Learning : 1.41 ± 0.23 Cued : 1.37 ± 0.51	$F_{2,18}=0.20$; $p=0.82$
Adol-DGNs	3 M	Control : 11068 ± 1752 Learning : 11680 ± 783 Cued : 10700 ± 975	$F_{2,16}=0.20$; $p=0.83$	Control : 2.75 ± 0.59 Learning : 2.59 ± 0.55 Cued : 2.17 ± 0.47	$F_{2,16}=0.32$; $p=0.74$
Adu-DGNs	1 M	Control : 10013 ± 2713 Learning : 9583 ± 1212	$t_7=0.17$; $p=0.86$	Control : 3.87 ± 1.36 Learning : 3.83 ± 0.73	$t_7=0.03$; $p=0.98$
Adu-DGNs	2 M	Control : 9360 ± 1655 Learning : 11240 ± 887	$t_6=-1.11$; $p=0.31$	Control : 6.67 ± 2.79 Learning : 17.69 ± 1.50	$t_6=-3.86$; $p=0.008$
Adu-DGNs	3 M	Control : 5792 ± 1038 Learning : 5580 ± 717	$t_{10}=0.17$; $p=0.86$	Control : 11.75 ± 0.74 Learning : 19.28 ± 1.55	$t_{10}=-3.84$; $p=0.003$
Emb-DGNs	3 M	Control : 132508 ± 9219 Learning : 152655 ± 14733 Cued : 136854 ± 14310	$F_{2,25}=0.50$; $p=0.61$	Control : 5.34 ± 1.12 Learning : 6.44 ± 0.73 Cued : 5.14 ± 0.55	$F_{2,25}=0.92$; $p=0.41$
Neo-DGNs	10 W	Control : 19407 ± 2115 Learning : 23476 ± 1268 Cued : 21663 ± 1262	$F_{2,25}=0.77$; $p=0.19$	Control : 8.72 ± 1.30 Learning : 8.03 ± 0.99 Cued : 7.83 ± 0.87	$F_{2,25}=0.14$; $p=0.87$

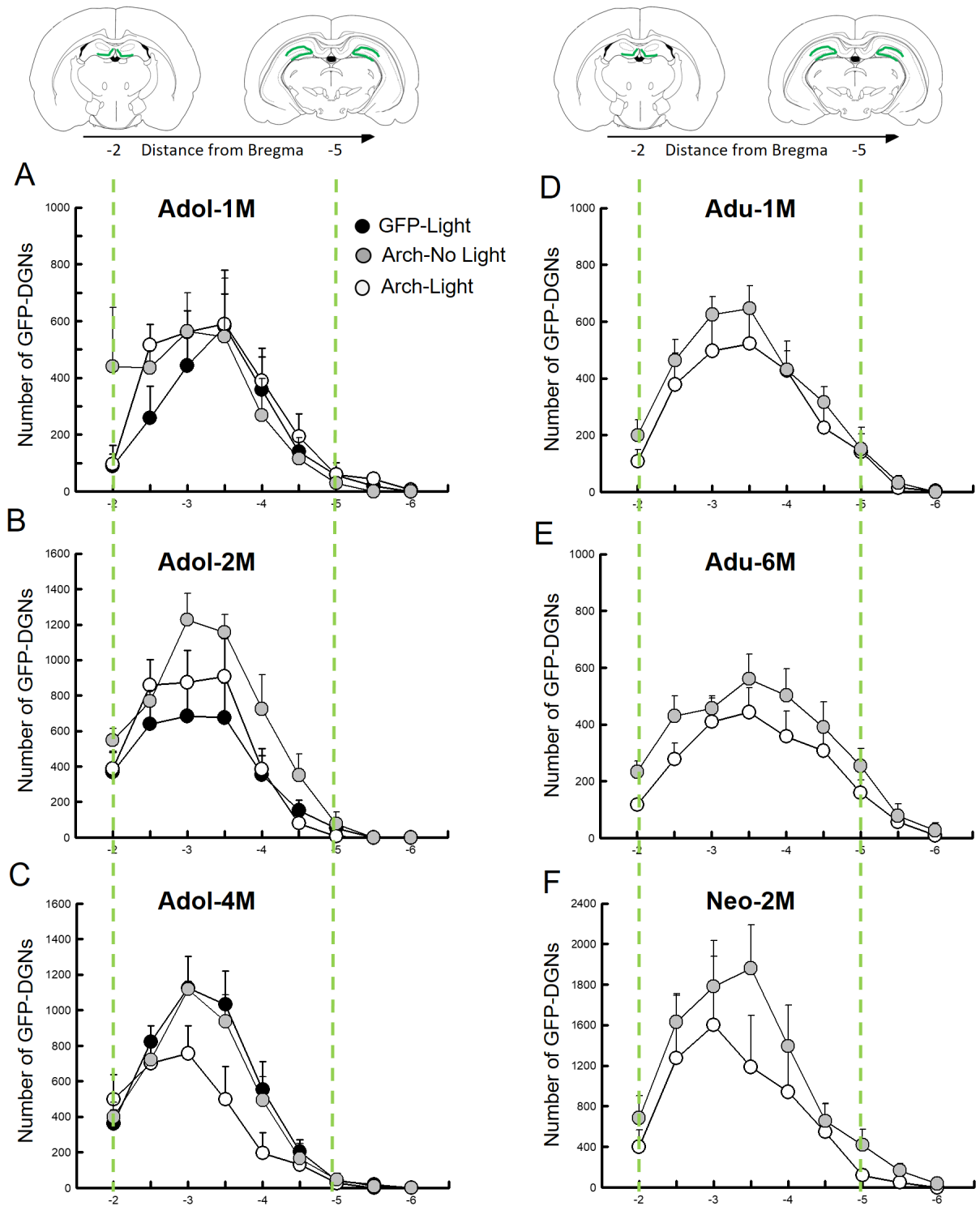
Supplementary Table 1: Statistical analysis of the expression of Zif268 in DGNs.

DGNs population	Delay	Number of GFP-IR cells	Analysis of GFP-IR cell numbers (Group effect)	Analysis of time spent in the quadrants (Quadrant effect)	Comparison of TQ to chance level
Adol-DGNs-Arch-No-Light		2454 ± 400		$F_{3,39}=8.59$, $p<0.001$ with TQ<SE $p=0.0013$; TQ<NE $p<0.001$; TQ<SW $p=0.0016$.	$t_{13}=4.07$, $p=0.0013$
Adol-DGNs-Arch-Light	1M	2395 ± 418	$F_{2,29}=0.37$, $p=0.69$	$F_{3,15}=17.23$, $p<0.001$ with TQ<SE $p<0.001$; TQ<NE $p<0.001$; TQ<SW $p<0.001$	$t_8=5.35$, $p=0.0031$
Adol-DGNs-GFP-Light		1945 ± 691		$F_{3,33}=10.29$, $p<0.001$ with TQ<SE $p<0.001$; TQ<NE $p<0.001$; TQ<SW $p<0.001$	$t_{11}=3.89$, $p=0.003$
Adol-DGNs-Arch-No-Light		3498 ± 670		$F_{3,36}=19.9$, $p<0.001$ with TQ<SE $p<0.001$; TQ<NE $p<0.001$; TQ<SW $p<0.001$	$t_{12}=5.86$, $p<0.001$
Adol-DGNs-Arch-Light	2 M	4849 ± 541	$F_{2,36}=2.45$, $p=0.10$	$F_{3,36}=1.84$, $p=0.16$	$t_{12}=1.53$, $p=0.15$
Adol-DGNs-GFP-Light		2925 ± 675		$F_{3,36}=15.12$, $p<0.001$ with TQ<SE $p<0.001$; TQ<NE $p<0.001$; TQ<SW $p<0.001$	$t_{12}=5.17$, $p<0.001$
Adol-DGNs-Arch-No-Light		2801 ± 634		$F_{3,27}=5.8$, $p=0.003$ with TQ<SE $p=0.0176$; TQ<NE $p=0.0185$; TQ<SW $p=0.0024$	$t_9=3.29$, $p=0.009$
Adol-DGNs-Arch-Light	4 M	3885 ± 457	$F_{2,24}=1.55$, $p=0.23$	$F_{3,21}=5.6$, $p=0.005$ with TQ<SE $p=0.013$; TQ<NE $p=0.0068$; TQ<SW $p=0.0068$	$t_7=3.40$, $p=0.01$
Adol-DGNs-GFP-Light		4151 ± 617		$F_{3,24}=14.85$, $p<0.001$ with TQ<SE $p<0.001$; TQ<NE $p<0.001$; TQ<SW $p<0.001$	$t_8=5.06$, $p=0.001$
Adu-DGNs-Arch-No-Light	1 M	2315 ± 581	$t_{19}=0.85$, $p=0.41$	$F_{3,27}=4.477$, $p=0.011$ with TQ<SE $p=0.019$; TQ<NE $p=0.016$; TQ<SW $p=0.010$	$t_9=2.51$, $p=0.033$
Adu-DGNs-Arch-Light		2863 ± 320		$F_{3,30}=53.897$, $p<0.001$ with TQ<SE $p<0.001$; TQ<NE $p<0.001$; TQ<SW $p<0.001$	$t_{10}=10.85$, $p<0.001$
Adu-DGNs-Arch-No-Light	6 M	2129 ± 365	$t_{21}=1.49$, $p=0.15$	$F_{3,30}=6.4$, $p=0.001$ with TQ<SE $p=0.005$; TQ<NE $p=0.002$; TQ<SW $p=0.003$	$t_{10}=3.82$, $p=0.003$
Adu-DGNs-Arch-Light		2929 ± 389		$F_{3,33}=2.2$, $p=0.1$	$t_{11}=1.62$, $p=0.13$
Neo-DGNs Arch-No-Light	2 M	6117 ± 2876	$t_{18}=1.01$, $p=0.33$	$F_{3,18}=12.24$, $p<0.001$ with TQ<SE $p<0.001$; TQ<NE $p<0.001$; TQ<SW $p<0.001$	$t_6=5.18$, $p=0.002$
Neo-DGNs Arch-Light		9126 ± 1561		$F_{3,36}=6.18$, $p=0.0017$ with TQ<SE $p=0.002$; TQ<NE $p=0.003$; TQ<SW $p=0.021$	$t_{12}=3.23$, $p=0.0072$

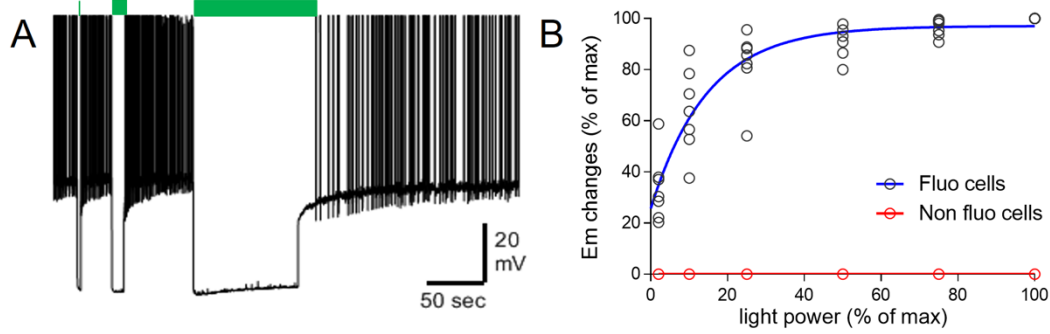
Supplementary Table 2: Statistical analysis of behaviour performance in optogenetic experiments.

DGN population	Delay	Number of rats	Number of DGNs analyzed	Sholl analysis (Light effect)	Ddendritic length (Light effect)
Adol-DGNS-Arch-No-Light Adol-DGNS-Arch-Light	1 M	6 6	34 36	$F_{1,68}=0.1490, p=0.7007$	$t_{68}=0.3878, p=0.6994$
Adol-DGNS-Arch-No-Light Adol-DGNS-Arch-Light	2 M	10 13	59 68	$F_{1,125}=1.752, p=0.1880$	$t_{125}=1.325, p=0.1876$
Adol-DGNS-Arch-No-Light Adol-DGNS-Arch-Light	4 M	8 8	45 42	$F_{1,85}=1.807, p=0.1824$	$t_{85}=1.344, p=0.1824$
Adu-DGNS-Arch-No-Light Adu-DGNS-Arch-Light	1 M	7 7	41 37	$F_{1,76}=0.1539, p=0.6960$	$t_{76}=0.3970, p=0.6925$
Adu-DGNS-Arch-No-Light Adu-DGNS-Arch-Light	6 M	6 11	31 54	$F_{1,83}=12.96, p=0.0005$	$t_{83}=3.603, p=0.0005$
Neo-DGNS Arch-No-Light Neo-DGNS Arch-Light	2 M	5 5	32 30	$F_{1,60}=0.011, p=0.9136$	$t_{60}=0.1084, p=0.9140$

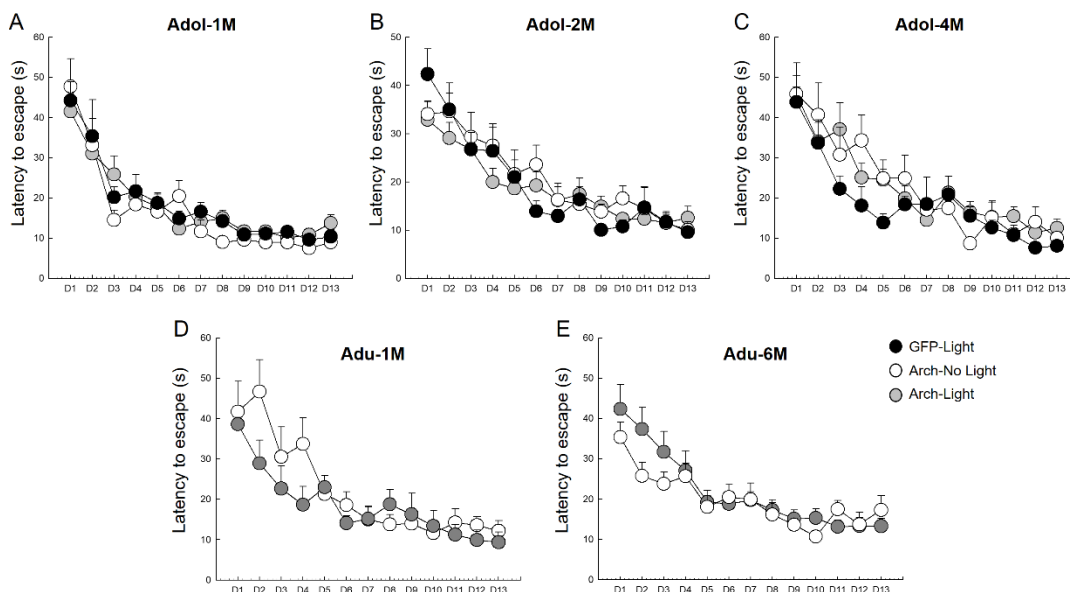
Supplementary Table 3: Statistical analysis of light effect in dendritic morphology of DGNS population



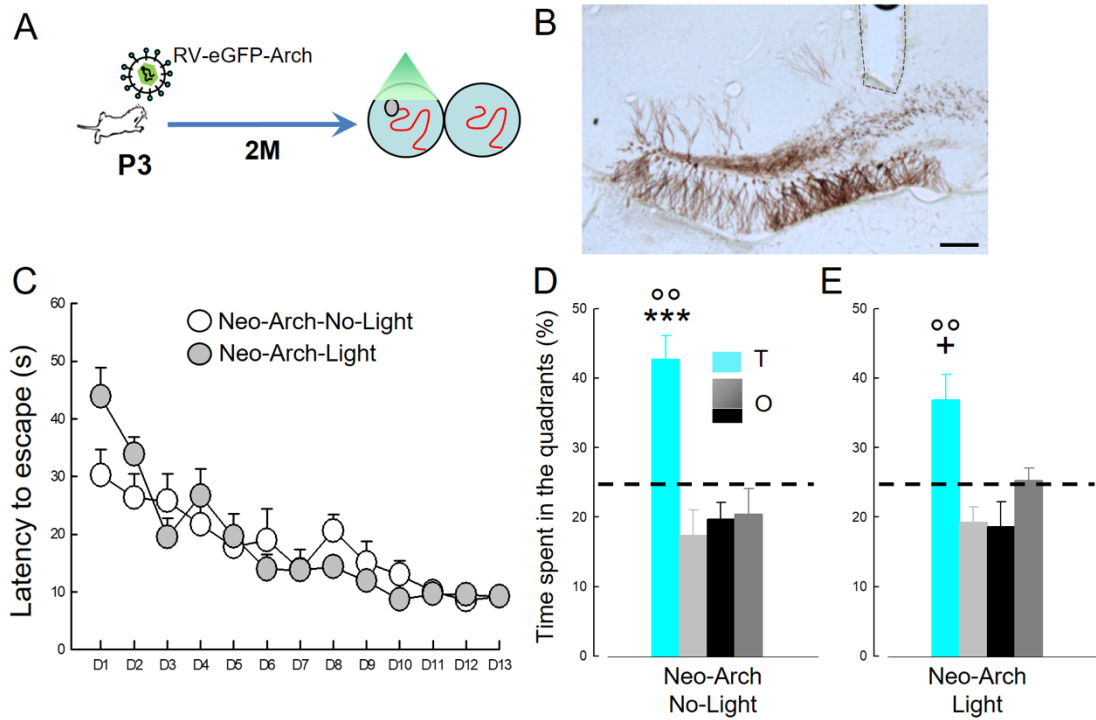
Supplementary Fig. 1: Septotemporal distribution of Arch-eGFP or GFP DGNs. Number of GFP-IR DGNs along the septotemporal axis of Adol-1M (A), Adol-2M (B), Adol-4M (C), Adu-1M (D), Adu-6M (E) and Neo-DGNs (F) experiments.



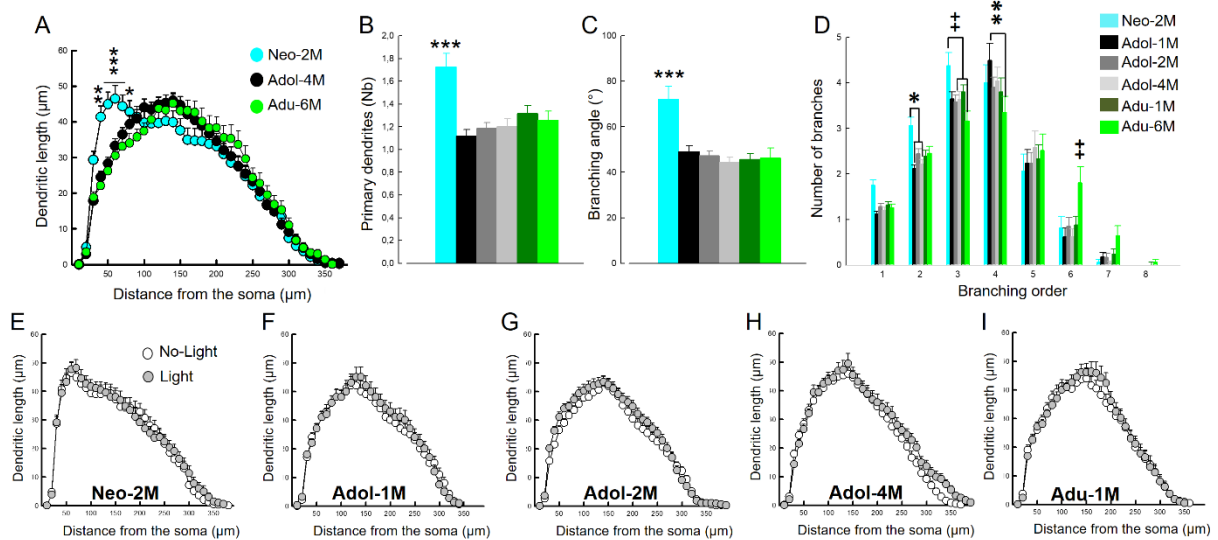
Supplementary Fig. 2: Whole cell recordings of DGNs in rats infected with RV-Arch-eGFP. (A) Representative tracing showing a whole cell patch-clamp recording under current clamp mode. Positive currents were injected in order to make the cell fire artificially. When the light was turned on (2, 10 and 90 sec, green rectangle), cell potential was instantly reduced and neurons stopped firing. **(B)** Light effect into neuronal activity, evaluated by monitoring membrane potential (Em), was power-dependent and reached a maximal effect with low power. Importantly, no effect was observed in non-fluorescent cells, in which Em was not changed.



Supplementary Fig. 3: Optical silencing of DGNs born in adolescent or adult rats does not influence acquisition of spatial memory. RV-GFP or RV-eGFP-Arch were injected bilaterally in the DG of adolescent rats at P28 and animals were trained 1 (A), 2 (B) or 4 (C) months after the infection. RV-Arch-eGFP were injected bilaterally in the DG of 2-month-old rats and animals were trained 1 (D) or 6 (E) months later. Rats were trained to find a hidden platform in the water maze with half of the group trained with “Light” and the other half with “No Light”. In all groups, illumination during training did not influence learning. [Light effect: (A) $F_{2,29}=0.2964$, $p=0.7451$; (B) $F_{2,36}=0.3257$, $p=0.7241$; (C) $F_{2,24}=1.241$, $p=0.3070$; (D) $F_{1,19}=0.6424$, $p=0.4328$; (E) $F_{1,21}=0.5850$, $p=0.4529$].

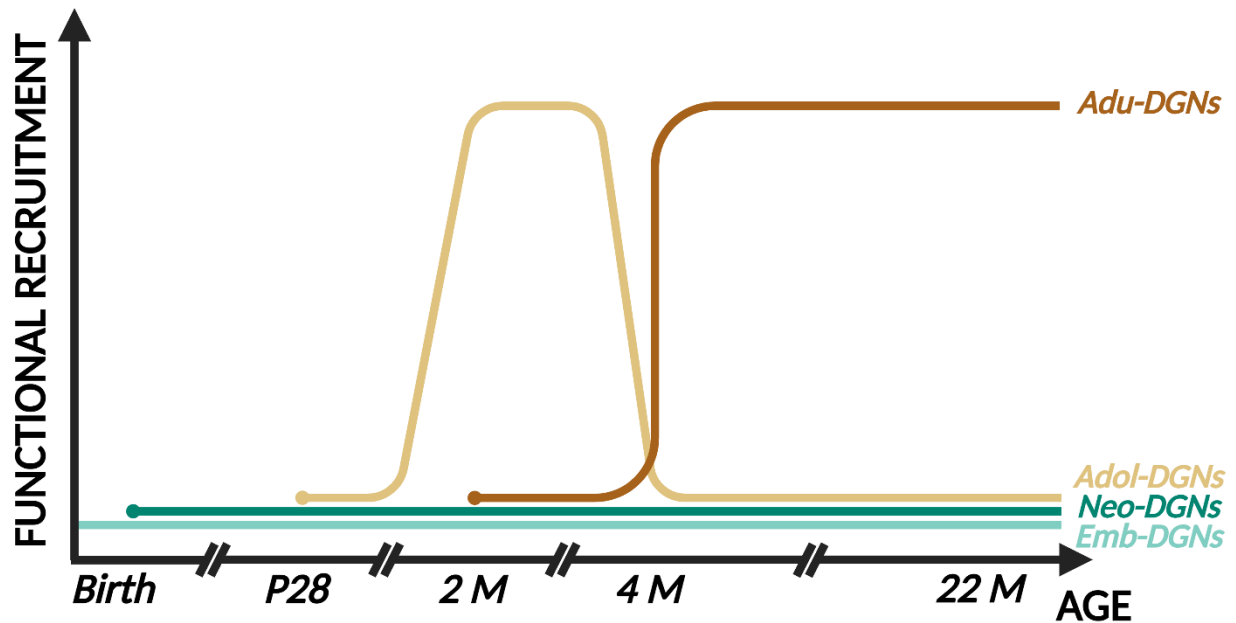


Supplementary Fig. 4: Optical silencing of DGNs born in neonates does not influence learning or remembering. (A) Experimental design: RV-Arch-eGFP were injected bilaterally in the DG of neonates at P3 (Neo). (B) Illustration of Neo-DGNs infected with RV-Arch-EGFP; dashed line, position where the optic fiber was placed (Bar Scale= 200μm). (C) Rats were trained to find a hidden platform in the water maze with half of the group trained with “Light On” and the other half with “Light Off”. Illumination during training did not influence learning (Light effect: $F_{1,18}=0.01421$, $p=0.9064$). (D, E) Silencing 2-month-old Neo-DGNs during learning does not impaired the ability of the animals to remember the platform location. + $p<0.05$ compared to light grey and *** $p<0.001$ compared to the other quadrants. °° $p<0.01$ compared to chance level. T: target quadrant. O: other quadrants.



Supplementary Fig. 5: DGNs generated during development are not affected by the light and present distinct morphological characteristics. (A) Sholl distribution of the three mature DGNs population analyzed (interaction group x distance to the soma: $F_{78, 4095}=3.36$, $p<0.001$) shows a different profile in Neo-DGNs. (B) Neo-DGNs have a higher number of primary dendrites (Group effect $F_{5,236}=8.258$, $p<0.0001$) and (C) a broader branching angle (Group effect $F_{5,236}=10.35$, $p<0.0001$) compared to Adol- and Adu-DGNs. Branching order distribution (D) shows different profile between the three DGNs population analyzed (interaction group x branching order : $F_{35,1652}=2.116$, $p<0.0002$), of note, no differences were observed between the delays analyzed in the Adol-DGNs (Group effect $F_{2,135}=1.151$, $p=0.3194$). (E-I) Sholl analysis shows that Neo-, Adol- nor Adu-1M DGNs are not affected by the light * $p\leq 0.05$, ** $p\leq 0.01$, *** $p\leq 0.001$; + at least at $p<0.05$ ++ at least at $p\leq 0.01$.

