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Synaptotoxicity in Alzheimer's disease: Influence of APP processing on excitatory synapses

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THÈSE

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Préparée au sein de l'**institut des Neurosciences de Grenoble
INSERM U1216 – Equipe Neuropathologies et Dysfonctions
Synaptiques**

Dans l'**École Doctorale de Chimie et Sciences du vivant**

Synaptotoxicité dans la maladie d'Alzheimer : Influence du processing de l'APP sur les synapses excitatrices

Thèse soutenue publiquement le **6 décembre 2019**, devant le jury
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**Synaptotoxicity in Alzheimer's disease:
Influence of APP processing
on excitatory synapses**

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To my mams,

Résumé

La maladie d'Alzheimer (MA) est définie comme une maladie neurodégénérative où des altérations synaptiques mènent à la perte neuronale parallèlement à des défauts de mémoire et d'apprentissage. Il est établi que les dysfonctions synaptiques observées dans la MA sont initiées par les formes oligomériques du peptide β -amyloïde ($A\beta$), un dérivé protéolytique de l'Amyloid Precursor Protein (APP). Cependant, le chemin qu'empreinte $A\beta$, selon son origine intra- ou extracellulaire, afin d'induire ces effets délétères et la façon dont ses effets sont maintenus et se propagent dans le cerveau restent encore à définir.

Dans cette étude, nous avons utilisé plusieurs formes mutées de l'APP qui conduisent à des peptides $A\beta$ avec des signatures moléculaires uniques, tel que : la mutation Swedish (K670M/N671L) (APP_{swe}) qui augmentent la sécrétion (extracellulaire) d' $A\beta$; la mutation Osaka (E693 Δ) (APP_{osa}) qui cause une accumulation intraneuronale (intracellulaire) d' $A\beta$; ainsi que la mutation Icelandic (A673T) (APP_{ice}) qui a été établi comme diminuant la production d' $A\beta$ et protégeant contre la MA. Ces formes mutées d'APP ont été surexprimées dans des cultures de neurones corticaux murins et on permit : i) d'étudier la morphologie et fonction des épines dendritiques, l'élément post-synaptique, par microscopie confocale; ii) de tenter de mieux comprendre comment la pathologie se développe et se propage dans le cerveau et iii) d'identifier un nouveau partenaire d'interaction avec l' $A\beta$ faisant la lumière sur un possible rôle physiologique de ce peptide dans les neurones.

Nous montrons qu'une accumulation pathologique d' $A\beta$, due à la surexpression d' APP_{wt} , APP_{swe} et APP_{osa} mais pas APP_{ice} , induit une diminution significative de la densité synaptique particulièrement celle des épines les plus fonctionnelles, dites « mushroom ». Ses épines mushroom restantes présentent également une augmentation significative de leur volume et il semblerait que l' $A\beta$ intracellulaire soit suffisant pour induire ses effets. Ses épines mushroom élargies présentent également une plasticité structurale altérée puisqu'elles n'ont pas augmenté d'avantage de volume à la suite d'une activation synaptique. Il semblerait que ceci soit la résultante d'un défaut de la dynamique activité-dépendante du cytosquelette d'actine dans les épines. Ces altérations de la morphologie, structure et plasticité synaptique serait dû à une interaction, nouvellement identifiée, de l' $A\beta$ avec l'actine et pourrait faire lumière sur un possible rôle physiologique de l' $A\beta$ dans la plasticité synaptique activité-dépendante. De plus, nous montrons que le clivage amyloïde de l'APP est aussi activité-dépendant et que la séquence du peptide $A\beta$ généré est aussi importante, dans l'induction de la synaptotoxicité, que sa concentration. En effet, car nous montrons que des concentrations pathologiques du peptide $A\beta_{ice}$ n'engendrent pas de perte ou de gonflement des épines mushroom. Enfin, nous mettons en lumière que l' $A\beta$ sécrété dans le milieu extracellulaire affecte, non seulement le neurone sécrétant lui-même, mais aussi la densité synaptique des neurones sains avoisinant (qui ne surexpriment pas d'APP) d'une manière APP-dépendante, rappelant un mécanisme de propagation du type prion. L'ensemble de ces données démontrent que le clivage protéolytique de l'APP et la production d' $A\beta$ qui en découle est un processus finement accordé, impliqué dans le remodelage de l'actine dans la plasticité synaptique activité-dépendante et ouvre de nouvelles voies pour le développement de stratégies thérapeutiques contre la MA.

Abstract

Alzheimer's disease (AD) is defined as a neurodegenerative disorder where synaptic defects lead to neuronal loss and concurrent memory impairments. It is now well-established that synaptic dysfunction in AD is initiated by oligomeric forms of the amyloid- β peptide ($A\beta$), a proteolytic derivative of Amyloid Precursor Protein (APP). However, the pathway by which $A\beta$ induces its deleterious effects, whether it is due to intra- and/or extracellular $A\beta$ pools, and how these effects are sustained and propagated throughout the brain, are still unclear.

In this study, we used several mutated forms of APP which give rise to $A\beta$ peptides with unique molecular signatures, such as: the Swedish mutation (K670M/N671L) (APP_{swe}) which increases secreted (extracellular) $A\beta$; the Osaka mutation (E693 Δ) (APP_{osa}) which causes intraneuronal (intracellular) accumulation of $A\beta$; and the Icelandic mutation (A673T) (APP_{ice}) which has been reported to decrease $A\beta$ production and protect against AD. These mutated forms of APP were overexpressed in cultured mouse cortical neurons in order to: i) study the morphology and function of dendritic spines, the post-synaptic element of synapses, by confocal microscopy, ii) get a better insight into pathology development and propagation and iii) identify a novel interacting partner bringing to light the possible physiologic role of $A\beta$ in neurons.

We report that pathological $A\beta$ accumulation, due to APP_{wt} , APP_{swe} and APP_{osa} overexpression but not APP_{ice} overexpression induces a significant decrease in spine density especially mushroom spines, accompanied by a significantly increased volume of the remaining mushroom spines, and that intracellular $A\beta$ is sufficient to induce these effects. These enlarged mushroom spines have impaired structural plasticity as they did not increase in volume following synaptic activation seemingly as a result of defective activity-dependent actin dynamics in the spines. This alteration of synaptic morphology, structure and plasticity seems to be due to a newly-identified interaction between actin and $A\beta$, hinting a possible physiological role for $A\beta$ in activity-dependent synaptic plasticity. We also show that synaptic activity modulates amyloidogenic APP processing which, in pathological conditions, further exacerbates these synaptic defects. Furthermore, we show that $A\beta$ sequence is as important as $A\beta$ concentration in inducing synaptic alterations since pathological concentrations of $A\beta$ harbouring the Icelandic mutation had no effect on spine density or volume. Lastly, we bring to light that secreted $A\beta$, not only affects the $A\beta$ -secreting neuron itself, but also affects spine density of nearby neurons in an APP-dependent manner, reminiscent of a prion-like mechanism. Together these results demonstrate that APP processing is a finely tuned equilibrium involved in actin-remodelling during activity-dependent synaptic plasticity and opens a new route for AD therapeutic strategies.

List of Abbreviations

4-AP: 4 aminopyridine	Cdk5: Cyclin-dependent kinase 5	mGluRs: metabotropic Glutamate receptor coupled to G proteins
ABP: Actin-binding protein	CJD: Creutzfeldt-Jacob disease	MMSE: Mini Mental Status Evaluation
AC: Adenylate cyclase	CREB: cAMP response element binding protein (Erk activated transcription factor)	MRI: Magnetic Resonance Imagery
AChE: Acetylcholinesterase	CSF: Cerebrospinal Fluid	MTs: Microtubules
AD: Alzheimer's disease	CTE: Chronic Traumatic Encephalopathy	NEP: Neprilysin
ADAM: A Disintegrin and Metalloprotease (α -secretase)	C-ter: Cterminal	NFTs: Neurofibrillary Tangles
ADDL/Aβ-derived diffusible ligand/synonym of Aβ peptide	DAG: Diacylglycerol	NMDA: N-Methyl-D-Aspartic Acid
AICD: APP Intracellular domain	EE: Early Endosome	NMDAr: NMDA receptor
AMP/ADP/ATP: Adenosine Mono/Di/Tri-phosphate	EOFAD: Early-onset Familial Alzheimer disease	N-ter: N-terminal
AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	EPSP: Excitatory Postsynaptic potential	PAK: p21-activated kinase
AMPAr: AMPA receptor	ER: Endoplasmic Reticulum	Pen2: Presenilin Enhancer 2 homolog (γ -secretase)
AMPK: AMP-activated protein kinase	ERF: Extracellular signal related kinase	PET-FDG: Positron Emission Tomography
Aph1: Anterior Pharynx defective 1 homolog (γ -secretase)	EZ: Endocytic zone	PKA/C: Protein kinase A/C
APLP1/2: APP-like Protein 1/2	F- or G-actin: Filamentous or globular Actin	PLC: Phospholipase C
APOE: Apolipoprotein E	FADs: Familial Alzheimer's disease	PM: Plasma membrane
APP: Amyloid Precursor Protein	GABA(r): α -aminobutyric acid (receptor)	PP1/2(A or B): Protein phosphatase 1/2(A or B)
AP-V: D,L-2-amino-5-phosphonopentanoic acid 5	GSK3β: Glycogen Synthase kinase 3 β	PR: polyribosomes
ARF6: ADP Ribosylation factor 6	GWAS: Genome-wide Association Study	PrPc: Prion protein c
Aβ: Amyloid Beta peptide	HEK: Human Embryonic Kidney cells	PS1/2: Presenilin 1/2 (γ -secretase)
BACE1: Beta-site Cleaving Enzyme 1 (β -secretase)	iGluRs: Ionotropic Glutamate receptor	PSD: Postsynaptic Density
Bic: Bicuculline	IP3: Inositol-1,4,5-triphosphate	RE: Recycling Endosome
βsecl: β -secretase Inhibitor	KO: Knock-out	sAPPα/β: soluble APP α / β (N-terminal fragment)
C83/α-CTF: α C-terminal Fragment (Non-Amyloidogenic pathway)	LA: Life-act or Life-actin	Ser: Serine
C99/β-CTF: β C-terminal Fragment (Amyloidogenic pathway)	LE: Late Endosome	SPECT: Single Photon Emission Computed Tomography
CA1/3: Hippocampal Cornu Ammonis Region 1/3	LFU: Low-frequency Uncaging	SSH1: Slingshot protein phosphatase 1
CAA: Cerebral Amyloid Angiopathy	LOAD: Late-Onset Alzheimer disease	STEP: Striatal enriched phosphatase
CaMKII: Calcium/calmodulin-dependent protein kinase II	LTD: Long-term Depression	Tau: Tubulin-associated Unit
cAMP: cyclic Adenosine monophosphate	LTP: Long-term Potentiation	TGN: Trans-Golgi Network
CCV: Clathrin-coated vesicle	MAP2: Microtubule-associated Protein	TTX: Tetrodotoxine
	MAPK: Mitogen-activated protein kinase	
	MCI: Mild Cognitive Impairments	

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I. Alzheimer's disease

A. A brief bit of history

On the 25th of November 1901 at Frankfurt Hospital, German medical doctor Aloïs Alzheimer (**Figure 1**, left panel) receives a new patient. This 51 year-old woman named Auguste Dieter (**Figure 1**, right panel) has a set of marked cognitive disorders. Dr Alzheimer will note a reduction of memory and the comprehension of language, a very pronounced aphasia, an unpredictable behaviour, paranoia and auditory hallucinations (Maurer et al., 1997).

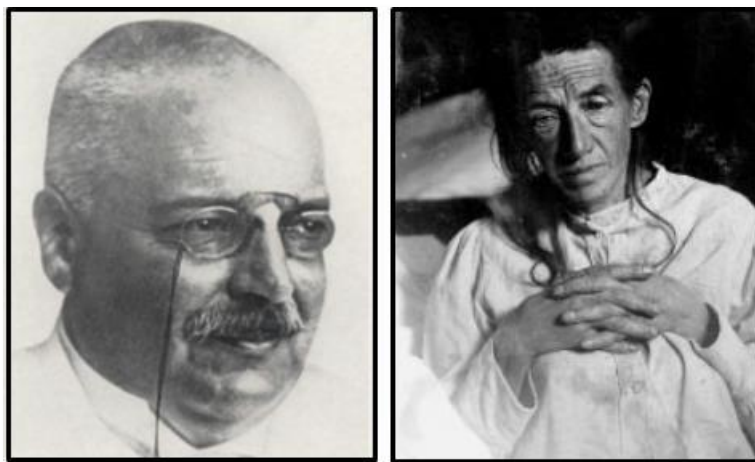


Figure 1: Portraits of Aloïs Alzheimer (left) and Auguste Dieter (right).

He observed, for example, that Mrs. Dieter was incapable of remembering the colour or shape of an object presented to her a few minutes before. She also had an Amnesic writing disorder and her spontaneous speech was full of paraphrasic derailments.

In 1906, Dr. Alzheimer will present this particular cognitive pathology that will take on his name in his article “Über einen eigenartigen schweren Erkrankungsprozeß der Hirnrinde” or “About a peculiar serious disease process of the cerebral cortex”. The post-mortem autopsy of Auguste D, in 1911, will allow the description of two particular histological markers, present in her brain, characteristics of her pathology: neurofibrillary tangles and senile plaques.

B. A worldwide issue for public health

With a world population growing older, developed, as well as developing, countries are facing new major social and economic challenges which are neurodegenerative diseases. The emergence of these pathologies is characteristic of aging societies. Between the years 2000 and 2050, the number of people of over the age of 60 will rise from 65 million to 2 billion. Such a rapid increase will require important economic, social and medical measures in certain countries. This rise in the proportion of older people is due to a “demographic transition”, corresponding to a decline in mortality and fecundity.

One of the principal consequences of this worldwide aging is the rise of pathologies such as dementia.

C. Dementia

Dementia is a chronic and evolving syndrome, where a person’s cognitive capacities are more strongly affected than someone aging normally. These cognitive functions such as memory, learning, reasoning, orientation and attention decline progressively and irreversibly. This will elicit sensory, motor and behavioural impairments, affecting an elderly person’s autonomy, creating a worldwide issue for public health; as this entails major medical and social costs. Several types of dementia exist, some can be classified and differ depending on what causes them.

The rise of Alzheimer’s disease (AD) sparked a strong interest in the scientific and medical community. Unknown to the general public four decades ago, AD is actually at the origin of the majority of dementias encountered in elderly people, roughly 60 to 70 %. This neurodegenerative pathology usually starts with marked memory deficits followed by a progressive decline of all the other cognitive functions like language, judgement and mood, ultimately leading to the death of the patient (the symptoms will be further described in **Part I.E.1**).

D. Statistics of Alzheimer’s disease

Since the early 80s, the scientific and medical community has been focusing on the identification of the symptoms, causes and risk factors of AD as well as potential therapeutic strategies. Although

the knowledge on this disease is considerably expanding, the biological alterations leading to AD are still unclear.

Amongst all the different types of dementia, 6 out of 10 cases are due to AD. Linked to aging, only 2 % of people under the age of 65 develop the disease and this is usually due to hereditary forms of the pathology which will be discussed further. However, AD affects more than 4 % of people over the age of 65, and 15 % of people over 80.

According to World Alzheimer Report, someone in the world develops dementia every 3 seconds. There were an estimated 46.8 million people worldwide living with dementia in 2015 and this number is believed to be close to 50 million people in 2017. These numbers will almost double every 20 years, reaching 75 million in 2030 and 131.5 million in 2050. Approximately 70 % of these cases will be attributed to AD.

According to “France Alzheimer & maladies apparentées”, in France, more than 850 000 people are affected by AD or a related disease. It is estimated that 1 in 4 people aged over 65 will develop AD by 2020, making this disease a true pandemic.

E. Clinical aspects of Alzheimer’s disease

1. Symptoms

- **Memory disorders**

One of the most obvious symptoms of AD is memory loss. Though forgetting someone’s name or not remembering where you put your keys is quite natural, the development of AD is characterised by the appearance of memory deficits. These deficits range from light anterograde amnesia (forgetting recent facts, such as forgetting an appointment or forgetting where an everyday object is in the house) to severe retrograde amnesia (forgetting older facts, such as historical events or the name of a family member...). The person is going to become more and more dependent on their entourage and is going to have to ask several times the same information without being able to retain it (Jahn, 2013; Sultzer et al., 2014).

- **Temporal-spatial disorientation**

Patients can get lost in familiar environments and lose notion of time. They can also have difficulties to understand something if it doesn’t happen immediately or forget where they are or how they got there.

- **Mood disorders**

AD patients are prone to mood swings and personality disorders. They can be delusional, get confused, anxious, depressed or even aggressive towards their close environment for no apparent reason (Mograb and Morris, 2014).

- **Apathy, indifference**

AD patients who suffer from apathy turn in on themselves; lose their interest and their motivation even for activities and hobbies they usually enjoy. They can appear indifferent and depressed, expressing very little or no emotions towards something whether it be good or bad (Mograb and Morris, 2014; Nobis and Husain, 2018).

- **Aphasia, language disorders**

People affected by AD can have difficulties in participating or following a conversation. They can have a hard time finding their words or sometimes just stop in the middle of a sentence without knowing how to finish it (Kirshner, 2012; Whitwell et al., 2015).

- **Agnosia, recognition impairments**

AD sufferers can have difficulties in recognising objects or people of their close environment without presenting any sensory impairment. Agnosia is often at the root of many behaviour disorders where patients have ill-adapted attitudes towards certain objects which they are no longer capable of recognising (Davis et al., 2012).

- **Apraxia, gesture impairments**

Apraxia, which is difficult to perceive in early stages of AD, is characterised by the patient's growing difficulties to accomplish gestures which require motor coordination. Over time the AD patient can forget acquired movements, lose dexterity and eventually lose the ability to accomplish elaborate tasks such as hand writing. At advanced stages of the disease, the patient can even forget how to execute the most simple of tasks such as brushing their teeth or chewing their food (Lesourd et al., 2013).

- **Progression of the disease**

Though the appearance of symptoms is gradual, this can greatly vary from one individual to another. In general, AD patients progressively lose their autonomy thus becoming more and more reliant on their entourage. Since the evolution of the pathology can take several decades,

establishing a general timeline of the progression of the disease which fits all patients is difficult. However, the medical community have distinguished three phases over the course of which the pathology develops (Sperling et al., 2014).

Firstly, there is the asymptomatic phase which can last more than a decade during which the patient undergoes anatomical as well as biological modifications, such as loss of neuronal density or variations in cerebrospinal fluid (CSF) composition. At this stage, these modifications do not induce any symptoms, more than likely due to compensatory mechanisms. From a clinical stand point, at this stage, the patient could only be distinguished from a healthy individual via elaborate neuropsychological tests.

Then comes the pre-dementia symptomatic phase which lasts 3 to 5 years, called MCI, for Mild Cognitive Impairments, during which the patient can still accomplish everyday tasks although certain cognitive impairments appear but without loss of autonomy. During this phase, the patient suffers from light executive function and memory deficits but not yet dementia (Eshkoo et al., 2015; Popp et al., 2015) (**Figure 2**).

Finally comes the symptomatic phase of dementia, the most severe stage of AD where the MCIs have progressed into full blown cognitive deficits with the emergence of behaviour disorders (Aisen et al., 2010; Tan et al., 2014).

2. Diagnostic

In order to identify whether a person is affected by AD or not, doctors firstly need to identify the presence of some form of dementia. For that, they use neurophysiological and behavioural tests. The most common test is the MMSE (for Mini Mental Status Evaluation) which globally evaluates cognitive functions (Derouesne et al., 1999).

Since dementia is not always due to AD, doctors must then investigate for specific signs of the pathology. They usually resort to magnetic resonance imagery (MRI) (Colliot et al., 2013) which allows the following of the evolution of cerebral atrophy. They can also use Positron Emission Tomography with Fluorodeoxyglucose (PET-FDG) (La Joie et al., 2013) in order to obtain functional imagery and bring to light a potential hypo-metabolism of certain areas of the brain. Single Photon Emission Computed Tomography (SPECT) can also be used to detect cerebral hypoperfusion in the temporal areas which is a characteristic lesion of AD (Valotassiou et al., 2010). Lumbar puncture can also be performed, as well as amyloid PET scans, in order to verify for the presence of Amyloid Beta

42 peptide ($A\beta_{42}$) and Tau protein (both are histopathological biomarkers associated with AD which will be described in **Part I.G**) in the cerebrospinal fluid (CSF) and brain, and confirm the diagnosis for AD (Olsson et al., 2016; Palmqvist et al., 2015). $A\beta$ accumulation starts early on in the development of the pathology and reaches a plateau when clinical symptoms occur. Biomarkers of synaptic dysfunction appear after and are strongly correlated with the severity of clinical symptoms. Tau protein accumulates later in the CSF and is correlated with Neurofibrillary Tangles (NFTs) and neuronal death (**Figure 2**).

Finally, other exams are performed in order to discard other causes of dementia (vitamin deficiency, hormone imbalance, infection, stroke...). Whilst these sets of exams enable doctors to assess quite specifically whether a patient is affected by AD, to this day there still isn't a formal diagnosis other than post-mortem brain autopsy.

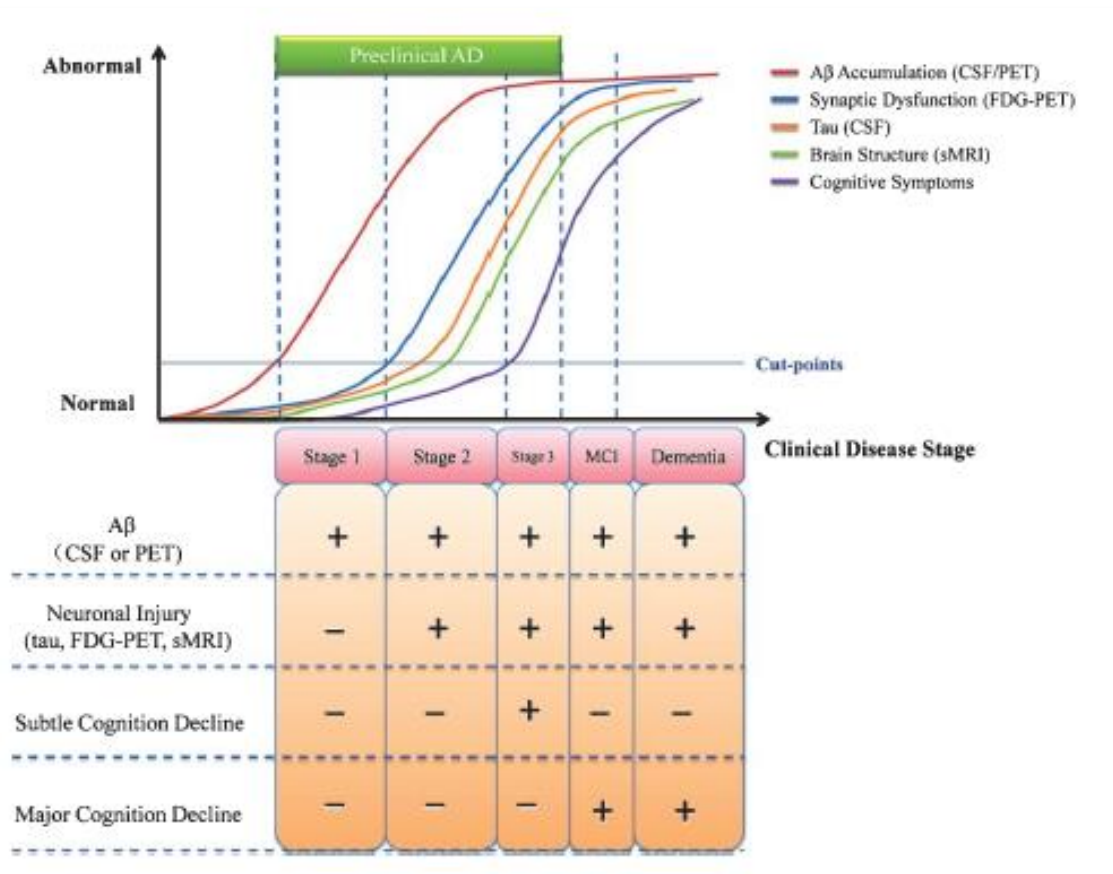


Figure 2: Timeline of the apparition of AD biomarkers and cognitive impairments at preclinical stages (adapted from Tan et al., 2014). The horizontal axis indicates clinical stages of AD: preclinical AD, Mild Cognitive Impairments (MCI), and dementia. The vertical axis indicates the relative values of each biomarker. $A\beta$ is identified in cerebrospinal fluid (CSF) $A\beta_{42}$ ELISA assays or PET amyloid imaging. Synaptic dysfunction is evidenced by functional imagery (FDG-PET or MRI). The horizontal "cut-points" line represents the threshold for the identification of the different stages.