Graphical abstract



To summarize these data; mature adu-DGNs are required for spatial learning and do not retire from this function. Adol-DGNs are involved in setting up the function and emb-DGNs and neo-DGNs are not involved in this type of learning. Interestingly, optogenetic silencing of the adol-DGNs and adu-DGNs during the learning task had no effect on the learning performances, but only on memory retention later on. In addition, we observed dendritic morphological differences according to the ontogenic origin of the DGNs, confirming these observations in rats.

II. ASSESSMENT OF THE SURVIVAL & ACTIVITY OF DEV-DGNS AND ADU-DGNS DURING AGING

A. General presentation

After having shown that mature adu-DGNs are required for spatial learning and do not retire from this function, we aimed at determine whether they stayed functional in the course of aging. Spatial learning abilities significantly decline with age, yet some individuals exhibit resilience and maintain good abilities while others are vulnerable with reduced capacities. Therefore, it seems that this specific ontogenic population could be the one supporting resilience to cognitive aging. Indeed, it has been demonstrated that the levels of neurogenesis at old age are predictive of the spatial learning abilities (Drapeau et al., 2003). Furthermore, as summarized in the introduction, manipulating adult neurogenesis to boost the population of immature adu-DGNs in the aged hippocampus has led to restored cognitive functions and improved spatial learning and memory performances. While such observations strongly highlight the contribution of immature adu-DGNs in preserving cognitive abilities during aging, their role once mature has been somewhat overlooked. Thus, also considering mature adu-DGNs within the aged network as a potential source of resilience against cognitive decline appears to be a promising strategy, especially as they are numerous and functionally significant for spatial learning.

Therefore, we addressed both the survival and recruitment of mature adu-DGNs born at different time of adulthood. In the continuity of our precedent study, we also investigated the survival and recruitment of dev-DGNs, to control for their non-involvement in the context of aging. Using an approach based of thymidine analogues and Zif268 expression we were able to target neurons born during embryogenesis, adolescence, the beginning of adulthood, at middle-age and at old age, all of these neurons having reach maturity at the time of learning.

Article 2:

**Responsiveness of dentate neurons
generated throughout adult life is
associated with resilience to
cognitive aging**



Responsiveness of dentate neurons generated throughout adult life is associated with resilience to cognitive aging

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Abstract

During aging, some individuals are resilient to the decline of cognitive functions whereas others are vulnerable. These inter-individual differences in memory abilities have been associated with differences in the rate of hippocampal neurogenesis measured in elderly. Whether the maintenance of the functionality of neurons generated throughout adult life is linked to resilience to cognitive aging remains completely unexplored. Using the immediate early gene *Zif268*, we analyzed the activation of dentate granule neurons born in adult (3-month-old), middle-aged (12-month-old), or senescent (18-month-old) rats ($n = 96$) in response to learning when animals reached 21 months of age. The activation of neurons born during the developmental period was also examined. We show that adult-born neurons can survive up to 19 months and that neurons generated 4, 10, or 19 months *before* learning, but not developmentally born neurons, are activated in senescent rats with good learning abilities. In contrast, aged rats with bad learning abilities do not exhibit activity-dependent regulation of newborn cells, whatever their birthdate. In conclusion, we propose that resilience to cognitive aging is associated with responsiveness of neurons born during adult life. These data add to our current knowledge by showing that the aging of memory abilities stems not only from the number but also from the responsiveness of adult-born neurons.

KEYWORDS

adult neurogenesis, aging cell, hippocampus, resilience, spatial memory, successful aging

1 | INTRODUCTION

Brain and cognition change with age, and although patterns of decline are evident at the population level, the rates of change differ among individuals as well as across brain regions and cognitive domains (Gray & Barnes, 2015; Nyberg, Lovden, Riklund, Lindenberger, & Backman, 2012). Indeed, some old individuals exhibit cognitive

abilities similar to those of younger ones (optimal/successful aging) whereas others show a clear and substantial cognitive decline without signs of pathologies (suboptimal/accelerated aging). Episodic memory is particularly sensitive to aging, and investigations conducted so far both in humans and in animal models have revealed that the preservation of episodic memory abilities is correlated with the structural and functional integrity of the hippocampal

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formation (Gonzalez-Escamilla, Muthuraman, Chirumamilla, Vogt, & Groppa, 2018). Several models and theories (maintenance, reserve, compensation) emerged in an effort to account for variability in cognitive outcome across old subjects, and high level of neural plasticity has been proposed for brain reserve and resilience to cognitive aging (Nyberg et al., 2012).

The ability of the adult brain, and in particular the dentate gyrus (DG) of the hippocampus, to create new neurons is a peculiar form of plasticity to protect the aging brain. Briefly, new dentate granule neurons (DGNs) generated throughout the entire life of an individual (Aimone et al., 2014), humans included (Moreno-Jiménez et al., 2019), are integrated into functional circuits and play a crucial role in complex forms of learning and memory (Clelland et al., 2009; Dupret et al., 2008; Tronel et al., 2012). In particular, both the addition and the elimination of new neurons *before*, *during*, or *after* learning were found to be important for learning and forgetting in young adult rodents (Akers et al., 2014; Dupret et al., 2007).

During aging, the rate of cell proliferation (and thus neurogenesis) decreases (Drapeau & Abrous, 2008), a process associated with the progressive loss or phenotypic and functional changes of neural stem cells (NSCs), (Martin-Suarez, Valero, Muro-Garcia, & Encinas, 2019; Schouten et al., 2019), or their niche (Diaz-Moreno et al., 2018). Inter-individual differences in the rate of adult neurogenesis (ANG) have been linked to variability in spatial memory abilities of senescent animals: Preserved memory functions are associated with the maintenance of a relatively high neurogenesis level measured *after* learning, whereas memory deficits are linked to exhaustion of neurogenesis (Drapeau et al., 2003). Moreover, we have found that corticosterone dampening from middle age on has a beneficial effect on both the rate of neurogenesis and spatial memory measured once animals have reached senescence (Montaron et al., 2006). Altogether this dataset raises the fascinating hypothesis that neurons generated throughout adult life

could constitute a mechanism that promotes resilience to cognitive aging.

To tackle this question, we examined whether the activation of DGNs generated throughout adult life was related to the maintenance of memory function by imaging them when animals reached senescence. DGNs born in adult (3-month-old), middle-aged (12-month-old), or senescent (18-month-old) rats were labeled with analogs of thymidine, and their activation in response to spatial learning was measured using Zif268, an immediate early gene (IEG) (Tronel, Lemaire, Charrier, Montaron, & Abrous, 2015), when animals have reached senescence. The activation of DGNs born during development was also examined.

2 | RESULTS

2.1 | Fate of dentate granule neurons born in old rats

In a first step, we sought to determine whether new neurons born during *senescence* are recruited by spatial learning. To do so, 18-month-old rats were injected with BrdU according to a previously described protocol (Table 1) and were trained 4 months later in the water maze using a reference memory protocol (Drapeau et al., 2003). Animals were trained for 11 days (Figure S1a,b) until the aged-unimpaired rats (AU) learned the task (day effect on Latency: $F_{11,66} = 2.35, p = .016$; day effect on Distance: $F_{11,66} = 2.76, p = .005$) and reached asymptotic levels of performances (with no statistical significant differences over the last 3 days). In contrast, the aged-impaired (AI) rats did not learn the task although they were searching and finding the platform most of the time (2 or 3 trials out of 4) (day effect on Latency: $F_{11,66} = 1.25, p = 1.25$; day effect on Distance: $F_{11,66} = 0.96, p = .48$). Ninety minutes after the last trial, animals (and their age-matched control group) were sacrificed for

TABLE 1 Summary of the procedures

Batch	Experiment	XdU	Rats' age at XdU injections	Rats' age at time of sacrifice	Neurons' age at time of sacrifice	Group size
1	Recruitment of 4-month-old Adu-DGNs	BrdU	18 months	22 months	4 months	C = 5 AI = 7 AU = 7
2	Recruitment of 10-month-old Adu-DGNs	IdU	12 months	22 months	10 months	C = 10 AI = 11 AU = 11
	Recruitment of 19-month-old Adu-DGNs	CldU	3 months	22 months	19 months	C = 10 AI = 11 AU = 11
3	Recruitment of DGNs born in adolescent rats	CldU	PN28	22 months	21 months	AI = 5 AU = 5
4	Recruitment of DGNs born in adolescent rats	CldU	PN28	14 months	15 months	AI = 5 AU = 5
5	Recruitment of DGNs born in embryos	CldU	Ed18.5	15 months	15 months	AI = 6 AU = 7

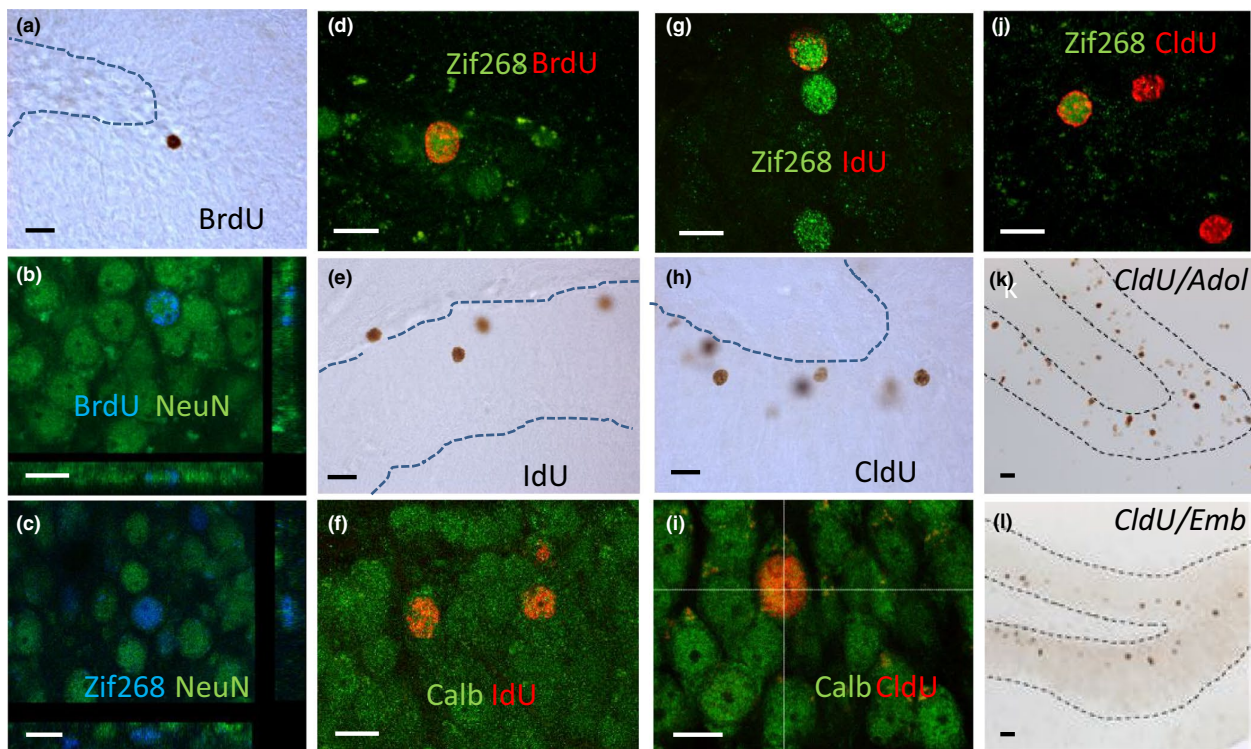


FIGURE 1 Granule neurons in the DG of aged rats. (a) Illustration of 4-month-old BrdU-IR neurons in an animal with preserved memory. (b) Confocal photomicrographs of 4-month-old BrdU-IR cells (blue) expressing NeuN (green). Confocal photomicrographs of (c) neurons (NeuN, green) expressing Zif268 (blue) and of (d) 4-month-old BrdU-IR cells (red) expressing Zif268 (green). (e) Illustration of 10-month-old IdU-IR neurons. Confocal photomicrographs of IdU-IR cells (red) expressing (f) calbindin (green) or (g) Zif268 (green). Illustration of 19-month-old CldU-IR neurons. (h) Confocal photomicrographs of CldU-IR cells (red) expressing (i) calbindin (green) or (j) Zif268 (green). (k) Illustration of CldU-IR neurons born in adolescent rats (PN28). (l) Illustration of CldU-IR neurons born in embryos (ED18.5). Bar scale for DAB = 20 μm . Bar scale for confocal illustration = 10 μm

immunohistochemistry. At the time of sacrifice, BrdU-IR cells were 4 months old and their majority was located within the granule cell layer (GCL) (Figure 1a).

These cells were more numerous in the GCL of animals with good learning abilities (AU) compared to animals with memory deficits (AI) (Figure 2a, $F_{2,16} = 7.64$, $p = .05$ with $C = \text{AI} < \text{AU}$ at $p < .01$). This finding is consistent with our previous study showing that the number of neurons generated 1 month *after* learning is higher in AU compared to AI (Drapeau et al., 2003) senescent rats. More than fifty percent of BrdU-IR cells in the GCL expressed NeuN (Figure 1b), and neuronal differentiation was not different among groups (Figure 2b, $F_{2,16} = 2.07$, $p = .15$).

To determine whether newborn neurons are recruited by learning, we used Zif268 since this IEG is still expressed in the old DG (Gheidi, Azzopardi, Adams, & Marrone, 2013). Given that a substantial fraction of cells generated during senescence did not express NeuN, we verified in trained animals that Zif268-expressing cells were expressing NeuN (Figure 1c). We found that the vast majority of activated cells (Zif268) were neurons (NeuN) and that this ratio was similar in good and bad learners (AI: 96.4 ± 0.5 ; AU: 96 ± 1.3 , $p > .05$). Then, we examined the activation of adult-born cells, meant to be neurons, in response to learning (Figure 1d). We found that

the percentage of BrdU-IR cells expressing Zif268-IR in aged animals with good learning abilities was greater than that of aged animals with memory deficits and that of the untrained control group (Figure 2c, $F_{2,16} = 3.70$, $p = .05$ with $C = \text{AI} < \text{AU}$ at $p < .05$). In contrast, the total number of Zif268-IR nuclei did not differ between groups (Figure 2d, $F_{2,16} = 0.25$, $p = .78$). These results show that neuronal cells in the senescent DG are recruited by spatial learning and not by nonspecific effects of training (swimming, stress) as revealed by the lowest level of recruitment of 4-month-old cells in aged-impaired and control animals.

2.2 | Fate of dentate granule neurons born in middle-aged and young rats

Then, we asked whether neurons born earlier, that is, in middle age or young adulthood, are also recruited by learning during aging. For this purpose, animals were injected with CldU when 3 months old and with IdU when middle aged (12 months old; Table 1). Animals were trained 10 months later for 11 days until the AU learned the task (day effect on Latency: $F_{10,100} = 22.08$, $p < .001$; day effect on Distance: $F_{10,100} = 18.77$, $p < .001$) and reached 3 days of stable

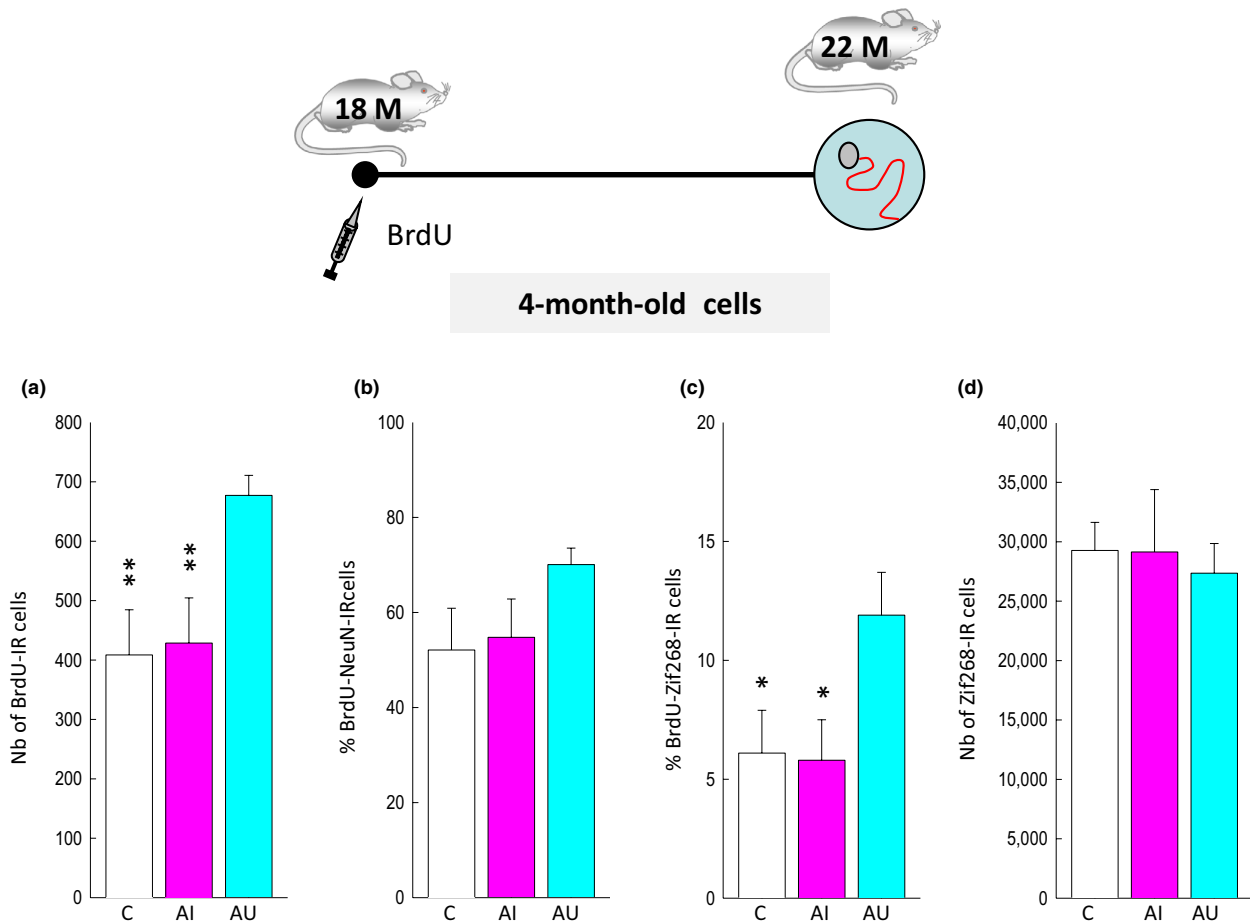


FIGURE 2 Neurons produced during old age are activated by spatial learning. Top: Experimental design. (a) The number of BrdU-IR cells is higher in the aged rats that learned the task (AU) compared to those with spatial memory deficits (AI) or to control animals (C). (b) The percentage of cells differentiating into neurons (BrdU-IR cells expressing NeuN) is similar in the three groups. (c) The expression of Zif268 in BrdU-IR cells generated in senescent DG is increased in AU compared to AI rats and C rats. (d) The number of neurons expressing Zif268 is similar in the three groups. * $p < .05$, ** $p < .01$ compared to AU

performances (Figure S1c,d). In this batch, the AI showed a dramatic improvement of their performances on the last training day (day effect on Latency: $F_{10,100} = 6.67$, $p < .001$; day effect on Distance: $F_{10,100} = 22.08$, $p < .001$). Trained animals (and their age-matched control group) were sacrificed 90 min after the last trial. At the time of sacrifice, IdU cells were 10 months old (Figure 1d). Their number was not influenced by training or by the cognitive status of the animals (Figure 3a, $F_{2,29} = 0.87$, $p = .43$). More than eighty percent of IdU cells expressed the neuronal marker calbindin (Figures 1f and 3b, $F_{2,28} = 4.21$, $p = .02$ with $C = AI < AU$ at $p = .02$). The percentage of neurons born during middle age and expressing Zif268 was greater in the AU group than that measured in AI and C groups (Figures 1g and 3c, $F_{2,29} = 4.87$, $p = .02$ with $C = AI < AU$ at $p < .01$ and $p < .05$, respectively).

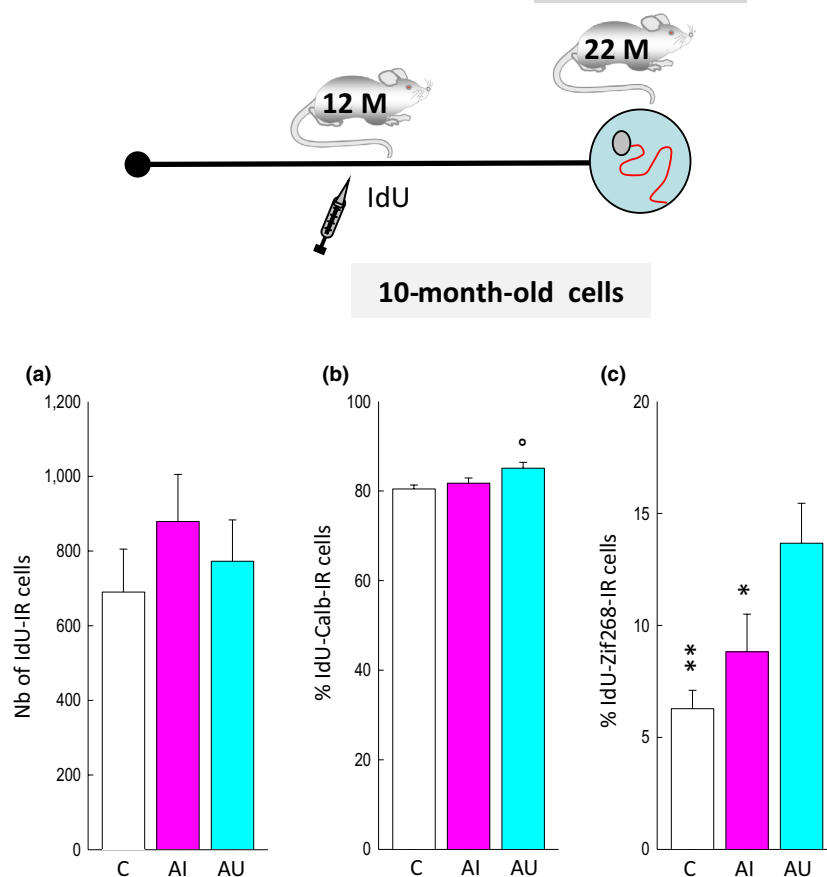
Nineteen-month-old CldU-IR cells were examined in the same animals (Figure 1h). Their number was not influenced by training or the cognitive status of the animal (Figure 4a, $F_{2,29} = 0.52$, $p = .6$). Their phenotypic analysis revealed that exposure to the water maze

slightly increased neuronal differentiation (CldU-calbindin co-expressing cells; Figures 1j and 4b,c: $82.8 \pm 0.1\%$ AI: $86.9 \pm 0.8\%$; AU: $86.2 \pm 0.7\%$; $F_{2,29} = 6.54$, $p < .01$ with $C < AI = AU$ at $p = .01$). Again, we found that the percentage of CldU-IR cells expressing Zif268 was greater in the AU group than that measured in AI and C groups (Figures 1j and 4c, $F_{2,29} = 6.96$, $p = .004$ with $C = AI < AU$ at $p < .01$ and $p < .05$, respectively). The total number of cells expressing Zif268-IR (C: $29,270.02 \pm 2,360.54$; AI: $26,068.94 \pm 2,366.78$; AU: $28,739.22 \pm 3,095.74$, $F_{2,29} = 0.42$, $p = .65$) did not differ between groups.

2.3 | Fate of dentate granule neurons born during development

Finally, we explored the role of dentate granule born during development of the DG by tagging neurons born in adolescent rats (PN28, Figure 1k) and neurons born in embryos (E18.5, Figure 1l) with CldU.

FIGURE 3 Neurons produced during middle age are activated by spatial learning in aged good learners. Top: Experimental design. (a) The number of IdU-IR cells generated at mid-age is independent of the memory abilities measured when rats reached senescence. (b) The percentage of cells differentiating into neurons (IdU-IR cells expressing calbindin) is slightly increased in AI (compared to C and AU). (c) The expression of Zif268 in IdU-IR cells is increased in AU rats compared to AI rats and C rats. * $p < .05$, ** $< .01$ compared to AU. ^o $p < .05$ compared to c



Animals were sacrificed when 22 or 15 months old, and for both groups, the number of CldU-IR cells and the percentage of CldU-IR cells expressing Zif268 were similar in AU and AI rats (Table S1).

3 | DISCUSSION

To determine whether neurons generated during adult life participate in learning abilities in old age, the expression of the IEG Zif268 was assessed in new neurons. We found that cells generated during young adulthood, middle age, and senescence survive for a long period of time and are functionally integrated into the dentate network. When taking into account individual differences in memory abilities, we highlight that although the number of new cells generated in 12-month-old animals (IdU-IR cells) is decreased 10-fold compared to 3-month-old rats (CldU-IR cells), the total number of CldU-IR or IdU-IR cells measured when animals reached senescence is similar in AU and AI and not different from untrained control animals.

These conclusions have been obtained using the hippocampal-dependent version of the Morris water maze (measuring reference memory with variable starting points), one of the most widely used behavioral tests to study normal aging in rats; this task does not involve food restriction or the administration of shock to encourage participation of old animals and is very sensitive to the aging

process (Kennard & Woodruff-Pak, 2011; Lubec et al., 2019; Mota et al., 2019). Although inter-individual differences in memory in aged rats have been demonstrated in other type of mazes (Barrett, Bennie, Trieu, Ping, & Tsafoulis, 2009; Temido-Ferreira et al., 2018), in object location memory tasks (Lux, Masseck, Herlitz, & Sauvage, 2017), and in the hole-board task (Lubec et al., 2019), we chose to use the water maze because it is the most widely used task to study the implication of adult-born neurons in aged rats (our own work, (Bizon & Gallagher, 2003; Marrone, Ramirez-Amaya, & Barnes, 2012)) whereas their role remains largely unexplored using the other tasks.

To map neuronal activity, Zif268 was preferred over many other markers such as c-Fos or Arc that have been frequently used in the memory field (Gallo, Katche, Morici, Medina, & Weisstaub, 2018). Zif 268 presents the advantage to have a high “basal” expression which increases the probability to map new neurons, the number of which decreases with aging. In addition, we have previously shown using Zif 268 and c-Fos that new neurons are recruited during both spatial memory acquisition and retrieval (Tronel, Charrier, et al., 2015; Tronel, Charrier, et al., 2015), indicating that either one or the other IEG can be used to analyze adult-born neurons’ activation in response to learning.

To study adult-born neuronal activity, we used immunohistochemistry, the only method available to study their role without impairing their activity. This technical approach is dependent on

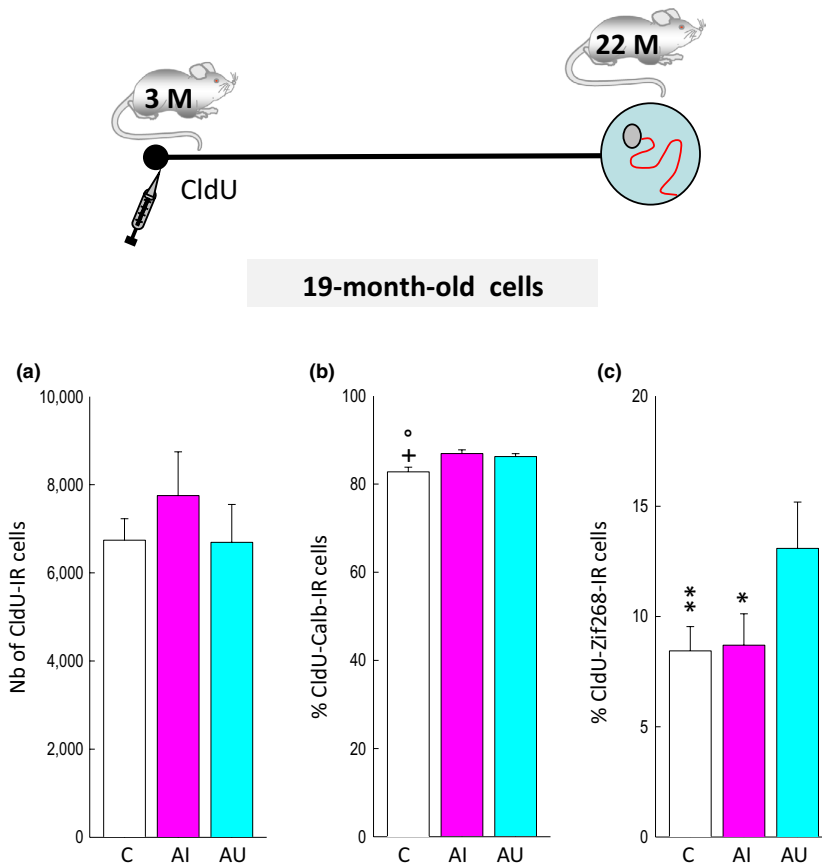


FIGURE 4 Neurons produced during young adulthood are activated by spatial learning in good learners. Top: Experimental design. (a) The numbers of CldU-IR cells generated when animals are young adult are independent of the memory abilities measured when rats reached senescence. (b) The percentage of CldU-IR cells expressing calbindin is increased by training. (c) The expression of Zif268 in CldU-IR cells generated in young adult DG is increased in AU compared to AI rats and C rats. $^{\circ}p < .05$ compared to AU, $+p < .05$ compared to AI. $*p < .05$, $**p < .01$ compared to AU

multiple factors that can bias sensitivity/detection of the antigens, and “old age” is a critical factor as it is accompanied with many changes—among which lipofuscin accumulation—that can interfere with antigen visualization (Moreno-Jiménez et al., 2019) for discussion (Lucassen, Fitzsimons, Salta, & Maletic-Savatic, 2020). Although it cannot be excluded that signal detection could be improved (Moreno-Jiménez et al., 2019), given that all old brains within a batch were processed together from perfusion to labeling, we tend to exclude a technical bias. However, visualizing the activity of new neurons during learning using calcium imaging is now the best available technic to confirm our conclusions in behaving rats in the course of aging.

Our conclusion that the responsiveness of dentate neurons generated throughout adult life is associated with resilience to cognitive aging has been obtained using two different cohorts of rats. The first one was utilized to study Adu-DGNs generated in senescent DG and the second one to study Adu-DGNs generated in young adult and middle-aged DG. When comparing the behavior of the two batches of rats, it appears that deficits in the aged-impaired rats were much more pronounced in the first batch of animals. This cohort effect, a well-known phenomenon in aging research (Schaie & Willis, 2015), could be related to housing conditions. Indeed, the first batch was raised in the vendor facilities until 16 months of age whereas the second one was raised in-house. Supporting this, in our previous

experiments performed in rats not aged in-house, the difference between AU and AI was more pronounced than that observed in the second experiment (Drapeau, Montaron, Aguerre, & Abrous, 2007). However, independently of the cohort of rats the same profile of activation was observed (C = AI < AU).

While the process of neurogenesis has been well characterized in young adult rodents (Aimone et al., 2014), information about their aging and their function is less abundant (Drapeau & Abrous, 2008; Encinas & Fitzsimons, 2017; McAvoy & Sahay, 2017). The number of stem cells, their rate of proliferation, and neuronal differentiation dramatically decrease with age, and the number of immature neurons (<4 weeks old) is thus significantly decreased. Recently, the development and functional integration of these cells have been described to be delayed by age. Indeed, 3-week-old neurons generated in middle-aged mice (10–14 months) displayed shorter and simpler dendrites and a dramatic reduction in spine number compared to those generated in 2-month-old mice and exhibited immature neuronal electrophysiological properties as revealed by the lack of functional glutamatergic synaptic inputs (Trincherio et al., 2017). In fact, their overall mature excitability and maximal glutamatergic connectivity are delayed compared to neurons born in younger animals (Trincherio, Herrero, Monzon-Salinas, & Schinder, 2019). The long-term destiny of adult-born neurons generated in young adult animals has not been explored in depth. We and others have shown that

contrary to what was initially hypothesized, new neurons survive for several months (Tronel, Charrier, et al., 2015) and even years in the DG (present results) and do not show signs of decline in excitability when they age: 5-month-old neurons are as excitable as 1-month-old-cells; they can even exhibit high levels of excitability following either enriched environment exposure or induction of LTP (Ohline et al., 2018). This latest very exciting result supports our hypothesis that even when several months old, Adu-DGNs are still plastic, they do not retire and participate in memory functions (Abrous & Wojtowicz, 2015), and even more so their persistence is not passive, but a result of their activity.

Here, we found that between middle age and senescence, the number of cells is further decreased, but then a difference between the AU and AI groups appears. Based on previous data, it is likely that the emergence of such a difference results from a difference in cell proliferation (Drapeau et al., 2003), neuronal differentiation (Drapeau et al., 2003; Qiao et al., 2019), cellular senescence (Hernandez-Segura, Nehme, & Demaria, 2018; Micheli et al., 2019), or changes in the neurogenic niche and/or to the systemic milieu (Mahmoudi, Xu, & Brunet, 2019; Villeda et al., 2011). Interestingly, we have shown that the senescent neurogenic niche is capable to rejuvenate upon removal of corticosterone (Montaron et al., 1999) or addition of pregnenolone sulfate (Mayo et al., 2005), indicating that neural stem cells are not depleted and keep their abilities to divide.

The main finding of our study is that there is a strong link between the ability for newborn cells to be recruited by learning and memory abilities in aged rats. Indeed, the percentage of adult-born cells expressing Zif268 was higher in animals that learned the task compared to animals that did it to a lesser extent. This finding is in accordance with our previous data showing that (a) when compared to control rats (naïve rats or rats trained to find a visible platform), adults required to use an hippocampal-dependent strategy in the water maze (or the dry maze) exhibit an increased percentage of mature adult-born neurons expressing Zif268 (Tronel, Charrier, et al., 2015), and (b) ablating mature adult-born neurons generated 4 months before training (when animals were 3 months old) delays the ability of rats to learn such a task (Lemaire et al., 2012). In the present experiment, the percentage of adult-born cells expressing Zif268 in each experimental group was similar in the three neuronal populations studied. It was thus independent of the age of the animals at the time of labeling (3, 12, and 18 months) and of the age of the cells at the time of training (4, 10, and 19 months). It was also independent of the total number of XdU cells. Note that even if in the first batch of animals, fifty percent of BrdU-IR cells differentiated into neurons, 96% of Zif268 cells were neurons suggesting that all BrdU-Zif268 cells were meant to be activated new neurons.

It could be argued that neurons born during development, which represent a major part of the DG, are also involved in differences in spatial memory abilities in old age. However, three arguments seem to rule out this hypothesis. First, the total number of granule cells is similar in AU and AI groups (Drapeau et al., 2003, 2007; Rapp & Gallagher, 1996). Second, if neurons generated during development (prenatal and postnatal periods) were activated by spatial learning,

given their high numbers, differences in the total number of Zif268 cells should have emerged as a function of the cognitive status. Third, we have shown that neurons born in neonates are not activated by spatial learning when they are mature compared to neurons of the same age born in adults. Indeed, the former are not recruited by spatial learning in the water maze when animals are tested at 7 months (Tronel, Charrier, et al., 2015). Here, we extended this observation showing that neurons born during the juvenile (PN28) or the embryonic (E18.5/19.5) period are not differentially recruited in good and bad learners. Recently, we began to explore the reasons for which developmentally and adult-generated neurons do not respond in the same way. By comparing the dendritic arbor of neurons born at different ontogenetic stages (embryonic, neonatal, adolescence, adulthood), we found that they display distinct morphological features and also different location (Kerloch, Clavreul, Goron, Abrous, & Pacary, 2019; Mathews et al., 2010) that may underlie different inputs and functions.

One question that we did not address is whether the three neuronal populations studied participate to the same extent to learning. To address this point, sophisticated models that allow to selectively tag new neurons generated within a defined period of time (adulthood, middle age, or senescence) and to ablate them during training performed at senescence are required. One possibility would be to take advantage of the recently developed pharmacogenetic or optogenetic approaches or in order to tag specifically neurons born in young adult rats and manipulate them when animals have reached senescence.

A previous study has shown that 4-month-old neurons generated in old rats exhibiting spatial memory deficits are recruited in response to spatial exploration behavior with the same probability than 4-month-old neurons generated in aged good learners or in young adult rats (Marrone et al., 2012). From this dataset, it was concluded that disrupted information processing at old age may be linked to a reduced number of adult-generated granule neurons and not to a deficit in their functionality. However, in this study the activation of adult-generated neurons was evaluated in response to a simple form a learning (spatial exploration). Taking the present data into consideration, we rather suggest that adult-born neurons in AU are sufficiently connected to integrate simple stimulations generated during simple forms of learning but insufficiently integrated to process the complex stimulations generated during spatial navigation.

Zif268 is known to be regulated in an activity-dependent manner by learning (for review, see Gallo et al., 2018; Veyrac, Besnard, Caboche, Davis, & Laroche, 2014). It is overexpressed in response to different types of learning in distinct structures and circuits that are processing the ongoing information, and several arguments indicate that it is required for the stabilization (and not acquisition) of long-lasting memories. Although the mechanisms are not fully understood, the activation of Zif268 may strengthen/stabilize the memory trace. It can be hypothesized that during learning the activation of Zif268 in adult-born neurons may be involved in the formation, stabilization, and reactivation of place cells in the hippocampal network, events known to support spatial learning.

Here, we hypothesize that adult-born neurons that do not exhibit activity-dependent regulation of Zif268 become functionally silent in the course of aging, leading to memory deficits. Although the firing patterns that are sufficient to induce Zif268 in adult-born neurons in "behaving" animals are so far unknown, adult-born neurons silencing may have several origins. It may result from a loss of synaptic inputs (Geinisman, Toledo-Morrell, & Morrell, 1986) altering the ability to fire properly (Ahlenius, Visan, Kokaia, Lindvall, & Kokaia, 2009); these synaptic alterations of Adu-DGNs could be linked to the acceleration of senescence through epigenetic changes (Penner, Roth, Barnes, & Sweatt, 2010), decreased autophagy activity (Glatigny et al., 2019), or changes of the local and systemic milieu (Villeda et al., 2011).

The HPA axis (and corticosterone) deserves a special attention in relation to its role in age-related memory disorders (Sapolsky, 1992). In his pioneer work, Issa and colleagues showed that AU rats were characterized by high level of corticosterone in basal condition and in response to a restraint stress (Issa, Rowe, Gauthier, & Meaney, 1990). We replicated and extended this finding by showing that animals with the heaviest adrenal glands, indicative of chronic HPA axis hyperactivity, exhibited the worst memory performance and the lowest number of proliferating cells or 3-week-old surviving cells in comparison with AU rats (Drapeau et al., 2003). Although some reports failed to support a relationship between cell birth and corticosterone levels during aging (using different rat strain (Heine, Maslam, Joels, & Lucassen, 2004), we can hypothesize that in the present experiment AU and AI rats exhibit different HPA axis activity both in basal condition and in response to training. This raises the possibility that differences in the reactivity of adult-born neurons in these animals are related to differences in the activity of the HPA axis. The influence of learning in the water maze on HPA axis activity is largely unknown at the exception of one study showing that corticosterone secretion is increased (in adult rats) immediately after the first training session and that corticosterone has pro-mnesic effect on learning (Sandi, Loscertales, & Guaza, 1997). Whether or not learning still increases corticosterone at the end of training when animals get habituated to the task is not known. But independently of this, AI exhibit a hyperactive HPA axis and it cannot be excluded that excessive levels of corticosterone may impair adult-born neurons' functioning and memory processing. As a consequence, blocking/removing glucocorticoid receptors expressed by adult-born neurons generated early in life at the time of training may increase their responsiveness to learning and promote memory. In this context, we have previously shown that blocking age-related increased in HPA axis activity from middle-age onward rejuvenates memory and the neurogenic niche in senescent subjects (Montaron et al., 2006).

Other strategies are also promising to prevent the development of memory disorders during the course of aging. To name a few, transfusion of aging individuals with young plasma, using metabolic drugs (rapamycin/metformin) or intracellular metabolic cascade (mTor), increasing mitochondrial fitness, ablating senescent cells (with senolytics), cellular reprogramming through epigenomic remodeling, transplanting cells, or other noninvasive environmental

approaches may promote successful brain aging (Mahmoudi et al., 2019; Villeda et al., 2011) (Fakouri, Hansen, Desler, Anugula, & Rasmussen, 2019; Munoz-Espin & Serrano, 2014; Shetty, Kodali, Upadhyaya, & Madhu, 2018).

In conclusion, our results highlight the importance of neurons born throughout adult life in providing resilience to age-related memory disorders. They reveal a novel perspective for developing therapies to promote resilience to age-related memory disorders or to rejuvenate the DG by acting throughout adult life on adult-born dentate neurons.

4 | EXPERIMENTAL PROCEDURES

4.1 | Animal

For these experiments, a total of 96 male Sprague Dawley rats (OFA, Janvier, France) were used. Animals were housed collectively until behavioral testing under a 12-hr:12-hr light/dark cycle with ad libitum access to food and water. Temperature (22°C) and humidity (60%) were kept constant.

In the first experiment, male rats ($n = 19$) were 16 months old on delivery. *In the second experiment*, rats ($n = 32$) were 2 months old on delivery. *In the third and fourth experiments*, rats ($n = 25$) were 21 days old on delivery. In the fifth experiment, pregnant Sprague Dawley female rats ($n = 4$) were individually housed in transparent cages. After delivery, litters were raised by their biological mothers until weaning (21 days after birth). After weaning, only the male progeny ($n = 20$) was kept. Rats were individually housed before the beginning of behavioral training. Animals with a bad general health status or tumors were excluded. Experimental procedures have been planned respecting the European directive of the parliament and the conceal of September 22, 2010 (2010/63/UE, 5012006A).

4.2 | Thymidine analog injections

Newly born cells were labeled by the incorporation of synthetic thymidine analogs (XdU, Sigma-Aldrich, Table 1). *In the first experiment*, rats were injected with 5-bromo-2'-deoxyuridine (BrdU) according to a previously described protocol (Drapeau et al., 2003, 2007). These animals received one daily BrdU injection (50 mg kg⁻¹ day⁻¹; ip) for 5 days when 18 months old, that is, 4 months before training. *In the second experiment*, rats received five injections of 5-chloro-2'-deoxyuridine (CldU) when 3 months old and five injections of 5-iodo-2'-deoxyuridine (IdU) when 12 months old (Dupret et al., 2007), both at equimolar doses of 50 mg BrdU/kg. *In the third and fourth experiments*, animals received one injection of CldU when 28 days old (equimolar dose of 50 mg BrdU/kg). *In the fifth experiment*, pregnant female rats received two injections of 5-chloro-2'-deoxyuridine (CldU, equimolar dose of 50 mg BrdU/kg 50 mg/kg) at E18.5 and E19.5.

4.3 | Water-maze training

Rats were tested in the water maze when 22 months old (experiments 1, 2, and 4) or 15 months old (experiments 3 and 5). The apparatus consisted of a circular plastic swimming pool (180 cm diameter, 60 cm height) that was filled with water ($20 \pm 1^\circ\text{C}$) rendered opaque by the addition of a white cosmetic adjuvant. Before the start of training, animals were habituated to the pool for 2 days for 1 min per day. During training, the *Learning* group (L) was composed of animals that were required to locate the submerged platform, which was hidden 1.5 cm under the surface of the water in a fixed location, using spatial cues available within the room. Rats were all trained for four trials per day (90 s with an inter-trial interval of 30 s and released from 3 different starting points that varied randomly each day). If an animal failed to locate the platform, it was placed on it at the end of the trial. The time necessary to reach the platform was recorded using a video camera that was secured to the ceiling of the room and connected to a computerized tracking system (Videotrack, Viewpoint). Daily results were analyzed in order to rank animals according to their behavioral score calculated over the last 3 days of training (when performances reached an asymptotic level). The behavioral scores calculated over the whole training duration of aged-unimpaired (AU) rats were below the median of the group, whereas those of aged-impaired (AI) animals were above the median of the group. Control groups consisted of animals that were transferred to the testing room at the same time and with the same procedures as trained animals but that were not exposed to the water maze.

4.4 | Immunohistochemistry

Animals were sacrificed 90 min after the last trial (Table 1). The different age-matched control groups were sacrificed within the same period. Free-floating sections (50 μm) were processed using a standard immunohistochemical procedure to visualize thymidine analogs (BrdU, CldU, IdU) on alternate one-in-ten sections using different anti-BrdU antibodies from different vendors (for BrdU: 1/200, Dako; CldU: 1/500, Accurate Chemical; IdU: 1/200, BD Biosciences) and Zif268 (1:500, Santa Cruz Biotechnology). The number of XdU-immunoreactive (IR) cells in the granule and subgranular layers (GCL) of the DG was estimated on a systematic random sampling of every tenth section along the septo-temporal axis of the hippocampal formation using a modified version of the optical fractionator method. Indeed, all XdU-IR cells were counted on each section and the resulting numbers were tallied and multiplied by the inverse of the section sampling fraction ($1/\text{ssf} = 10$ for BrdU and IdU cells that were counted in both sides of the DG and $1/\text{ssf} = 20$ for CldU-IR cells that were counted in the left side). The number of Zif268-IR cells (left side) was determined using a $100\times$ lens, and a $60 \times 60 \mu\text{m}$ frame at evenly spaced x-y intervals of $350 \mu\text{m}$ by $350 \mu\text{m}$ with a Stereo Investigator software (MicroBrightfield).

4.5 | Activation of new cells

The activation of adult-born cells was examined using immunohistochemistry. To visualize cells that incorporated thymidine analogs, one-in-ten sections were incubated with different anti-BrdU antibodies (BrdU and CldU, rat primary antibodies at 1/200 Accurate Chemical; IdU, mouse primary antibodies at 1/200, BD Biosciences). Sections were also incubated with Zif268 (rabbit, 1:500, Santa Cruz Biotechnology). Bound antibodies were visualized, respectively, with Cy3-goat anti-rat (1:1,000, Jackson) or Cy3-goat anti-mouse (1:1,000, Jackson) and Alexa 488 goat anti-rabbit antibodies (1:1,000, Jackson). CldU-Zif268 and IdU-Zif268 labeling were analyzed on different sections because of some cross-reactivity between secondary antibodies made in mice or rat (Figure 1 in Tronel, Charrier, et al., 2015). All BrdU⁻, CldU⁻, or IdU-labeled cells expressing Zif268 (one side) were analyzed using a confocal microscope with HeNe and Arg lasers (Leica, DMR TCSSP2AOBS), with a plane apochromatic 63X oil lens (numerical aperture 1.4; Leica). The percentage of BrdU⁻, CldU⁻, or IdU-labeled cells that expressed Zif268 was calculated as follows: $(\text{Nb of Xd}^+/\text{IEG}^+ \text{ cells}) / [(\text{Nb of XdU}^+/\text{IEG}^- \text{ cells}) + (\text{Nb of XdU}^+/\text{IEG}^+ \text{ cells})] \times 100$. All sections were optically sliced in the Z plane using 1- μm interval, and cells were rotated in orthogonal planes to verify double labeling.

4.6 | Analysis of phenotype

One-of-ten series was incubated with a rat monoclonal anti-BrdU antibody (1/200, Accurate Chemical) and with a mouse monoclonal anti-NeuN antibody (1:500, Millipore). Bound anti-BrdU and anti-NeuN antibodies were visualized with a Cy3-goat anti-rat (1:1,000, Jackson) and an Alexa 488 goat anti-mouse IgG antibody (1:1,000, Jackson). The phenotype of IdU-IR cells and CldU-IR cells was determined using rabbit anti-calbindin antibodies (1/200, Millipore) that were revealed with Alexa 488 goat anti-rabbit IgG antibodies (1/500, Jackson). We also analyzed the phenotype of Zif268 cells by incubating one-in-ten sections with a rabbit anti-Zif268 antibody (1:500, Santa Cruz Biotechnology) and a mouse monoclonal anti-NeuN antibody (1:500, Millipore). Bound anti-Zif268 and anti-NeuN antibodies were visualized with a Cy3-goat anti-rabbit (1:1,000, Jackson) and an Alexa 488 goat anti-mouse IgG antibody (1:1,000, Jackson).

4.7 | Statistical analysis

All data are expressed as mean \pm SEM. Data were analyzed using an ANOVA or Student's *t* test (2 tails) when necessary.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

MFM performed experiments. VC analyzed the recruitment of DGNs of rats from batches 4 and 5 and revised the paper. NB analyzed the recruitment of DGNs of animals from batch 3. PG performed experiment from batch 1. DNA conceived experiments, analyzed data and wrote the paper.

DATA AVAILABILITY STATEMENT

The authors elect to not share data.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

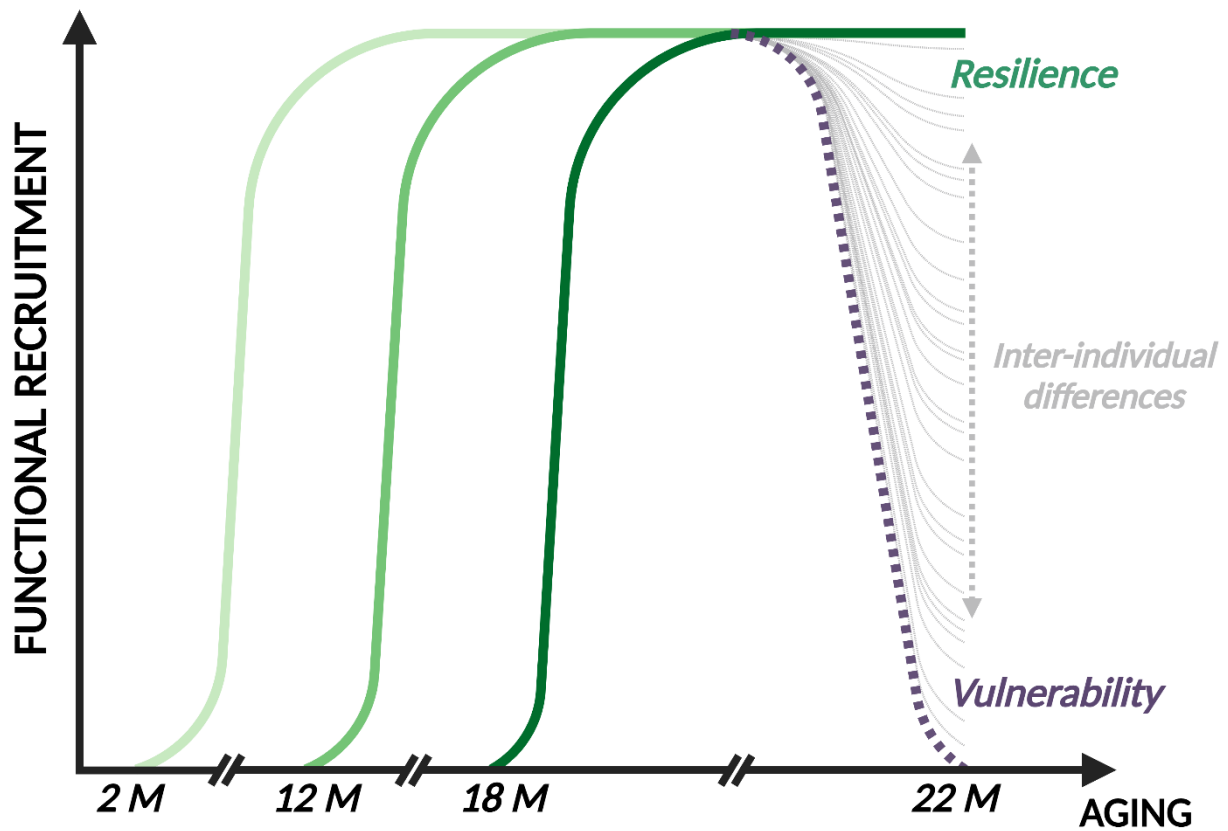
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SUPPLEMENTARY INFORMATION

Experiments	Batch	Nb of Cldu-IR cells	Statistical analysis	% of CldU-Zif-268-IR cells	Statistical analysis
Ado-DGNs	3	AU : 10496 ± 1219 AI : 8626 ± 1027	$t_8=1.17, p=0.27$	AU : 2.08 ± 0.63 AI : 3.21 ± 0.76	$t_8=-1.14, p=0.29$
Ado-DGNs	4	AU : 6928 ± 2131 AI : 7724 ± 1473	$t_8=-0.31, p=0.77$	AU : 3.52 ± 1.51 AI : 2.62 ± 0.13	$t_8=0.59, p=0.57$
Embryo-DGNs	5	AU : 160510 ± 10375 AI : 149371 ± 25974	$t_{11}=0.42, p=0.68$	AU : 7.84 ± 0.63 AI : 6.74 ± 0.35	$t_{11}=1.45, p=0.17$

Supplementary Table 1 : DGNs produced in adolescent rats or embryos are not activated by spatial learning in aged rats.