Recently, several promising lesser-invasive biomarkers have been brought to light as potential predictors of AD and brain amyloidosis. Cofilin 2 has been shown to be upregulated in the serum of AD animal models, AD patients and patients with MCI compared to controls (Sun et al., 2019). Also, high-precision plasma $A\beta_{42/40}$ in combination with age and APOE $\epsilon 4$ status (a genetic risk factor for AD, which will be further described in **Part I.E.2.a**) has been shown to be a very accurate predictor for AD and could be used in prevention trials to screen for individuals likely to be amyloid PET-positive and at risk for AD dementia (Schindler et al., 2019).

F. Different forms of AD & risk factors

For several decades, AD was classed into two possible clinic cases depending on the age of the onset of the pathology. If a person younger than 65 years of age was diagnosed with AD, it was considered a “presenile dementia”, where as if a similar diagnosis was given to a person over 65 it was considered an “Alzheimer-type senile dementia” (Roth et al., 1967, 1966; Tomlinson et al., 1970). To this day, there isn’t any formal evidence showing that AD is different depending on the age of onset. More recently, neuro-imaging, epidemiology and neuropathology research have highlighted the fact that AD is a multifactorial disease. On one hand, certain genetic factors are responsible for “familial forms of AD” (FADs) and generally result in an early onset of the pathology, some of these genetic factors will be further discussed below. On the other hand, other factors such as environmental factors seem responsible for “sporadic” AD, much more prevalent and with a later onset. Nevertheless, age still seems to be a factor in the development of AD and may reflect the effect of accumulating different risk factors throughout life.

1. Familial Alzheimer’s disease (FAD)

Familial Alzheimer's disease (FAD) or early-onset familial Alzheimer's disease (EOFAD) is an uncommon form of Alzheimer's disease that usually strikes earlier in life, usually between 40 and 50 years of age and is inherited in an autosomal dominant fashion (Bertram and Tanzi, 2005). Familial AD requires the patient to have at least one first-degree relative with a history of AD. Nonfamilial cases of AD are referred to as "sporadic" AD, and encompass the majority of AD cases where genetic risk factors are minor or unclear. The genetic mutations which induce FADs are all localised on the genes coding for proteins involved in the production of $A\beta$: the Amyloid Precursor Protein (APP), Presinilin 1 (PS1) and Presinilin 2 (PS2) which are situated on chromosomes 21, 14 and 1 respectively.

A mutation on one of these genes will affect the production, the metabolism, the sequence and/or stability of the A β peptides found in AD brains (see Part II.F.).

Gene Symbol	Location	Function	Pathway
APP	21q21.3	Neuronal development, Synaptic formation and repair, β -Amyloid production	APP processing
PS1	14q24.3	γ -Secretase activity, Intracellular signalling, β -Amyloid production	APP processing
PS2	1q42.13	γ -Secretase activity, β -Amyloid production, Synaptic plasticity	APP processing

Table 1: Genes implicated in risk of early-onset Alzheimer's disease (adapted from Giri et al., 2016).

a) Presenilins PS1 and PS2

Presenilins 1 and 2 are part of the γ -secretase complex, essential to A β production. Mutations on these genes will alter the formation process of the A β peptide in favour of an increased synthesis and aggregation.

PS1 gene is located on chromosome 14q24.3, and it is a vital component of the γ -secretase complex, which cleaves APP into A β fragments. To date, 215 pathogenic mutations have been identified in PS1 and account for up to 50% of EOFAD, with early age of onset. Mutant γ -secretase increases A β 42 level while it decreases A β 40 level, leading to an increase in the A β 42/40 ratio. In addition to their role in γ -secretase activity, PS1 mutations may compromise neuronal function, affecting GSK-3 β activity and kinesin-I-based motility, thus leading to neurodegeneration (Pigino et al., 2003).

PS2 gene is located on chromosome 1q31-q42, and it is very similar in structure and function to PS1. PS2 mutations are very rare, and to date only 13 pathogenic PS2 mutations have been detected in 29 families (Cruts et al., 2012a). PS2 is a main component of the γ -secretase complex (Wakabayashi and De Strooper, 2008) and mutation of this protein alters the γ -secretase activity and results in an increase of the A β 42/40 ratio in a similar manner to the PS1 mutation (Steiner, 2004; Tanzi and Bertram, 2005). People bearing PS2 mutations have a later onset of the pathology. It has been shown that β -secretase activity is enhanced by PS2 mutation, through reactive oxygen species-dependent activation of extracellular signal-regulated kinase (Park et al., 2012). Although, PS2 shows close homology to PS1, less amyloid peptide is produced by PS2.

b) APP

Mutations in the gene coding for APP were first brought to light by the observation that people with Down syndrome (trisomy 21) presented similar neuropathological features as people with AD. This raised the question of the possible existence of genes located on chromosome 21 involved in AD pathogenesis. For the first time in 1991, a region containing the APP gene was identified and enabled the identification of a mutation implicated in an autosomal dominant form of AD (Goate et al., 1991). Currently, out of the 30 mutations observed on the APP gene, 25 of them are pathogenic. Indeed, most of the mutations on the APP gene are situated near or around the secretases' cleavage sites (α , β and γ). These types of mutations tune APP cleavage and, as a result, modulate not only A β peptide production but can also modulate A β 's three-dimensional folding and aggregation capacities (Streltsov et al., 2011). These APP mutations will be described in more detail in **Part II.F**.

While early-onset familial AD is estimated to account for only 3.5% of total Alzheimer's disease (Harvey et al., 2003), it has presented a very useful model in studying various aspects of the disease. Currently, the early-onset familial AD gene mutations guide the vast majority of animal model-based therapeutic discovery and development for AD.

2. Sporadic Alzheimer's disease

Sporadic or Late-Onset Alzheimer's disease (LOAD) is considered multifactorial and is genetically far more complex than EOFAD with the possible involvement of multiple genes and environmental factors. Most cases of LOAD are sporadic with no family history of the disease.

a) Apolipoprotein E

Allelic variations for the gene coding for Apolipoprotein E (APOE, the major cholesterol carrier in the brain), on chromosome 19, represents the main genetic risk factor for LOAD (Strittmatter et al., 1993). The APOE gene has three allelic variations: APO ϵ 2 allele (5-10%), APO ϵ 3 allele (70-75%) and APO ϵ 4 allele (15-20%). It has been shown that the APO ϵ 4 allele increases the risk for AD by 20% (Corder et al., 1994) and is associated with both early- and late-onset AD (Borgaonkar et al., 1993). The presence of one ϵ 4 allele is enough to increase three-fold the risk for AD whereas the presence of both copies increases the risk 12-fold (Michaelson, 2014). The APOE gene codes for a protein essential to lipid metabolism but also for hepatic clearance of A β peptides (Kline, 2012). Notably, a

study has shown that patients with AD because of APO ϵ 4 tend to have less A β in the Cerebrospinal Fluid (CSF) than healthy people, but this A β was found mostly in oligomeric form, suggesting a link between APO ϵ 4 and oligomeric forms of A β (Tai et al., 2013). Other studies have highlighted that the APO ϵ 2 allelic variant may have a neuroprotective effect compared to its homolog APO ϵ 4 (Corder et al., 1994).

b) Other genetic factors

Before the era of large-scale Genome-Wide Association Study (GWAS), ϵ 4 allele of the APOE gene was the only well-established risk factor for the pathogenesis of LOAD, but with technological advances, researchers have identified a number of regions of interest in the genome that may increase a person's risk for LOAD to varying degrees. It was striking to note that most of the genes identified by GWAS that could be linked with the A β cascade or tau pathology roughly cluster within three pathways: Lipid metabolism, Inflammatory response and Endocytosis (Giri et al., 2016) (**Figure 3**).

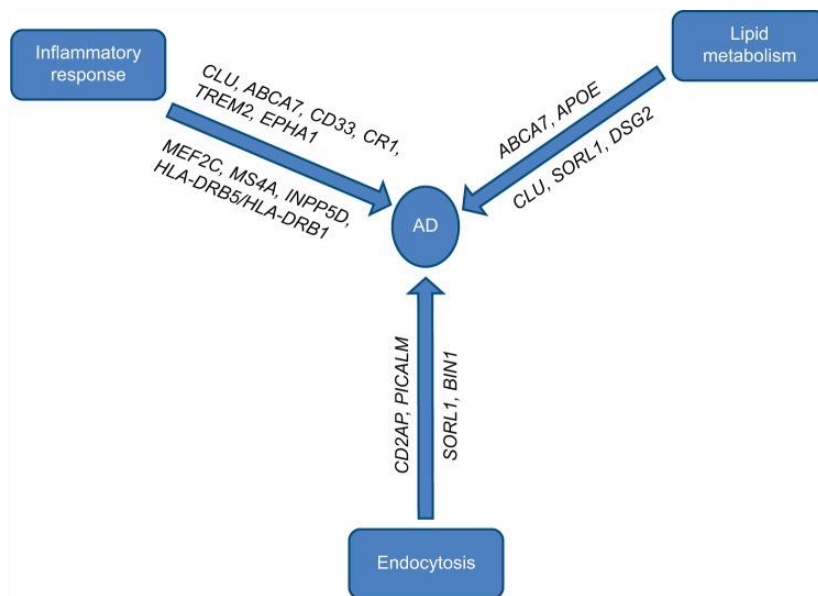


Figure 3: Major pathways involved in AD and affected genes (adapted from Giri et al., 2016)

These 29 or so genes and their role are briefly described in the table below (**Table 2**).

Gene	Function
APOE	Lipid transport, synaptic vesicle endocytosis, cytoskeletal dynamics
CLU	Synapse turnover, complement regulation, chaperone protein
ABCA7	Phagocytosis, lipid homeostasis
SORL1	Endocytosis, receptor for APOE, processing of APP
CR1	Amyloid β clearance, complement activation
CD33	Clathrin-mediated endocytosis, cell signalling
MS4A	Signal transduction, immune function
TREM2	Inflammatory response
BIN1	Synaptic vesicle endocytosis, APP trafficking, cytoskeletal dynamics
CD2AP	Receptor-mediated endocytosis, cytokinesis, cytoskeletal dynamics
PICALM	Clathrin-mediated endocytosis
EPHA1	Synaptic development, immune function, neural development
HLA-DRB5/ HLA-DRB1	Immune function, histocompatibility
INPP5D	Cytokine signalling, immune function
MEF2C	Myogenesis, synapse formation
CASS4	Cell migration, cell adhesion
PTK2B	Calcium homeostasis, MAP kinase signalling
NME8	Ciliary function, neuronal cell proliferation
ZCWPW1	Epigenetic regulation, neural development
CELF1	mRNA editing, pre-mRNA splicing
FERMT2	Cell–cell adhesion, angiogenesis
SLC24A4/RIN3	Cell signalling, neural development
DSG2	Cell–cell adhesion
PLD3	Signal transduction, epigenetic modification
UNC5C	Neural development
AKAP9	Signal transduction
ADAM10	Hippocampal neurogenesis, cell adhesion

Table 2: Common and rare gene variants associated with Alzheimer’s disease identified by GWAS (adapted from Giri et al., 2016).

c) Environmental factors

Despite the fact that sporadic or late-onset AD is the most frequent form of the pathology; its origin is still to be understood. From a clinical stand point the elements which spark LOAD are various and heterogeneous. Nevertheless, several environmental risk factors have been identified and associated to disease development. One of the predominant risk factors is lifestyle. Indeed, an improper diet rich in saturated fats, salt and refined sugars may lead to obesity, hypertension and type II diabetes. These metabolic disorders raise the level of inflammation in the body and increase the incidence of AD. Alcohol and tobacco consumption are also aggravating factors as they are known to accelerate cellular aging. Other factors such as environmental pollution due to neurotoxic metals (lead, mercury, aluminium, cadmium and arsenic) and/or pesticides may alter A β production

and Tau phosphorylation thus increasing the incidence of AD also (Chin-Chan et al., 2015). Furthermore, social context is also a determining factor in the development of AD. The level of education, physical activity and post-menopause treatments are amongst the parameters susceptible in modulating the risk of developing sporadic forms of the pathology (Dosunmu et al., 2007; Barnes and Yaffe, 2011).

Nonetheless, several neuroprotective environmental factors susceptible to slow down or even decrease the incidence of AD have been brought to light. Performing regular physical activity, consuming foods rich in omega-3 and foods rich in antioxidants (fatty fish, nuts, seeds, fruit, vegetables, green tea...), the use of non-steroid anti-inflammatories and having a cognitively stimulating environment are some of them.

G. Histopathological aspects of Alzheimer's disease

1. Macroscopic lesions

On a macroscopic level, AD is characterised by a cerebral atrophy mainly localised in the entorhinal cortex, hippocampus, amygdala, and in the temporal and frontal gyri. This translates as a weight reduction of the temporal, parietal and frontal lobes. Often a dilation of the cerebral ventricles can also be observed (Perl, 2010) (**Figure 4**).

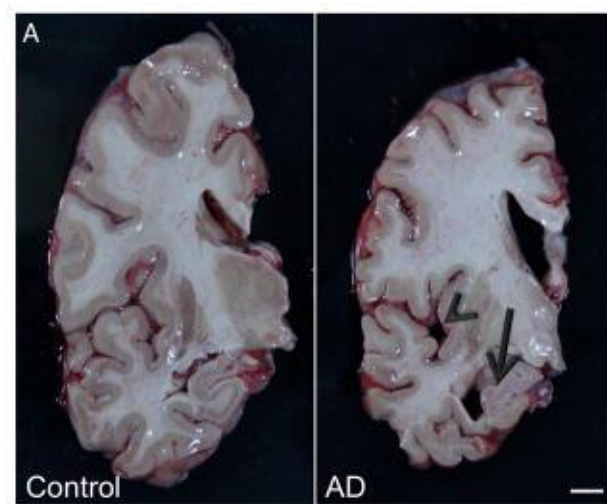


Figure 4: Cross section of a normal brain versus AD brain (adapted from Crimins et al., 2013). AD patient brains are characterised by gross atrophy of the hippocampus (arrow in A) and cortical thinning (arrowhead in A).

2. Microscopic lesions

Microscopic lesions that appear in the brain are not homogenous. The topography and the kinetics of the apparition of the lesions are crucial to identify the pathology. Two types of lesions can be distinguished, on one hand the ones associated with protein aggregation and on the other hand ones associated with synaptic and neuronal loss (Duyckaerts et al., 2009).

On a tissue level, two main histopathological lesions, hallmarks of AD, can be identified: intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated Tau protein, and extracellular senile plaques composed of aggregated A β peptides that form insoluble antiparallel β -sheets called fibrils (**Figure 5**).

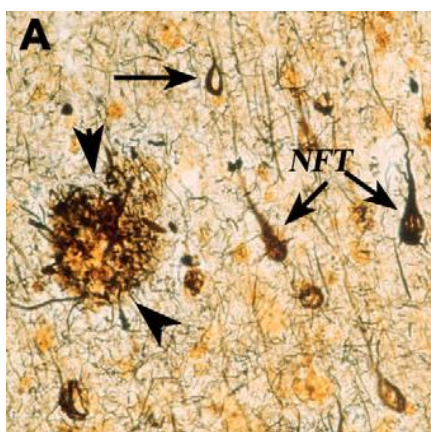


Figure 5: Hallmarks of AD (adapted from Nixon et al., 2007). AD brain stained with Bielschowsky silver revealing extracellular β -amyloid plaques (arrowheads) and intracellular neurofibrillary tangles (arrows).

It has been shown that these two hallmarks evolve differently in the brain during AD progression (Braak and Braak, 1991). A model of AD development, in 6 different stages, has been established (**Figure 6**).

NFTs first accumulate in the entorhinal cortex and hippocampus (**Figure 6**, stages I and II). During these so called “silent” stages, Mild Cognitive Impairments (MCIs) appear but are not determining signs of AD as these cognitive symptoms may also point towards potential other forms of dementia. Then, NFTs progress to the limbic (**Figure 6**, stages III and IV) and cortical (**Figure 6**, stages V and VI) areas of the brain. At this stage, Fully Developed Alzheimer is attained and cognitive deficits are significant.

As for amyloid deposits, which constitute senile plaques, they are initially found in the cortical areas (**Figure 6**, stage A). They then progress to the associated cortical areas such as the hippocampus (**Figure 6**, stage B) and finally to the rest of the cortex (**Figure 6**, stage C) including the sensori-motor cortex.

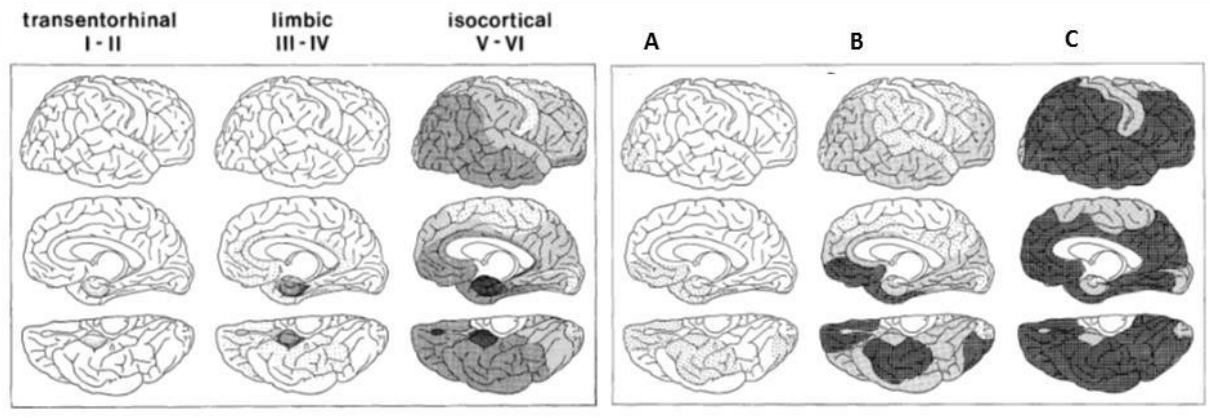


Figure 6: Evolution of the spatial distribution of NFTs and senile plaques during AD (adapted from Braak and Braak, 1991). The shades of grey show low (white) to high (black) density of NFTs (left panel) and senile plaques (right panel).

a) Intracellular Tau and neurofibrillary tangles

These lesions were first described by Alois Alzheimer in the early 20th Century, during the study of his patient's brain (Alzheimer et al., 1907, translated into English in 1995). However, it wasn't until the 1960s that electron microscopy studies revealed the exact composition of these lesions; namely: neurofilaments, microtubule associated proteins (MAP2), vimentin and tropomyosin proteins, elements of the proteasome, proteoglycans, inflammatory molecules, amyloidogenic proteins (APP, presenilin, APE) and elements of the cytoskeleton such as Tau protein (Smith et al., 1996).

Tau (Tubulin Associated Unit) protein is an organiser and stabiliser of microtubules (MTs). These MTs are, in a sense, the scaffold of cells as they shape and structure them. Tau phosphorylation allows the regulation of its binding to MTs. Unphosphorylated Tau has a higher affinity for MTs and so phosphorylated Tau remains detached from the MTs. The MT stabilisation and assembly role of Tau highlights its importance in axonal growth and cytoskeletal structure stability in the mature neuron. Interestingly, Tau is not the only MT-associated protein. *In vivo* studies in mice show that knocking out Tau does not significantly alter the stabilisation of the MTs. This suggests early developmental compensation mechanisms by other MAPs, mainly MAP1A which is found to be increased (D Ke et al., 2012). This compensation doesn't seem to occur in adult brains (D Ke et al., 2012), which might explain why Tau pathology causes neuronal MT instability.

In immature neurons, Tau is mainly present in axonal and somatodendritic compartments. In mature neurons, however, Tau is mainly found in the axon (Burack and Halpain, 1996) where they allow transport of cargo from the cell body to the axon terminals (Avila et al., 2016) by interacting with transport proteins like dynein and kinesin (Dixit et al., 2008). In addition, it has also been shown that the N-terminal projection of Tau has the ability to bind to the Annexin A2 protein at the level of the axonal plasma membrane, highlighting the ability of Tau to interact with cell membranes (Sotiropoulos et al., 2017). Through this membrane interaction, Tau is believed to play a role in various cell signalling pathways by interacting with transmembrane receptors (Guo et al., 2017). Interestingly, Tau has also been found in the nucleus, where it is believed to bind to DNA and plays a role of protection against stressful stimuli such as hypothermia or hyperthermia which can cause DNA damage (Guo et al., 2017). Additionally, Tau has been described to play a role in the regulation of genetic and epigenetic expression by binding and changing the conformation of DNA and its binding to histones. Furthermore, other roles have been found for Tau, including a role in the insulin signalling pathways (Jolivald et al., 2008) (**Figure 7**).

Although Tau is mainly found in axons, it is also present in somatodendritic compartments and post-synaptic densities (Wang and Mandelkow, 2016). In dendrites and dendritic spines, Tau has an important role in synaptic plasticity. Tau acts as a scaffold at the level of the synapses, both in pre- and post-synaptic compartments. Tau is known to play a role in synaptic activity, as it interacts with Fyn tyrosine kinase and binds to PSD-95, which regulates the function of post-synaptic receptors, such as NMDA receptors (NMDAR) (Ittner et al., 2010; Arendt et al., 2016) (**Figure 7**).

In pathological conditions, it is precisely at the level of the postsynaptic density that Tau has been proposed to exert its deleterious effects. In the case of AD, studies using live microscopy and confocal imaging have demonstrated that A β causes the mislocalisation and abnormal phosphorylation of Tau at the postsynaptic level, abnormally increasing its abundance in this compartment (Frandsen et al., 2014; Amar et al., 2017). This may increase the interaction of Tau with Fyn tyrosine kinase and PSD-95, increasing the synaptic activity through NMDAR, which might lead to excitotoxicity. Furthermore, a study showed that Tau is required to cause Long-term Depression (LTD) in the hippocampus (Kimura et al., 2014). This could mean Tau plays a role in downregulating AMPA receptors (AMPA), possibly leading to synapse pruning (**Figure 7**).

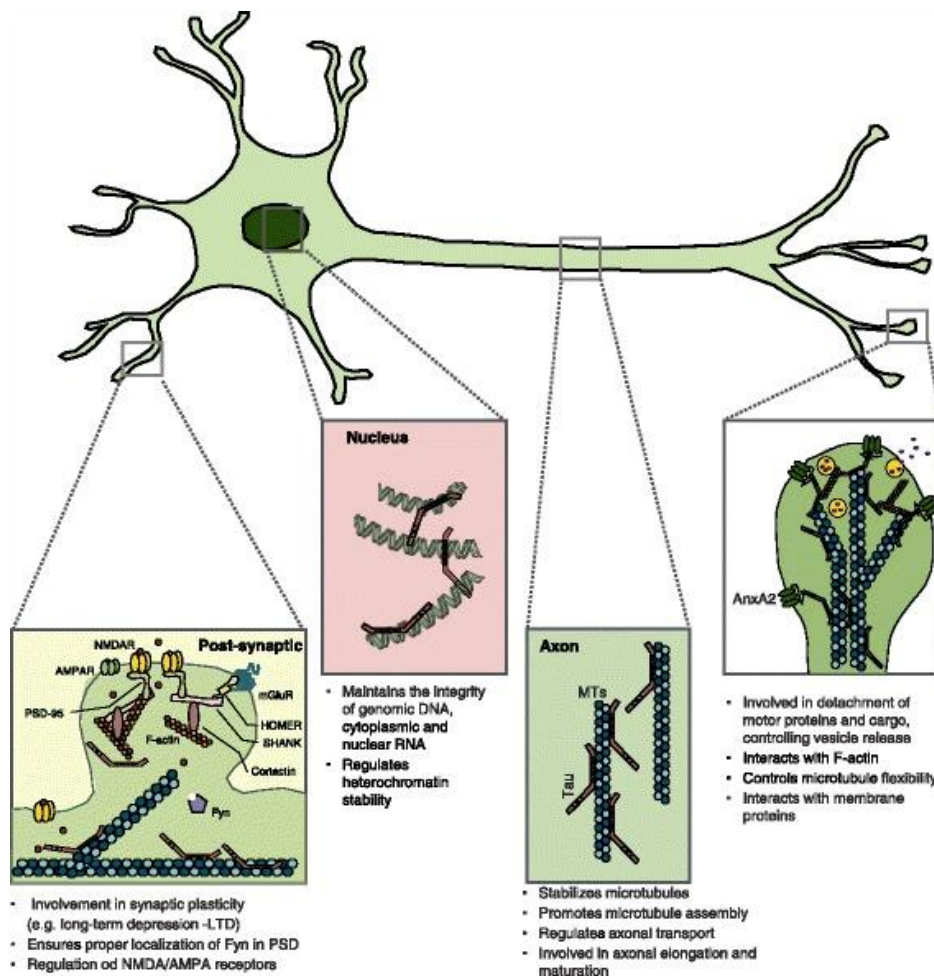


Figure 7: Identified functions and localisations of Tau in neurons (adapted from Sotiropoulos et al., 2017). Tau protein is located in several compartments in the neuron. In axons, Tau stabilises MTs and regulates axonal transport. At the synapses, Tau interacts with membrane proteins and plays a role in neuronal activity as well as synapse plasticity. In the nucleus, Tau protects DNA and nuclear RNA, while regulating genetic and epigenetic expression.

NFTs appear when Tau is abnormally phosphorylated and starts forming aggregates within the neuron. Studies have also reported that Tau cleavage by caspases generates peptides that are more prone to form aggregates (Gamblin et al., 2003). These peptides aggregate with the non-cleaved Tau around MTs, where Tau gets hyperphosphorylated. This causes Tau to unbind from the MTs and cause their destabilisation whilst simultaneously aggregating into NFTs and accumulating in the cytosol (Rissman et al., 2004).

It is now widely accepted that Tau protein is the major component of NFTs. However, NFTs aren't a determining factor for AD since they are also found in frontotemporal dementia and other neurodegenerative pathologies known as tauopathies (Delacourte and Buée, 2000).

b) A β peptide and extracellular senile plaques

Although described for the first time by Alois Alzheimer in the early 20th Century (Alzheimer et al., 1907, translated into English in 1995) after brain autopsy of his patient, the principal constituent of the extracellular senile plaques wasn't established until 1984 by Dr. Glenner's team. These plaques showed to be aggregates of a peptide named Amyloid Beta 42 peptide or A β (Glenner and Wong, 1984).

A β is a physiological peptide, produced throughout the course of life. Senile plaques, however, are characteristic lesions of AD where A β peptides aggregate in an aberrant manner. Amyloid deposits are absent in young individuals and gradually appear with aging. In fact, it is only when these plaques grow considerably in number and size that they induce neuroinflammation, which is characterised by the activation of brain resident immune cells.

Although A β is the major constituent of senile plaques, there are other elements that participate in the making of these plaques. Indeed, during the formation of these plaques A β binds with ApoJ (or clusterin) (Martin-Rehrmann et al., 2005), ApoE, cholesterol (Lesser et al., 2011), with cathepsin D, with components of the extracellular matrix such as thrombospondin, ICAM1 (Inter-Cellular Adhesion Molecule 1) and heparan sulfate proteoglycans, but also Ca²⁺, iron (Everett et al., 2018) and other metals (copper, zinc...) (Ha et al., 2007).

Furthermore, these amyloid deposits can aggregate into different forms in the brain (Duyckaerts et al., 2009; LeVine and Walker, 2010).

The first correspond to "diffuse" forms where the deposits are relatively large, up to several hundred micrometres in diameter. These have little immunoreactivity and are difficult to identify by immunohistochemistry. Congo Red and Thioflavine S (two dyes which allow staining of aggregates) do not allow the visualisation of these diffuse plaques as they are mainly composed of A β 40. However, they can be revealed with antibodies against A β (Güntert et al., 2006). These types of plaques can be found in large numbers and sizes in individuals who allegedly have no cognitive deficits, which raises doubts as to their toxicity in the brain. In fact, it has been proposed that these forms of plaques are an early form of senile plaques in certain brain areas (Duyckaerts et al., 2009; LeVine and Walker, 2010) (**Figure 8**).

The second form of plaques are described as "focal" which are spherical deposits much smaller in size but also much more dense. As they contain a lot more A β 42 molecules, these focal deposits seem to form the core of senile plaques. They are surrounded by processes coming from nearby

neurons and astrocytes (Duyckaerts et al., 2009; LeVine and Walker, 2010) (**Figure 8**).

Other amyloid deposits, described as “stellar deposits” have also been identified. Smaller and likely associated to astrocytes, these deposits are less studied than the previous aforementioned forms although often observed (Duyckaerts et al., 2009) (**Figure 8**).

In some cases, amyloid β can also accumulate in the walls of blood vessels, particularly in the ones present in the core of senile plaques, which induces CAA (Cerebral Amyloid Angiopathy) also known as congophilic angiopathy (Yamada and Naiki, 2012).

It's the form and properties of the amyloid peptides which induce the formation of fibrils and eventually plaques on the outside of neurons. However, some A β can be found intracellularly (Bayer and Wirths, 2010; Tomiyama et al., 2010; Thal and Fändrich, 2015). Quite intriguingly, this intracellular accumulation of A β 42 correlates much more with neuronal cell death than the formation of extracellular senile plaques (Christensen et al., 2008) and the smaller oligomers seem more toxic than fibrillary A β deposits.

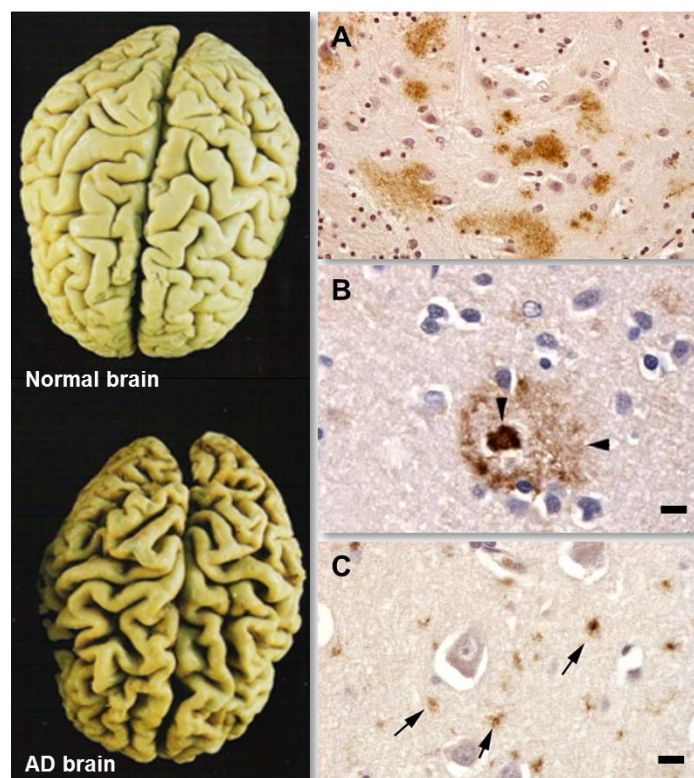


Figure 8: Macroscopic lesions and the different aspects of amyloid deposits (adapted from Duyckaerts et al., 2009). Left panel: Image of a normal brain versus an advanced stage of AD brain. Right panels: the different types of amyloid deposits identifiable by immunohistochemistry. (A) Diffuse amyloid peptide deposits. (B) Focal deposits (arrowheads) much denser, which form the core of amyloid plaques, surrounded by a halo of much less dense amyloid peptides. (C) Stellar deposits (arrows).

c) Links between A β and Tau

The hypothesis of the amyloid cascade originated from the observation that mutations which cause AD are always associated with alterations of the Amyloid Precursor Protein (APP) metabolism, leading to A β 42 overproduction particularly. On the contrary, Tau mutations do not lead to AD. Subsequently, it has been postulated that it may be the accumulation of neurotoxic amyloid peptides which drives Tau protein modifications leading to MT destabilisation, formation of NFTs, and eventually neuronal loss and memory deficits (Hardy and Higgins, 1992). Indeed, a study showed that A β 42 fibrils induce NFT formation (Götz et al., 2001). It has also been proposed that A β can induce Tau cleavage by caspases, generating Tau fragments prone to aggregate and form NFTs (Gamblin et al., 2003).

Nevertheless, NFTs have been observed in brains in absence of amyloid deposits (Braak and Braak, 1997) which suggests that both lesions occur independently and in parallel of one another, and that NFT formation might even precede amyloid plaque formation (Duyckaerts et al., 2009). Moreover, amongst the different transgenic mouse models of AD used in research, the ones that bare mutations only on APP and PS do not present any NFTs. On the other hand, the triple transgenic 3xTg-AD mouse model, developed by LaFerla's team, which bares the Swedish double mutation on APP (APP^{swe}), a mutation on PS1 (PS1 M146V) and a Tau mutation (MAPT P301L) develop NFTs. However, NFTs appear after amyloid plaque formation in this 3xTg-AD model (Oddo et al., 2003; Billings et al., 2005). The possibility of a synergistic interaction between both A β and Tau pathways in

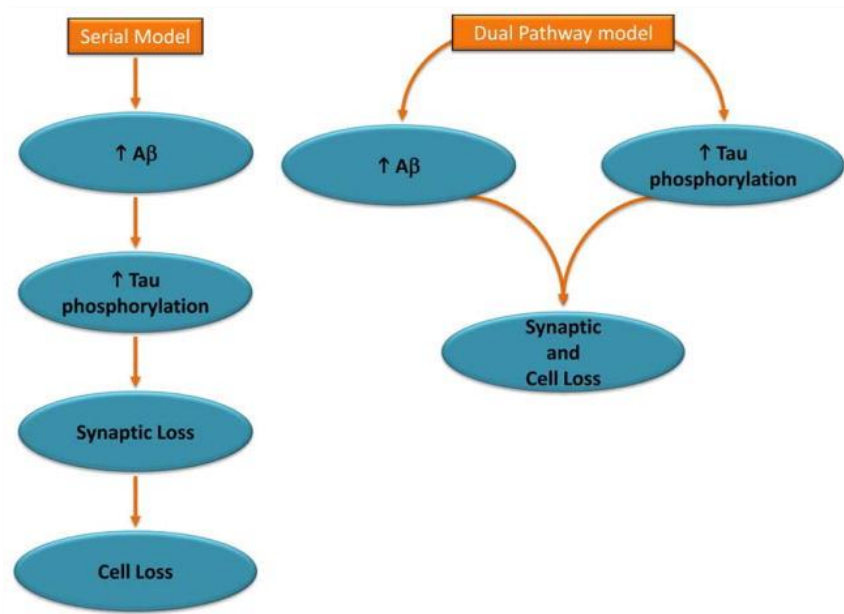


Figure 9: Two Hypothesized Models Linking Core Features of Alzheimer's Disease (adapted from Small and Duff, 2008). The amyloid hypothesis assumes a serial model of causality, whereby abnormal elevations in A β drive tau hyperphosphorylation and other downstream manifestations of the disease. According to the dual pathway hypothesis, A β elevations and tau hyperphosphorylation can be linked by separate mechanisms driven by a common upstream molecular defect.

the physiopathology of AD, driving synaptic dysfunction and neuronal degeneration, is widely accepted (Small and Duff, 2008) (**Figure 9**).

H. Propagation of the pathology throughout the brain

Extracellular A β deposits and intracellular Tau aggregates are both required to make the definite neuropathological diagnosis of AD. However, the interplay between these two proteins and the way they spread throughout the brain is still to be clearly determined. It is known that Tau pathology in the cell body of neurons precedes A β plaque formation and the two histopathological markers evolve and spread spatiotemporally differently in the brain. Indeed, Tau seems to spread outwards, from the centre to the cortex whereas A β deposits do the opposite, spreading from the cortex to the centre (Braak and Braak, 1991). As NFTs appear first, this was long used as an argument to say that Tau was the initiating factor of AD.

1. Propagation of Tau

From the observations made during brain autopsies of patients with tauopathies, that NFTs and other Tau aggregates progress in a stereotypical neuroanatomical pattern of spreading, came the hypothesis that Tau might spread from neuron to neuron. To support this hypothesis, several studies show that Tau is present in the extracellular compartment, that Tau can be released from donor cells and uptaken by recipient cells (Goedert et al., 2010; Mudher et al., 2017).

The secretion of Tau has been described to occur in multiple ways. Interestingly, the vast majority (90%) of secreted Tau is released as a free protein, whereas only a small fraction is bound to microvesicles such as exosomes or ectosomes (Katsinelos et al., 2018). The mechanisms of this secretion are still not fully understood, though it has been reported that Tau may exit the cell through diffusion or through the formation of nanotubes between cells (Tardivel et al., 2016). It has also been reported that phosphorylation of Tau increases Tau secretion through exosomes (Katsinelos et al., 2018). This makes sense in the light of the fact that phosphorylated Tau is more available for secretion as unphosphorylated Tau would rather tend to bind to MTs.

The uptake mechanism of Tau by recipient cells is not fully described yet but several studies seem to indicate that i) small oligomeric species of Tau, not monomeric, are spontaneously uptaken by the

recipient cells *in vitro* ii) this would occur through simple diffusion or endocytosis (Wu et al., 2013, 2016) (Figure 10).

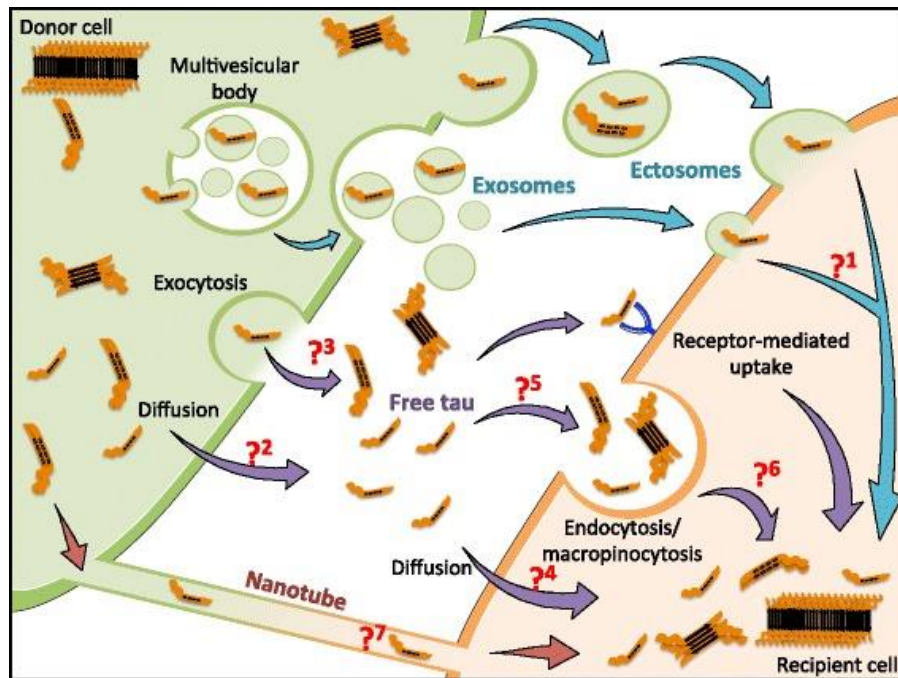


Figure 10: Different pathways identified for Tau propagation (adapted from Mudher et al., 2017). Tau proteins can be transferred from donor cells (green) to recipient cells (orange) using different routes. Pathway indicated by blue arrows: tau proteins are released in the medium by extracellular vesicles like exosomes and ectosomes. Violet pathway: Around 90% of tau in the extracellular space is found as free protein, passive diffusion facilitated by a membraneous transporter/receptor (?2) or active exocytosis (?3) might contribute to this process. Uptake of free tau species by recipient cells, including APP-mediated endocytosis has been reported. Whether free or aggregated tau is taken up by other mechanisms such as diffusion (?4) or non-receptor mediated endocytosis/macropinocytosis (?5) has not been resolved. Nor is it known how membrane-bound tau can escape from vesicles and enter the cytoplasm of recipient cells (?6). Red pathway: Tau was shown to be present inside nanotubes connecting cells *in vitro* and to allow its interneuronal transfer. This mechanism could potentially participate in prion-like propagation of tau pathology but whether it is a mode of transcellular transfer of seeding-competent tau species *in vivo* needs to be investigated.

Interestingly, Tau fibrils bind to transmembrane protein APP and increase its internalisation as well as increasing intracellular aggregation of Tau (Takahashi et al., 2015).

Most convincingly, several studies have showed that intracerebral injection of misfolded Tau (whether it be from brain extracts from transgenic mice with Tau P301S mutation or purified Tau fibrils from P301S brain extracts or preformed recombinant Tau fibrils) into the brain of transgenic mice expressing human wild-type Tau, not only induced Tau pathology but also the spread of this pathology along a path that is neuroanatomically connected (Ahmed et al., 2014; de Calignon et al., 2012; Iba et al., 2013; Dujardin et al., 2014; Mudher et al., 2017).

Additionally, Tau propagation is closely linked to neuronal activity (Wu et al., 2016). Indeed, an increased neuronal activity leads to an increase in the release and spread of Tau. The fact that Tau spreads along neuroanatomically connected tracts and that the spreading is neuronal activity-dependent strongly suggests that the propagation of Tau occurs in a transsynaptic fashion (Guo et al., 2017). Different conformers of Tau with a functional diversity have been identified and described as “strains” of Tau. Those strains of aggregated Tau seem to be stably transferred from neuron to neuron (Sanders et al., 2014). This means that an aggregated strain of Tau is able to leave a neuron, enter the recipient neuron and seed aggregation inside the new neuron in manner that templates its conformation (Kaufman et al., 2016).

Taken together, all these data point towards Tau as being a “prion-like” protein, as it has been proposed by Stanley Prusiner.

2. Propagation of A β

Although NFTs appear before A β deposits in the pathogenesis of AD, FADs are only provoked by mutations of genes implicated in APP cleavage; no mutations of Tau are able to induce or reproduce the symptoms of AD. Moreover, in the past twenty years, an increasing number of studies have highlighted the toxicity of the soluble oligomeric forms of A β that form long before the plaques, and NFTs, bringing oligomers of A β at the forefront of AD development (Walsh and Selkoe, 2007).

Intensive neuropathological examination of AD brains revealed that A β deposits appear to follow distinct pathways, progressively spreading through interconnected brain regions, rather than emerging randomly in the brain over time (Braak and Braak, 1991; Eisele and Duyckaerts, 2016). This gradual spread in the brain highlights an underlying, molecular mechanism evocative of A β acquiring a self-propagating conformation upon misfolding and aggregation (Condello and Stöehr, 2018).

The first hints that pointed towards A β as being a “prion-like” protein dates back from the 50s to the mid-80s. During that time, a number of children with conditions such as short stature were treated with growth hormone that had been isolated from cadaveric human pituitary glands. It was later discovered that some of these batches of hormones were contaminated with PrP prions, resulting in several children developing iatrogenic Creutzfeldt-Jacob disease (CJD) (Rudge et al., 2015). It has been recently discovered that some of these cases, who unfortunately died of CJD at ages ranging from 36 to 51, had significant amounts of A β in the form of A β plaques or A β deposits (Jaunmuktane et al., 2015). These findings raise the possibility that some batches of growth

hormones were not only contaminated by PrP prions, but also with A β seeds that originated from pituitary glands collected from patients who died with AD (Walker et al., 2016).

Though the most convincing evidence for A β self-propagation came from *in vivo* studies where A β aggregates derived from AD patients or aged transgenic mice were injected into young transgenic mice expressing human A β (TgAPP23) (Meyer-Luehmann et al., 2006). The injection of A β -containing brain homogenates was sufficient to induce potent deposition of endogenous, host expressed A β . Notably, the removal of aggregated A β by chemical denaturation or immunodepletion abrogated the seeding potential of these preparations, which designates A β directly as being the self-propagating entity. These experiments suggest a mechanism where A β aggregates form spontaneously in focal areas of the aging brain and start to self-propagate from neuron to neuron, whether it is via interconnected neural circuits or by physical proximity of the neurons (Wei et al., 2010).

More recently, further evidence for iatrogenic induction of A β suspected in patients exposed to pituitary-derived hormones, dural grafts or surgical instruments contaminated with A β has been brought to light. For the first time, long-term memory and learning impairments have been demonstrated in mouse lemurs (*Microcebus murinus*), a non-transgenic non-human primate model by inoculation with Alzheimer brain homogenates. The brains of these animals, which underwent intracerebral injections with Alzheimer human brain extracts, displayed parenchymal and vascular depositions of A β , as well tau lesions, close to the inoculation site (Gary et al., 2019). Furthermore, progressive cerebral atrophy and neuronal loss was also observed in the hippocampus and entorhinal cortex of these animals, which are interconnected brain structures. The results of this study further support the prion-like nature of A β and its self-propagation from neuron to neuron.