Graphical abstract



In summary, the survival and differentiation of adu-DGNs born in adult or middle-aged animals is independent of the cognitive status of the animals. In contrast, when adu-DGNs are generated in very old individuals, their survival and differentiation is lower in vulnerable individuals indicating a change in the neurogenic niche or the senescence of the stem cells. A similar result was obtained when adult neurogenesis was measured after learning (Drapeau et al., 2003). More importantly we have shown that adu-DGNs born 19, 10 and 4 months before learning are more functional and recruited by spatial learning in resilient animals and not in vulnerable ones. In contrast emb-DGNs (E18.5) and adol-DGNs (P28) are not recruited by spatial learning, a result consistent with what we observed in adult rats (article 1). For this reason, they will not be examined anymore in the following steps.

III. PRESERVATION OF ADU-DGNS HEALTH DURING AGING LEADS TO RESILIENCE AGAINST COGNITIVE DECLINE

A. General presentation

The two previous papers highlighted the essential role of mature adu-DGNs in spatial learning and their contribution to resilience against age-related decline in spatial abilities. Importantly, resilience was shown not to rely on the survival of mature adu-DGNs but rather on the maintenance of their ability to be recruited during learning at old age. This was specific of adu-DGNs and not emb-DGNs or adol-DGNs. This confirms that adu-DGNs indeed constitute a distinct population from dev-DGNs in the context of aging and spatial learning. Particularly, the specific higher recruitment of adu-DGNs in resilient animals has been observed for adu-DGNs born throughout the entire adulthood, and not only for the ones born at old age. This indicates that neurons born during young adulthood and middle-age also have to face and adapt to age-related challenges in order to support resilience to cognitive aging. Given that inter-individual differences can already be observed around middle-age, this suggests that mechanisms or resilience are already established by adu-DGNs during the first stages of aging.

Based on this observation, we adopted a pseudo-longitudinal strategy in order to examine the progressive evolution of adu-DGNs health in the course of aging. For technical purpose, and given that all adu-DGNs have similar preserved reactivity at old age, we focused on neurons born in young adults as they are easier to tag in higher quantities. Based on the observations made in the introduction, resilience to cognitive aging is associated with the preservation of the DG connectivity, and more specifically projections from the EC. These observations were made based on animals cognitively-assessed in the watermaze. Given the essential role of mature adu-DGNs in this type of learning, we propose that they undergo progressive disconnection from the network with age, altering their functionality. We addressed the evolution of their glutamatergic connectivity during aging and subsequently the evolution of their metabolism health, using their mitochondrial homeostasis as a proxy, for which impairments with age could underlie functional alterations. Finally, we adopted an optogenetic approach in which mature adu-DGNs were stimulated in order to see if we could restore memory functions in the vulnerable population.

Article 3:

**Old but strong: the key to successful
cognitive aging lies with adult-born
hippocampal neurons**

Old but strong: the key to successful cognitive aging lies with adult-born hippocampal neurons

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Abstract

Aging is associated with a decrease in memory abilities. Yet, some individuals remain resilient with preserved functions. Adult hippocampal neurogenesis is a unique form of plasticity in the hippocampus that plays a critical role in memory processes. However, the integration and role of adult-born dentate granule neurons (adu-DGNs) during aging and in relation to cognitive aging remain unclear. Here, we investigated the integration and role of very old adu-DGNs in animals that were cognitively characterized as resilient or vulnerable to cognitive aging. We report that 1-2-year-old adu-DGNs promote resilience to cognitive aging through the preservation of their synaptic inputs with mossy cells and neurons in the supra-mammillary area (SuM) of the hypothalamus. Specifically, we report a maintenance of the mitochondrial homeostasis at the location of mossy cells and SuM inputs on adu-DGNs. Furthermore, by-passing the reduced innervation of adu-DGNs in vulnerable animals by optogenetic stimulation leads to restored memory retrieval abilities. Altogether, our data point out that the maintenance of healthy adu-DGNs throughout life is essential for resilience against cognitive aging and that they represent a valuable tool for restoring cognitive functions at old age.

Introduction

Cognitive aging has become a burden worldwide with the expansion of elderly populations. This expansion is accompanied by a decline of memory abilities (Ando and Ohashi, 1991; Robitsek et al., 2008). Interestingly, this decline is unequal as some individuals remain resilient with preserved memory functions while others are vulnerable with decreased abilities (Rapp and Amaral, 1992; Gallagher et al., 1993; Gallagher and Rapp, 1997; Nyberg et al., 2012). Adult neurogenesis in the dentate gyrus of the hippocampus represents a unique form of plasticity supporting spatial memory (Anacker and Hen, 2017; Abrous et al., 2022). Aging is associated with an exhaustion of the pool of new neurons and their delayed maturation (Drapeau and Nora

Abrous, 2008; McAvoy and Sahay, 2017; Trinchero et al., 2017) and increasing this pool at old age was shown to improve memory abilities (Kempermann et al., 1998; Montaron et al., 2006; McAvoy et al., 2016; Berdugo-Vega et al., 2020). However, whether neurons born early in adult life are involved in the aging of cognitive functions remains largely unknown. The few data available indicate that these neurons survive for years and express markers of neuronal activity in response to learning (Kempermann et al., 2003; Montaron et al., 2020), suggesting that they may participate to the maintenance of spatial memory abilities during aging. Here, we hypothesized that successful aging depends upon the preservation of the health and connectivity of adu-DGNs, allowing the accurate reception of incoming information and their subsequent recruitment. In contrast, adu-DGNs in the vulnerable population may experience a progressive disconnection from the network, impeding their recruitment and learning abilities. Thereafter, we investigated whether their stimulation by optogenetics was sufficient to improve learning abilities in aged vulnerable animals.

A pseudo-longitudinal study of adu-DGNs

To analyze the lifelong survival of cells born in young adult rats (3 month-old), animals were injected with thymidine analog (Bromodeoxyuridine (BrdU), intraperitoneal injections) that incorporated into dividing cells. To specifically study the morphological features of adu-DGNs, Moloney leukemia virus-based retroviral vectors (M-rv) were injected in the dentate gyrus at the same period. Different M-rv were used to study the morphology (M-rv-GFP (Zhao et al., 2008)), glutamatergic post-synaptic density (M-rv-PSD95-GFP (Kelsch et al., 2008)) and mitochondrial network (M-rv-MitoDsRed (Steib et al., 2014)) of adu-DGNs. To examine the progressive mechanisms of resilience selected during aging, we adopted a pseudo-longitudinal strategy in which animals (n=35 per cohort) were submitted to a spatial navigation learning task in the Morris water maze either at the end of adulthood (8 month-old rats), middle-age (12 month-old rats) or old age (18 month-old rats). This task was chosen as a gold standard method to study cognitive aging as it depends upon adult neurogenesis (Dupret et al., 2008) and is highly sensitive to aging (Gallagher et al., 1993). Within each cohort of rats (adult, middle-aged, old), resilient and vulnerable animals were identified. Subsequently, the morphological analysis was performed in the most resilient (n=5) with the best memory abilities, and the most vulnerable (n=5) with the worst memory abilities (Fig S1). Using this strategy adu-DGNs aged either 5, 9 or 15 month-old in rats aged respectively 8, 12 and 18 month-old at the time of training and sacrifice were analyzed.

Cell death or cellular senescence does not account for inter-individual differences in cognitive status

We first examined whether the survival of adu-DGN born in young rats was similar or different between the three cohorts of rats cognitively characterized in the watermaze (Fig1A-B and Fig S1). At all time-points examined, the total number of adu-DGNs remained similar between resilient and vulnerable animals across all ages (Fig 1C). Because cellular senescence has been described in aged stem cells (Fatt et al., 2022) and in post-mitotic cells (Von Zglinicki et al., 2021), we used as a marker Senescence-Associated- β -Galactosidase (SA- β -Gal) (Dimri et al., 1995). Senescent adu-DGNs were detected and quantified with visualization of cells co-expressing BrdU and SA- β -Gal (Fig 1D). Yet, resilient and vulnerable animals showed similar levels of senescent adu-DGNs for all ages (Fig 1E). The same profile was observed for the total number of senescent cells (SA- β -Gal positive) in the granule cell layer (Fig S2). Therefore, while adu-DGNs do not die over the period of 1-2 years, they do not enter into cellular senescence arrest. The prevention and/or facilitation of cellular senescence does not appear to be a resilience mechanism employed by adu-DGNs.

The gross morphological architecture of adu-DGNs does not depend upon the cognitive status

One of the hallmarks of neuronal aging is an atrophy of the dendritic network associated with a loss of inputs in the cortex (Allard et al., 2012). We first studied the dendritic morphology of adu-DGNs, using a M-rv-GFP (Zhao et al., 2008)), allowing to label and analyze their somato-dendritic compartments (Fig 2A-2B) from three cohorts of rats cognitively characterized (Fig S1). The total dendritic length of adu-DGNs (Fig 2C), their primary dendrite length (Fig 2D), number of nodes (Fig S3A) and numbers of ends (Fig S3B) were similar and independent of the cognitive status of the animals. To better investigate the dendritic complexity, we analyzed the dendrites of adu-DGNs according to their branching order, shown as a good example for sub-dendrite complexity in DGNs (Kerloch et al., 2019). However, the adu-DGNs of resilient and vulnerable animals still displayed similar length and number of low and high-order dendrites (Fig 2E-2F and Fig S3C-S3D). Moreover, Sholl analysis revealed that their dendritic arbor had similar complexity (Fig 2G and Fig S3E). In order to further investigate the adu-DGNs integration within the dentate gyrus, the branching angle and path distance of the dendrites were calculated. Adu-DGNs in resilient and vulnerable animals displayed similar integration for these parameters as well (Fig 2H-2I). Given this apparent unchanged dendritic morphology, we also investigated the output

morphology of adu-DGNs at the level of their mossy fiber boutons (MFBs) in the CA3 at the oldest time point (18 month-old). No differences in the MFBs area nor in the number and lengths of filipodia could be observed, suggesting preserved synaptic outputs in both resilient and vulnerable rats (Fig 2J). Of note, the adu-DGNs in resilient and vulnerable animals showed similar cell body morphology (Fig S3F). Altogether, no major change in the morphology of adu-DGNs could be evidenced in the course of aging. However, it does not rule out more subtle changes in terms of connectivity.

Mossy cells and SuM glutamatergic inputs to adu-DGNs are maintained in resilient individuals

We refined the connectivity study to the glutamatergic inputs of adu-DGNs, as they represent the main excitatory source essential for neuronal activity and play a key role in spatial learning. During aging, glutamatergic inputs in the molecular layer (ML) of the dentate gyrus were shown to be decreased in vulnerable animals (Geinisman et al., 1986), suggesting a disconnection from the excitatory network with age. Glutamatergic inputs project to dendritic spines, which are characterized by the presence of the post-synaptic density (PSD), where the PSD95 scaffolding protein is present in high quantity (Chen et al., 2005). We used PSD95 as a proxy for glutamatergic inputs onto the spines of adu-DGNs by injecting a M-rv-PSD95-GFP expressing PSD95 coupled with eGFP (Kelsch et al., 2008) (Fig 3A). Since the dentate gyrus receives specific and identified inputs from different glutamatergic regions in each of the sub-layers of the ML [outer molecular layer (OML), middle molecular layer (MML) and inner molecular layer (IML)] (Figure 3B), the quantification of clusters of PSD95 was made for each sub-layer. For all cognitively assessed aged-population (Fig S1), the adu-DGNs of resilient and vulnerable animals displayed similar post-synaptic densities in the OML (Fig 3C) and MML (Fig 3D). However, adu-DGNs in resilient animals showed a specific preservation of their PSD95 density in the IML compared to adu-DGNs in vulnerable animals for all ages (Fig 3E). The glutamatergic inputs to the IML come from mossy cells and neurons in the supra-mammillary area (SuM) of the hypothalamus. Thus, our results suggest that the maintenance of mossy cells and SuM projections to adu-DGNs is essential for successful cognitive aging.

Mitochondrial homeostasis at the location of mossy cell and SuM projections is maintained in resilient adu-DGNs

Given that PSD95 is expressed onto spines, the previous results suggest that adu-DGNs in the vulnerable population have a reduced number of spines in the IML. Such spine disruption could be linked to metabolic alterations. The mitochondrial network contributes to the formation, maintenance, elimination and functionality of spines (Cameron et al., 1991; Datta and Jaiswal, 2021) and mitochondria depletion was shown to reduce spine density (Li et al., 2004). According to these observations, we followed the evolution of the mitochondrial network in adu-DGNs with age with the use of a M-rv-MitoDsRed (Steib et al., 2014) (Fig 4A), labelling their mitochondrial clusters. The quantification of the density of mitochondrial clusters along the dendrites was then performed in the IML, MML and OML as described previously (Fig 4B). For all cognitively characterized aged-populations (Fig S1), the adu-DGNs of resilient animals exhibited a preserved mitochondrial density in the IML compared to adu-DGNs of vulnerable animals (Fig 4E), an observation similar to the preserved post-synaptic density in the same sub-layer. On the contrary, the adu-DGNs in resilient and vulnerable animals showed similar mitochondrial densities in the OML and MML at the end of adulthood and middle-age (Fig 4C-4D). However, the decreased mitochondrial density started to spread to these layers at old age (Fig 4C-4D). These results indicate that a mitochondrial dysfunction may render vulnerable potentially via the disruption of spines and the subsequent degeneration of inputs projecting to the spines. Therefore, the preservation of mitochondrial homeostasis and health in adu-DGNs is essential to maintain good learning abilities with age.

Stimulation of adu-DGNs restores memory retrieval in vulnerable animals

Taken together, the previous data indicate that the observed rarefaction of inputs and disruption of mitochondrial homeostasis could be attributed, at least in part, to a reduction in the functionality of adu-DGNs. To by-pass these alterations, and given that adu-DGNs outputs are preserved, we artificially stimulated adu-DGNs with optogenetics according to a previously used protocol (Masachs et al., 2021). M-rv-ChannelRhodopsin-GFP was bilaterally injected in the dentate gyrus of 3 month-old rats. At old age (20 month-old), animals were submitted to learning in the watermaze during which adu-DGNs were stimulated. We observed similar levels of activation of adu-DGNs (aged 17 month-old) between the two cognitive populations (Fig S4). Control animals followed the same procedure but without light stimulation. Two days after the end of learning, a probe test (during which the platform is removed) was conducted without

stimulation in order to test the strength of memory (Fig 5A). Animals were not over-trained in order to increase the likelihood to enhance the memory trace. Optogenetic manipulation of adu-DGNs did not improve the learning performances of old animals (Fig S5), as previously shown in younger animals (Masachs et al., 2021), but improved the memory of the platform location. Indeed, we found that the stimulation of adu-DGNs during learning promoted the formation of a precise memory in vulnerable animals, as indicated by the decreased first latency to reach the platform position once the memory test started compared to the non-illuminated vulnerable group (Fig 5C). When the percentage of time spent in the target quadrant was analyzed, the stimulation of adu-DGNs was efficient only in resilient rats (Fig 5D-5G), as confirmed by the heatmaps analysis of the probe test (Fig 5H). This suggest that artificial stimulation of adu-DGNs in vulnerable rats is sufficient to restore memory retrieval but yet not enough to promote the formation of a strong and stable memory trace. We obtained similar results when the learning and memory test were performed at middle-age (12 month-old) (Fig S6). Altogether, these results indicate that the stimulation of adu-DGNs restored memory retrieval in vulnerable animals and increased the stability of memory in resilient animals.

Discussion

Our results highlight the essential role played by adu-DGNs in the resilience to cognitive aging. By using a novel strategy aiming at tagging adu-DGNs generated during early adulthood, we followed their “health” in the course of aging. We found that adu-DGNs progressively lose their glutamatergic inputs in individuals with reduced memory abilities, an effect starting around middle-age and maintained lifelong. The loss of inputs was not due to the death or gross morphological deterioration of adu-DGNs. This loss of excitatory innervation could represent the source of the loss of responsiveness of adu-DGNs during learning at old age in vulnerable animals (Montaron et al., 2020). This loss of responsiveness is specific to adu-DGNs as the other populations of granule neurons generated during development are not recruited by learning, a finding consistent with the fact that the population of adu-DGNs is the only one necessary for spatial learning in adult rats (Masachs et al., 2021). Therefore, given than adu-DGNs alone are needed for resilience in spatial learning abilities, we propose that this loss of inputs is relevant and specific to them only.

We reported for the first time a highly specific loss of inputs in the IML, suggesting the rarefaction of inputs from mossy cells and neurons in the SuM. We studied the mitochondrial network of adu-DGNs, given that they represent essential hubs for synaptic functioning and spine

maintenance (Li et al., 2004). Interestingly, we observed a specific reduction of the mitochondrial density in the IML as well. The reduced mitochondrial density could originate from mitochondrial dysfunctions or deposition, which is activity calcium-dependent (Wang and Schwarz, 2009). Local reduction in the activity could generate local deposition of mitochondria and prevent their trafficking to the IML.

These data suggest a link between the loss of glutamatergic inputs and spines (using PSD95 as a proxy) and mitochondria alterations. To date, only the MML and OML connectivity of the dentate gyrus had been addressed in the context of inter-individual variabilities. It has been shown using electronic microscopy that the number of axo-spinous synapses was reduced in the MML of vulnerable rats (Geinisman et al., 1986). In the same line of idea, the synaptophysin levels, a marker for pre-synaptic vesicles, indicated impaired connectivity in the MML and OML of vulnerable rats, corroborating the previous results even though synaptophysin is not specific of glutamatergic inputs (Smith et al., 2000). These results suggest that higher memory functions are linked to the preservation of inputs from the entorhinal cortex. We observed the emergence of alterations in the mitochondrial network in the OML and MML in old vulnerable animals (18 month-old). We propose that mitochondrial alterations act as precursors to spine disruption and the subsequent loss of inputs. Expanding upon this hypothesis, it appears justifiable to speculate that reduced PSD95 density would likely have been observed at timepoints beyond 18 month-old in the MML and OML. Indeed, the previous studies addressing the DG connectivity according to the cognitive status (Geinisman et al., 1986; Smith et al., 2000) were conducted on older rats aged 24-28 month-old, which likely allowed them to identify the eventual loss of inputs from the EC that we might have detected at a later time. Nevertheless, we cannot exclude that the tool used in our present study is more coarse-grained than electronic microscopy, suggesting that we may have missed some changes at the levels of inputs from the entorhinal cortex. In addition, we cannot exclude the involvement of other inputs. For example, cholinergic inputs from the medial septum terminates on granule cells and make asymmetric and presumably excitatory contacts on dendritic spines, chiefly in the IML (Amaral et al., 2007) and degenerate in vulnerable individuals (Fischer et al., 1989).

Nevertheless, we identified new players in cognitive aging; the mossy cells and neurons from the SuM. Mossy cells are known to play a role in regulating adult neurogenesis (Yeh et al., 2018) and memory retrieval (Li et al., 2021), such as projections from the SuM do (Aranda et al., 2008; Li et al., 2020, 2022). The loss of these projections could therefore account for the reduced abilities in the vulnerable population. We report that adu-DGNs generated 15 months before

training in the watermaze need to remain healthy to maintain the ability of the animals to learn the task. Indeed, stimulating adu-DGNs in vulnerable rats can restore memory retrieval abilities, although not to the same extent as in a resilient rat. Given the mentioned role of the mossy cells and the SuM, we propose that both adu-DGNs stimulation and preserved connectivity are necessary for the perseverance of the memory trace during the retrieval process.

In conclusion, we highlighted the essential role of old adu-DGNs in the resilience against cognitive decline. Our results indicate that brain resilience relies on maintenance of the connectivity between mossy cells/SuM and adu-DGNs. Specifically, we showed that adu-DGNs even very old represent a valuable tool for rejuvenating memory abilities at old age.

FIGURES

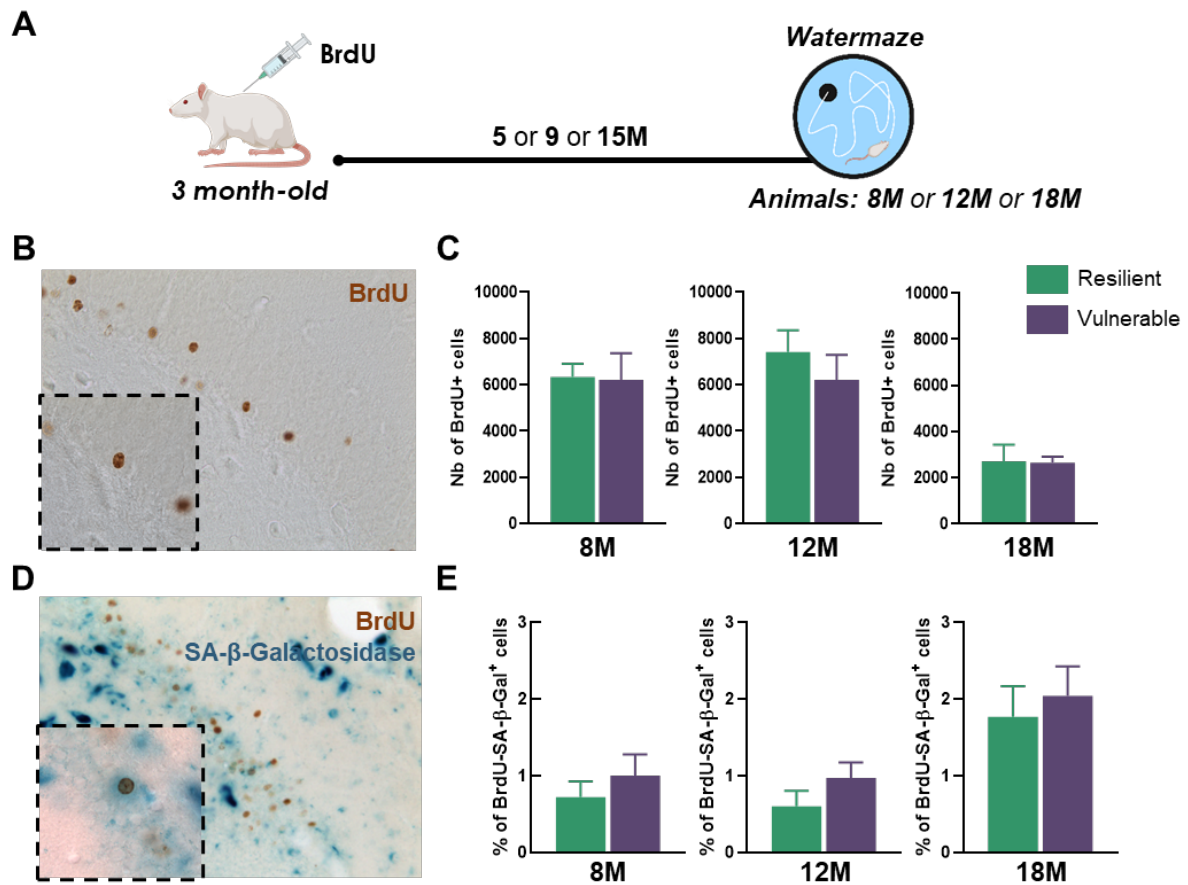


Figure 1: Resilience to cognitive aging is not associated with adu-DGNs death or entry into cellular senescence. (A) Schematic diagram of the experimental design. (B) Picture of a labelled adu-DGNs (BrdU⁺). (C) Resilience to cognitive aging is not linked to different survival of adu-DGNs at 8 month-old (unpaired *t* test: $t_8 = 0.09$, $P > 0.05$), 12 month-old (unpaired *t* test: $t_8 = 0.83$, $P > 0.05$) and 18 month-old (unpaired *t* test: $t_8 = 0.94$, $P > 0.05$). (D) Picture of a senescent adu-DGN (BrdU⁺-SA-β-Gal⁺). (E) Resilience to cognitive aging is not linked to higher levels of senescent adu-DGNs at 8 month-old (unpaired *t* test: $t_8 = 0.81$, $P > 0.05$); 12 month-old (unpaired *t* test: $t_8 = 1.29$, $P > 0.05$) or 18 month-old (unpaired *t* test: $t_8 = 0.48$, $P > 0.05$). Data are presented as mean ± S.E.M. from 5 extreme animals per group. Statistical significance * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$.