Another direct proof that A β oligomers are sufficient for self-propagation came from transmission studies using highly purified brain-derived A β or synthetic A β fibrils formed *in vitro* (Stöhr et al., 2012). The study highlighted that synthetic A β fibrils alone are sufficient for propagation in mice demonstrating quite evidently that A β shares the same self-propagating features as PrP prions. Moreover, A β shares additional similarities with PrP prions such as the rich β -sheet structure of A β fibrils as well as an increased stability towards chemical denaturation and protease digestion (Fritsch et al., 2014; Watts et al., 2014). Under harsh denaturing conditions, A β loses its self-propagating activity, highlighting that distinct structural motifs are required for templating and propagation (Meyer-Luehmann et al., 2006), bringing forward the notion of A β strains.

These A β strains and their impact on AD pathogenesis will be further described in **Part II.F.2**.

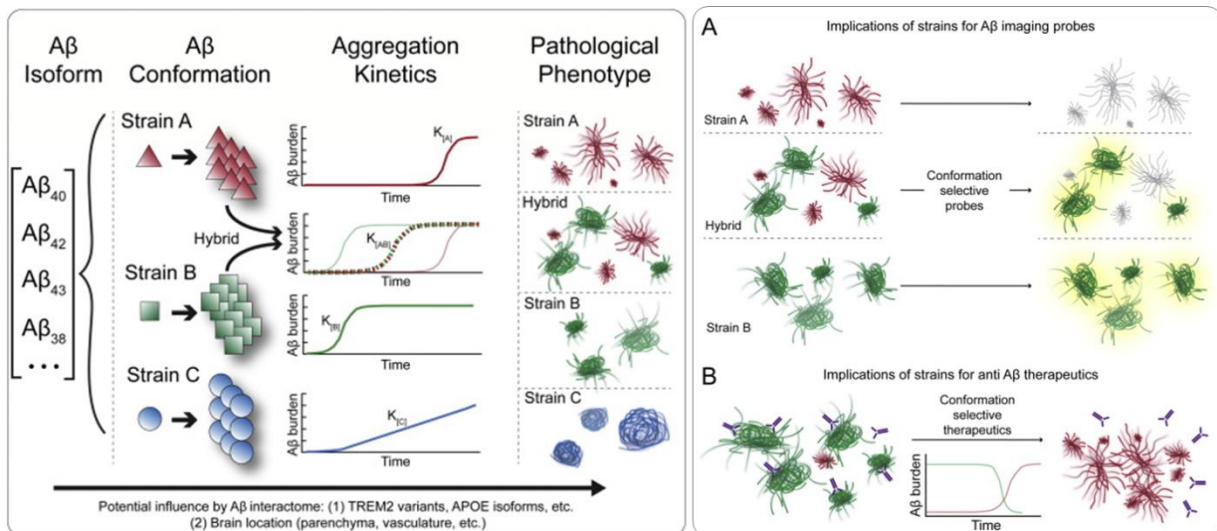


Figure 11: Self-propagating conformational strains of A β in AD and their potential impact on diagnostic tools and treatments (adapted from Condello et al., 2017). (Left panel) Different A β strains may lead to different pathological phenotypes and kinetics that may differ between patients. Given the wide heterogeneity seen sporadic AD, a mixture of strains could be observed in patients (“hybrid”) resulting in mixed/hybrid pathology. (Right panel) Differential strains of A β and potential hybrids make diagnosis and potential treatment even more complex.

3. APP-dependent propagation

Another possible mechanism for disease propagation involves the Amyloid Precursor Protein (APP), the transmembrane protein from which A β is cleaved, and a specific subset of 100nm diameter extracellular vesicles called exosomes. These exosomes, secreted by all mammalian cells, enable the transfer of material from one cell to another, allowing intricate intercellular communication. It has recently been shown in neuronal cells overexpressing APP harbouring the Swedish mutation (see **Part II. F. 1**) that a specific subset of exosomes originating from these neuronal cells is highly enriched with APP and its cleavage fragments, especially CTF- α and CTF- η (the proteolytic cleavage of APP will be further described in the next chapter). These exosomes specifically target and are endocytosed by other neurons where these CTFs and APP can undergo further processing. These results bring to light a new mechanism by which AD pathology may spread from neuron to neuron (Laulagnier et al., 2018).

II. Amyloid Precursor Protein processing and Amyloidogenesis

The Amyloid- β peptide ($A\beta$) is a cleavage product of a large transmembrane protein, namely the Amyloid Precursor Protein (APP), of which its role, function, trafficking and processing will be described below.

A. APP – Background

APP is a 130 kDa type-I transmembrane glycoprotein which is composed of a large extracellular N-terminal domain and a short cytoplasmic C-terminal tail (Müller et al., 2017). The APP gene is located on the long arm of chromosome 21 and contains 18 exons that are alternatively spliced. It is part of a protein family which includes APP-like protein 1 (APLP1) and APLP2, many of which are conserved across several species including invertebrate *C. elegans* and *D. melanogasters* (Shariati and De Strooper, 2013). Prokaryotes, plants, and yeasts do not appear to possess members of the APP family of proteins. The evolutionary appearance of the APP family of proteins therefore seems to coincide with the evolution of the earliest nervous systems with functioning synapses (Shariati and De Strooper, 2013) but also coincides with other events such as the appearance of lipoprotein receptors (Dieckmann et al., 2010). Interestingly, APLP1 is found only in mammals and lacks two exons found in both APP and APLP2, suggesting that it diverged during evolution from the latter gene (Zheng and Koo, 2011). Amongst these three genes, only APP contains the motif required for $A\beta$ formation. The APP splice variants range from 365 to 770 amino acids with three major $A\beta$ -containing isoforms: APP770, APP751 and APP695, with the number indicating amino acid length (Zheng and Koo, 2011).

APP770 and APP751 isoforms are expressed in most tissues. APP770 is the full length isoform which hasn't undergone any splicing. This isoform has a Kunitz domain, inhibitor of serine proteases, and an OX-2 domain, an antigen found on the surface of neurons and certain immune cells. These domains are found on exons 7 and 8 respectively. APP751 isoform undergoes splicing of exon 8 and so loses the OX-2 domain; whereas APP695, which is predominantly expressed in neurons, undergoes splicing of exons 7 and 8, losing both Kunitz and OX-2 domains (Coburger et al., 2013).

The extracellular domain of APP contains E1 and E2 domains. The E1 domain is reported to function as the major interaction interface for dimerization of cellular APP/APLPs (Soba et al., 2005). Although some studies suggest E2 domain has the same trans-dimerization capacity (Wang and Ha, 2004), biochemical assays have failed to confirm this phenomenon (Soba et al., 2005).

Besides its implication in different pathologies such as AD or Chronic Traumatic Encephalopathy (CTE), APP is also involved in numerous physiological processes, although its actual function remains unclear. These processes include synapse formation and repair (Priller et al., 2006), anterograde transport to synapses (Satpute-Krishnan et al., 2006), transport of iron (Duce et al., 2010), cell adhesion, via an RHDS motif similar to the adhesion region of integrins which can be found along with APP at the surface of axons, as well as neurite outgrowth via its interaction with integrin β 1 (Young-Pearse et al., 2008). APP also has binding domains to bind to different metals such as copper and zinc, and to bind to the extracellular matrix (heparin, collagen and laminin) (Thinakaran and Koo, 2008). In all, a trophic role as well as a cell adhesion role for APP has perhaps been the most consistently and arguably the best-established functions for the molecule.

As its name states, APP is a precursor, of $A\beta$ but also of other peptides. These peptides are produced via cleavage of APP by several enzymes called secretases. Amongst these secretases there are: I) the α -secretases, part of the ADAM (A Desintegrin And Metalloprotease) enzyme family (Vincent and Checler, 2012), II) the β -secretases, also known as BACE1 (Beta-site Cleaving Enzyme 1) (Chami and Checler, 2012), and III) the γ -secretases, composed of 4 proteins, Aph1 (Anterior pharynx defective 1 homolog), Pen2 (Presenilin enhancer 2 homolog), Nicastrin, and the Presenilins (PS1 and PS2) which form the catalytic domain of the γ -secretase complex (Wolfe, 2013; Gertsik et al., 2014). Depending on which secretase and in what order they will cleave APP, two main cleavage pathways can be distinguished: an amyloidogenic pathway, which yields $A\beta$ peptides, and a non-amyloidogenic pathway.

B. APP processing and trafficking

1. The amyloidogenic pathway, β - and γ -secretases

The amyloidogenic pathway is the pathway that generates $A\beta$ peptides by consecutive cleavage by β -secretase and γ -secretase. The first step in $A\beta$ generation is cleavage of APP at the β -cleavage site by β -secretase. The putative β -secretase BACE1 is a membrane-bound aspartyl protease with a characteristic type I transmembrane domain near the C-terminus (Vassar et al., 1999) and is thought to be the rate-limiting factor in $A\beta$ generation from APP. β -secretase is mainly found in Golgi membranes and endosomes where the environment is acidic, providing an optimal environment for BACE1 activity (Rajendran et al., 2006). Following β -cleavage, two fragments are generated: the large N-terminal sAPP- β fragment and the C-terminal fragment C99, also called β -CTF. sAPP- β has been

given a role in axonal pruning and neuronal death since a study showed that this soluble fragment may be a death receptor-6 ligand which activates caspase 6 (Nikolaev et al., 2009). However, the question about sAPP- β remains since some studies demonstrate that sAPP- β also stimulates neurite outgrowth (Chasseigneaux and Allinquant, 2012).

After β -cleavage, the C-terminal fragment β -CTF is then cleaved, at the transmembrane level, by the γ -secretase complex which completes the amyloidogenic pathway by rendering an A β peptide ranging from 38 to 43 aa depending on the location of γ -cleavage, and a final C-terminal fragment of APP called AICD (APP IntraCellular Domain) (**Figure 12**).

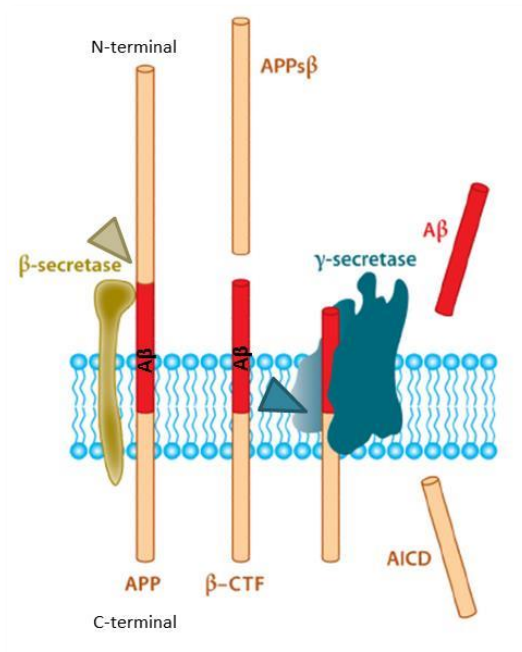


Figure 12: The amyloidogenic pathway (adapted from O'Brien and Wong, 2011). APP cleavage by β -secretase generates sAPP- β and β -CTF fragments. Subsequent γ -cleavage by γ -secretase generates A β peptide and AICD.

The γ -secretase complex is not specific to APP and has other substrates vital for the organism such as Notch (De Strooper et al., 1999). However, several modulators of γ -secretase activity have been identified to decrease A β production without interfering with cleavage of the other substrates (Kounnas et al., 2010), showing that A β production can be modulated without major side effects.

This final C-terminal fragment, which is common to both APP cleavage pathways, may act as a transcription factor as it translocates to the nucleus to regulate certain genes such as: p53 (a tumour suppressor), GSK-3 β (a kinase implicated in Tau phosphorylation) but also APP and BACE (Pardossi-Piquard and Checler, 2012).

2. The non-amyloidogenic pathway, α - and γ -secretases

This pathway is characterised by the cleavage of APP by α -secretase, the α -cleaving site is within the A β sequence of APP, precluding A β generation. Following α -secretase cleavage, two fragments are generated: the N-terminal fragment sAPP- α and the C-terminal fragment named C83 or α -CTF. This cleavage occurs at the plasma membrane. Studies report that the soluble sAPP- α fragment has neurotrophic properties and improves neuron survival *in vitro* and protects from excitotoxicity (van der Kant and Goldstein, 2015) despite having only 17 aa difference with sAPP- β . The α -CTF fragment then undergoes γ -cleavage and generates two new fragments: P3, which contains the aa 17 to 43 of the A β sequence, and the AICD fragment (**Figure 13**).

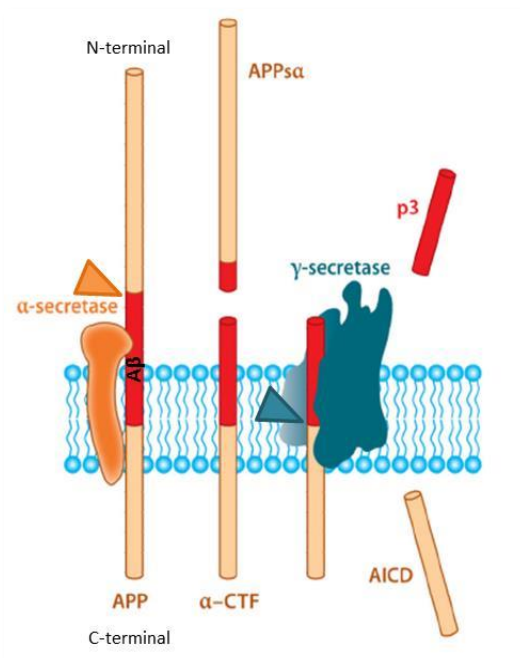


Figure 13: The non-amyloidogenic pathway (adapted from O'Brien and Wong, 2011). APP cleavage by α -secretase generates sAPP- α and α -CTF fragments. Subsequent γ -cleavage by γ -secretase generates P3 peptide and AICD.

3. Other cleavage pathways of APP

This binary vision of APP cleavage has recently evolved since the recent discovery of new cleavage pathways of APP. One particular pathway is the η -secretase (eta-secretase) pathway whereby APP is first cleaved by MT1-MMP and MT5-MMP (Membrane-Type Matrix Metalloproteinases) which have the η -secretase activity (Ahmad et al., 2006; Baranger et al., 2017, 2016) releasing a N-terminal

fragment sAPP- η and a C-terminal fragment CTF- η . CTF- η is then cleaved by γ -secretase, releasing an amyloid- η (A η) peptide and AICD. This new A η peptide has been found in brains of AD mouse models as well as in human AD brains (Willem et al., 2015). This discovery was made in a study with mice treated with a BACE1 inhibitor where the inhibition effectively decreased A β production but led to an accumulation of A η which induced significant neuronal impairments. In particular, Long-Term Potentiation (LTP), the electrophysiological correlate of memory and learning, was decreased in the hippocampi of these A η burdened mouse brains. These findings bring to light the possible negative outcome of using BACE1 inhibition as a therapeutic strategy to reduce A β load in AD brains in an attempt to alleviate memory deficits; and also strengthen the notion of the multitude of A β strains and their consequences, as well as the complexity of sporadic AD pathogenesis.

Other minor alternative cleavage pathways have also been discovered such as the δ -secretase pathway (Zhang et al., 2015) and cleavage by meprin (Jefferson et al., 2011) which won't be described in this manuscript.

4. Intracellular trafficking of APP

- Biosynthesis and progression through the secretory pathway

In non-polarised mammalian cells, APP follows the canonical protein maturation pathway (**Figure 14**). It is synthesised in the Endoplasmic Reticulum (ER) and then transported through the Golgi Apparatus to the trans-Golgi Network (TGN) and eventually to the Plasma Membrane (PM) (Haass et al., 2012). During its transit to the PM, newly synthesised APP undergoes post-translational modifications such as *N*- and *O*-linked glycosylation, ectodomain and cytoplasmic phosphorylation, as well as tyrosine sulphation. Based on APP overexpression studies in cultured cells, only around 10% of newly synthesised APP reaches the PM, whereas the majority of APP at steady-state is localised in the Golgi apparatus and the TGN. APP which eventually reaches the PM is internalised within minutes of arrival to the cell surface because of the "YENPTY" internalisation motif near the C-terminus of APP (residues 682-687 of APP695 isoform) (Lai et al., 1995; Marquez-Sterling et al., 1997). After endocytosis, APP is sorted to endosomes, a portion of endocytosed molecules is recycled to the cell surface and some are also sorted to lysosomes for degradation (Haass et al., 1992).

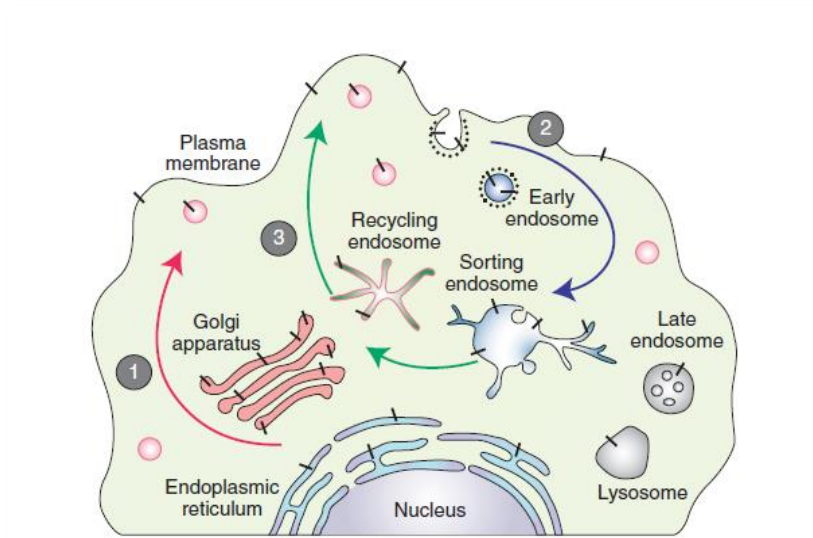


Figure 14: Intracellular trafficking of APP (adapted from Haass et al., 2012). Newly synthesised APP molecules (black bars) mature through the constitutive secretory pathway (1). Once APP reaches the plasma membrane, it is rapidly internalised (2), and sorted through the endocytic and recycling compartments back to the cell surface (3) or degraded in the lysosome.

- APP trafficking in neurons

Neurons are highly polarised into soma, axons, and dendrites, all of which perform different functions and therefore are equipped with distinct sets of proteins and lipids that finely regulate protein trafficking. Disturbances in this system could affect APP processing and have been linked to AD pathogenesis (Morfini et al., 2009). The transport from the ER to Golgi and TGN is thought to be similar in non-polarised mammalian cells and in the neuronal soma. However, after leaving the TGN in neurons, APP is transported to axons and dendrites in post-Golgi transport vesicles (Kins et al., 2006). APP delivery to the axons makes use of the fast axonal transport system, with kinesin-1 as the microtubule motor protein (Kins et al., 2006). APP vesicles continuously move unidirectionally, with an average speed of 4.5 $\mu\text{m/s}$, reaching maximal speeds up to 10 $\mu\text{m/s}$. This is among the fastest transport velocities measured in cultured neurons. Significant retrograde transport with slightly slower kinetics was also observed (Kaether et al., 2000). Little is known about the fate of the axonal transport carrier vesicles. Where do they fuse with axonal plasma PM? Where do the retrograde carriers go? A small fraction of the axonal APP has been suggested to undergo transcytotic transport to dendrites (Simons et al., 1995) but the significance and kinetics of this process need to be determined. Likewise, a detailed study of dendritic transport kinetics of APP is lacking. Furthermore, the sorting signals mediating axonal and/or dendritic transport of APP remain elusive as it has been reported that APP is transported into axons and dendrites without apparent sorting signals (Back et al., 2007). At this point, the only certainty is that APP trafficking in neurons is fundamentally different

from that of other cells, and more work has to be done to fully understand polarized APP sorting in neurons.

C. A β production and clearance

1. A β production sites

Although the amyloidogenic and non-amyloidogenic pathways of APP are well described, the actual localisation of APP and the α -, β - and γ -secretase complexes are still to be clearly identified as much as APP and A β trafficking in neurons. Notably, numerous studies show that A β accumulates intracellularly however the precise production site of the peptide is still under debate.

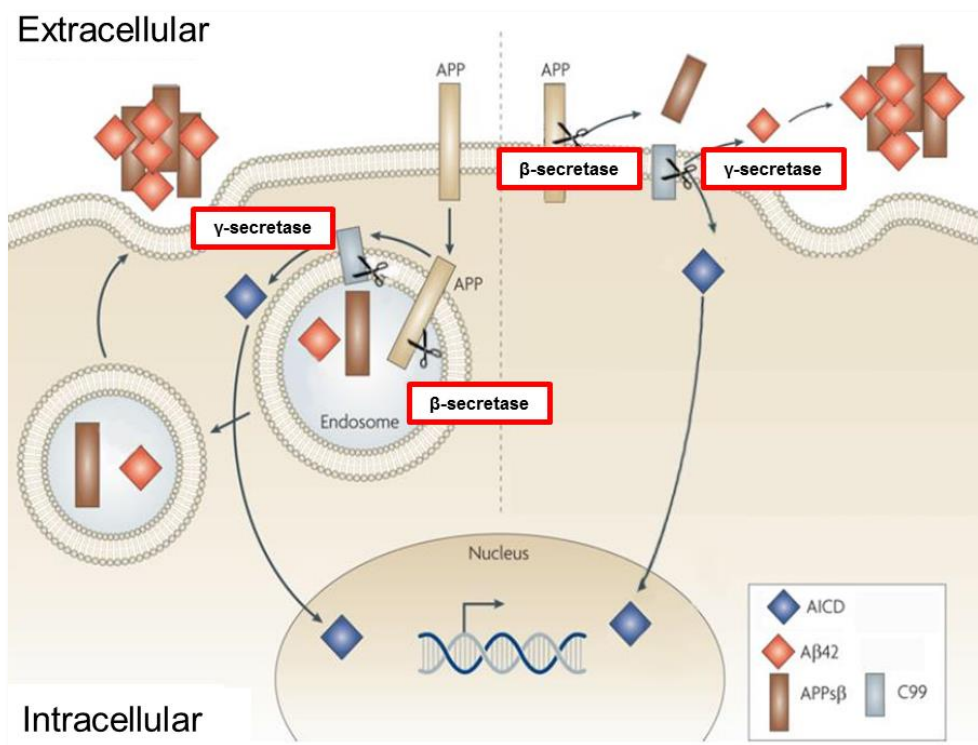


Figure 15: Possible A β production pathways (adapted from Aguzzi and O'Connor, 2010). APP is cleaved firstly by β -secretase, generating two fragments, sAPP- β (brown bars) and a membrane peptide C99 or β -CTF (grey bars). C99 is then cleaved by γ -secretase generating the A β peptide (orange squares). APP may be cleaved in two ways: either inside endosomes (left side) or directly at the plasma membrane (right side).