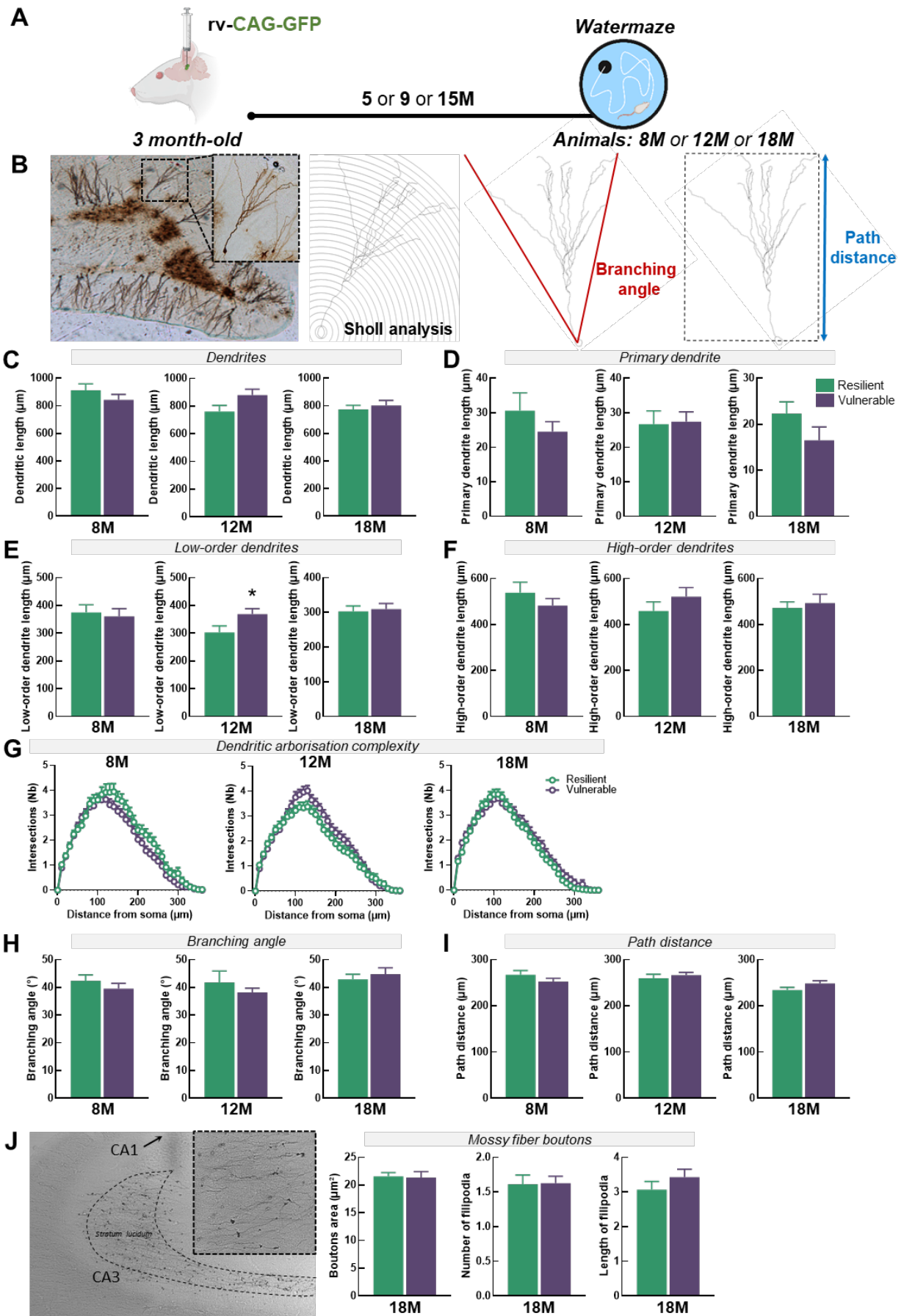


Figure 2: The gross morphological architecture of adu-DGNs does not depend upon the cognitive status. (A) Schematic diagram of the experimental design. (B) Picture of a labelled mAdu-DGN and examples of Sholl analysis for dendritic complexity, branching angle and path distance calculations. (C) Adu-DGNs in the two cognitive populations show similar total dendritic length at 8 month-old (unpaired t test: $t_{90} = 1.15$, $P > 0.05$), 12 month-old (unpaired t test: $t_{102} = 1.98$, $P > 0.05$) and 18 month-old (unpaired t test: $t_{111} = 0.61$, $P > 0.05$). (D) Adu-DGNs have similar primary dendritic length in the two cognitive populations at 8 month-old (unpaired t test: $t_{90} = 1.11$, $P > 0.05$), 12 month-old (unpaired t test: $t_{102} = 0.15$, $P > 0.05$), and 18 month-old (unpaired t test: $t_{111} = 1.50$, $P > 0.05$). (E) Adu-DGNs have similar low-order dendrites length in the two cognitive populations at 8 month-old (unpaired t test: $t_{90} = 0.34$, $P > 0.05$) and 18 month-old (unpaired t test: $t_{111} = 0.76$, $P > 0.05$). A small increase in vulnerable animals could be observed at 12 month-old (unpaired t test: $t_{102} = 2.12$, $P \leq 0.05$). (F) Adu-DGNs show similar high-order dendrites length between the two cognitive populations at 8 month-old (unpaired t test: $t_{90} = 1.11$, $P > 0.05$), 12 month-old (unpaired t test: $t_{102} = 0.15$, $P > 0.05$) and 18 month-old (unpaired t test: $t_{111} = 1.50$, $P > 0.05$). (G) Adu-DGNs of resilient and vulnerable individuals have similar dendritic complexity at 8 month-old (RM-two-way ANOVA, $F_{36,3240}=1.09$, $P > 0.05$), 12 month-old (RM-two-way ANOVA, $F_{36,3408}=1.93$, $P < 0.001$) and 18 month-old (RM-two-way ANOVA, $F_{36,3996}=0.84$, $P > 0.05$). (H) Adu-DGNs in the two cognitive populations show similar branching angles at 8 month-old (unpaired t test: $t_{90} = 0.99$, $P > 0.05$), 12 month-old (unpaired t test: $t_{102} = 0.87$, $P > 0.05$) and 18 month-old (unpaired t test: $t_{111} = 0.64$, $P > 0.05$). (I) Adu-DGNS show similar path distance between the two cognitive populations at 8 month-old (unpaired t test: $t_{90} = 1.30$, $P > 0.05$), 12 month-old (unpaired t test: $t_{102} = 0.69$, $P > 0.05$) and 18 month-old (unpaired t test: $t_{111} = 1.63$, $P > 0.05$). (J) Adu-DGNs show similar MFBs area (unpaired t test: $t_{28} = 0.17$, $P > 0.05$), number (unpaired t test: $t_{28} = 0.08$, $P > 0.05$) and length (unpaired t test: $t_{28} = 1.10$, $P > 0.05$) of filipodia between resilient and vulnerable animals at 18 month-old. Data are presented as mean \pm S.E.M. from 5 extreme animal per group (a min of 4 neurons were traced per animal, with 8M-Res = 37 neurons and 8M-Vul = 55 neurons; 12M-Res = 48 neurons and 12M-Vul = 58 neurons; 18M-Res = 63 neurons and 18M-Vul = 50 neurons. Statistical significance * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$.

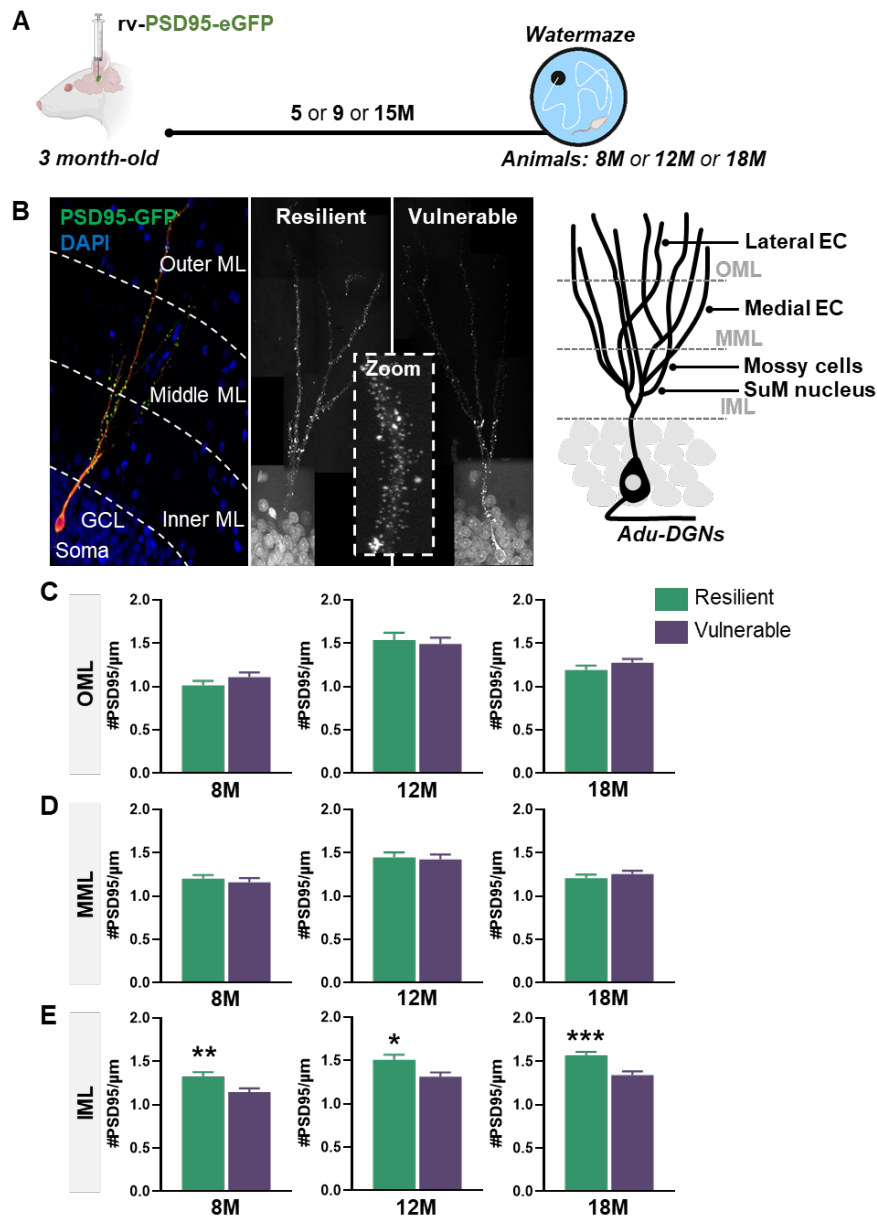


Figure 3: Resilience to cognitive aging is linked to the maintenance of specific glutamatergic inputs of adu-DGNs. (A) Schematic diagram of the experimental design. (B) Picture of a labelled adu-DGN and the subsequent molecular layer (ML) division in three sub-layers; inner (IML), middle (MML) and outer (OML) molecular layer with their different glutamatergic inputs. (C) Resilience to cognitive aging is not linked to the preservation of glutamatergic inputs in the OML at 8 month-old (unpaired t test: $t_{104} = 1.27$, $P > 0.05$), 12 month-old (unpaired t test: $t_{87} = 0.38$, $P > 0.05$) or 18 month-old (unpaired t test: $t_{154} = 1.20$, $P > 0.05$). (D) Resilience to cognitive aging is not linked to the preservation of glutamatergic inputs in the MML at 8 month-old (unpaired t test: $t_{193} = 0.65$, $P > 0.05$), 12 month-old (unpaired t test: $t_{199} = 0.29$, $P > 0.05$) or 18 month-old (unpaired t test: $t_{210} = 0.83$, $P > 0.05$). (E) Resilience to cognitive aging is related to the preservation of glutamatergic inputs in the IML at 8 month-old (unpaired t test: $t_{195} = 2.73$, $P < 0.01$), 12 month-old (unpaired t test: $t_{220} = 2.51$, $P \leq 0.05$) and 18 month-old (unpaired t test: $t_{206} = 3.62$, $P < 0.001$). Data are presented as mean \pm S.E.M. from 5 extreme animals per group (a min of 3 neurons were analyzed per animal, with 8M-Res = 27 neurons and 8M-Vul = 27 neurons; 12M-Res = 21 neurons and 12M-Vul = 33 neurons; 18M-Res = 30 neurons and 18M-Vul = 29 neurons. # for density. Statistical significance * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$.

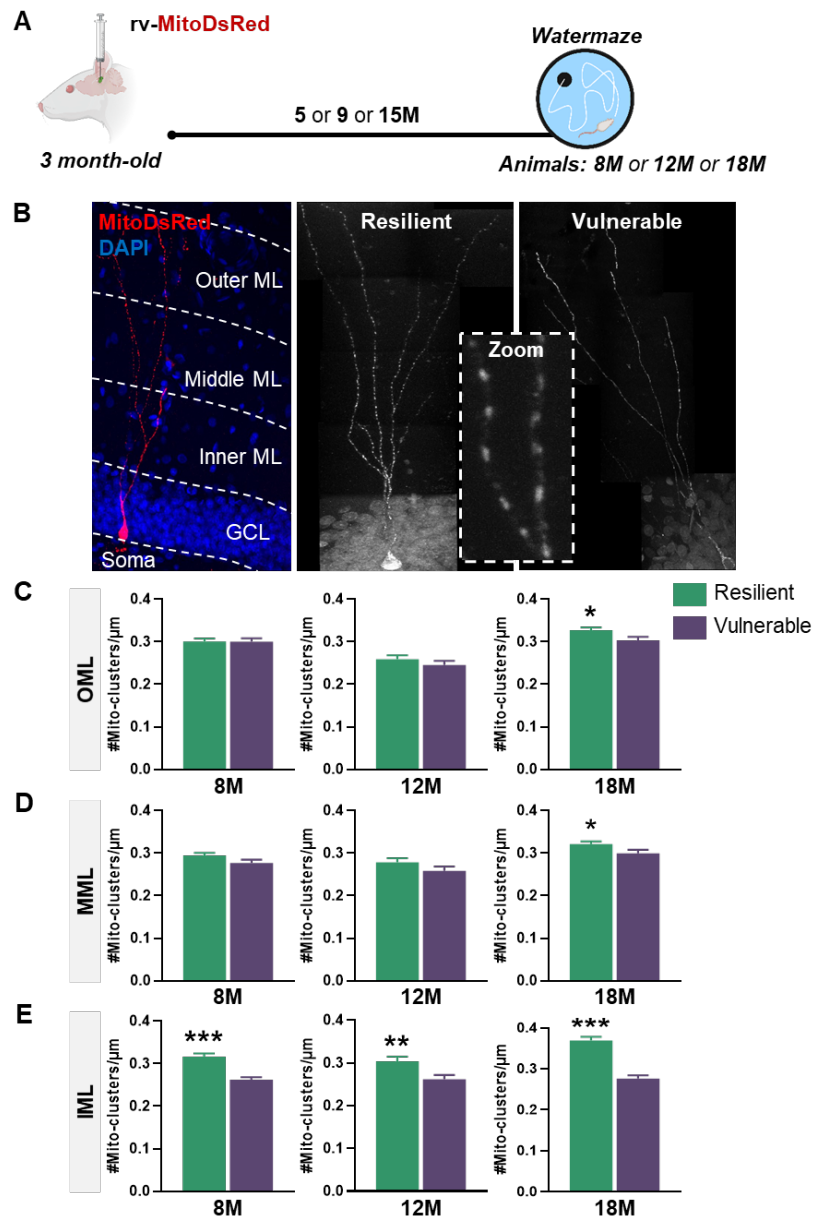


Figure 4: Resilience to cognitive aging is linked to the maintenance of the mitochondrial density in adu-DGNs. (A) Schematic diagram of the experimental design. (B) Pictures of labelled adu-DGNs and illustration of the molecular layer (ML) division in three sub-layers; inner (IML), middle (MML) and outer (OML) molecular layer. (C) Resilience to cognitive aging is linked to the preservation of the mitochondrial density of the OML at 18 month-old (unpaired t test: $t_{171} = 2.33$, $P \leq 0.05$) but not earlier at 8 month-old (unpaired t test: $t_{154} = 0.01$, $P > 0.05$) or 12 month-old (unpaired t test: $t_{134} = 0.97$, $P > 0.05$). (D) Resilience to cognitive aging is linked to the preservation of the mitochondrial density of the MML at 18 month-old (unpaired t test: $t_{198} = 2.01$, $P \leq 0.05$) but not earlier at 8 month-old (unpaired t test: $t_{203} = 1.81$, $P > 0.05$) or 12 month-old (unpaired t test: $t_{168} = 1.41$, $P > 0.05$). (E) Resilience to cognitive aging is related to the preservation of the mitochondrial density of the IML at 8 month-old (unpaired t test: $t_{208} = 5.53$, $P < 0.001$), 12 month-old (unpaired t test: $t_{183} = 2.91$, $P < 0.01$) and 18 month-old (unpaired t test: $t_{202} = 7.42$, $P < 0.001$). Data are presented as mean \pm S.E.M. from 5 extreme animals per group (a min of 3 neurons were analyzed per animal, with 8M-Res = 28 neurons and 8M-Vul = 25 neurons; 12M-Res = 20 neurons and 12M-Vul = 20 neurons; 18M-Res = 27 neurons and 18M-Vul = 27 neurons). # for density. Statistical significance * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$.

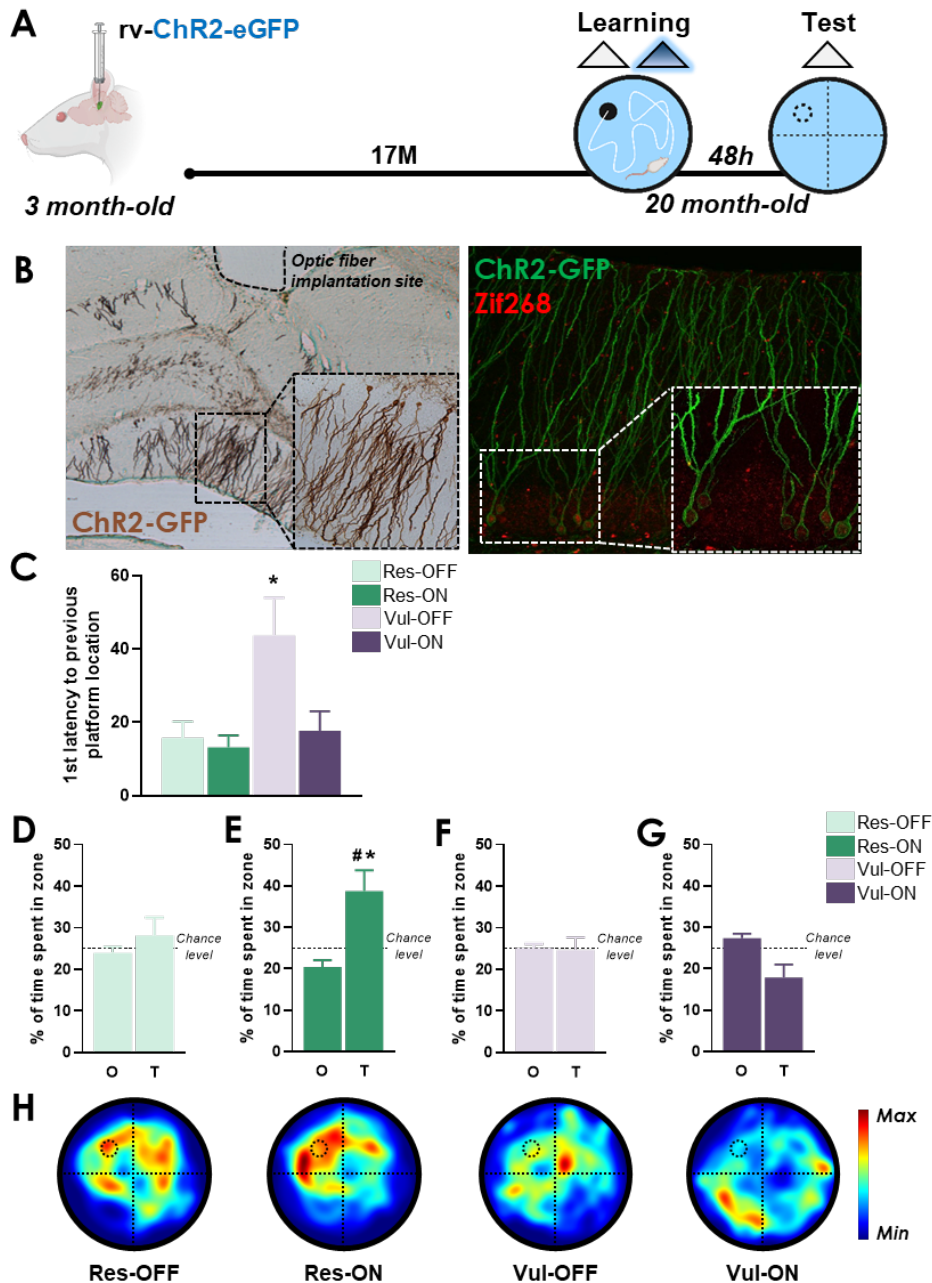


Figure 5: Optogenetic stimulation of adu-DGNs during learning restores memory precision in old vulnerable animals. (A) Schematic diagram of the experimental design. (B) Picture of labelled adu-DGNs (GFP⁺) and optogenetically stimulated adu-DGNs (GFP⁺-Zif268⁺). (C) Stimulation of adu-DGNs in old stimulated vulnerable (Vul-ON) animals promoted the formation of a precise memory trace similar to resilient animals (Res-OFF and Res-ON) (One-way-ANOVA, $F_{(3, 13)} = 5,23$, $P < 0,05$). (D) Old non stimulated resilient (Res-OFF) animals did not form a stable memory trace (paired t test: $t_3 = 0,72$, $P > 0,05$). (E) Stimulation of adu-DGNs in old stimulated resilient (Res-ON) animals promoted the formation of a strong memory trace (paired t test: $t_4 = 2,77$, $P \leq 0,05$ and one sample t test against #chance level $t_5 = 3,23$, $P \leq 0,05$). (F) Old non-stimulated vulnerable (Vul-OFF) animals did not form a stable memory trace (paired t test: $t_3 = 0,12$, $P > 0,05$). (G) Stimulation of adu-DGNs did not restore the formation of a stable memory trace in Vul-ON animals (paired t test: $t_3 = 2,28$, $P > 0,05$). (H) Heatmaps depicting the animals search location and occupancy during the memory test. Data are presented as mean \pm S.E.M. Statistical significance * $P \leq 0,05$, ** $P < 0,01$, *** $P < 0,001$.

SUPPLEMENTARY FIGURES

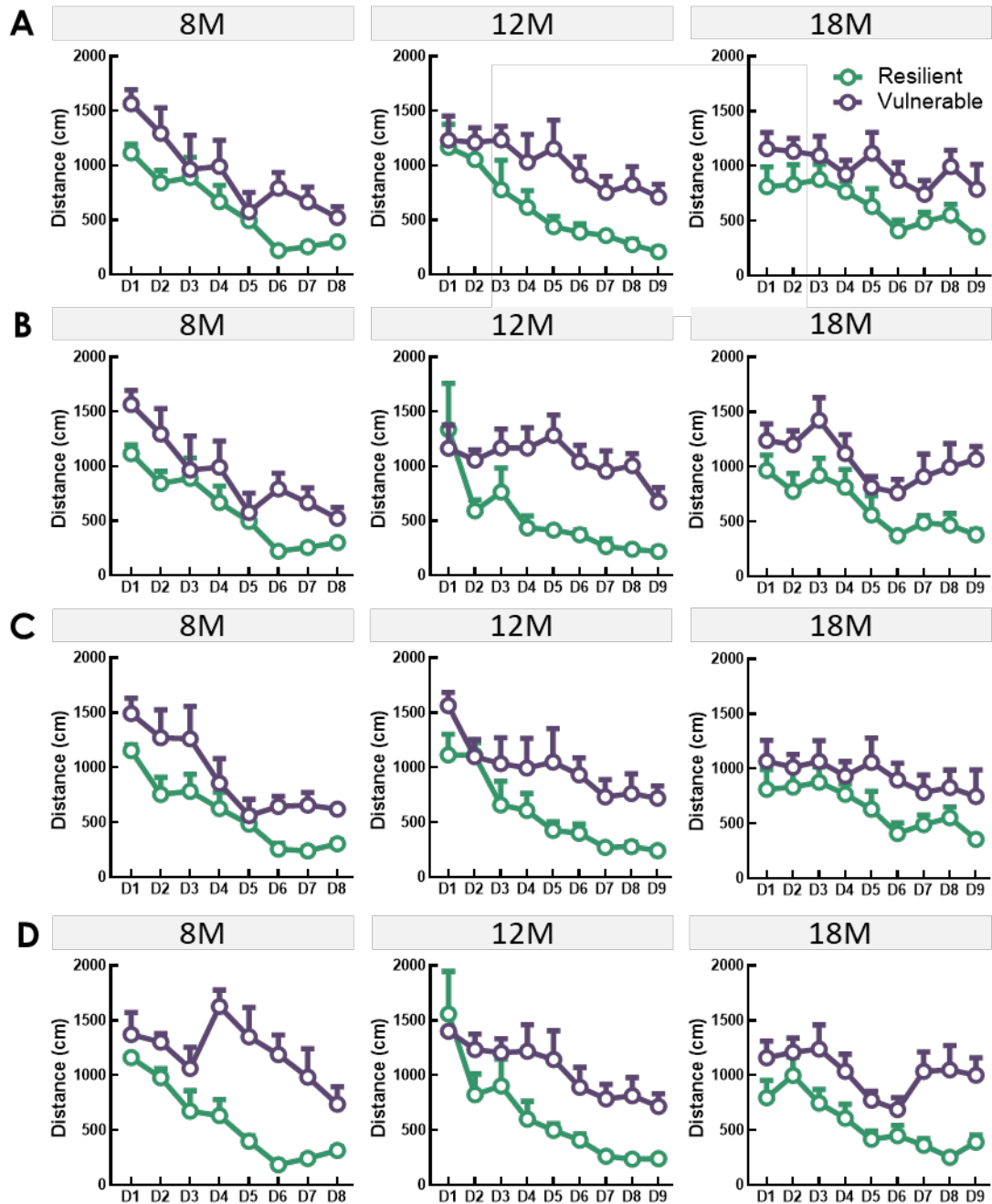


Figure S1: Resilient animals show better learning abilities than vulnerable animals at each age. (A) Extremes animals for the two cognitive populations analyzed in the morphology study with rv-CAG-GFP. (B) Extremes animals for the two cognitive populations analyzed in the glutamatergic innervation study with M-rv-PSD95-GFP. (C) Extremes animals for the two cognitive populations analyzed in the mitochondrial network study with M-rv-MitoDsRed. (D) Extremes animals for the two cognitive populations analyzed in the senescence study with BrdU-Sa β Gal

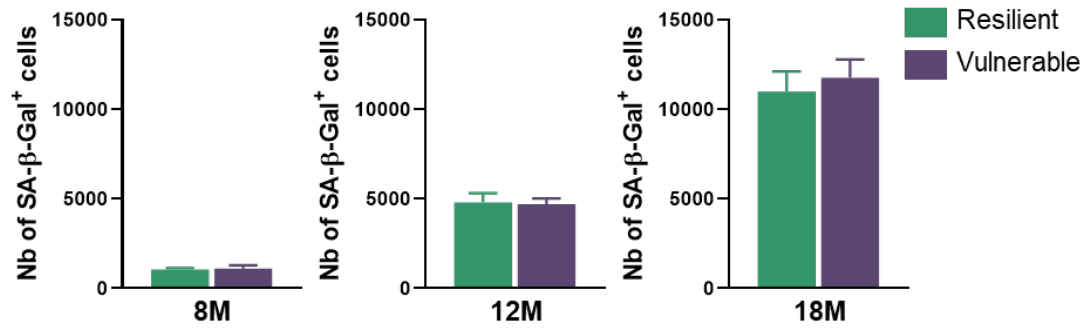


Figure S2: Resilience to cognitive aging is not linked to different levels of cellular senescence in the granule cell layer. (A) Resilient and vulnerable animals show similar number of senescent cells in the granule cell layer at 8 month-old (unpaired t test: $t_8 = 0.28$, $P > 0.05$), 12 month-old (unpaired t test: $t_8 = 0.14$, $P > 0.05$) and 18 month-old (unpaired t test: $t_8 = 0.51$, $P > 0.05$). Data are presented as mean \pm S.E.M. from 5 extreme animals per group. Statistical significance * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$.