Two hypotheses have been proposed for A β production (LaFerla et al., 2007; Aguzzi and O'Connor, 2010):

- Either APP is endocytosed then cleaved, leading to an intracellular A β production possibly followed by A β exocytosis (**Figure 15**, left side).
- Or β - and γ -cleavage occurs directly at the neuron's plasma membrane and the release of A β into the extracellular space is immediate (**Figure 15**, right side). In this case, it is still to be determined whether A β or at least a fraction of the secreted A β is then uptaken by the surrounding cells to form intracellular pools of A β .

This is why it is important to understand where the APP fragments and the β - / γ -secretases are located in order to determine exactly where A β is produced during APP trafficking. Indeed, A β production may potentially occur where APP and β - : γ -secretase complexes are (for review: LaFerla et al., 2007; van der Kant and Goldstein, 2015).

- [Non-amyloïdogenic pathway](#)

On one hand, the APP found at the plasma membrane is mainly cleaved by α -secretase, releasing the sAPP- α fragment into the extracellular space and leaving the C83 fragment (or α -CTF) within the plasma membrane (**Figure 16**, 2) before being internalised and addressed to early endosomes (**Figure 16**, 3). Since α -cleavage occurs within the A β sequence of APP, it precludes A β production and therefore constitutes the non-amyloïdogenic pathway.

- [Amyloïdogenic pathway](#)

On the other hand, the APP that still hasn't been processed (**Figure 16**, 1) is endocytosed then internalised in early endosomes (**Figure 16**, 3). Early endosomes have optimal pH for β -secretase activity. When APP is addressed to endocytosis sites via its sequestration within lipid rafts, this precludes α -cleavage and therefore promotes the amyloïdogenic pathway.

- [β-secretase cleavage:](#)

Although BACE1 and APP can be found in separate intracellular vesicle pools they, however, can be found in the same somato-dendritic compartments in conditions of neuronal activity (Das et al., 2013). BACE1 and APP can also be internalized separately from the plasma membrane and interact with one another if they are in the same early endosome (Sannerud et al., 2011). Notably, while APP

is internalised via a clathrin-dependent endocytosis, BACE1 however is internalised via an ARF6-dependent (ADP Ribosylation Factor 6) endocytosis (Sannerud et al., 2011). As the pH at the plasma membrane (pH \approx 7.5) is not optimal for β -secretase activity, BACE1 cleaves APP only once it is internalised in the most acidic environment of the endosomal system (optimal pH \approx 4.5–5.5). Together these data suggest that APP cleavage by BACE1 takes place within early endosomes to generate sAPP- β and C99 (β -CTF) within the endosomal lumen. The sAPP- β fragment is then sorted to recycling endosomes to be readdressed to the plasma membrane (Figure 16, 4) or it can be sorted to the lysosome for degradation (Figure 16, 8).

C99 from β -cleavage and C83 from α -cleavage are retained within the membranes of early endosomes (Figure 16, 3) before being sorted to late endosomes (Figure 16, 5). Once they are in the late endosomes, these fragments are either addressed to the Trans-Golgi network (Figure 16, 6) and then exocytosed (Figure 16, 7) or sorted to lysosomes for degradation (Figure 16, 8).

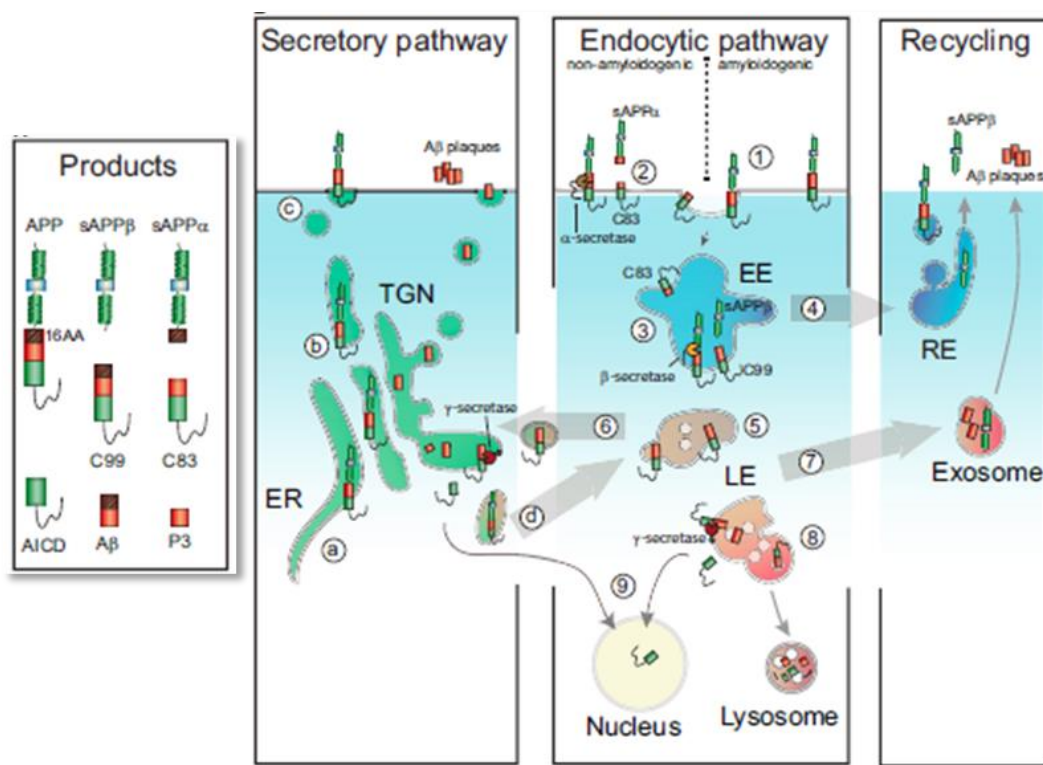


Figure 16: APP Proteolytic Products and Intracellular Trafficking of APP (adapted from Van der Kant and Goldstein, 2015). APP is synthesized in the ER (a) and trafficked via the TGN (b) to the PM (c) or endosomes (d). Full-length APP can be endocytosed from the PM (1) or cleaved by the α -secretase (2) to release sAPP α in the extracellular environment as well as generating C83 that is also endocytosed. In the early endosomes (EE), full-length APP is cut by the β -secretase generating C99 and sAPP β (3). Full-length APP and soluble APP fragments generated in the endosome might be recycled to the PM via recycling endosomes (RE) (4) or can be trafficked toward late endosomes (LEs) (5). In the LEs, these fragments can subsequently be sorted toward the TGN (6), exocytosed (7), or further trafficked to the lysosome for degradation (8). Cutting of C99 and C83 by the γ -secretase might occur in the TGN, the LE, or in both compartments, therefore possibly releasing A β and P3 either via the secretory pathway or via exocytosis. Cutting by the γ -secretase also releases the AICD that can translocate to the nucleus (9).

- [γ-secretase cleavage:](#)

According to the literature, the exact location of γ -cleavage is still currently under debate and it suggests that C99 (β -CTF) and C83 (α -CTF) cleavage by the γ -secretase complex occurs within the TGN and/or in late endosomes. Following this cleavage, A β or P3 is released via the secretory pathway or exocytosis depending on the initial β - or α -cleavage respectively. The third released fragment is AICD and may act as a transcription factor (**Figure 16, 9**).

The exact fragments generated by γ -cleavage vary in size. Studies have identified several A β peptides made up of 34 to 50 aa and approximately 90% of these A β peptides are A β_{40} . Although A $\beta_{42/43}$ peptides represent the smallest fraction generated by γ -cleavage, these peptides are the ones that are the most aggregation-prone and induce most of the neurotoxic effects observed in AD. These peptides can be found in senile plaques and are more inclined to undergo oligomerisation and fibrillogenesis (Kim et al., 2007).

In the light of these data, there is no consensus concerning the production site of A β . It can be produced in endosomes, in the TGN where APP undergoes strong glycosylation or both structures. It is possible that the location of β -CTF γ -cleavage, in late endosomes or in the TGN, might influence where the γ -secretase complex cleaves β -CTF and influence the size of the generated A β peptide; indeed, one location might favour A β_{42} production over A β_{40} . Furthermore, cleavage within the TGN could lead the generated A β peptide towards the constitutive secretory pool and therefore release A β into the extracellular space, facilitating amyloid plaque formation. Cleavage in late endosomes could release A β in the lumen of the organelle from which it could either be degraded later in the endosome or exocytosed to be secreted. It is also important to keep in mind that mutations on APP itself might also influence these parameters.

2. A β peptide degradation and clearance

A β peptides can be degraded by neuropeptidases, by truncation of their N-terminus in the cerebral interstitial environment (extracellular space) or cleared via specific receptors which allow them to cross the blood-brain barrier. Several A β degrading neuropeptidases have been identified (Miners et al., 2011) including neprilysin (NEP), insulin-degrading enzyme, and endothelin-converting enzyme. However, the neuropeptidase recognised as being the most efficient in degrading A β is NEP, a zinc-dependent metalloprotease enzyme (Iwata et al., 2000). It has been shown, for example, that A β_{42} degradation in conditions of synaptic activity occurs mainly via NEP but this clearing process

declines over time as it has been reported that NEP declines with age (Tampellini et al., 2009). Despite having identified the enzymes involved in A β degradation and clearance their regulation is still to be clearly determined. It has been reported that NEP could be regulated by AICD generated from γ -cleavage of APP (Grimm et al., 2013). In this light, γ -secretase could indirectly participate in the regulation of clearing mechanisms of A β peptides by NEP.

Taken together, this set of A β degrading enzymes constitute potential therapeutic targets capable of modulating endogenous A β concentration.

D. Toxic and physiologic roles of A β

A β peptides are naturally produced by neuronal metabolism and can be detected in the plasma or CSF of non-AD patients (Haass et al., 1992; Walsh et al., 2000). In these conditions, the physiological presence of A β peptides does not induce any neurodegenerative processes. Studies have shown that in physiological conditions a “normal” production of A β occurs but the proportion of the different forms of A β differ from the proportions found in pathological conditions. Particularly, the A β_{40} /A β_{42} ratio is modified in pathological conditions. Some studies suggest that the A β_{40} /A β_{42} ratio is a determining factor of toxicity, fibrillogenesis and pathological distribution of A β and highlight the fact that A β_{40} and A β_{42} have very different roles. A β_{42} promotes accumulation and deposition of amyloid plaques whereas A β_{40} does not (Kim et al., 2007; Jan et al., 2008).

In pathologic conditions, the A β_{40} /A β_{42} ratio is reduced. A number of studies have shown that during AD, A β_{42} is increased due to a hyper-activation of the amyloidogenic pathway and/or due to mutations affecting the actors of A β_{42} synthesis in both animal models and humans (Li et al., 2007) and/or due to defects in A β clearance mechanisms. Other studies have shown that A β load in the CSF varies according to synaptic activity (Cirrito et al., 2005) and circadian rhythms (Musiek et al., 2015). Notably, inhibition of synaptic activity decreases the amount of A β secreted into the extracellular space whereas increased synaptic activity or disrupted circadian rhythm provokes an increase of A β secretion into the extracellular space.

It has been proposed that, in physiologic conditions, A β selectively depresses excitatory synaptic transmission via NMDAr and inversely, synaptic activity modulates β -cleavage. This activity-dependent modulation of endogenous A β production, at the synaptic level, may normally participate in a negative feedback loop that could keep neuronal hyperactivity in check (Kamenetz et al., 2003). In this light, it is possible that synaptic depression from excessive A β could contribute to cognitive

decline during early AD by disrupting this feedback system and disease progression (**Figure 17**). This points towards APP as being the gun targeting the synapse, A β is the bullet and synaptic activity is the trigger.

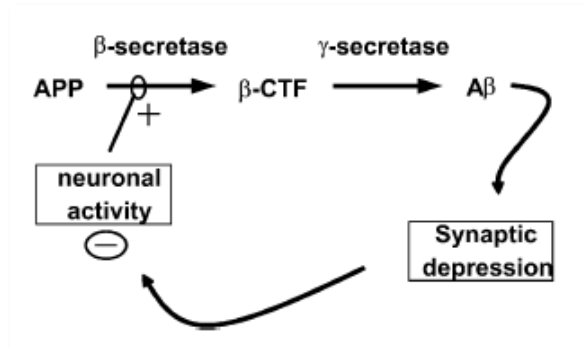


Figure 17: Negative feedback model indicating proposed interaction between neural activity and APP processing (adapted from Kamenetz et al., 2003). Neural activity regulates B-secretase activity on APP. Formation of A β depresses synaptic transmission. Synaptic depression decreases neural activity.

E. The different forms of A β

The A β peptide can be found under different conformations: alpha-helices, intermediate soluble forms such as monomers and oligomers, but also insoluble beta-sheet rich forms such as protofibrils and fibrils which are at the origin of amyloid plaques (**Figure 18**).

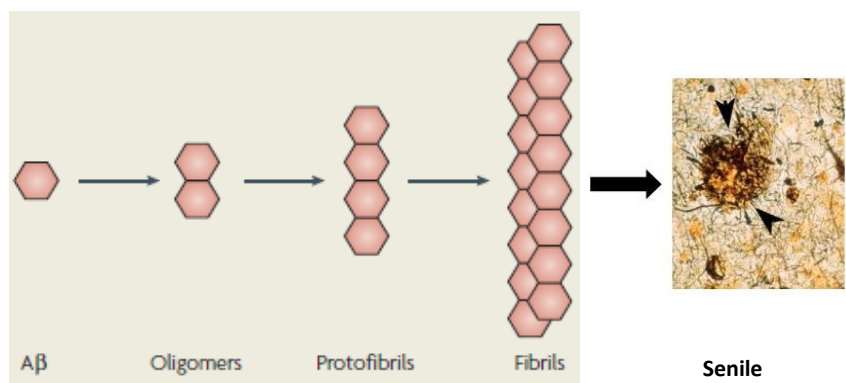


Figure 18: A β assembly states (adapted from La Ferla et al., 2007). A β can exist in multiple assembly states: monomers, oligomers, protofibrils and fibrils. It is the ability of this peptide to fibrils and other intermediate states that impart the unique pathophysiological characteristics of AD pathology. Fibril formation is a complex, nucleation-dependent process. The mechanism driving this process, particularly in the elderly brain, is not yet understood, but it appears to be closely related to protein misfolding. In its monomeric state, A β does not appear to be neurotoxic. In contrast, oligomeric and protofibrillar species impede synaptic plasticity processes.

A β peptides have a tendency to aggregate. This is why, in most cases, the majority of A β in AD brains is aggregated in the form of fibrils which themselves tend to form amyloid plaques. Several

studies have investigated the aggregation properties of A β peptides. The A β_{42} peptide is the most hydrophobic compared to its shorter counterparts. Its solubility is therefore lower and its aggregating capacity higher (approximately 70 times more rapid than A β_{40}). The two extra residues in the C-terminal of A β_{42} give it a more structured organisation, compared to A β_{40} , responsible for its toxicity and more potent aggregating properties (Sgourakis et al., 2007). The reason why these extra two residues on A β_{42} , compared to A β_{40} , change its toxic and aggregating properties is still to be discovered. However, the capacity of A β to aggregate and form organised fibrillary structures not only depends on the nature of A β (40/42), the pH, temperature, and concentration of the peptide but mostly depends on the hydrophobic interactions of the peptides. These hydrophobic interactions enable them to go from an α -helix monomeric form to a “random coil” monomeric structure, whereby the monomeric units adopt random conformations, to eventually a β -sheet structure (Zagorski and Barrow, 1992; Ahmed et al., 2010). NMR spectroscopy based studies have shown that A β oligomers do not possess this β -sheet conformation, characteristic of fibrils, but are rather composed of loosely aggregated strands with a turn conformation, placing Phe19 in contact with Leu34 (Ahmed et al., 2010) (**Figure 19, A**). It is the association of several β -sheet units that polymerise in a parallel manner that lead to the formation of protofibrils which themselves will associate into insoluble “coiled” fibrils (**Figure 19, B**) that will eventually aggregate into extracellular amyloid plaques.

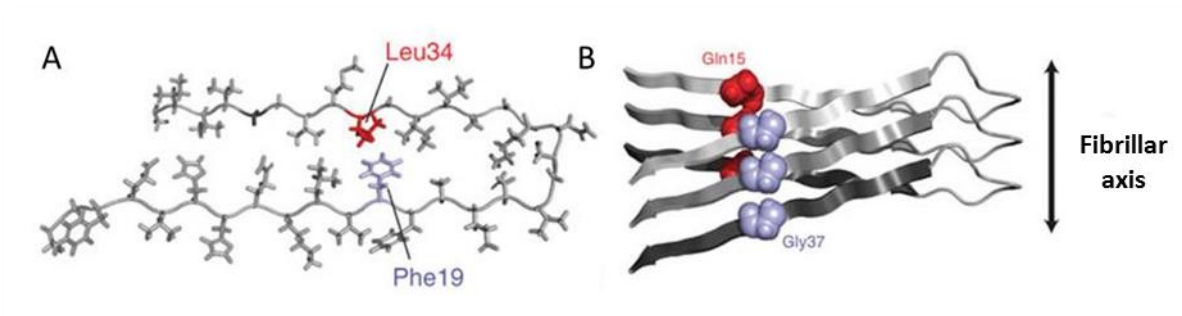


Figure 19: Illustration of the molecular model of in turn conformation (adapted from Ahmed et al., 2010). (A) Oligomeric A β_{42} has a structural conformation whereby Phe19 and Leu34 are in contact. (B) Stacked model of fibrillar A β_{42} that contact via Gln15 and Gly37 interaction between the N- and C-termini of each β -strands.

It has therefore been shown that soluble monomers aggregate to form oligomers and that these soluble oligomeric forms of A β range in size from 4 to 100 kDa. It is these forms that are described as being the most toxic forms and responsible for AD cognitive deficits (Lambert et al., 1998; Haass and Selkoe, 2007; Tomiyama et al., 2010). Molecular biology studies on mouse primary neuronal cultures have shown the enhanced toxicity of A β oligomers compared to protofibrils and fibrils (Ahmed et al., 2010). Several types of globular oligomers have been identified ranging from dimers, trimers,

tetramers up 24-mers, with a higher molecular weight. These globular oligomers are coined ADDLs for “A β -derived diffusible ligands”.

So far, a detailed description of the toxicity of wild-type oligomeric A β has been given. However, the complexity of AD pathophysiology goes beyond the wild-type form of the peptide. Indeed, although most AD cases are sporadic, FADs are usually far more aggressive forms of the pathology and are very informative in understanding AD pathogenesis. The different pathogenic mutations that have been identified on APP either lead to increased wild-type A β production or generate mutated toxic forms of A β . These mutated A β s can each adopt different conformations, have different aggregating properties, can be located differently inside and outside the cell, and generally behave differently. Studying these mutated forms of the peptides and their consequences, through transgenic animal models and human cases, have helped tremendously in understanding AD in the past four decades. These studies have brought to light the notion of A β strains, that each strain may behave and evolve in its own way and that AD patients might even have a combination of several strains within the brain. This has added a level of difficulty in understanding and finding therapeutic strategies for sporadic and familial AD. Nevertheless, using these mutated forms of A β and studying their consequences on biological processes might give an insight into the common denominator which gives rise to the cognitive impairments observed in AD.

Some of these most remarkable mutations on APP, with unique signatures, will be described in the next section.

F. The different mutations of APP

Over the last couple of decades, numerous mutations on APP have been found in FADs. These mutations usually lead to aggressive forms of AD with an early onset and can be filed roughly into two categories: i) mutations that affect A β production; ii) mutations that affect A β conformation.

1. APP mutations affecting A β production

To date, about 50 pathogenic mutations of APP have been reported (“AD&FTD Mutation Database,” <http://www.molgen.ua.ac.be/admutations>), most of which affect the proteolysis of APP in such a way that A β ₄₂ levels are changed relative to other A β isoforms (Suzuki et al., 1994; Scheuner et al., 1996; Kwok et al., 2000; De Jonghe et al., 2001; Di Fede et al., 2009; Cruts et al., 2012). Most of

the pathogenic mutations of APP (**Figure 20**) are autosomal dominant and occur near the β -secretase cleavage site (aa 670 to 682), near the γ -secretase cleavage site (aa 713 to 724) (Ringman et al., 2014), or within the A β sequence of APP (the latter will be described in **Part II.F.2**).

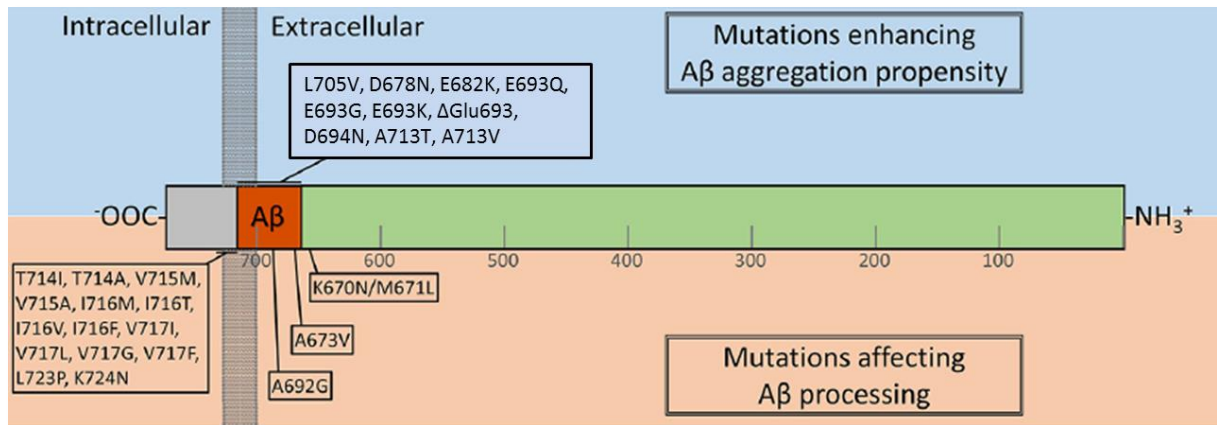


Figure 20: Sequence and mutations of APP (adapted from Bemporad et al., 2019). The extracellular N-terminal domain (residues 18–671), the A β 42 sequence (residues 672–713), and the intra-cellular C-terminal domain (residues 714–770) are shown in green, orange, and grey, respectively. The transmembrane domain encompasses residues 700–723. Mutations that have been shown to induce an enhancement of the Aggregation propensity are reported above. Mutations that alter the processing of the APP sequence are reported below.

a) Mutations affecting β -cleavage

- Swedish (K670M/N671L)

The Swedish (Swe) double mutation K670M/N671L, located just before the β -cleavage site, is probably the most renowned and used mutation in AD research. The most commonly used transgenic AD mouse models usually carry this mutation, such as Tg2576 mice, J20 mice (which also carry the V717F Indiana mutation on APP), APP/PS1-21 (which also carry a mutation on PS1) and 3xTg mice (which also carry mutations on PS1 and Tau). This mutation leads to a general 20% increase of A β ₄₀ and A β ₄₂ production and secretion without affecting A β ₄₀/A β ₄₂ ratio (Citron et al., 1992; Cai et al., 1993).

- Taiwanese (D678H)

The Taiwanese D678H mutation increases general A β ₄₀ and A β ₄₂ levels and also favours the amyloidogenic pathway, decreasing A β ₄₀/A β ₄₂ ratio. The mutant A β is also more susceptible to the formation of ion-induced A β oligomers and exhibits greater toxicity in vitro compared with wild-type A β 42 (Chen et al., 2012).

- Leuven (E682K)

The Leuven E682K mutation at the β' site in APP shifts BACE1 cleavage toward the β -site and causes a significant increase in total A β levels and also decreases A β_{40} /A β_{42} ratio (Zhou et al., 2011).

b) Mutations affecting γ -cleavage

- **Austrian (T714I)**

This mutation is thought to affect APP processing by γ -secretase and alters the A β_{40} /A β_{42} ratio approximately 11-fold, simultaneously increasing A β_{42} and decreasing A β_{40} secretion (Kumar-Singh et al., 2000).

- **German (V715A) and French (V715M)**

The German V715A increases A β_{42} and decreases A β_{40} levels. It has been observed that A β_{40} /A β_{42} ratio is increased fourfold in HEK293 cells and primary neuronal cultures (De Jonghe et al., 2001; Cruts et al., 2003).

The French V715M mutation is unusual as it was found to reduce total A β production. However, this mutation caused significant reduction in A β_{40} levels with no change in A β_{42} levels, leading to a decreased A β_{40} /A β_{42} ratio (Ancolio et al., 1999).

A study using NMR spectroscopy found that these two mutations at the same position, altered the structure and dynamics of the transmembrane domain in APP, making it more accessible to γ -secretase for cleavage in position 42 of A β sequence, and consequently shifting production toward A β_{42} (Chen et al., 2014).

- **Iberian (I716F) and Florida (I716V)**

Both of these mutations decrease A β_{40} /A β_{42} ratio by increasing γ -secretase specificity for cleavage in position 42 of A β sequence (De Jonghe et al., 2001; Eckman et al., 1997; Guardia-Laguarta et al., 2010; Herl et al., 2009).

- **Indiana (V717F), V717G, London (V717I), V717L**

The aa corresponding to codon 717 is within the transmembrane region of APP, mutations at this position all alter relative levels of A β peptides leading to decreased A β_{40} /A β_{42} ratio, usually by increasing A β_{42} levels and decreasing (or not changing) A β_{40} levels (De Jonghe et al., 2001; Eckman et al., 1997; Herl et al., 2009; Suzuki et al., 1994; Tamaoka et al., 1994).

The London V717I missense mutation, the first described and best characterised of all APP mutations actually affects both β - and γ -secretase cleavage sites, resulting in increased $A\beta_{42}$ levels. It is to note, the initial clinical case of a Chinese AD patient with the London mutation had a more aggressive form of AD than Western AD patients with the same mutation. This finding brings to light the fact that the phenotypes of AD patients with identical APP mutations are affected by ethnic differences, environment or possibly other unknown factors (Zhang et al., 2017).

- **Australian (L723P) and Belgian (K724N)**

Both of these mutations also decreased $A\beta_{40}/A\beta_{42}$ ratio by increasing $A\beta_{42}$ production and decreasing $A\beta_{40}$ production (Kwok et al., 2000; Theuns et al., 2006).

- **Flemish (A692G) and A693V**

Although these two mutations are actually located within the $A\beta$ sequence, they render APP a better substrate for β -secretase cleavage consequently increasing overall $A\beta$ production (Farzan et al., 2000; Di Fede et al., 2009). The A673V mutation will be further discussed in **Part II.F.3**.

2. **APP mutations affecting $A\beta$ sequence**

Mutations found within the $A\beta$ sequence of APP (aa 692 to 705) are more complex as they mostly result in a variety of mutated $A\beta$ peptides with each mutant having their own degree of toxicity, aggregating properties, localisation inside/outside the cell and/or resistance to degradation.

*a) **The hotspot for $A\beta$ mutations (aa 693 to 694 of APP)***

Most mutations within the $A\beta$ sequence of APP are located on amino acids 693 and 694 of APP. These mutations are clustered around the central hydrophobic $A\beta$ core near the α -secretase cleavage site, resulting in an array of polymorphic aggregates in a mutation-dependent manner (Dai et al., 2018).

- **Osaka (E693 Δ)**

The Osaka E693 Δ mutation is a deletion of a glutamate at position 693. This particular mutation was initially found in a Japanese pedigree where the patients had severe AD-like cognitive deficits but PET-scans using Pittsburgh compound-B showed they lacked the $A\beta$ -plaque hallmark of the disease.

Although the secretion of total A β is markedly reduced by this mutation, the variant A β is more resistant to proteolytic degradation. Furthermore, the A β -osaka (A β osa) peptide accumulates intracellularly and shows unique aggregation properties of enhanced oligomerisation but no fibrillisation conferring it with high synaptotoxicity as it has been shown to inhibit LTP more potently than wild-type A β (Nishitsuji et al., 2009; Takuma et al., 2008; Tomiyama et al., 2010, 2008; Umeda et al., 2015).

Moreover, studies using A β ₄₀ with the Osaka deletion showed that the internalization and binding of this variant to PC12 neurons was enhanced 6-fold which would in part explain the high level of intraneuronal A β seen in this Japanese pedigree (Poduslo et al., 2012).

- Arctic (E693G), Italian (E639K) and Dutch (E693Q)

These three point missense mutations cause an overall decrease of A β ₄₀ and A β ₄₂ levels in plasma. Additionally, low levels of A β ₄₂ are detected in condition media from HEK293 cells transfected with APP_{E693G} and APP_{E693K}. However, fibrillisation studies demonstrate no difference in fibrillisation rate, but Arctic, Italian and Dutch A β form protofibrils at a much higher rate and in larger quantities than wild-type A β with higher stability (Nilsberth et al., 2001; Poduslo and Howell, 2015).

In addition, these mutations are found to confer resistance to neprilysin-catalysed proteolysis of A β (Tsubuki et al., 2003).

- Iowa (D694N)

The Iowa D694N autosomal dominant mutation has been shown to affect A β peptide structure in vitro resulting in the formation of a turn rather than a bend motif (Krone et al., 2008). Additional in vitro experiments have shown that the Iowa mutation promotes fibrillogenesis of A β and results in greater A β -induced toxicity (Van Nostrand et al., 2001, 2002).

b) Other

- Tottori (D678N)

The D678N mutation alters an amino acid within the A β region of APP, specifically at position 7 (D7N). The mutation does not affect A β generation from APP (Hori et al., 2007), but does alter the assembly kinetics of the peptide. Mutant A β displays accelerated secondary structure transitions and an increased propensity to form relatively large oligomers. The oligomers are also more efficient nucleators of fibril formation, and are significantly more cytotoxic than wild-type peptides (Ono et al., 2010).

3. *Not all mutations on APP are toxic*

- **Icelandic (A673T) the protective mutation**

This rare mutation was initially found in one Finnish subject who lived until age 104.8 years and showed little beta-amyloid pathology. Although dementia was noted at 104 years of age, this was attributed to likely hippocampal sclerosis (Kero et al., 2013). Because of the low amount of parenchymal plaque pathology at 104.8 years of age, Kero et al. (2013) suggested that this variant protects against amyloid pathology and AD.

It has been reported that some of the reasons why this mutation is allegedly protective against AD is because: i) $A\beta_{ice}$ has a lower rate of aggregation (Poduslo and Howell, 2015) ii) it has a lower rate of β -cleavage. Indeed, studies using APP_{ice} overexpression in HEK293 cells, as well as in human iPSC lines that express APP_{ice} at endogenous levels, showed an approximate 30 to 40% reduction in the formation of amyloidogenic peptides (Jonsson et al., 2012; Maloney et al., 2014). Although this reduction might seem quite significant, it is important to keep in mind that the amyloidogenic pathway accounts for only 10% of total APP processing (Sinha and Lieberburg, 1999).

- **A673V the mutation with two faces**

A673V is a particular recessive mutation whereby the homozygous state is very amyloidogenic and the heterozygous state is not. In the homozygous state this mutation not only leads to increased $A\beta$ production but also enhances $A\beta$ aggregation and toxicity. However, in the heterozygous state, when there is a mixture of mutated and wild-type $A\beta$ peptides they both aggregate more slowly than either peptide alone (Di Fede et al., 2012).

These $A\beta$ peptides, with different aggregation states or fibrillary structures, imply multiple pathways of binding/internalisation for the different $A\beta$ peptides. These findings bring to light how subtle changes in the primary structure of $A\beta$ translate into dramatic changes in the clinical expression and distinct neuropathology. Therefore these different variants will provide greater insight into the mechanisms of the disease.

G. **Therapeutic strategies**

To this day, the only treatments that exist to treat AD are directed towards alleviating the symptoms more than treating the causes. Amongst these treatments, four of them are

acetylcholinesterase inhibitors and the fifth is an NMDAr antagonist. Older studies had shown that cholinergic neurons were particularly damaged in AD (Coyle et al., 1983) and the number of remaining cholinergic neurons correlated with AD patients' cognitive performances (Baskin et al., 1999). Acetylcholinesterase inhibitors (AChE) block the enzymes responsible for acetylcholine degradation inducing an increase in acetylcholine concentration in the synaptic cleft, thus prolonging the action of the neurotransmitter on the post-synaptic element. This allows counterbalancing the decrease of synaptic influx of acetylcholine due to selective neurodegeneration of presynaptic neurons. However, these treatments are effective only when there is still synthesis and release of acetylcholine meaning they lose their efficacy when neuronal degeneration is too advanced (Ellis, 2005). In parallel of AChE, a non-competitive NMDAr antagonist, memantine, is used for treating AD especially in more advanced and severe stages of the pathology. This molecule is able to block NMDAr during low but continuous release of glutamate, which occurs during neuronal death. Although these treatments are currently used in AD therapy, their efficacy is only relative.

More recently, new therapeutic strategies have been developed to target AD's abnormal A β peptide accumulation in order to decrease/restore these levels back to physiological levels in the brain (Giacobini and Gold, 2013):

1. Decreasing A β production

One of the therapeutic strategies is to target the proteolytic enzymes of APP processing and therefore targeting A β production (γ - and β -secretases).

a) *Inhibition of γ -secretase*

Several inhibitors of the γ -secretase complex have been tested, but none of them showed any significant therapeutic efficacy and their development has been aborted during clinical trials. Also, several problems were recurrently encountered, such as difficulties in making the molecule cross the blood-brain barrier. Amongst these γ -secretase inhibitors, two molecules that modulate γ -secretase activity without altering Notch's signalling pathway have been developed: Tarenflurbil and Avagacestat. Tarenflurbil was abandoned during phase 3 of the clinical trials due to lack of effect on patients that were currently at a mild stage of the pathology. Avagacestat was also abandoned because of cognitive deterioration of the patients. Another inhibitor of the γ -secretase,

Semagacestat, 30 times stronger than Tarenflurbil but also interferes with Notch signalling, was also abandoned due to lack of selectivity but also because of cognitive deterioration of the patients.

b) Inhibition of β -secretase

Another very promising therapeutic strategy, which has been undergoing clinical trials in the last few years, is BACE inhibitors. Since BACE1 is the β -cleaving enzyme of APP and is thought to be the rate limiting factor in A β production, both academia and industry have invested substantial resources into developing chemical compounds to inhibit BACE1 function. Several BACE inhibitors are currently being tested in Phase 2 and/or 3 trials (**Table 3**).

Compound	Company	Trial Phase	NCT Number ^a	Doses (mg)	A β Reduction, % (mg) ^b	Patient Population	Expected Completion Year
CNP520	Novartis, Amgen	2/3	NCT02565511	50	~ 60 (10)	Asymptomatic at-risk persons (APOE4)	2023
			NCT03131453	15/50	~ 80 (35)		2024
AZD3293 (LY3314814)	Eli Lilly, AstraZeneca	2/3	NCT02783573	20/50	~ 60 (20)	Early and mild AD	2021
			NCT02972658		~ 80 (50)		2020
			NCT02245737		2019		
			NCT03019549		2017		
LY3202626	Eli Lilly	2	NCT02791191		~ 50 (1)	Mild AD	2019
Elenbecestat (E2609)	Eisai, Biogen	2/3	NCT02322021	50	~ 50 (5)	Early AD	2020
			NCT03036280		~ 80 (50)		2020
			NCT02956486		2020		
JNJ-54861911	Janssen	2/3	NCT02569398	5/25	~ 50 (5)	Asymptomatic at-risk persons and early AD	2023
			NCT02406027	5/10/25	~ 80 (25)		2022
Verubecestat (MK-8931)	Merck	2/3	NCT01739348	12/40/60	~ 50 (12)	Prodromal AD	2017
		3	NCT01953601	12/40	~ 80 (40)		2018

Table 3: Ongoing BACE-Inhibitor Clinical trials (adapted from Zhu et al., 2018). NCT: National Clinical Trial. ^aNumbers refer to the study codes in the ClinicalTrials.gov database. ^bData from preclinical human studies or phase 1 studies.

Although BACE inhibition effectively reduces A β levels with all of these different molecules, most of them failed to show any therapeutic benefits. Recently, clinical trial for Verubecestat (MK-8931), a BACE1 and 2 inhibitor, failed to rescue cognitive impairment in patients with prodromal AD. Several other trials have been discontinued before the end of trial period such as LY3202626, Lanabecestat (AZD3293) and Atabecestat (JNJ-54861911). LY3202626, although a potent BACE1 inhibitor, alone induced no cognitive improvement in AD patients. It was combined with an immunotherapy using injection of a monoclonal antibody against a pyroglutamate form of A β aggregated in plaques Donanemab, in another trial which also failed to show any improvement. Lanabecestat (AZD3293), a BACE1 and 2 inhibitor, effectively reduced A β levels in plasma and CSF of patients up to 70% but the trial was prematurely discontinued in 2018 due to lack of efficacy on cognitive symptoms. Atabecestat (JNJ-54861911) was also discontinued in 2018 because treatment group not only had worsened cognitive symptoms than placebo group; they also reported more depression, anxiety, and

sleep problems than controls. Only two trials are still ongoing, Elenbecestat (E2609), which is coming to term in 2020, and Umibecestat (CNP520), which is terminating in 2023/2024 (<http://www.alzforum.org/therapeutics>).

One of the main issues with this therapeutic strategy is that BACE1 has only been recently discovered, and the research community are only starting to unravel its role outside APP processing. It has been shown that BACE1 has many other substrates, indicating it may be involved in various other physiological functions (Zhu et al., 2018a). Notably, it has several roles at the synapse including functions in synaptic structure and synaptic plasticity (**Table 4**).


BACE1 Cleavage	Name (Abbreviation)	Localisation	Functions	Reference
High 	Seizure protein 6 (SEZ6)	Dendrite, dendritic spine	Dendritic arborisation, maintenance of dendritic spine density, synaptic transmission, and LTP	Gunnerson et al., 2007; Zhu et al., 2018b
	Amyloid precursor-like protein 1 (APLP1)	Pre- and postsynapse	Synaptogenesis, dendritic spine density	Schilling et al., 2017
	Close homologue of L1 (CHL1)	Axon, presynaptic boutons	Axon guidance	Cao et al., 2012; Rajapaksha et al., 2011
	Neuroigin 4 (NLGN4)	Glycinergic postsynapses	Synaptic transmission	Hoon et al., 2011
	Neuroigin 2 (NLGN2)	Inhibitory synapses	Synaptic transmission	Nguyen et al., 2016
	Contactin 2	Axon, presynaptic boutons	Axon guidance	Gautam et al., 2014
	Amyloid precursor protein (APP)	Pre- and postsynapse	Dendritic spine morphology, density, and dynamics	Weyer et al., 2014
Low	Neuroigin 1 (NLGN1)	Dendritic spine of excitatory synapses	NMDAR-mediated synaptic transmission and LTP	Jiang et al., 2017; Song et al., 1999
	n.a.	Neuregulin 1 (NRG1)	Axon, presynaptic boutons	Myelination, dendritic spine density

Table 4: BACE1 substrates involved in synaptic plasticity (adapted from Zhu et al., 2018).

Indeed, BACE1 can be found at the pre- and post-synaptic structures and recent studies of *Bace1*^{-/-} mice have been very informative in order to understand these roles (**Table 5**). These findings highlight the complexity of using BACE1 inhibition as a therapeutic strategy and give insight into why these BACE1 inhibitor trials have failed to improve cognitive deficits, if not worsen them.

	<i>Bace1</i> ^{-/-}	<i>Bace1</i> ^{+/-}	BACE Inhibition	Reference
Synaptic structure				
Presynaptic terminals	Normal in mossy fibre terminals	n.a.	n.a.	Kandalepas et al., 2013
Spine density	Reduced in CA1, normal in cortical L5 neurons	n.a.	Reduced in cortical L5 neurons	Filser et al., 2015; Savonenko et al., 2008; Zhu et al., 2018b
Spine plasticity	Normal in cortical L5 neurons	Normal in cortical L5 neurons	Impaired in cortical L5 neurons	Filser et al., 2015; Zhu et al., 2018b; Zou et al., 2016
Synaptic function				
Basal synaptic transmission	Reduced in CA1	Normal in CA1	Reduced in CA1	Filser et al., 2015; Giusti-Rodríguez et al., 2011; Kamikubo et al., 2017
Presynaptic function	Impaired in CA1 and CA3	Normal in CA1 and CA3	Normal in CA1	Giusti-Rodríguez et al., 2011; Kandalepas et al., 2013; Wang et al., 2014, 2008; Zhu et al., 2018b
LTP	Reduced in CA1 and CA3	Normal in CA1 and CA3	Reduced in CA1	Filser et al., 2015; Giusti-Rodríguez et al., 2011; Kamikubo et al., 2017; Wang et al., 2014; Zhu et al., 2018b
LTD	Normal in CA1; slight deficits in CA3	n.a.	n.a.	Laird et al., 2005; Wang et al., 2008

Table 5: Consequences of genetic deletion and pharmacological inhibition of BACE1 on synapses (adapted from Zhu et al., 2018).

Although BACE1 inhibition yielded underwhelming results during clinical trials, at this point, the only proposed solution to minimise negative side effects due to BACE1's other functions within the brain is to only lower the activity of BACE1 by 50% (*Bace1*^{+/-} mice) as this suppresses A β generation by 40% without inducing synaptic deficits (Devi and Ohno, 2015; Sadleir et al., 2015) (**Table 5**).

2. Immunotherapies

This second therapeutic strategy is based on the development of active or passive immunotherapies, against the A β peptide. These approaches aim at promoting the clearance of already existing A β peptides in extracellular amyloid plaques and/or to reduce its accumulation via cellular immunity.

Despite encouraging results from trials using non-specific immunologic approaches (immunoglobuline, IVIg, gammagard) on AD transgenic animals; these effects weren't reproduced in AD patients. A first active vaccine against the A β peptide (AN1792) was developed then discontinued due to harmful inflammatory reactions. Furthermore, post-mortem studies showed that there was

no correlation between the amount of A β clearance in amyloid plaques in the brains of the patients and their synaptic integrity. Afterwards, a monoclonal antibody against soluble and insoluble (fibrillary) A β , Bapineuzumab, was developed and showed promising results on transgenic AD animals as it significantly reduced the accumulation of A β . However, clinical trials were discontinued due to lack of therapeutic efficacy. Another monoclonal antibody was developed, targeting an epitope in the central region of A β , Solanezumab. Despite Solanezumab having shown positive results in AD transgenic animals with an improvement of cognition, it failed to reproduce these effects in humans. Regardless of these failures, several new monoclonal antibodies and six new vaccines are currently under clinical trials and focus on patients in early stages of AD and even prodromal AD. However, the lack of biomarkers to detect early preclinical phases of AD and the complexity of the measures to track the cognitive evolution of patients with presymptomatic/early AD constitutes a real challenge to demonstrate the beneficial effects of these therapeutic approaches since these patients present no clinical symptoms of AD yet.

Although many doubts have been expressed regarding the validity of the amyloid hypothesis due to the lack of effect of these therapeutic strategies targeting A β , a new study shows encouraging preliminary results and reinforces the hypothesis of the amyloid cascade (Sevigny et al., 2016). This study is based on the development of a new human monoclonal antibody, coined Aducanumab, which selectively targets A β aggregates and more specifically soluble oligomers and insoluble fibrils. Preclinical tests carried out on transgenic AD animals show that an analogue of Aducanumab crosses the blood-brain barrier, binds to parenchymal A β in the brain and decreases soluble and insoluble A β in plaques in a time- and dose-dependent manner, which enabled the launch of clinical trials. These trials were performed on patients with mild-AD which were administered a placebo or 1, 3, 6 or 10 mg/kg doses of Aducanumab every month for 2 years. The results seem encouraging since several reports show that patients treated with Aducanumab present a significant reduction of amyloid plaques in a time- and dose-dependent manner. The development of this antibody is currently in phase 3 of clinical trials and should reinforce the importance of targeting the A β peptide in the treatment of AD.

Breaking news

At the time of finishing writing this manuscript, biotechnology firm Biogen and its partner Eisai announced plans to abandon the two clinical trials for Alzheimer's treatment using the drugs Elenbecestat and Aducanumab. The decision to end the studies came after a data safety monitoring board found that the benefits of administering these drugs "did not outweigh the risks".

<https://alzheimersnewstoday.com/aducanumab/>

<https://www.the-scientist.com/news-opinion/biogen--eisai-end-two-late-stage-trials-for-alzheimers-treatment-66431>

3. Decreasing A β aggregation

A β aggregation into fibrils, which is at the basis of amyloid plaque formation, has driven researchers to develop anti-aggregating compounds, which preclude the oligomerisation of A β peptides, or promote the clearance of these aggregates.

Several compounds, such as Tramiprosat or Scyllo-inositol, have been developed but without any successful results once tested on AD patients.

4. Increasing A β clearance

The fourth therapeutic approach is based on the use of compounds which decrease A β levels in the plasma. Notably, Neprilysine, a zinc metallo-protease which degrades A β peptides in the brain, has been the subject of many studies *in vitro* and also using transgenic AD animals. Despite positive results in decreasing plasma A β levels *in vitro*, the concentration of A β in AD transgenic mouse models was not decreased. This suggests that this therapeutic approach will have limited efficacy.

5. Counteracting the toxic effects of A β

Another major route in the development of therapeutic strategies is to counteract the deleterious effects induced by A β , especially the toxic effects inflicted on synapses and synaptic plasticity processes. Indeed, it is now well-established that A β induces a reduction in the number of synapses (*i.e.* a decrease in dendritic spine density) and a decrease of Long-term Potentiation, the experimental correlate of memory and learning processes (both will be further described in the next chapters) which leads to AD-related cognitive and memory defects. Many studies have investigated potential diffusible factors that could counteract these effects. One of which being Vascular Endothelial cell Growth Factor (VEGF), which is already known to be required for the action in antidepressant therapies. This molecule, and its receptor VEGFR2, are involved in increasing NMDA-dependent postsynaptic responses (De Rossi et al., 2016). This molecule has been shown to be abundant in the CNS but decreased in the CSF of AD patients as well as transgenic AD animal models, due to an interaction of VEGF with A β , impeding VEGF function. It has been shown that VEGF treatment on AD transgenic mouse hippocampal neurons counteracts A β -induced synaptic dysfunction and restores long-term synaptic plasticity processes (Martin, 2018), opening a new route for therapeutic molecule development.