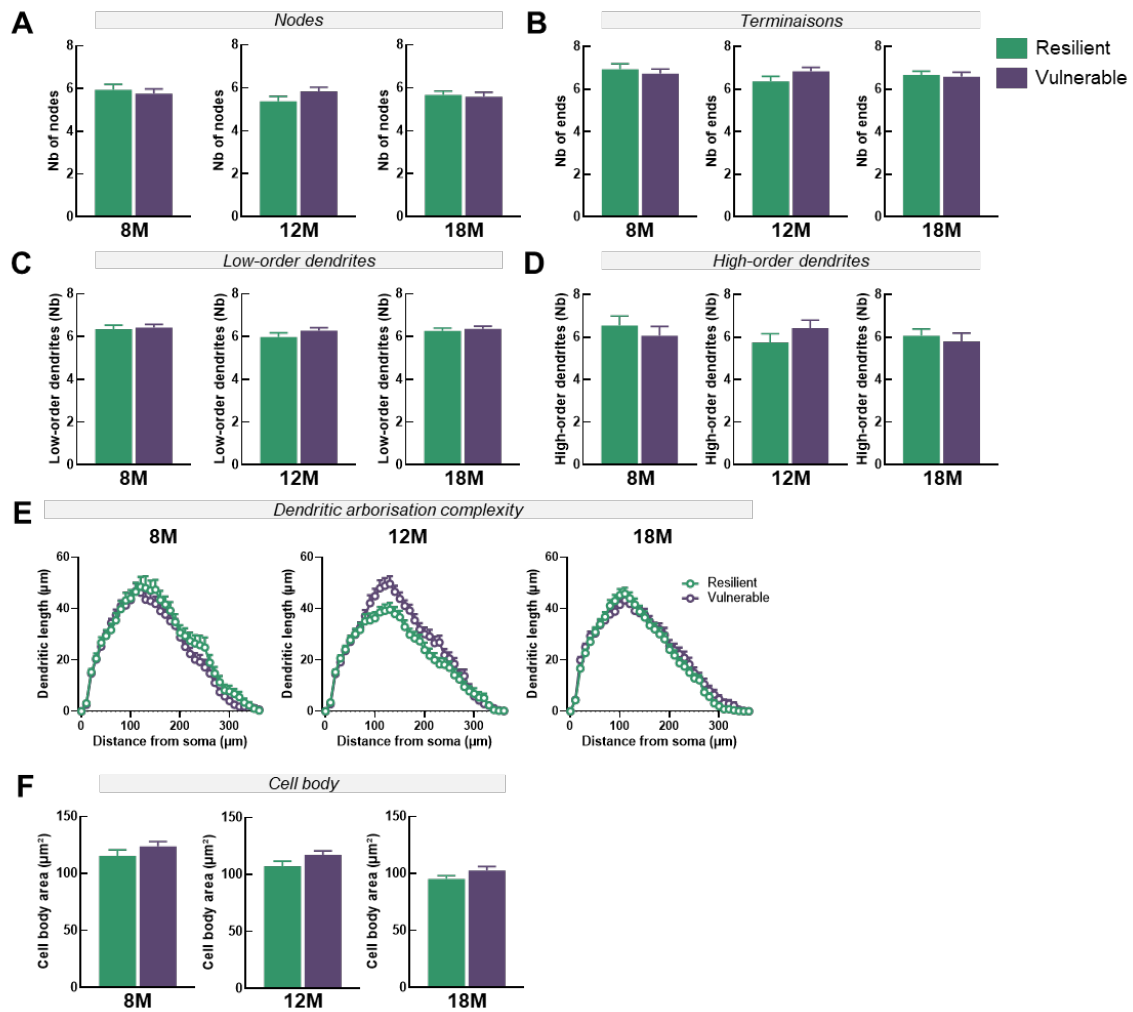


Figure S3: The gross morphological architecture of adu-DGNs does not depend upon the cognitive status. (A) Adu-DGNs in the two cognitive populations show similar number of nodes at 8 month-old (unpaired t test: $t_{90} = 0.55$, $P > 0.05$), 12 month-old (unpaired t test: $t_{102} = 1.59$, $P > 0.05$) and 18 month-old (unpaired t test: $t_{111} = 0.31$, $P > 0.05$). (B) Adu-DGNs have similar number of ends in resilient and vulnerable animals at 8 month-old (unpaired t test: $t_{90} = 0.64$, $P > 0.05$), 12 month-old (unpaired t test: $t_{102} = 1.53$, $P > 0.05$) and 18 month-old (unpaired t test: $t_{111} = 0.31$, $P > 0.05$). (C) Adu-DGNs in the two cognitive populations show similar low-order dendrites number at 8 month-old (unpaired t test: $t_{90} = 0.28$, $P > 0.05$), 12 month-old (unpaired t test: $t_{102} = 1.33$, $P > 0.05$) and 18 month-old (unpaired t test: $t_{111} = 0.48$, $P > 0.05$). (D) Adu-DGNs show similar high-order dendrites number at 8 month-old (unpaired t test: $t_{90} = 0.72$, $P > 0.05$), 12 month-old (unpaired t test: $t_{102} = 1.22$, $P > 0.05$) and 18 month-old (unpaired t test: $t_{111} = 0.02$, $P > 0.05$). (E) Adu-DGNs have similar dendritic complexity between the two cognitive populations at 8 month-old (RM-two-way ANOVA, $F_{36,3240}=0.68$, $P > 0.05$) and 18 month-old (RM-two-way ANOVA, $F_{36,3996}=0.82$, $P > 0.05$). At 12 month-old, a small differences could be detected between the resilient and vulnerable populations (RM-two-way ANOVA, $F_{36,3708}=2.59$, $P < 0.001$). (F) Adu-DGNs in the two cognitive populations show similar cell body area at 8 month-old (unpaired t test: $t_{90} = 1.22$, $P > 0.05$), 12 month-old (unpaired t test: $t_{102} = 1.84$, $P > 0.05$) and 18 month-old (unpaired t test: $t_{111} = 1.66$, $P > 0.05$). Data are presented as mean \pm S.E.M. from 5 extreme animal per groups (a min of 4 neurons were traced per animal, with 8M-Res = 37 neurons and 8M-Vul = 55 neurons; 12M-Res = 48 neurons and 12M-Vul = 58 neurons; 18M-Res = 63 neurons and 18M-Vul = 50 neurons. Statistical significance * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$).

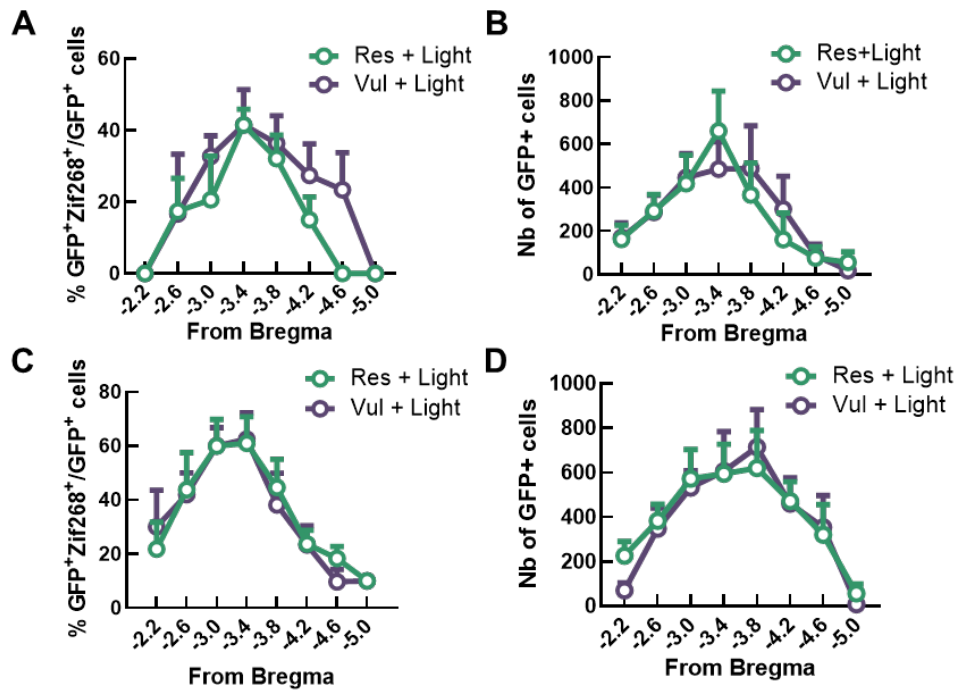


Figure S4: Similar levels of adu-DGNs stimulation between resilient and vulnerable animals. (A) Similar percentage of adu-DGNs stimulation between resilient and vulnerable animals at 20 month-old. (B) Similar number of GFP labelled adu-DGNs between stimulated resilient and vulnerable animals at 20 month-old. (C) Similar percentage of adu-DGNs stimulation between resilient and vulnerable animals at 12 month-old. (D) Similar number of GFP labelled adu-DGNs between stimulated resilient and vulnerable animals at 12 month-old. Data are presented as mean \pm S.E.M for 20M-Res-Light: 5 animals, 20M-Vul-Light: 4 animals, 12M-Res-Light: 6 animals, 12M-Vul-Light: 5 animals. For the percentage of activation, data expressed as $(\text{Nb of GFP}^+\text{-Zif268}^+ \text{ cells}) / (\text{Nb of GFP}^+\text{-Zif268}^+ \text{ cells} + \text{Nb of GFP}^+\text{-Zif268}^- \text{ cells}) \times 100$.

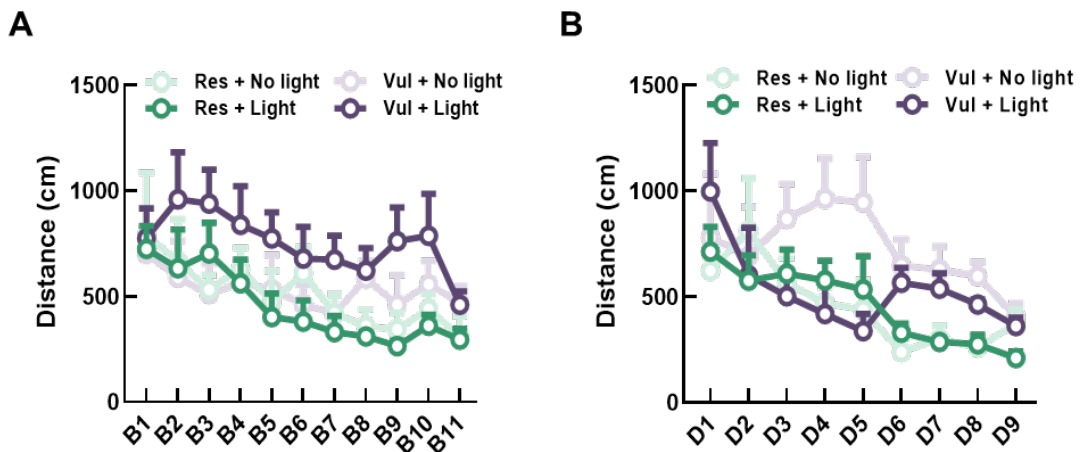


Figure S5: Stimulation of adu-DGNs during the learning phase has no effect on the performances. (A) Learning performances of each group over several days of training at 20 month-old. Days of learning are presented in blocks; one block corresponds to two days of learning (B) Learning performances of each group over several days of training at 12 month-old. Data are presented as mean \pm S.E.M for: 5 animals, 20M-Res-Light: 5 animals; 20M-Vul-Light: 4 animals, 20M-Res-NoLight: 4 animals, 20M-Vul-NoLight: 4 animals, 12M-Res-Light: 6 animals, 12M-Vul-Light: 5 animals, 12M-Res-NoLight: 5 animals and 12M-Vul-NoLight: 4 animals.

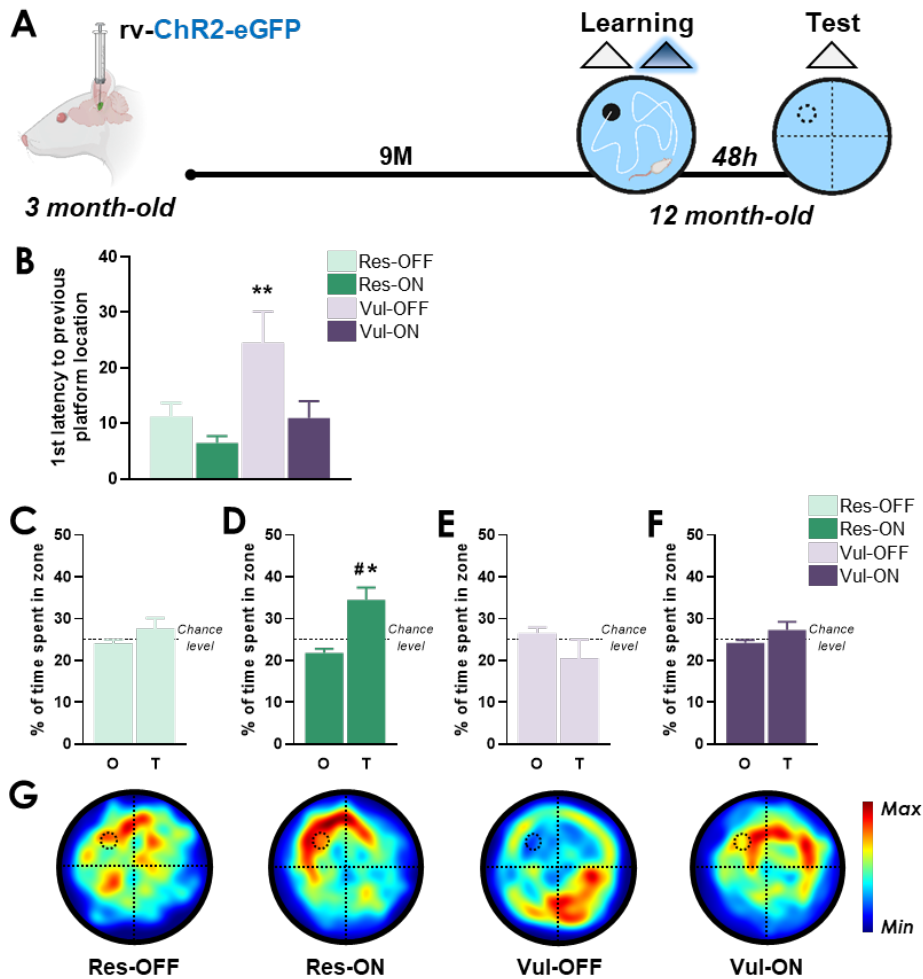


Figure S6: Optogenetic stimulation of adu-DGNs during learning restores memory precision in middle-aged vulnerable animals. (A) Schematic diagram of the experimental design. (B) Stimulation of adu-DGNs in middle-age stimulated vulnerable (Vul-ON) animals promoted the formation of a precise memory trace similar to resilient animals (Res-OFF and Res-ON) (One-way-ANOVA, $F_{(3,16)} = 5,94$, $P < 0.01$). (C) Middle-age non-stimulated resilient (Res-OFF) did not form a stable memory trace (paired t test: $t_4 = 1.08$, $P > 0.05$). (D) Stimulation of adu-DGNs in middle-age stimulated resilient (Res-ON) animals promoted the formation of a strong memory trace (paired t test: $t_5 = 3.23$, $P \leq 0.05$ and one sample t test against #chance level $t_5 = 3,23$, $P < 0,001$). (E) Middle-age non-stimulated vulnerable (Vul-OFF) animals did not form a stable memory trace (paired t test: $t_3 = 1.01$, $P > 0.05$). (F) Stimulation of adu-DGNs did not restore the formation of a stable memory trace in Vul-ON animals (paired t test: $t_4 = 1.15$, $P > 0.05$). (G) Heatmaps depicting the individuals search location and occupancy during the memory test. Data are presented as mean \pm S.E.M. Statistical significance * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Material & Methods

Rats

A total number of 281 male Sprague-Dawley rats (OFA, Janvier, France) were used for these experiments. Rats aged 3 month-old at the time of delivery were grouped housed in standard cages under a 12/12h light/dark cycle with ad libitum access to food and water. Temperature (22°C) and humidity (60%) were kept constant. Rats were individually housed before the beginning of training at either the end of adulthood (8 month-old), middle-age (12 month-old) or old age (18 or 20 month-old). Experimental procedures have been carried out following the European directive of the parliament and the conceal of September 22, 2010 (2010/63/UE). Animal studies were approved by the ethical committee of the University of Bordeaux (ID #10864, #11155, #21501, #5012006A).

Thymidine analog injections

BrdU (5-bromo-2'-deoxyuridine) was dissolved in a Phosphate Buffer (pH 8.4). Rats received four injections (50mg/kg) intra-peritoneally during four consecutive days when 3 month-old.

Retroviruses

The M-rv-CAG-GFP has been described previously (Zhao et al., 2008) and is a gift from Prof. F.H. Gage (Salk Institute, La Jolla, CA). The M-rv-PSD95-GFP has been described previously (Kelsch et al., 2008) and is a gift from Dr C. Lois (Caltech, Pasadena, CA). The M-rv-MitoDsRed has been described previously (Steib et al., 2014) and is a gift from Dr D.C. Lie (Friedrich-Alexander Universität Erlangen-Nürnberg, Erlangen, Germany). The M-rv-Channelrhodopsin-GFP was kindly provided by the Dr S. Ge (Stony Brook University, New York City, NY). High retroviruses titers (between 5×10^8 and 5×10^9 Transduction Units/mL) were prepared with a human 293-derived retroviral packaging cell line (293GPG) kindly provided by Dr D.C. Lie. Virus-containing supernatant was harvested three days after transfection with Lipofectamine 2000 (Thermofisher). This supernatant was then cleared from cell debris by centrifugation at 3500 rpm for 15 min and filtered through a 0.45 μm filter (Millipore). Viruses were concentrated by two rounds of centrifugation (19 500 rpm 2 h) and resuspended in PBS.

Surgery

Rats were anaesthetized with 3% isoflurane and placed in the stereotaxic frame, where they were maintained asleep with 2% isoflurane for the duration of the surgery. Analgesia was provided

by an injection of Metacam (1ml/kg, sub-cutaneously, Boehringer Ingelheim) and lidocaine (0.1 ml, sub-cutaneous, Vetoquinol) at the incision site.

Retroviral injections

A retroviral mix of half M-rv-GFP and half M-rv-MitoDsRed was prepared before injection. The retroviral injections (2 μ L per injection site at 0.3 μ L/min) were stereotaxically made into the dentate gyrus of 3 month-old adult rats with a microcapillary pipette connected to a Hamilton syringe placed into a micro injector (KDS legato 130) directly attached to the stereotaxic frame. Cannulas were maintained in position for 3 minutes after the end of the injections to let the suspension infuse. The Moloney leukemia virus-based retroviral vector M-rv-PSD95-GFP and mixed M-rv-GFP/M-rv-MitosDsRed were injected to the right and left dorsal dentate gyrus respectively. For the optogenetic experimental cohorts, the M-rv-Channelrhodopsin-GFP was injected bilaterally to the dorsal area of the dentate gyrus. Injections were administered using the following coordinates: [AP]:-3,5mm; [L]:-/ +1,7mm; [P] from skull:-4,3mm.

Optic fiber implantations

Home-made optic fibers were made following a protocol previously described (Sparta et al., 2011). The implantation surgical procedure was carried out one month before the start of behavior, following the same protocol as that described for the retroviral injection. The skull was scratched and three anchor screws were fixed (one in the anterior area and two in the posterior area). The two optic fibers [length: 4.5 mm, diameter: 200 μ m, numeric aperture (NA: 0.37)] were lowered with a speed of \sim 2 mm/min upwards to the M-rv injection site with the following stereotaxic coordinates: [AP]:-4,1mm; [L]:-/ +1,8mm; [P] from dura-mater:-3mm. Fibers were stabilized using dental cement. When the cement was dry, scalps were sutured and disinfected with local antiseptic treatment (Betadine). All rats were followed up until the start of the behavior. During handling, fur was checked and the scar disinfected systematically. Rats were handled every day to habituate them before the behavioral procedure.

Watermaze procedures

The apparatus consisted of a circular plastic swimming pool (180 cm diameter, 60 cm height) filled with water (20 ± 1 °C) rendered opaque by the addition of a white cosmetic adjuvant. Two days before training, the animals were habituated to the pool for 1 min. For old animals, we followed previous protocol published for aging experiments in the laboratory (Drapeau et al., 2003). The capacity to swim and to see correctly were assessed during a training phase where

animals had to locate a visible platform within the swimming pool, all rats with deficits were removed from the experiments (i.e. rats that did not find the platform in less than 20s after 5 day of platform training). During training, animals were required to locate a submerged platform hidden 1.5 cm under the surface of the water in a fixed location, using the spatial cues available in the room. Rats were trained for four trials per day (90 s with an inter-trial interval of 30 s and released from three different starting points that varied randomly each day). If an animal failed to locate the platform, it was placed on the platform at the end of the trial for the duration of the inter-trial interval. The distance crossed to reach the platform was recorded using a video camera that was secured to the ceiling of the room and connected to a computerized tracking system (Videotrack, Viewpoint). Daily results were analyzed in order to rank animals according to their behavioral score calculated over the last 3 days of training. Training stopped before the resilient animals reached plateau phase of learning.

Water maze for optogenetic manipulation of adu-DGNs

The watermaze was equipped with a blue laser source (OptoDuet 480 nm, 200mW, Ikecool) connected to a rotatory joint to avoid tangling of the patch cords connected to the intra-dentate gyrus optic fibers. Light intensity at the optic fiber cable tip was controlled every day before the beginning of the session (8-10 mV). Illumination was applied throughout the session at 20 Hz pulses of 15 ms. The time to reach the platform was recorded using a video camera connected to a computerized tracking system (Polyfiles, Imetronic). Light stimulation was provided during the 4 trials each day for the entire learning phase. When resilient animals reached the plateau phase, training was stopped and 48h later animals were tested during a probe test for 60 s where no animals were under no-light stimulation. During the probe test, the hidden platform was removed and the time spent in each quadrant was quantified. Control animals consisted of animals that underwent the same procedure but without light stimulation.

Resilient and vulnerable extremes populations

Following the end of learning, animals were cognitively attributed according to the mean performances of their three last days of training. From the whole population submitted to the learning task, the five animals with the higher score (i.e. lowest mean distance to reach the platform) were attributed as extreme resilient and the five animals with the lowest score (higher mean distance to reach the platform) were attributed as extreme vulnerable. Only the extremes were kept for analysis as considered the most representative of their cognitive abilities, as already published in the laboratory (Drapeau et al., 2003).

Immunohistochemistry and analysis

For animals injected with M-rv-CAG-GFP, M-rv-MitoDsRed, M-rv-PSD95-GFP and M-rv-Channelrhodospin, animals were perfused transcardially with a phosphate-buffered solution of 4% paraformaldehyde 90 min after the last training session. After 24h fixation, optogenetic brains were cut using a vibratome. For PSD95-GFP and mitoDsRed, after 24h of fixation, brains were transferred in a PBS-sucrose (30% solution for 24h and then cut using a vibratome.