The main difficulty in the development of all these therapeutic strategies is the discrepancy between the clinical efficacy of these therapies on AD transgenic animals and the efficacy on humans. Together these data and studies strongly suggest an evolution of the amyloid hypothesis in the coming years; and that finding a one-size-fits-all therapeutic approach is proving to be very difficult. Thus, a more made-to-measure, personalised therapeutic strategy might be the way forward to treat this multi factorial pathology that is AD.

H. A β and Synaptotoxicity

Historically, senile plaques, made up of A β fibrils, were considered as the initiating factor and at the root cause of neurotoxic and neurodegenerative phenomena observed during AD (Hardy and Higgins, 1992). However, the cognitive deficits observed, and the significant loss of synapses, do not correlate with this hallmark of AD. Rather, it is now widely accepted that it is the soluble oligomers of A β that are responsible of the first synaptic and cognitive alterations that are at the origin of AD pathogenesis. Insoluble amyloid deposits that constitute the plaques are seen, these days, more as a reservoir of bioactive oligomers (Haass and Selkoe, 2007).

This is why, the recent evolution of the amyloid cascade hypothesis (**Figure 21**) brings to light that it is the oligomers of A β that are responsible for the first synaptic and cognitive deficits observed in AD. This hypothesis has become the dominating model of the development of this pathology (Glennner and Wong, 1984; Hardy and Higgins, 1992) albeit the linearity of this cascade is still controversial (De Strooper and Karran, 2016). Studies have revealed that synapse loss is the best suited marker of observed cognitive deficits (Selkoe, 2002; Shankar et al., 2007). Since, numerous studies have confirmed the synaptotoxic effects of the soluble oligomeric forms of A β (for review: Shankar and Walsh, 2009).

However, even though the A β peptide has been identified in the mid-80s as the main component of extracellular senile plaques, studies carried out on cultures of primary neurons treated with synthetic or human brain extracted A β peptides as well as studies using transgenic AD animal models have shown the existence of intracellular A β (Gouras et al., 2000; Grundke-Iqbal et al., 1989; for review: LaFerla et al., 2007; Ripoli et al., 2014; Tampellini et al., 2011) and particularly inside synapses (Pickett et al., 2016). It seems the intracellular accumulation of A β oligomers is an upstream event in the formation of senile plaques in AD pathogenesis, and is responsible for the early dysfunctions in AD. Several studies show that the intraneuronal accumulation of A β peptides coincides with synaptic dysfunction along with the memory alterations dependent of the

hippocampus, the cerebral structure implicated in spatial memory (Billings et al., 2005; LaFerla et al., 2007; Umeda et al., 2015).

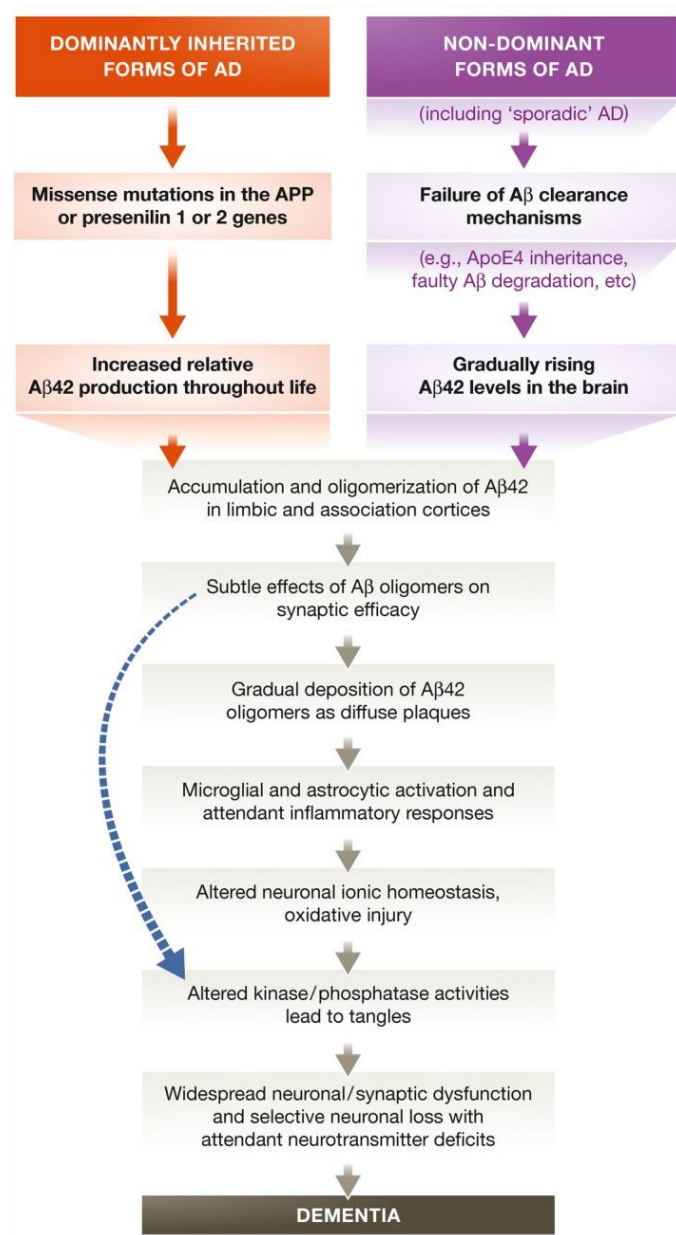


Figure 21: The amyloid cascade hypothesis (adapted from Selkoe and Hardy, 2016). Illustration of the sequence of major events leading to the development of AD.

The production site and localisation of the Aβ peptide is controversial, as detailed previously in **Part.II.C.1**. It is still to be determined whether the majority of produced Aβ comes from an intracellular production within the membrane of intraneuronal organelles or whether this production occurs at the plasma membrane and the extracellular release of Aβ is immediate. Nevertheless, internalisation of Aβ peptides and its inherent effects are regulated by several receptors and

transporters with which A β can interact (**Table 6**). Notably, A β can interact with post-synaptic receptors such as cholinergic nicotinic receptors α 7nAChR but also ionotropic glutamatergic NMDA (N-methyl-D-aspartic acid) receptors (Dinamarca et al., 2012). It is important to note that these receptors interact with oligomeric and not fibrillary forms of A β (Nimmrich et al., 2008) and that their function is altered upon this interaction which leads to synaptic defects. Currently, it is well accepted that the prime target of A β is the synapse (Li et al., 2013; Ripoli et al., 2014; Shankar and Walsh, 2009), which subsequently induces the synaptic alterations and neuronal defects observed during the development of AD, and ultimately results in memory impairments.

A β binding receptors/transporters	Effects	Reference
NMDA receptors	<ul style="list-style-type: none"> • \nearrow neurotoxic calcic influx • Endocytosis of NMDAr 	De Felice et al., 2007 Snyder et al., 2005
AMPA receptors	Calcineurin-dependent endocytosis of AMPAR	Zhao et al., 2010
GABA receptors	Inhibition of GABA _r	Ulrich, D., 2015
αv type Integrins	Inhibition of LTP (Long Term Potentiation)	Wang et al., 2008
Prion protein receptors (PrP^c)	<ul style="list-style-type: none"> • Binding at lipid rafts • Activates Src Kinase Fyn 	Um et al., 2013
Nicotinic α7nACh receptors	<ul style="list-style-type: none"> • Receptor internalisation • Intracellular accumulation of Aβ • Induces NMDAr endocytosis 	Wang et al., 2000
Lipoprotein-related receptors (LRP1)	<ul style="list-style-type: none"> • Binding to receptor or via its ligand ApoE • Induces LRP1 endocytosis • Intracellular accumulation of Aβ 	Fuentealba et al., 2010

Table 6: Examples of receptors and transporters that interact with A β

III. The excitatory glutamatergic synapse

A. The chemical synapse

In the central nervous system, synaptic transmission between two neurons occurs through distinct contact points called synapses, which can be electrical or chemical. Electrical synapses are a minority in vertebrate CNS; communication occurs via ion exchanges and a direct passage of current through gap junctions. The majority of synapses found in the CNS are chemical. They are at the basis of neurotransmission and can be excitatory or inhibitory. These chemical synapses are constituted of pre- and postsynaptic elements separated by a gap called the synaptic cleft. Together these elements form the functional unit that transmits nervous messages from one neuron to another (**Figure 22**). In this part, we will only focus on the architecture and function of the excitatory glutamatergic synapse.

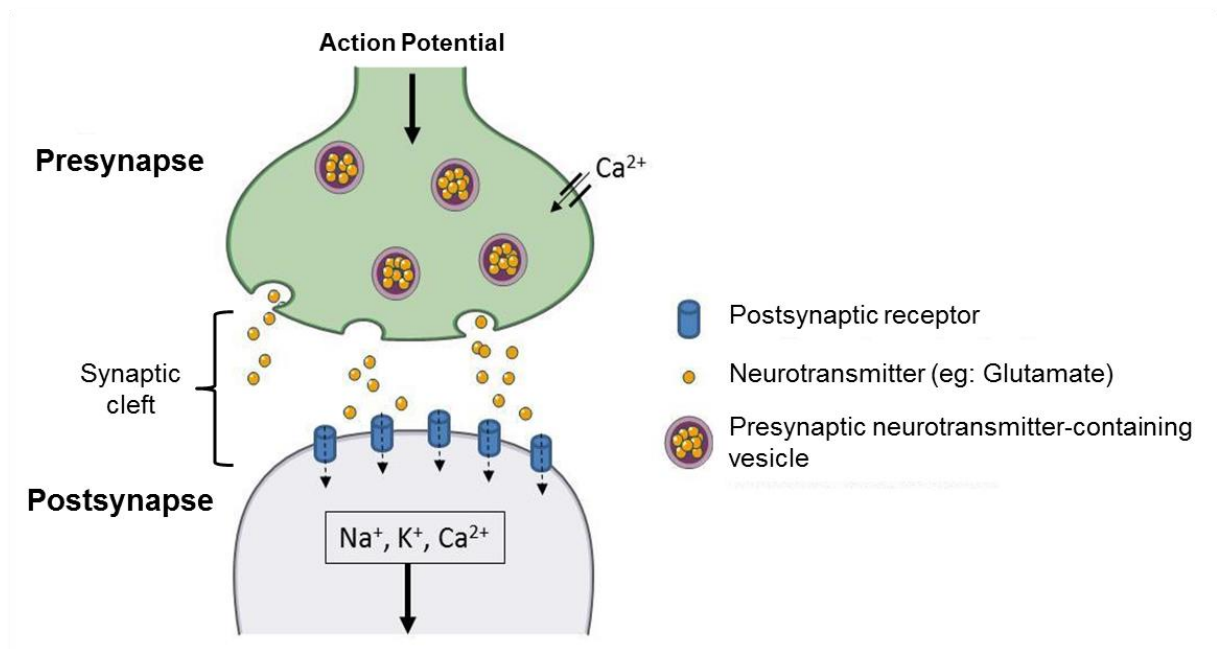


Figure 22: Illustration of a chemical synapse. The action potential arrives at the presynaptic axonal terminal (black arrow) and provokes the opening of voltage-gated calcium channels. Calcium entry induces neurotransmitter-containing vesicle fusion to the presynaptic plasma membrane and release of neurotransmitter into the synaptic cleft. Neurotransmitter (eg: Glutamate) then binds to postsynaptic receptors. This binding allows the opening of postsynaptic ion channels creating an influx of cations (Na^+ , K^+ , Ca^{2+}) in the postsynaptic element which will induce a depolarisation of the membrane whereas an influx of anions (Cl^-) will induce a hyperpolarisation. This ion influx enables the transmission of the nervous signal.

Electron microscopy studies have enabled to identify excitatory glutamatergic synapses by the presence of: i) a presynaptic area encompassing numerous small vesicles which contain the neurotransmitter (glutamate) and ii) an electron-dense postsynaptic area known as the postsynaptic density (PSD) (Harris et al., 1992). The pre- and postsynaptic membranes are approximately 20 nm

apart, this gap delimitates the synaptic cleft. This narrow space is spread across a relatively wide area and enables a rapid increase of glutamate concentration whilst limiting its diffusion outside the synaptic cleft (Isaacson and Nicoll, 1993; Savtchenko and Rusakov, 2007). The structure of the synapses is maintained by adhesion proteins which link the pre- and postsynaptic elements together and organise the different components of the synapse. This architecture enables to minimise excitotoxicity phenomena and optimises the transmission of electrochemical signals.

B. Glutamatergic neurotransmission

There are several types of chemical synapses depending on the neurotransmitter that is released (**Table 7**). The majority of the excitatory neurotransmission in the CNS is carried out by glutamatergic excitatory synapses. These types of synapses are very abundant in the hippocampus and cortex.

Neurotransmitter	Postsynaptic effect
Glutamate (GLU)	Excitatory
Acetylcholine	
Catecholamine (Dopamine, Adrenalin...)	
Histamine	
Adenosine triphosphate (ATP)	
Serotonin	
γ -aminobutyric acid (GABA)	Inhibitory
Glycine	
Neuropeptides	Excitatory and inhibitory
Nitric oxide (NO)	

Table 7: Examples of neurotransmitters and their postsynaptic effects.

Glutamatergic neurotransmission plays an essential role in brain development and in the molecular mechanisms of memory and learning. This neurotransmitter activates two types of receptors, which are pharmacologically different and are classed depending on their transduction modalities, namely: metabotropic receptors and ionotropic receptors (Dhami and Ferguson, 2006; Zhu and Gouaux, 2017).

C. Glutamate receptors and synaptic transmission

The presynaptic element contains the machinery which enables the release of glutamate by regulating synaptic glutamate-containing vesicle transport and fusion to the plasma membrane. Neurotransmitter release from this presynaptic element is calcium-dependent and relies on both activation of voltage-gated channels (Katz and Miledi, 1967) and intracellular calcium release from reservoirs present in the presynaptic element (Emptage et al., 2001). The glutamate released into the synaptic cleft binds and activates different types of receptors located on the pre- and postsynaptic elements. Two broad categories of receptors can be distinguished: i) metabotropic receptors coupled to G proteins (mGluRs) ii) ionotropic receptors coupled to ion channels which include AMPAR (α -amino3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor), NMDAR (N-methyl-D-aspartate receptor) and kainate receptors.

1. Metabotropic receptors

Metabotropic receptors (mGluRs) are G-protein coupled receptors. There are eight sub-types of mGluRs subdivided into three families depending on their sequence homology and their coupling to secondary messengers (**Figure 23**) (for review: Nicoletti et al., 2011).

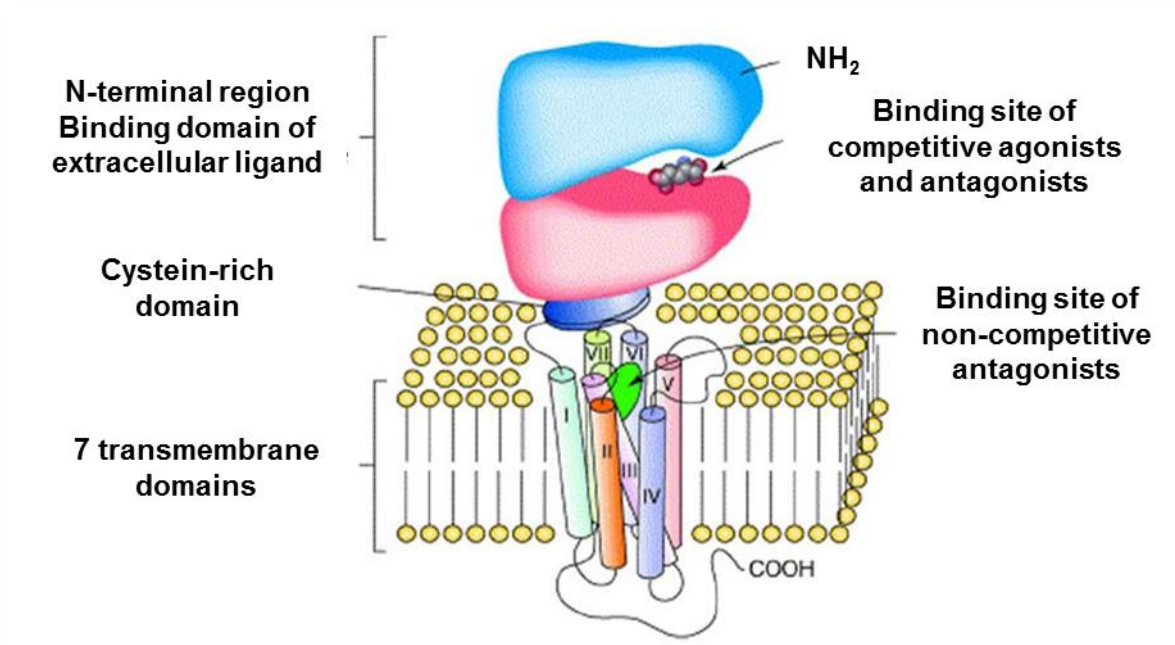


Figure 23: Illustration of a metabotropic glutamate receptor (adapted from: Spooren et al., 2001). The neurotransmitter (glutamate) binding domain in the N-terminal region is followed by a heptahelix domain (the region with 7 transmembrane domains) and a cytosolic C-terminal domain (COOH).

mGluR1 and mGluR5 belong to group I; mGluR2 and mGluR3 belong to group II; and mGluR4, mGluR6, mGluR7 and mGluR8 belong to group III (**Table 8**; mGluRs section).

Group I includes receptors positively coupled to phospholipase C (PLC) via Gq-type G-proteins. PLC activation in turn induces the release of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 will induce intracellular release of Ca²⁺ stored in the endoplasmic reticulum (Endoh, 2004).

Metabotropic receptors from group II and III are negatively coupled to adenylate cyclase (AC) via inhibitory G-proteins which will reduce the activity of AC and decrease the amount of cyclic adenosine monophosphate (AMPC) in the cell (for review: Conn and Pin, 1997). AMPC is implicated in the activation of protein kinase A (PKA), phosphorylates kainate receptors, but also AMPAR and potentiates their activity.

The mGluRs regulate and participate in synaptic transmission via several effectors such as protein kinase C (PKC), inositol triphosphate receptors or membrane ion channels.

Amongst these three groups of receptors, metabotropic receptors from group I are localised at the postsynaptic level, whereas receptors from group II are localised at the pre- and postsynaptic level, and receptors from group III are preferentially expressed at the presynaptic level where they modulate the release of neurotransmitters by acting as presynaptic autoreceptors (**Table 8**, mGluRs section). A number of studies have shown that metabotropic receptors from group I are involved in numerous psychiatric disorders and in synaptic plasticity (for review: Bhattacharyya, 2016). The variety of roles held by glutamate metabotropic receptors reflects in the diversity of their localisation around the glutamatergic synapse but also in their pharmacological characteristics as well as their sensitivity to agonists/antagonists (**Table 9**, mGluRs section).

Family	iGluRs			mGluRs		
Receptor	AMPA	Kainate	NMDA	Group I	Group II	Group III
Structure of complex	Homo/hetero- oligomeric			Homo/hetero- oligomeric		
Subunits	GluA1 to 4	GluK1 to 5	GluN1 GluN2 (A, B, C, D) GluN3 (A, B)	mGluR1 and 5	mGluR2 and 3	mGluR4, 6, 7, 8
Permeability (iGluRs) or induced effects (mGluRs)	Na ⁺ , K ⁺ , (Ca ²⁺)	Na ⁺ , K ⁺ , (Ca ²⁺)	Na ⁺ , K ⁺ , Ca ²⁺ +++	↑ PLC	↓ AC	
Localisation	Postsynaptic	Pre- and postsynaptic	Postsynaptic	Postsynaptic	Presynaptic	

Table 8: Overview of the principal characteristics of ionotropic and metabotropic glutamate receptors.

Metabotropic glutamate receptors have slow activation kinetics and are not involved in fast synaptic transmission. Indeed, their activation via glutamate does not result in the opening of a channel but rather in the activation of signalling cascades implicated in relatively slow modulations of synaptic transmission (Niswender and Conn, 2010).

2. Ionotropic receptors

Ionotropic receptors (iGluRs) are channel receptors permeable to sodium (Na^+), calcium (Ca^{2+}) and potassium (K^+) ions (**Table 8**, iGluRs section).

In the CNS, there are three groups of ionotropic receptors. They are classed depending on their pharmacological characteristics, their selective sensitivity to agonists, their voltage sensitivity and their localisation (**Table 9**, iGluRs section). These receptors were named after the agonist that enabled to identify them: AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NMDA (N-methyl-D-aspartate) and kainate (KA).

Family	iGluRs			mGluRs		
Receptor	AMPA	Kainate	NMDA	Group I	Group II	Group III
Particularities	- GluA2 impermeable to Ca^{2+} - Fast excitatory synaptic transmission	- 2 binding sites with different affinities - Involved in epilepsy - Modulates glutamate release	- Channel blocked by Mg^{2+} at resting potential - Learning, memory, induction of synaptic plasticity +++	Regulation of neuronal excitability via ion channel modulation	Involved in inhibition of glutamate and other neurotransmitter release	
Agonists	AMPA	Kainate	NMDA	(S)-3,5-DHPG	DCG-IV	
Antagonists	DNQX, NBQX, CNQX	GYKI 53655	AP-5	AIDA, MPEP	LY341495	
Voltage sensitivity	Voltage-dependent	Voltage-dependent	Voltage-dependent	G-Protein coupled receptor		

Table 9: Particularities that differentiate metabotropic and ionotropic glutamate receptors.

Each subunit of iGluRs is composed of four domains: an N-terminal domain, an agonist binding site (Glutamate or Glycine) formed by the spatial folding of two extracellular loops S1 and S2. These two loops are linked to the transmembrane domain (TM) composed of hydrophobic regions (TM I-IV) that defines a part of the pore of the tetrameric receptor. Lastly, there is the cytosolic C-terminal domain (**Figure 24**). This domain is the most variable between the different subunits of iGluRs and

participates in the trafficking and anchoring of iGluRs to scaffold proteins at the plasma membrane. The function of iGluRs is conditioned by their localisation in the synaptic complex, at the presynaptic level they modulate neurotransmitter release (kainate type receptors), and at the postsynaptic density (PSD) they participate in synaptic transmission (AMPA, NMDA and KA). Furthermore, iGluRs can also be extrasynaptic and be activated either by glutamate diffusion outside the synaptic cleft, this phenomenon is coined a “spillover”, or by glutamate release for glial cells.