CAG-GFP expressing cells

Free-floating 50- μ m-thick sections were processed according to a standard immunohistochemical procedure on alternate one-in-ten sections to visualize the retrovirus-expressing GFP with an anti-GFP primary antibody (Rabbit, 1:12000; Millipore, Cat#AB3080P). Bound antibodies were visualized using the biotin-streptavidin technique (ABC kit, Vector Laboratories Inc., Cat#PK-4000) and 3,3'-diaminobenzidine as a chromogen with a biotinylated goat anti-rabbit antibody (1:200, Vector Laboratories Inc., Cat#BA-1000). The morphometric analysis of virus-labelled neurons was performed with a \times 100 objective. Measurements of dendritic parameters as well as Sholl analysis were performed with the NeuroLucida (software Microbrightfield, Colechester, VT, USA). The analysis of the dendritic arborization (low and high order repartition, branching angle and path distance calculations) was performed as previously described (Kerloch et al., 2019) blind by the person who carried it out. Only animals with at least 4 neurons who could be reconstructed and neurons with a minimum of 4 ramifications point were kept for analysis.

PSD95-GFP and mitoDsRed expressing cells

Free-floating 50- μ m-thick sections were processed according to standard immunohistofluorescence procedure on alternate one-in-ten sections to visualize the retrovirus-expressing eGFP and mitoDsRed with an anti-GFP primary antibody (Rabbit, 1:500; Millipore, Cat#AB3080P) and anti-DsRed primary antibody (Rabbit, 1:1000, Clontech Takara, Cat#632496). Bound antibodies were visualized with with Cy3 goat anti-rabbit (Jackson Immuno Research, 1:1000, Cat#111-167-003) secondary antibody. Primary antibodies were incubated simultaneously at 4°C for 72h, and secondary antibodies were incubated simultaneously at room temperature (RT) for 2h. Fluorescence was studied using a SPE confocal system with a plane apochromatic X 63 oil lens (numerical aperture 1.4; Leica) and a digital zoom of 2,5. For both approaches, MosaicJ plugin from ImageJ was used to reconstruct the neuron as a whole. Mosaic

reconstruction allowed to determine the length of the molecular layer and to delimitate the inner, middle and outer sub-layers of the molecular layer. For each layer, dendritic segments of minimum 15 μm and maximum 50 μm were analyzed (minimum of 3 neurons per animals, 5 animals per group). The number of clusters of PSD95-GFP and mitoDsRed was determined either manually or semi-automatically using an intensity threshold in Image J. The density of clusters was obtained by dividing the number of clusters by the length of the corresponding dendritic segment. The analysis was performed blind by the person who carried it out.

BrdU and Senescence-Associated- β -Galactosidase (SA- β -Gal) positive cells

BrdU+ cells throughout the entire granular layer of the dentate gyrus were revealed using the biotin-streptavidin technique with a horse anti-mouse antibody (1:200, Vector Labs, Peterborough, UK. #BA-2001). SA- β -Gal+ cells were revealed using the Senescence β -Galactosidase Staining Kit (Cell Signaling, #9860) according to the manufacturer's instructions. The number of BrdU+ cells and SA- β -Gal+ cells in the dentate gyrus was estimated with systematic random sampling of every tenth section along the septo-temporal axis of the hippocampal formation using a modified version of the optical fractionator method. Indeed, all of the BrdU-SA- β -Gal+ cells were counted on each section and the resulting numbers were tallied and multiplied by the inverse of the section sampling fraction ($1/\text{ssf} = 10$). The percentage of colocalization BrdU-SA- β -Gal+ cells was calculated as follows: $(\text{Nb of BrdU}^{++}\text{-SA-}\beta\text{-Gal}^{+}) / (\text{Nb of BrdU}^{++}\text{-SA-}\beta\text{-Gal}^{+} + \text{Nb of BrdU}^{++}\text{-SA-}\beta\text{-Gal}^{-}) \times 100$. The analysis was performed blind by the person who carried it out.

Statistical analysis

The data (mean \pm SEM) were analyzed using the Student t-test (two-tailed, paired or unpaired when appropriate) or one way and repeated-measures two-way ANOVA, then followed by Tukey's comparison test when necessary. Data were tested for normality. All analyses were carried out using the software GraphPad Prisms 8.

Supplementary Material & Methods

Channelrhodospine-GFP expressing cells

Free-floating 50- μm -thick sections were processed according to a standard immunohistochemical procedure on alternate one-in-ten sections to visualize the retrovirus-expressing GFP with an anti-GFP primary antibody (Rabbit, 1:2000; Millipore, Cat#AB3080P). Bound antibodies were visualized using the biotin-streptavidin technique (ABC kit, Vector

Laboratories Inc., Cat#PK-4000) and 3,3'-diaminobenzidine as a chromogen with a biotinylated goat anti-rabbit antibody (1:200, Vector Laboratories Inc., Cat#BA-1000). Channelrhodopsin-GFP+ cells were counted under a ×100 microscope objective throughout the entire septotemporal axis of the granule layer of the dentate gyrus. The total number of cells was estimated using the optical fractionator method, and the resulting numbers were tallied and multiplied by the inverse of the sections sampling fraction (1/ssf10). The analysis was performed blind by the person who carried it out.

Channelrhodopsin-GFP and Zif268 colocalisation

The activation of Channelrhodopsin-GFP+ cells was studied using immunohistofluorescence procedure on alternate one-in-ten sections. The retrovirus-expressing GFP was visualized with an anti-GFP primary antibody (Chicken-1:1000, Abcam, Cat#ab13970) and cellular activity with an anti-Zif268 antibody (Rabbit-1:100, Santa Cruz Biotechnology, Cat#sc-189). Bound antibodies were visualized with Alexa488 goat anti-chicken (Thermo Fisher, 1:1000, Cat#A-11039) and Cy3 goat anti-rabbit (Jackson Immuno Research, 1:1000, Cat#111-167-003) secondary antibodies. Primary antibodies were incubated simultaneously at 4 °C for 72h, and secondary antibodies were incubated simultaneously at RT for 2h. Fluorescence was studied using a SPE confocal system with a plane apochromatic X 63 oil lens (numerical aperture 1.4; Leica) and a digital zoom of 2,5. The percentage of GFP cells expressing IEG (all along the temporal–septal axis) was calculated as follows: $(\text{Nb of GFP}^+\text{-IEG}^+\text{ cells}) / (\text{Nb of GFP}^+\text{-IEG}^-\text{ cells} + \text{Nb of GFP}^+\text{-IEG}^+\text{ cells}) \times 100$. In middle-age experiments, every GFP+ were analyzed in the left and right dentate gyrus. In old age experiments, every GFP+ cells were analyzed in the left dentate gyrus. The analysis was performed blind by the person who carried it out.

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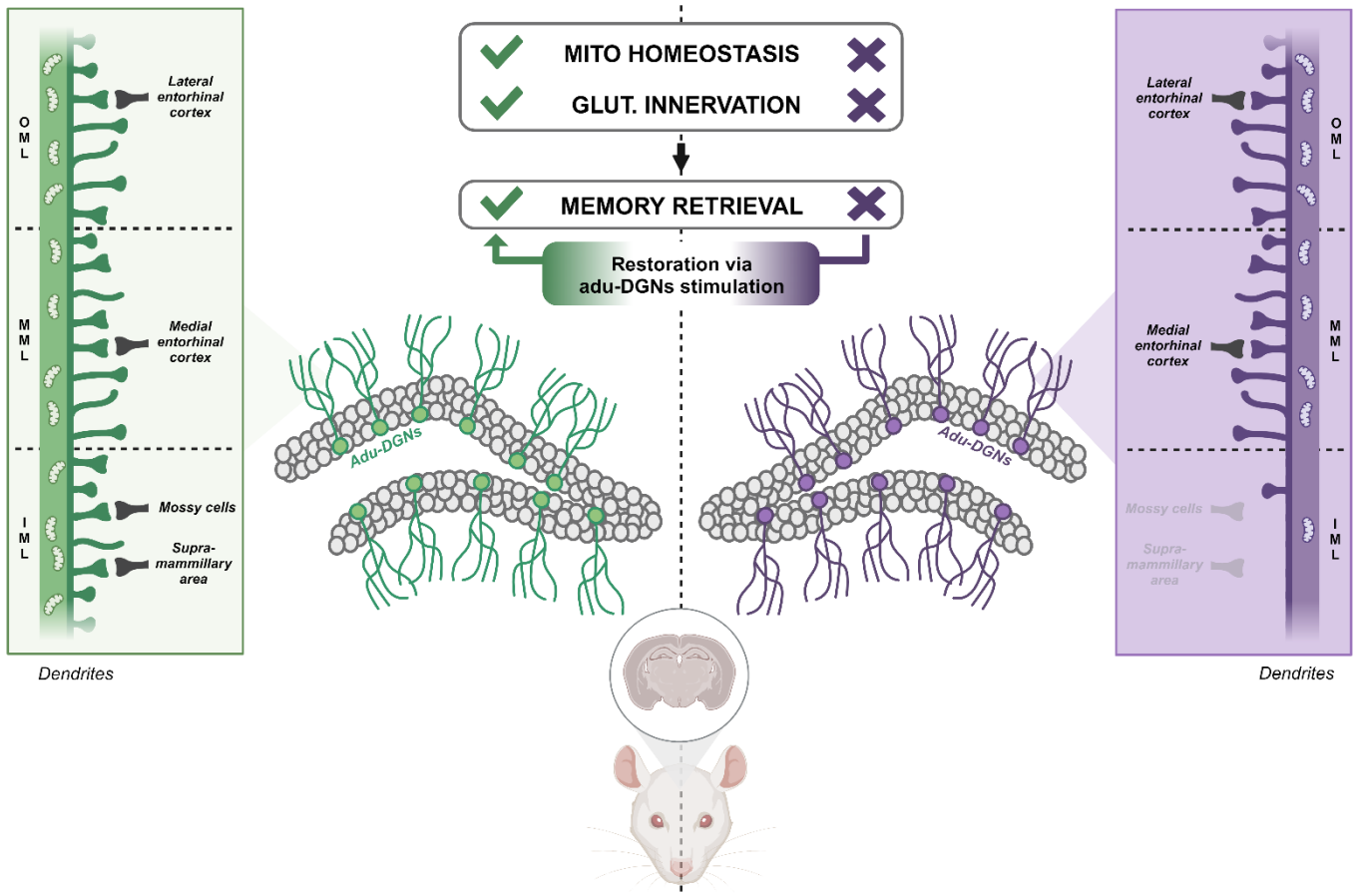
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Graphical abstract

RESILIENCE

VULNERABILITY



In summary, resilience to cognitive aging has been shown to rely on very old adu-DGNs. The maintenance of their “health” throughout aging represents a key process to preserved abilities at old age. More particularly, the maintenance their innervation from mossy cells and neurons in the SuM at early age together with the preservation of the mitochondrial homeostasis at the location of these projections is essential. Nevertheless, despite such alterations with age, it is possible to counteract them by optogenetic stimulation and subsequently to restore memory functions in old vulnerable animals. Altogether, old adu-DGNs represent a valuable tool to fight cognitive aging.

CHAPTER III:

DISCUSSION

The work presented in this thesis identified several key points; i) mature adu-DGNs are functionally needed for spatial learning and memory in the established adult network, with a prior transient role of adol-DGNs, ii) resilience to cognitive aging is associated with the ability for mature adu-DGNs to be recruited during learning at old age, iii) their inability to do so in vulnerable animals is associated to a loss of inputs coming from mossy cells and neurons of the SuM and an alteration of their metabolic health (using their mitochondrial homeostasis as a proxy), iv) despite these signs of “unhealthy” aging the artificial stimulation of mature adu-DGNs allows to restore retrieval abilities, indicating that manipulating very old adu-DGNs represents a good potential tool to counteract cognitive decline.

I. BOTH THE ONTOGENIC ORIGIN AND THE NEURONAL MATURITY DETERMINE FUNCTIONAL INVOLVEMENT

A. Morphological differences between ontogenic populations

Before delving into functional involvement, it seems interesting to come back to the morphological differences discussed in the introduction between granule neurons according to their ontogenic origin. In the work presented here, we addressed the morphological differences in rats (Masachs et al., 2021) to compare with what has been done in mice (Kerloch et al., 2019). As a reminder, emb-DGNs and neo-DGNs in mice were shown to be morphologically distinctive from one another and from adol-DGNs and adu-DGNs, which are more similar. In our rat experiments, even though we lack information about emb-DGNs, we compared the morphology of neo-DGNs, adol-DGNs and adu-DGNs ([Article 1 – Supplementary Figure 5](#)). We observed that neo-DGNs stand out from the two other granule neurons populations, with a higher number of primary dendrites and a wider branching angle. They also displayed more complex proximal dendrites, but similar distal ones. Although these data were obtained in animals trained in the watermaze, they confirm that the temporal origin of granule neurons dictate their dendritic shape.

These findings represent the first signs of differences between ontogenic populations. It would be interesting to go further into their comparison. For example; as we focused here on the dendritic arborization, it would be interesting to study the development of the granule neurons

axonal projections. Each population being added one after another, they could have a different proximo-distal projection organization in the CA3 or even CA2. Such different downstream projections could therefore relate to different functional implications. Similarly, it would be interesting to compare the MFBs between the ontogenic populations, given that the latter ones integrate in an already established network, they might have to compete for space and synaptic connections. Similarly, a complete electrophysiological comparative study of each ontogenic population remains to be done. The difficulty of these approaches relies on molecular tools to tag and target each ontogenic population from one another. In this way, molecular profiling, either via RNAsequencing or proteomics for each ontogenic population would be a great opportunity to better characterize and differentiate them.

B. Ontogenic origin and maturity for functional involvement in spatial learning: time matters

As discussed in the introduction, it tested the hypothesis that the ontogenic origin of a neuron dictates its functional role in the network. Altogether, the development of memory is not a singular ontogenetic process but instead follows a precise calendar with learning capacities emerging sequentially from simple to complex. Based on these observations, we have proposed that dev-DGNs would be responsible for fundamental functions and basic behaviors, whereas adu-DGNs would undertake more complex and nuanced roles needed for adaptive behaviors. In this line of thought, dev-DGNs would therefore undertake the functions/abilities established in newly born and adolescent individuals, referred to as first-order capacities while adu-DGNs would undertake more complex adaptive inferential function (Abrous et al., 2022).

Concerning the populations of dev-DGNs, some investigations hint toward an involvement of dev-DGNs in elemental associative memory, a function that appears early during development (Esmorís-Arranz et al., 2008). Besides, neo-DGNs were shown to be specially needed for “simple” contextual discrimination when animals have to distinguish two different contexts (Tronel et al., 2015b). Unfortunately, the populations of emb-DGNs or neo-DGNs were not much more investigated in higher-order functions, such as complex associative memory, memory discrimination, cognitive flexibility or forgetting. Concerning adol-DGNs, the few studies which addressed their role observed their implication in more complex forms of memory, such as complex associative memory (Arruda-Carvalho et al., 2011; Farioli-Vecchioli et al., 2008) or cognitive flexibility (Garthe et al., 2009, 2016). These observations support the progressive setting-up of complex functions with age. The only study addressing them in forgetting did not show

their implication (Kodali et al., 2016) but neither did they show the involvement adu-DGNs in this study, which contrasts with the rest of the literature. Concerning adu-DGNs, our review of the literature indicates that they have a clear role in complex associative memory and memory discrimination (**Introduction - Table 5 and 6**). Similarly, both cognitive flexibility and forgetting can be attributed to adu-DGNs (**Introduction – Table 8 and 9**). However, their implication when immature or mature in these functions remains elusive and calls for specific targeting and manipulation at both stages.

Concerning spatial learning and memory, which has been the focus in the work presented in this thesis, things are highly complex (**Introduction – Table 7**). One should start by mentioning that the findings in the literature are disparate probably due to the methodological approach used to target granule neurons. The impact of killing a neuron and silencing it by optogenetics are not the same on behavior. Killing a neuron is a permanent action that can lead to irreversible changes in the brain and behavior. However, once the different studies are classified according to their approach targeting either one or another ontogenic population or within the adult population only immature or both immature and mature adu-DGNs, a working hypothesis can be elaborated.

Concerning dev-DGNs, neo-DGNs were shown not to be recruited by spatial learning using IEGs (Tronel et al., 2015b) and their chemogenetic stimulation during spatial memory retrieval had no effect on performances, indicating their lack of involvement in spatial memory (Lods et al., 2022). We confirmed their lack of involvement in spatial learning in our first and second article. Concerning adol-DGNs, their irradiation-mediated ablation suggested that they could be involved in spatial memory retention while not in spatial learning (Snyder et al., 2005). However, the irradiation-mediated ablation-to-learning delay also covers the beginning of adulthood. Therefore, some immature adu-DGNs were also ablated, which could be at the origin of the altered performances. Nevertheless, later studies showed the involvement of adol-DGNs by transgenic alterations in the learning phase (Farioli-Vecchioli et al., 2008) and by chemogenetic silencing or pharmacological ablation in spatial memory (Gu et al., 2012; Martinez-Canabal et al., 2013a). Yet, other studies did not find any implication of adol-DGNs in spatial learning by their chemogenetic silencing (Gu et al., 2012; Ash et al., 2023). Therefore, there are conflicting results for adol-DGNs involvement in spatial memory, which called for more investigations. We adopted a dual approach relying on both the study of adol-DGNs neuronal activity following learning with the use of IEGs, and their inhibition by optogenetic. By doing so, we detected an involvement of adol-DGNs but only during a specific window while they are aged between 1 and 2 months (**Article 1**). Interestingly, our findings resolve most of the conflicting results in the literature listed above

as adol-DGNs aged less than a month or barely aged 1 month-old were shown as not involved (Snyder et al., 2005; Gu et al., 2012; Ash et al., 2023) and adol-DGNs aged between 1 and 2 months were shown to be involved (Snyder et al., 2005; Farioli-Vecchioli et al., 2008; Gu et al., 2012). We studied adol-DGNs in the context of spatial learning because allocentric learning, which is hippocampal-dependent, is put in place around late adolescence. We highlighted an unknown transient role for adol-DGNs in setting-up the ability of spatial learning. However, it seems worth mentioning that even though adolescent animals start to navigate through space, whether or not they use allocentric strategy remains unclear. In order to conclude, it would be interesting to determine if the inactivation of the DG (or the new neurons) during adolescence impairs the emergence of allocentric memory. Given that adol-DGNs lose their implication in spatial learning once adu-DGNs overtake this function, it seems adequate to suggest that they must have other functions in the network. Intriguingly, it has been shown that adu-DGNs inhibit neo-DGNs (Ash et al., 2023), which raises the question of the interaction between the different cohorts of granule neurons during memory processing.

Concerning adu-DGNs, short-term delay ablation (by irradiations, pharmacological or transgenic approaches) of immature adu-DGNs (and only them due to the short delay) often concluded on their non-involvement during the spatial learning phase (Shors et al., 2002; Wojtowicz et al., 2008; Lemaire et al., 2012; Groves et al., 2013; Martinez-Canabal et al., 2013a; Mateus-Pinheiro et al., 2021). However, the reduction in their number by ablation or transgenic manipulation resulted in spatial memory retrieval impaired abilities (Deng et al., 2009; Zhang et al., 2008). Besides, enhancing neurogenesis via EC stimulation was shown to improve spatial memory performances once the stimulated-produced neurons reached some degree of maturity (Stone et al., 2011a). Additionally, our laboratory has shown a functional involvement of adu-DGNs that are immature during learning in long-term memory retrieval performances once they also reach some degree of maturity (Lods et al., 2021, 2022). Therefore, while immature adu-DGNs seem to be irrelevant to navigate through space, they seem to be needed for memory retrieval. Only one study argue in contradiction with this working hypothesis, as immature adu-DGNs alteration did impair the spatial learning phase (Farioli-Vecchioli et al., 2008). However, this study altered their adu-DGNs population by prematured maturation, which could therefore tend to classify them as mature adu-DGNs. While this separation seems clear, it seems important to mention that the role of immature adu-DGNs in spatial learning does not stop at their own functional involvement but also encompasses their influence on other granule neurons. Indeed, immature adu-DGNs were shown to modulate the remapping of mature granule neurons which

acquired place fields by altering their firing field, which helps non-overlapping representations (Tuncdemir et al., 2023).

Finally, long-term ablation by irradiation (which encompassed both immature and mature adu-DGNs) did not impair spatial learning, spatial memory retention, or both (Saxe et al., 2006; Meshi et al., 2006). Similarly, transgenic ablation of mature adu-DGNs by GCV injection had no effect on spatial learning or memory retention (Deng et al., 2009). However, when we manipulated mature adu-DGNs in the laboratory by different means (pharmacological (Ara-C), transgenic manipulation, chemogenetic inhibition), we observed impaired performances during both spatial learning (Dupret et al., 2008; Lemaire et al., 2012) or memory retrieval testing (Lods et al., 2021). Similarly, their chemogenetic stimulation during the memory test was shown to improve retrieval performances (Lods et al., 2022). Therefore, in our hands, there seems to be an involvement of mature adu-DGNs in both spatial learning and memory retention, a working hypothesis supported by IEGs studies (Kee et al., 2007; Tronel et al., 2015a).

To confirm this working hypothesis, we adopted the same dual approach for adu-DGNs as we did for adol-DGNs taking into account if adu-DGNs were immature or mature. We showed that immature adu-DGNs are not recruited by spatial learning. We also showed that their inhibition during learning did not impact learning performances or memory retrieval. It seems worth noting that inhibition was only done during the learning phase. Therefore, it could also be interesting to inhibit immature adu-DGNs during retrieval as well, to determine if it has any impact, given that the literature suggest their function during this phase. On the other hand, our results showed that mature adu-DGNs are recruited by learning using IEGs and that their inhibition during learning impacted memory retrieval. However, their inhibition did not impact the learning performances themselves. Additionally, in our aging experiments ([Article 3](#)), mature adu-DGNs stimulation did not improve learning performances either, but only had an effect on memory retrieval afterwards. We propose that this lack of effect of mature adu-DGNs manipulation during learning could be due to the few neurons infected by retroviral injections and therefore manipulated. It could be hypothesized that a larger population of neurons manipulated would result in learning alterations. However, it seems to be enough to impact memory performances. Altogether, we identified mature adu-DGNs as the population needed for spatial memory, the memory function we aimed at studying in the context of cognitive aging. Therefore, they became our main target to restore memory abilities with age.

II. EVOLUTION OF ADU-DGNS HEALTH DURING AGING

A. Early and progressive de-afferentiation of adu-DGNs

The DG “connectivity” has been poorly investigated in the context of inter-individual differences during cognitive aging ([Introduction – Table 1](#)). To summarize, the overall activity of granule neurons (as well as their number) has been shown to be similar between resilient and vulnerable animals. In contrast, the number of perforated synapses was shown to be reduced in the MML of vulnerable animals (Geinisman et al., 1986), which are believed to be representative of a structural modification resulting in a sustained augmentation of synaptic efficacy (Siekevitz, 1985). Given that the MML receives inputs from the MEC, it was hypothesized that resilience to cognitive aging is linked to the preservation of these inputs. In the same line of idea, the synaptophysin levels, a marker for pre-synaptic vesicles, indicated impaired connectivity in the MML and OML of vulnerable rats, corroborating the previous results even though synaptophysin is not specific of glutamatergic inputs (Smith et al., 2000). Astonishingly, the excitatory synaptic strength of the LEC inputs was similar according to the cognitive status (Tran et al., 2019). Yet, a drastic reduction in the plastic properties of entorhinal-DG synapses in middle-aged animals (8-10 month-old) was reported, placing limits in the communication of information between the these two regions (Amani et al., 2021).

Altogether, these data suggest a need for the preservation of EC projections to granule neurons for resilience to cognitive aging. However, there is a clear lack of investigation about the IML and the inputs projecting to this sub-layer. Here, we report for the first time a specific loss of glutamatergic inputs in the IML of adu-DGNs starting early in adult life around middle-age. We also studied the mitochondrial network of adu-DGNs, as they represent essential hubs at the levels of spines. The spines dynamics highly rely on mitochondria, which contribute to their formation, maintenance, elimination and functionality (Cameron et al., 1991; Li et al., 2004; Kasthuri et al., 2015; Datta and Jaiswal, 2021). Additionally, mitochondria depletion was shown to reduce spine density (Li et al., 2004). Interestingly, we also observed a reduction of the mitochondrial density in the IML across all studied ages. Unfortunately, given that we observed similar reduction of both PSD95 and mitochondrial density in the IML at our earliest time point (8 month-old), we cannot conclude on which one starts first, a situation resembling of a "chicken or the egg" dilemma.

On the contrary to the IML, we found a similar PSD95 density in adu-DGNs for the MML and OML between resilient and vulnerable animals across all studied ages, indicating no alterations in the EC to adu-DGNs connectivity. These results seem to be in contradiction with what has been described in the literature. Yet, while the mitochondrial density in the MML and OML remained similar according to the cognitive status around middle-age (8 and 12 month-old), we observed a spread of the decreased mitochondrial density to the MML and OML at old age (18 month-old). We propose that the mitochondrial alteration represent the “egg” in our dilemma, acting as a precursor to spine disruption and to the subsequent loss of inputs on the former spine. Expanding upon this hypothesis, it appears justifiable to speculate that reduced PSD95 density would likely have been observed at timepoints beyond 18 month-old in the MML and OML. Indeed, the two studies previously cited (Geinisman et al., 1986; Smith et al., 2000) were conducted on older rats aged 24-28 month-old, which likely allowed them to identify the eventual loss of inputs from the MEC that we might have detected at a later time.

In that working hypothesis of mitochondria alterations being the “egg”, the fact that mitochondria start by being disrupted only in the IML suggests differences between mitochondria networks within the dendritic compartment itself. Differences in mitochondria morphology and dynamics were already shown between cell types (Fecher et al., 2019) or even between the axon and the dendrites in a neuron (Duarte et al., 2023). However, our results would indicate an even further level of complexity in the mitochondrial network. This working hypothesis relying on mitochondria being the “egg” would require further experiments to confirm it, such as the specific rescue of mitochondria in adu-DGNs with the help of specific retroviruses. Additionally, this raises the question of the causes responsible for this mitochondrial deficiency in adu-DGNs, and more particularly why only in the IML. Although these questions remain largely unanswered, we can begin to speculate about potential reasons. The reduction of mitochondrial density could originate from mitochondrial mechanisms dysfunctions, such as fission or fusion. It could also originate from mitochondrial deposition (stop in their trafficking), which is dependent on the local calcium activity (Wang and Schwarz, 2009). As discussed in the introduction, the aging of the DG is highly sensible to changes in its milieu and to changes in circulating factors. From all the factors that have been shown to impact NSCs in the neurogenic niche, many could also alter mitochondria homeostasis in adu-DGNs, starting with corticosteroids. Indeed, glucocorticoids have been shown to stop mitochondria trafficking in neurons and lead to their accumulation around the soma and subsequently to energy deficiency and impaired synaptic transmission (Choi and Han, 2021). Therefore, the beneficial effects observed upon adrenalectomy and reduced corticosterone

exposition could also be linked to effect on mature adu-DGNs and not only NSCs as discussed in the introduction (Montaron et al., 1999). This mitochondrial deficiency in vulnerable adu-DGNs at early age clearly calls for more investigations to understand its causes but also to prevent it as a potential therapy for cognitive aging.

Nevertheless, given our lack of results about PSD95 density at later time points, we cannot exclude the possibility that we would not have observed a reduced density in the MML and OML. At least 3 non-exclusives hypotheses could explain these differences: i) the most likely hypothesis, as mentioned earlier, is that the animals in our experiments were younger than that the ones investigated in the other studies, ii) Geinisman and colleagues used electron microscopy on samples of small size and may have seen differences that we could have missed using our tool, especially given than it does not allow to distinguish between perforated and non-perforated synapses, iii) the differences between our results and the literature are due to the specific study of adu-DGNs, which have a different connectivity pattern than other ontogenic population and therefore possible different aging profiles. Indeed, differences in connectivity patterns between neo-DGNs and adu-DGNs have been evidenced in the young DG network: neo-DGNs were shown to have a higher mushroom density in the MML and OML compared to the IML. On the contrary, mature adu-DGNs display similar mushroom density in the IML, MML and OML (Cole et al., 2020). Given these differences to start with, it could be hypothesized that they do not evolve similarly. This hypothesis suggests that both Geinisman and Smith studies investigated dev-DGNs with different aging profiles than the adu-DGNs we investigated. It could indeed be hypothesized given that dev-DGNs are believed to be the most abundant population in the aged DG (Lagace et al., 2007; Ninkovic et al., 2007). In that case, the probability for Geinisman and colleagues to investigate dev-DGNs (and not the less numerous adu-DGNs) is much higher. However, by tagging neo-DGNs (aged P6), it was recently shown that 20% of the cells die within six months (Cahill et al., 2017), suggesting a possible renewal of the granule neurons population with age. If we consider that dev-DGNs may be prone to cell death during aging, then adu-DGNs may be more abundant in the aged DG. In that case, Geinisman and Smith would indeed have studied adu-DGNs, which brings us back to our initial hypothesis. Clearly, more investigations are needed to understand the heterogeneity in aging profiles in terms of connectivity patterns of the different ontogenic populations in face to aging.

Finally, we should also highlight the possibility that both mitochondria and inputs are consequences of primary alterations leading to the results we have observed. In this line of thoughts, a third possibility could rely on alterations of the environment of the synapses in the

IML itself. A synapse is not only composed of the pre-synaptic and post-synaptic compartment, but is enveloped by an astrocytic process, forming the tripartite synapse (Ventura and Harris, 1999; Witcher et al., 2007). Astrocytes play a role in synapses formation, maintenance and even pruning (Clarke and Barres, 2013). They are linked to adult neurogenesis, as they control the synaptic integration of newborn cells as well as spine density and maturation by supplying D-serine through vesicular release (Sultan et al., 2015). Interestingly, the synthesis of D-serine precursor, L-serine, is branching from glycolysis (Le Douce et al., 2020), a process that can be altered during aging and associated to memory impairments as discussed in the introduction (Gage et al., 1984b). Additionally, astrocytes processes were shown to be altered in the context of aging (Popov et al., 2021) and their functional decline during aging may contribute to cognitive deficits (Verkhatsky and Semyanov, 2023). In Alzheimer's disease, astrocyte dysfunction eventually leads to synapse loss and cognitive decline (Hulshof et al., 2022). In the context of inter-individual differences during cognitive aging, it could therefore be hypothesized that astrocytic dysfunction leads to the observations made in the IML. Thus, once the synapses are lost, the inputs projecting to it would degenerate and the mitochondria would migrate to other locations where they are needed. Unfortunately, nothing is known about aging astrocytes between resilient and vulnerable animals, especially in relation to adu-DGNs and according to the sub-layers of the ML, which calls for more investigations.

B. New players in the maintenance of memory abilities: mossy cells and SuM

We detected new early players in cognitive aging by conducting a pseudo-longitudinal study: the mossy cells and/or the SuM. Very little is known in the context of inter-individual variabilities about these two types of projections to the DG. However, some investigations about these cells have been made in the young adult network and have shown their involvement in spatial learning memory. Mossy cells were shown to have multiple firing fields on the contrary to granule neurons which only have one and sparsely fire (Danielson et al., 2017; GoodSmith et al., 2017; Senzai and Buzsáki, 2017). Even though the functional implication of this multiple firing pattern of mossy cells remains unknown, it indicates that they have a spatial implication. In terms of spatial memory retrieval, a function impacted in our aging experiments, mossy cells were shown to control the efficacy of the retrieval process (Li et al., 2021). In the context of aging and in models of Alzheimer's disease, mossy cells alterations led to spatial cognitive deficits (S. Li et al., 2022) and memory retrieval imprecision (Deng et al., 2020). While these studies suggest that these effects

are indirect with interneurons in between, we propose that in our results, impairments in the mossy cells projections to the granule neurons in the vulnerable population leads to spatial learning deficits and alterations in the retrieval of the memory.

The first hints towards an involvement of the SuM region in modulating the DG functions and more particularly spatial learning was due to the observation that the SuM is responsible for the theta frequencies needed for spatial learning in the DG (Pan and McNaughton, 1997). Later on, pharmacological inactivation of the SuM was shown to lead to spatial learning and memory retrieval deficits (Shahidi et al., 2004; Aranda et al., 2008). It has also been shown that the SuM-to-DG projections are needed for spatial memory retrieval due to the release of glutamate that helps coordinate the activity of both regions (Li et al., 2020). This SuM-DG relation needed for retrieval has been linked to adult neurogenesis as SuM-induced stimulation on the DG promoted proliferation and maturation of immature adu-DGNs (Li et al., 2022). Besides, this study suggests that higher retrieval abilities can also be achieved by stimulating SuM-to-DG inputs. This aligns with our results showing that stimulated resilient animals (with preserved inputs) are doing even better in the memory retrieval test. Additionally, the excitatory effect of SuM projections on granule neurons has been shown to facilitate their firing in response to EC stimulation (Hashimoto et al., 2018). Therefore, their loss around middle-age can also diminish the role of EC stimulation in memory, even though these inputs do not degenerate specifically.

C. Brain reserve, maintenance and compensation

If we place our findings on the evolution of adu-DGNs connectivity back into the context of brain reserve, maintenance and compensation discussed in the introduction (Nyberg et al., 2012; Stern et al., 2023), the three could actually be proposed here. None can be excluded given that we already observe preserved post-synaptic density in the resilient population at the shorter time point (animals: 8 month-old, adu-DGNs: 5 month-old). Following the brain reserve theory, resilient adu-DGNs would have more connections in the IML to start with compared to vulnerable adu-DGNs. It has been reported that immature adu-DGNs display a higher mushroom density in the MML and OML compared to the IML. They only reach balance between the three layers upon the completion of their maturation (Cole et al., 2020). It could be hypothesized that vulnerable adu-DGNs never finish their maturation and keep a connectivity pattern similar to the one they have while immature with higher density in the MML and OML than in the IML.

Alternatively, in the brain maintenance theory, both resilient and vulnerable adu-DGNs would have completed maturation and have balanced innervation in their three layers at the

beginning of adulthood, therefore starting on an equal footing. However, during aging, resilient animals would manage to maintain their connectivity while vulnerable ones would suffer from a progressive degeneration due to age-related challenges.

Finally, in the brain compensation theory, resilient and vulnerable adu-DGNs would also start on equal footing at the beginning of adulthood. However, resilient adu-DGNs would manage to compensate and increase their connections in order to cope with age-related challenges. This mechanism suggests a high potential for neuronal plasticity in already long mature and established neurons.

To start to tackle this question, we took advantage of experiments run in the lab (Charrier et al., *in preparation*) in which young adult rats injected with the M-rv-PSD95-GFP at 2 month-old were submitted to the watermaze at 4 month-old in the same protocol than for our aging experiments (**Figure 1A**). First, it is important to note that obtaining cognitively declined animals at this young age is difficult. Indeed, as depicted in **figure 1B** we can see that learning is much faster with only 6 days of training, and achieved by both cognitive populations. Nevertheless, we were able to extract “extreme” animals with small learning differences. We will keep naming them resilient and vulnerable, even though the designation here is extrapolated and the vulnerable animals actually only exhibit a one day-delayed learning profile. In this context, when the post-synaptic density is quantified in each sub-layer of the ML, we can observe no difference according to the cognitive status in any of them (**Figure 1C-1E**).

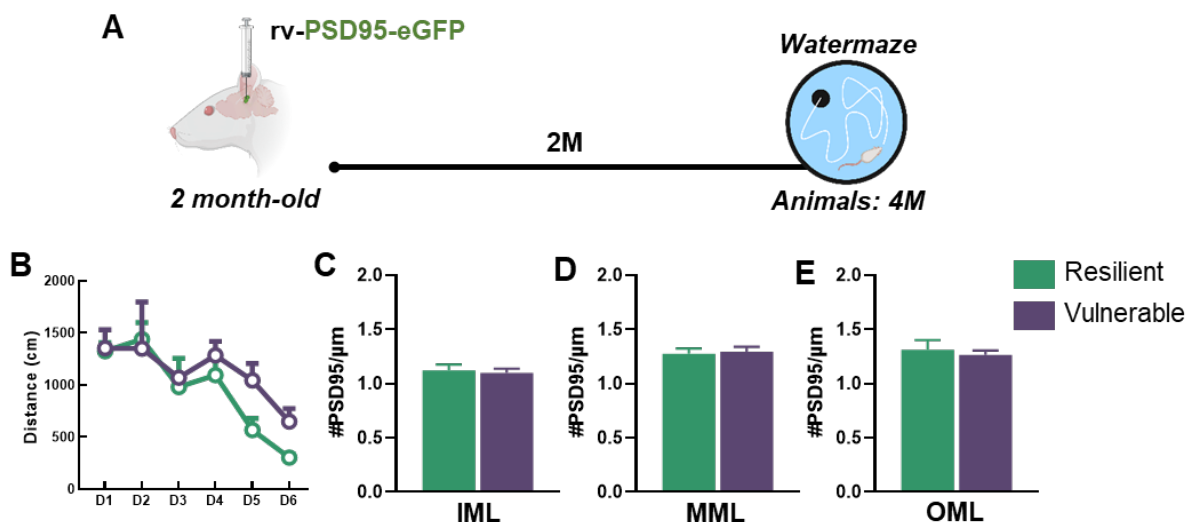


Figure 1: Young mature adu-DGNs display similar post-synaptic densities in each sub-layers between young adult resilient and vulnerable animals. (A) Schematic diagram of the experimental design. (B) Resilience to cognitive aging is not linked to the preservation of glutamatergic inputs in the IML at 4 month-old (unpaired t test: $t_{181} = 0.35$, $P > 0.05$). (C) Resilience to cognitive aging is not linked to the preservation of glutamatergic inputs in the MML at 4 month-old (unpaired t test: $t_{137} = 0.31$, $P > 0.05$). (D) Resilience to cognitive aging is not linked to the preservation of glutamatergic inputs in the OML at 4 month-old (unpaired t test: $t_{56} = 0.49$, $P > 0.05$). Data are presented as mean \pm S.E.M. from 3 extreme animals per group (a min of 3 neurons were analyzed per animal, with 4M-Res = 20 neurons and 4M-Vul = 19 neurons. Statistical significance * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$).

These observations rule out the brain reserve theory. It indicates that vulnerable adu-DGNs complete similar maturation than resilient adu-DGNs and that individuals start on equal footing for the criterion addressed here. It also indicates that in a period of 4 months, the deficits in the IML start to appear, either via brain maintenance or brain compensation.

D. Adu-DGNs and the hippocampal network

1. Inhibitory inputs

To conclude about the connectivity study made in the work presented in this thesis, it is important to mention that we focused on glutamatergic inputs, given the observations made in the literature about the EC inputs and the fact that adu-DGNs maintain their reactivity at old age in resilient animals. However, we have to acknowledge here the lack of investigation made about the inhibitory GABAergic inputs. Granule neurons receive many projections from a multitude of interneurons as discussed in the introduction, and impairments of this inhibitory connectivity could also lead to cognitive deficits due to the impairment of excitation/inhibition balance. Even though no differences were observed in the number of axo-dendritic synapses in the old DG according to the cognitive status (Geinisman et al., 1986), only the MML has been addressed, which does not rule out other loss in other layers, as they all receive different GABAergic projections (**Introduction – Figure 6**). Besides, a specific down-regulation of interneurons marker GAD67 and somatostatin has been reported in the hilus (Spiegel et al., 2013; Koh et al., 2014). Hilar interneurons project in the IML and OML (in addition to the MML), suggesting possible alteration in the GABAergic innervation of these layers, which could participate in the reduction of learning abilities with age.

Additionally, the DG also received long-range GABAergic projections from the SuM which co-release both glutamate and GABA (Pedersen et al., 2017; Hashimoto et al., 2018). Even though it remains a matter of debate if GABA is directly delivered to the spine or not, the

decreased innervation from this region could then also be correlated with decreased GABAergic release. Nevertheless, SuM projections were shown to mostly keep an excitatory effect on granule neurons (Hashimoto et al., 2018), which comforts us on the hypothesis suggesting that the loss of inputs from the SuM will prominently lead to decreased excitation of adu-DGNs. Other long range GABAergic projections from the septum (Amaral et al., 2007) and the EC (Germroth et al., 1989; Melzer et al., 2012) could also be at play. New tools available today, based on the same principle than the one used with rv-PSD95-GFP, relying on the expression of Gephyrin, a marker for inhibitory synapses, could be applied here. In the same way than for the glutamatergic innervation, each layer of the ML could be separately investigated to better differentiate between the different GABAergic inputs.

2. Whole hippocampal network

In our last optogenetic experiments, we observed an improvement of memory retrieval abilities in vulnerable animals when old adu-DGNs were stimulated during the learning phase. This indicates that vulnerable adu-DGNs are able to transmit the information that they integrated when stimulated, and therefore that their outputs are healthy. Indeed, we observed no alterations in their mossy fiber outputs morphology. These findings seem to be in contradiction with results reported in the literature, in which the DG-CA3 connections are replaced by auto-associational synapses CA3-CA3 in vulnerable animals (Buss et al., 2021). However, this paper investigated much older rats (28-29 month-old), and could therefore have detected alterations that we could not at earlier age. Nevertheless, the fact that MFBs are still morphologically similar between cognitive populations at our investigated age (20 month-old) indicates that adu-DGNs can transmit the information. Thus, it is not surprising that by optogenetically reactivating them we observed improved memory performances. The fact that the outputs eventually get deteriorated at older ages suggests that there is an age limit to which we can hope to restore memory functions, as the network for memory retrieval would be broken.

Indeed, memory retrieval is not a process that is confined to the DG but rather encompasses the whole hippocampus and some cortical regions, mostly the prefrontal one (Nakashiba et al., 2008; Wang and Morris, 2010; Zorzo et al., 2021). The multiple and vast information from the EC is received by the DG, which encodes it in an organized and pattern separated manner before encoding it in the CA3. If the network relation is interrupted, it could be suggested that memory retrieval would not be possible, even with adu-DGNs stimulation. Indeed, the disruption of the intra-hippocampal network communication between the DG and the CA3 has been shown to lead

to the loss of the memory trace (Madroñal et al., 2016). The granule neurons in the DG are needed during the retrieval process as our results and the literature indicate. The optogenetic inhibition of granule neurons activated during the learning of a fearful memory was shown to inhibit the expression of this fearful memory (Denny et al., 2014). In the same idea, stimulation of granule neurons necessary for the learning has been shown to be sufficient to elicit partial expression of the memory (Liu et al., 2012). Given these observations, we propose that the stimulation of mature adu-DGNs during spatial learning promoted the formation of a stronger memory trace than in non-stimulated animals. Upon memory retrieval, the memory trace was therefore strong enough to be retrieved. Interestingly, we still observed better memory retrieval performances in resilient stimulated animals. We propose that this is linked to the integrity of the inputs of resilient adu-DGNs, and more particularly the ones from the mossy cells and the SuM, which are as discussed before essential for spatial memory retrieval (Li et al., 2021, 2022). This suggests that inputs to the DG send spatial information during the retrieval test to promote the persistence of the memory and that preserved network in its integrity further promotes restored memory functions.

It would be interesting to investigate if the optogenetic stimulation of adu-DGNs was sufficient to counterbalance the loss of inputs in the IML, for example by promoting plasticity in the adu-DGNs and increasing their number of spines. Similarly, it might be possible that the CA3 hyper-activity and CA1 hypo-activity observed in vulnerable animals ([Introduction – Table 1](#)) has been compensated following stimulation. However, such a short-term manipulation of the network activity might not be sufficient to restore such large alterations in the network. Longer manipulation (by pharmacogenetics for example) might represent a good asset to restore the network on the long-term and obtain greater beneficial effects.

III. CONCLUDING REMARKS

A. Future directions

The research work described in this thesis mostly focused on the examination of *post-mortem* tissues. It allowed us to gain insights into the functional significance of the various ontogenic populations in spatial learning and to investigate the health of adu-DGNs in the aging brain. While these methods are valuable and suitable for addressing our initial scientific question, future research involving *in vivo* analysis on awake animals could add to the results presented. The first *in vivo* approaches that we adopted based on optogenetic manipulation allowed us to

better identify the function of granule neurons and to counteract our main focus in this work: cognitive aging. However, the necessity for tissues at each age made us adopt a pseudo-longitudinal study. While this approach is interesting as it allowed us to study early stages of aging around middle-age, we did not investigate the aging of the same animals nor the aging of the same population of adu-DGNs throughout the entire life. New technical approaches using miniscope and calcium imaging allow to image and follow neuronal activity at the single cell resolution. By applying this approach to our model, it would be interesting to follow the evolution of the activity of adu-DGNs within the same animals at different ages. Therefore, we could confirm *in vivo* the results reported in the second article indicating a reduced responsiveness of adu-DGNS in response to learning. Similarly, recent evidences highlighted that the *cfos* expression patterns in the DG change dramatically from day to day during watermaze learning (Lamothe-Molina et al., 2022). This suggests that the populations of vulnerable adu-DGNs we identified at the end of learning might be larger and that their recruitment vastly changes during learning. Additionally, this approach would also bring more insights about the evolution of the cognitive profile of an individual during its life, and would allow to conclude if the cognitive profile of an animal in one learning/memory test is the same in other types of memory. Nevertheless, it should be taken into account that multiple training might by itself change the cognitive profile of an individual. Interestingly, more recent technical innovations allow to merge both calcium imaging with optogenetics. In this way, it would for example be possible to visualize the activity of immature adu-DGNs during a task while inhibiting mature adu-DGNs, and vice versa, which would allow to better understand the interaction between the two populations.

Our findings did not conclude on the molecular mechanisms employed by adu-DGNs in the vulnerable population to undergo functional shutdown and leading to their reduced responsiveness during learning at old age. Our investigation on senescence remained inconclusive. We propose that the functional shutdown could be achieved by epigenetic regulation. As mention in the introduction, epigenetic modifications in the hippocampus are major indicators in the differences between resilient and vulnerable animals (**Introduction – Table 1**). The epigenetic reorganization allows the cell to control cellular activity via the down or up-regulation of gene expression. One of the most common epigenetic regulator consist of DNA methylation (Harman and Martín, 2020). Given that the hippocampus is characterized by a decline of methylation levels with age, a phenomenon that has been linked to decreased learning abilities (Gontier et al., 2018; Oliveira et al., 2012b), we propose that adu-DGNs in resilient animals should exhibit higher levels of methylation compared to adu-DGNs in vulnerable animals.

The work presented here allowed to highlight that old adu-DGNs produced during young adulthood play a part in maintaining memory abilities, which start to decrease around middle-age. This suggests that adu-DGNs start to face challenges early in their lives, and that they represent early indicators of cognitive aging. As discussed, such challenges can originate from the over (or absence) exposition to some circulating factors. Thus, establishing a molecular profiling of factors that can be predictive for future vulnerability to cognitive aging would be interesting.

Now that we have identified and validated that old adu-DGNs can help rescue memory performances at old age, more naturalistic approaches to preserve their health are needed in order to aim for translational research. In this way, it has been shown that environmental factors can have many deleterious effects, including the diet (Zainuddin and Thuret, 2012; Murphy and Thuret, 2015). Consequently, caloric restriction has been shown to have several beneficial aspects on adult neurogenesis. Therefore, one could suppose that the modulation of the diet adopted early in the adult age could have beneficial effects on the long-term.

B. Reframing cognitive decline

In the research conducted in this thesis, our primary objective was to gain a deeper insight into cognitive decline. Nevertheless, cognitive decline is an intricate and multifaceted phenomenon that actually involves various regions of the brain. With a focus only on the hippocampus, cognitive aging remains highly variable in terms of outcomes depending on the sub-region addressed. Because of this, we aimed at better identifying and study one of the many possible players in cognitive decline: adult neurogenesis and more particularly old adu-DGNs. However, it is essential to acknowledge that this process alone cannot account for the entirety of age-related impairments, nor can it single-handedly reverse the complexities of cognitive decline. In a collaborating brain-aging consortium with other research groups, we have identified several hallmarks that support resilience to cognitive aging, some already established and some that we believe are emerging in the field (**Figure 2**). These hallmarks differ from the well-established hallmarks of aging described in other organs, given the fundamental different properties of the central nervous system (López-Otín et al., 2013). Particularly, these hallmarks were defined mostly considering the hippocampus, and therefore might lack insights about the other brain areas.

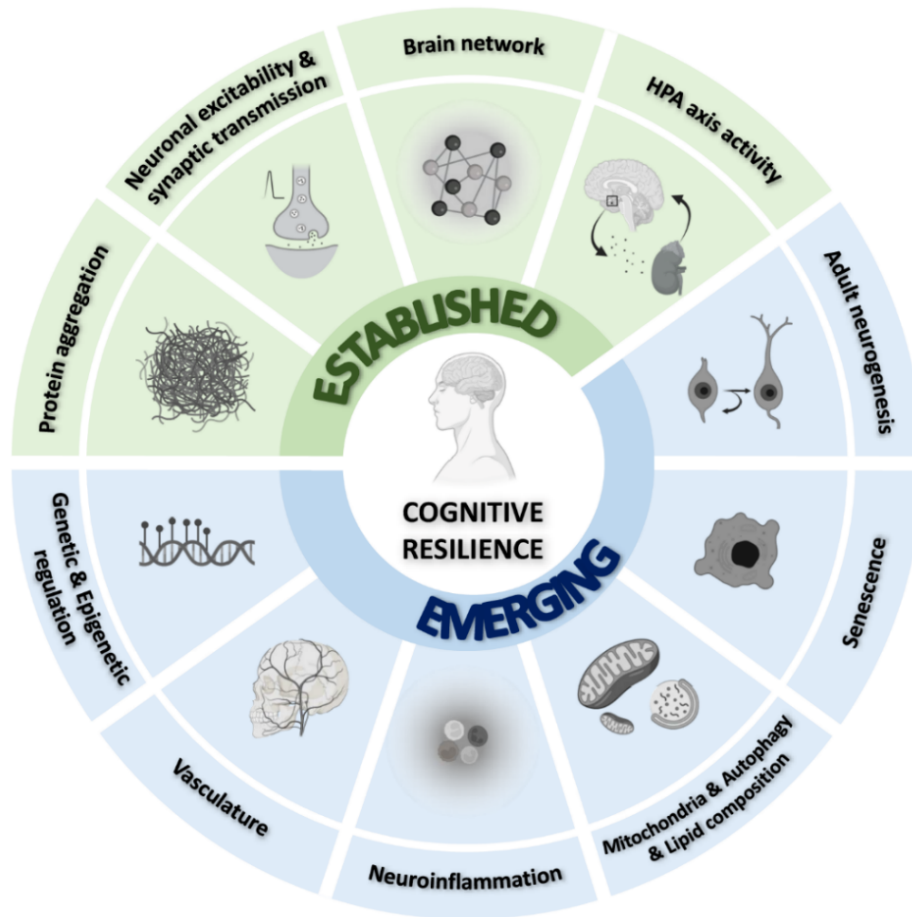


Figure 2: Hallmarks of resilience to cognitive aging.

This concluding remark therefore aimed at reminding that the adult neurogenesis alone, nor the DG or the hippocampus by themselves encompass the whole complexity of cognitive decline. The brain is an enormous network, and the ability of each region to interact and adapt according to the other represents the essence of its function in the body. Both the aging of each region and the age-related impact on the inter-regional communications have to be addressed and understood if we hope to one day help cognitive aging.

CHAPTER IV: REFERENCES

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