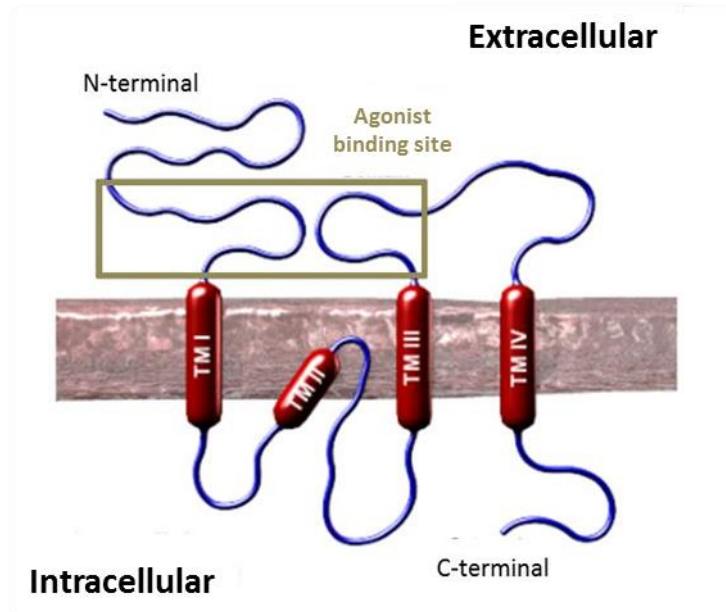


Figure 24: Schematic diagram of the general structure of ionotropic glutamate receptors (adapted from Bristol, n.d.). The N-terminal domain is extracellular whereas the shorter C-terminal domain is intracellular.

a) AMPA receptors

The AMPA receptor (AMPAr) is a complex formed of four subunits: GluA1, GluA2, GluA3 and GluA4 (**Table 8**, iGluRs section) produced by different genes (Hollmann and Heinemann, 1994; Traynelis et al., 2010). Therefore, it is a tetrameric glutamatergic receptor. The types of subunits which form the receptor dictate its electrophysiological properties. AMPAr forms an ion channel that is permeable to K^+ and Na^+ but is also permeable to Ca^{2+} . The GluA2 subunit is responsible for blocking the passage of calcium through the pore (**Table 9**, iGluRs section) therefore AMPAr that possess a GluA2 subunit are permeable to calcium ions.

AMPA receptors are the main actors in fast synaptic transmission and are present in all glutamatergic synapses. These receptors are, therefore, rapidly activated. They trigger glutamatergic neurotransmission and have high motility within the membrane. They enable rapid conversion of glutamate release from presynaptic vesicle into electrical activity in the post synaptic neuron.

Therefore these receptors are mainly concentrated on the postsynaptic membrane of dendritic spines, within the PSD. However, their localisation at the subcellular level seems variable. Although they are present in large quantities at the synapse, they are also present outside the synapse where they diffuse freely in the plasma membrane. Once these extrasynaptic AMPAR reach the synapse, however, their motility is greatly reduced thus indicating that these extrasynaptic AMPAR might serve as a reservoir of postsynaptic receptors (for review: Groc and Choquet, 2006).

b) NMDA receptors

NMDA receptors (NMDAR), contrary to AMPA receptors, need simultaneous fixation of glutamate (or NMDA) and a co-agonist, glycine or D-serine, on two sites located on two different subunits, in order to be activated (Mothet et al., 2000). Furthermore, this receptor forms an ion channel that is permeable to Na^+ , K^+ but also Ca^{2+} , and is blocked by an extracellular Mg^{2+} ion when the membrane is at resting potential. This membrane potential-dependent Mg^{2+} makes this NMDAR a real sensor of simultaneous postsynaptic neuron depolarization and presynaptic glutamate release (Yuste et al., 1999). Simultaneous activation of both pre- and postsynaptic elements is therefore required to activate these receptors, and the subsequent Ca^{2+} entry allows them to play an essential role in the establishment of certain synaptic plasticity processes.

NMDA receptors are also heterotetramers composed of two mandatory GluN1 subunits associated to GluN2 (A to D) or GluN3 (A and B). Each of these subunits has an extracellular N-terminal domain (NTD), an agonist binding domain (ABD), a transmembrane domain that forms the channel pore, and an intracellular C-terminal domain of which the length varies depending on the type of subunit (Paoletti et al., 2013). The ABD forms a central pocket that binds to: the co-agonist (glycine or D-serine) for GluN1 and GluN3; and glutamate for GluN2 (A – D) (Paoletti et al., 2013; Yao et al., 2013). Each receptor subtype has specific biophysical, pharmacological and signalling pathway properties (Paoletti et al., 2013).

The spatiotemporal expression of the different subunit mRNAs varies significantly, particularly GluN2 (Monyer et al., 1994). During mouse embryonic development of the CNS, only receptors containing GluN2B and GluN2D are present. GluN2B is expressed throughout the brain whereas GluN2D is restricted to the diencephalon, mesencephalon and brain stem. Receptors with GluN2A subunits start progressively to be expressed after birth, contrary to GluN2D containing receptors that decrease rapidly (Monyer et al., 1994). This same inverted tendency is observed for GluN2A and GluN2B, with a maximal expression of GluN2B during the second postnatal week, then a progressive

decline to then be restricted to anterior brain structures. In parallel, GluN2A expression increases and spreads progressively throughout the cortex, hippocampus and cerebellum. Finally, GluN2C containing receptors are expressed after birth, mainly in the cerebellum and, to a lesser extent, in the olfactory bulb. Spatiotemporal expression modulation of these different subunits could play a crucial role in regulating neuronal circuit remodelling and adaptation to afferent sensory experiences (Sanz-Clemente et al., 2013).

Although NMDA receptors are always expressed at the postsynaptic density (PSD), whether the synapse is active or not (Takumi et al., 1999), some NMDAR are also expressed extra- and presynaptically. Extrasynaptic NMDAR are organised in clusters which can be mobilized to the synapse by lateral diffusion during synaptic plasticity processes (Papouin and Oliet, 2014). It is believed that extrasynaptic NMDAR are mainly composed of GluN2B subunits. Indeed, GluN2B preferentially interacts with SAP102 rather than PSD95, contrary to GluN2A, which increases its diffusion capacity thus increasing the extrasynaptic pool of GluN2B containing receptors. At the presynaptic level, NMDAR can be found in both excitatory and inhibitory synapses (Bouvier et al., 2015). These receptors modulate neurotransmitter release but can also be implicated in presynaptic signalling. Notably, it has been suggested that presynaptic receptors could be a source of calcium influx involved during LTD processes (Bidoret et al., 2009).

Together these data highlight that NMDA receptor subunit composition influence synaptic transmission by modulating their motility, their synaptic or extrasynaptic localisation, and even their conductance (Paoletti et al., 2013).

c) Kainate receptors

Kainate receptors are ubiquitously expressed in the CNS and can be found at both pre- and postsynaptic levels. Contrary to AMPA and NMDA receptors, kainate receptors are not much involved fast glutamatergic synaptic transmission. However, they play an important role in regulating neuronal network activity and plasticity (Sihra et al., 2014). They can also regulate postsynaptic membrane depolarisation as well as presynaptic neurotransmitter release (Huettner, 2003). Furthermore, these receptors also take part in the modulation of presynaptic plasticity processes in the hippocampus as they facilitate long-term potentiation (LTP) at the synapses between mossy fibres and CA3 neurons (Lauri et al., 2001).

In order to ensure proper synaptic transmission at the synapse, AMPA and NMDA receptors along with a host of other components are all finely organised on the postsynaptic element: the dendritic spine, which will be described in the next section.

D. The Dendritic spine

1. Background

Dendritic spines form the postsynaptic compartment which receives the nervous influx of excitatory glutamatergic synapses. These spines were initially discovered by Ramòn y Cajal in 1888. They noticed the presence of these distinct membrane protrusions all along the dendritic arborisation of neurons (**Figure 23**).

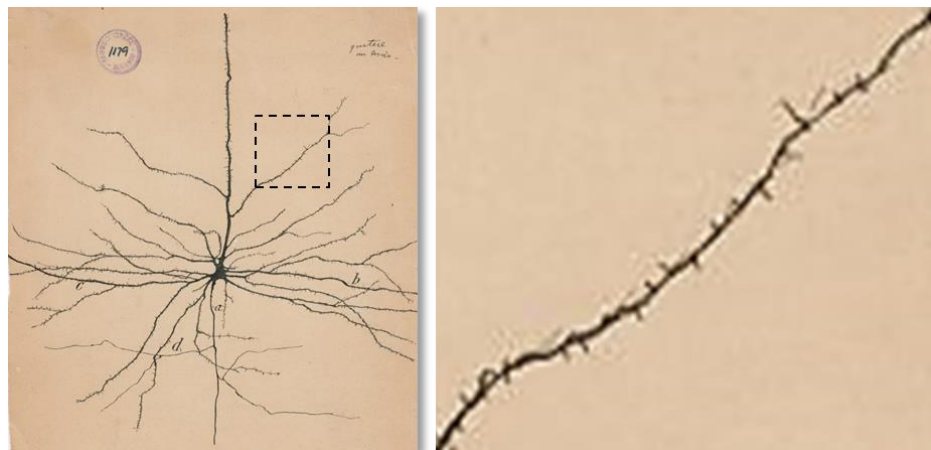


Figure 23: Original illustration of Ramon y Cajal. Silver-stained pyramidal neuron (left). Close-up of a dendritic branch with dendritic spines (right).

These protrusions were later coined “dendritic spines”. It wasn’t until the development of electron microscopy that more information was available about these structures. In 1959, George Gray showed, for the first time, the contact point upstream of the dendritic spine: the axon terminal or axonal bouton (**Figure 24**). Gray managed to distinguish two categories of synapses. The first was symmetrical synapses with two electron-dense zones: one on the presynaptic and one on the postsynaptic side; both of these zones were of same size (Guillery, 2005). These symmetrical synapses are now known to be characteristic of inhibitory synapses. The second category of synapses was asymmetrical synapses, with an electron-dense zone on the postsynaptic side only, and corresponds mainly to excitatory synapses.

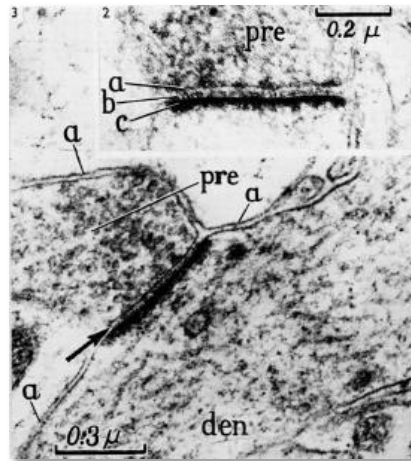


Figure 24: The difference in thickness of pre- and postsynaptic density is one of the first criteria to differentiate synapses (adapted from Guillery, 2000). Electron-microscopic image of an asymmetrical synapse. “a” is the presynaptic electron-dense zone. “b” is the dense material in the synaptic cleft and “c” is the postsynaptic density. “pre”: presynaptic; “den”: dendrite.

2. Dendritic spine morphology

Dendritic spines are small (typically 0.5 – 2 μm in length) membranous protrusions that house the essential postsynaptic components, including the PSD, actin cytoskeleton, and a variety of “supporting” organelles (Figure 25).

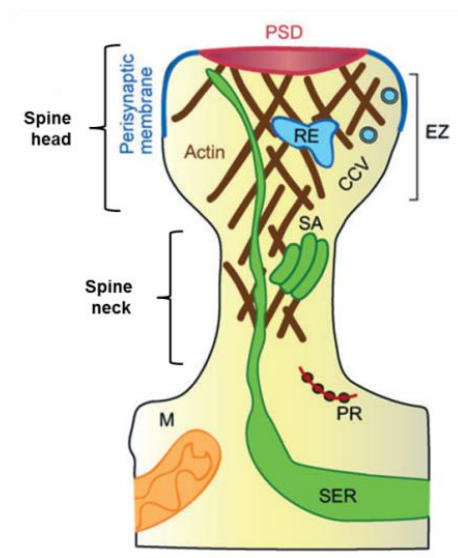


Figure 25: Schematic diagram of a mature mushroom-shaped spine, showing the PSD, the perisynaptic membrane and other organelles (adapted from Sheng and Hoogenraad, 2007). The endocytic zone (EZ) is located lateral of the PSD in extrasynaptic regions of the spine, where it may be associated with clathrine-coated vesicles (CCV) and recycling endosomes (RE). Smooth endoplasmic reticulum (SER), polyribosomes (PR) and mitochondria (M) are found mainly at the base of the spine neck but may extend into the spine. The abundant cytoskeleton (brown lines) is connected to the PSD and determines spine structure and motility. Other abbreviation: SA, spine apparatus.

Spines occur at a density of 1 to 10 spines per μm of dendrite length on principal neurons (Sorra and Harris, 2000). Typical spines have a bulbous head (receiving a single presynaptic bouton) connected to the parent dendrite through a thin spine neck (**Figure 25**).

The surface of the spine head can be divided into three concentric circles. The centre, averaging at 500 to 1000 nm in diameter, is the core of the PSD housing the AMPARs and NMDARs (**Figure 25**, red zone), just opposite the presynaptic release site. Around the core is a 100-200 nm ring (**Figure 25**, blue zone) called the perisynaptic domain, where mGluRs are preferentially enriched. Beyond this perisynaptic domain is the extrasynaptic domain (Scheefhals and MacGillavry, 2018). This highly organised spatial segregation has important functional implications for synaptic efficacy, where the peri- and extrasynaptic domains act as reservoirs for glutamate receptors.

Because the neck hinders diffusion of molecules to and from the parent dendritic shaft, spines serve as microcompartments in which biochemical changes in one individual synapse can be isolated from other synapses on the same neuron (Kennedy et al., 2005; MacGillavry and Hoogenraad, 2015). The geometry of the spine neck determines, for example, calcium efflux into the dendritic shaft, thus modulating the degree of calcium elevation in the spine head following NMDAR activation. This spine neck diffusion barrier is said to be modulated by synaptic activity (Tønnesen et al., 2014).

Dendritic spines are highly heterogeneous structures that show dynamic motility, especially during development and synaptic plasticity processes (Tada and Sheng, 2006). Their number, size and shape undergo plastic changes correlated with long-term modifications of synaptic strength and interneuronal connectivity (Hayashi and Majewska, 2005). Spine shape has been broadly categorised as “mushroom”, “thin” or “stubby” (which will be further described in **Part III.C.2.a, b and c**) (**Figure 26**). Though electron-microscopy studies tend to show more of a continuum between these categories, there is growing evidence that different spine shapes and sizes reflect different developmental stages and/or altered strength of synapses (Hayashi and Majewska, 2005; Yuste and Bonhoeffer, 2001). Sophisticated imaging experiments indicate that the volume of spine heads can increase with stimuli that strengthen synapses and can decrease with stimuli that weaken synapses (Hayashi and Majewska, 2005; Kasai et al., 2003). The molecular mechanisms that coordinate synaptic strength with spine morphogenesis are still under investigation.

Spines with large heads are generally stable, express large numbers of AMPAR, and contribute to strong synaptic connections. In contrast, spines with small heads are more motile, less stable, and contribute to weak synaptic connections (Holtmaat et al., 2006). *In vivo* timelapse studies show that spines turn over at various rates in the mouse brain; a large proportion of mushroom spines are persistent, with lifetimes up to several months (Trachtenberg et al., 2002). Nevertheless, it is

currently believed that a subset of spines can undergo changes in shape or number in response to experience and other factors in the adult brain, thereby relating spine morphology to synaptic plasticity and long-term memory formation.

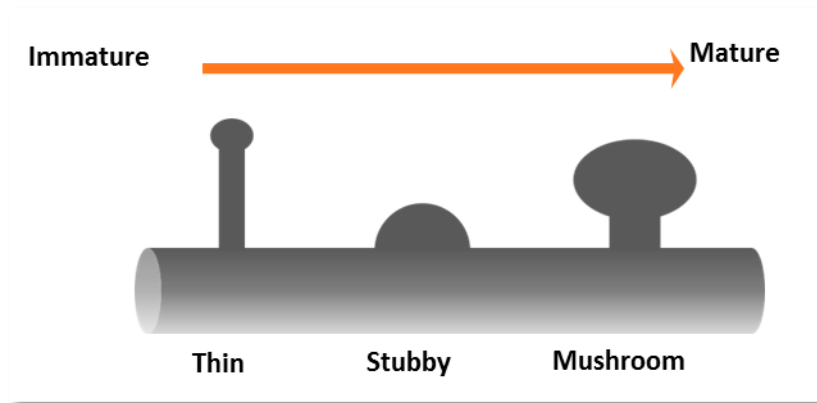


Figure 26: Schematic diagram of the different types of spines. Spine shape translates synaptic strength and functionality but also spine maturity. Thin spines are less mature and functional whereas mushroom spines are mature and functional. Stubby spines are at an intermediate stage.

a) Thin spines

Thin spines represent the majority of dendritic spines in the adult hippocampus and cortex (Harris et al., 1992). These spines are particularly dynamic and show the most spontaneous morphology changes. They have distinct morphological characteristics such as a defined neck, which is much longer than the diameter of the neck, and a defined small head, that is barely wider than the spine neck (**Figure 26**) and faces an axonal bouton. The dynamics of these spines enable them to maintain an excellent structural flexibility. These changes in shape are associated to neurotransmission modifications on the presynaptic side and enable the spines to adapt their shape according to an increased or decreased stimulus. Therefore, because of their plastic nature, these thin spines are also called “learning spines” (Bourne and Harris, 2007; Harris and Kater, 1994).

b) Stubby spines

Stubby spines are intermediate spines in the process of maturation. These spines are also considered as axon-dendritic contacts. They don't have a mature shape as they do not have a neck at the base of the dendrite (**Figure 26**). Their length is approximately the same as their head-width.

c) *Mushroom spines*

Mushroom spines represent approximately 25% of the spines present in the CNS (Bourne and Harris, 2007). These are characteristic of mature spines as they have a defined thin neck, a well-defined bulbous head with a diameter significantly superior to the neck diameter and this head is at least 0.6 μm (Bourne and Harris, 2007; Sorra and Harris, 2000). Spine head volume ranges from 0.01 to 0.8 μm^3 (Gipson and Olive, 2017).

These spines have the highest concentration of PSD components. Mushroom spines are the active/mature spines that ensure glutamatergic synaptic transmission.

How a thin spine matures into, eventually, a mushroom spine will be described in the following section.

3. Dendritic spine morphogenesis

Dendritic spines appear early on during post-natal development, after neurite formation. To date, there is no consensus in the scientific community about the formation process spines or spinogenesis. There are currently three models of spine development (**Figure 27**).

The earliest model (studied on pyramidal neurons) proposes that spine formation is initiated by the axon terminal that makes contact with the dendrite directly. Then, the postsynaptic element develops from this contact point and forms a dendritic spine (Miller and Peters, 1981).

The second model came from a study on Purkinje cells (Sotelo, 1991), where the proposed hypothesis is that the dendritic spine develops independently from the axon terminal. Dendritic spine would emerge and mature from the dendrite without axonal contact.

The last model, based on pyramidal neuron studies, proposes that filopodia (the most immature form of spine) are the precursors of spines. These filopodia emerge from the dendrite and make contact with a nearby axon. This contact initiates the maturation of both the axon terminal and the dendritic spine. More recent optogenetic and ultrastructure studies seem to support this last hypothesis (Hotulainen et al., 2009; Korobova and Svitkina, 2010).

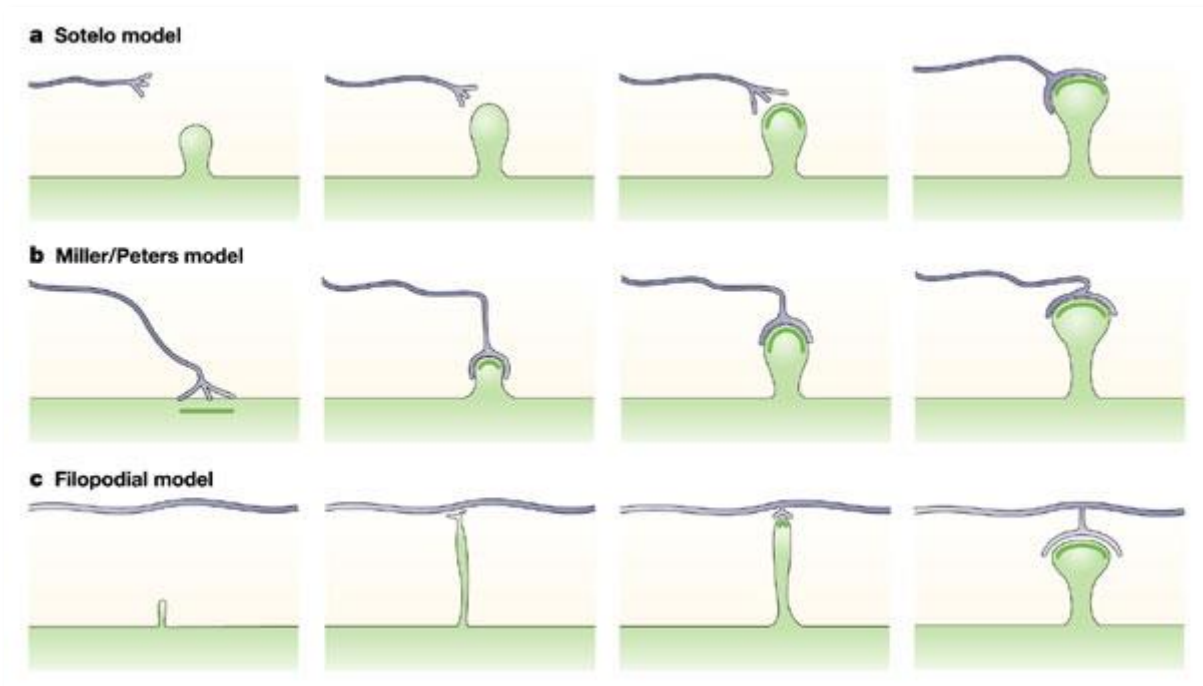


Figure 27: The different models of spinogenesis (adapted from: Yuste and Bonhoeffer, 2004). (a) Sotelo's model, 1990. This model proposes that spines from Purkinje cells develop independently from axonal fibres. (b) Miller & Peter's model, 1981. The authors propose a model where axon terminals stimulate spine formation. (c) Filopodia model, Vaughn, 1989. Vaughn proposes a "synaptotropic" hypothesis where filopodia associate to axons and form dendritic spines upon contact.

The size, shape, motility, maturation and stability of dendritic spines depend largely on actin, the primary cytoskeleton within spines. A complex network of regulatory proteins control actin arrangement and spine morphogenesis.

E. Actin cytoskeleton: the scaffold of dendritic spines

Despite having a small volume, dendritic spines are actin-rich protrusions with thousands of proteins, among other molecular components involved in different functions (Murakoshi and Yasuda, 2012) (**Figure 28**). Both monomeric (G-actin) and filamentous (F-actin) actin are present in spines, below the PSD. Whereas spine heads mainly consist of a network of branched F-actin, in the neck filaments they usually form long bundles lengthwise of the spine apparatus (Hotulainen and Hoogenraad, 2010).

In the last few years, some studies have proposed the existence of different actin pools within the dendritic spine. A dynamic pool is believed to be found below the spine surface, whereas another pool is thought to be more stable to support the overall structure of the spine. A third pool of stable

F-actin has been described upon glutamate uncaging or LTP induction (Honkura et al., 2008), and its confinement to dendritic spines seems to require CAMKII activity. Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) along with actin are important molecules involved in synapse structure and plasticity. Indeed, in addition to its signalling function, CaMKII plays a structural role via direct interaction with actin filaments, (Okamoto et al., 2009) (**Figure 28**).

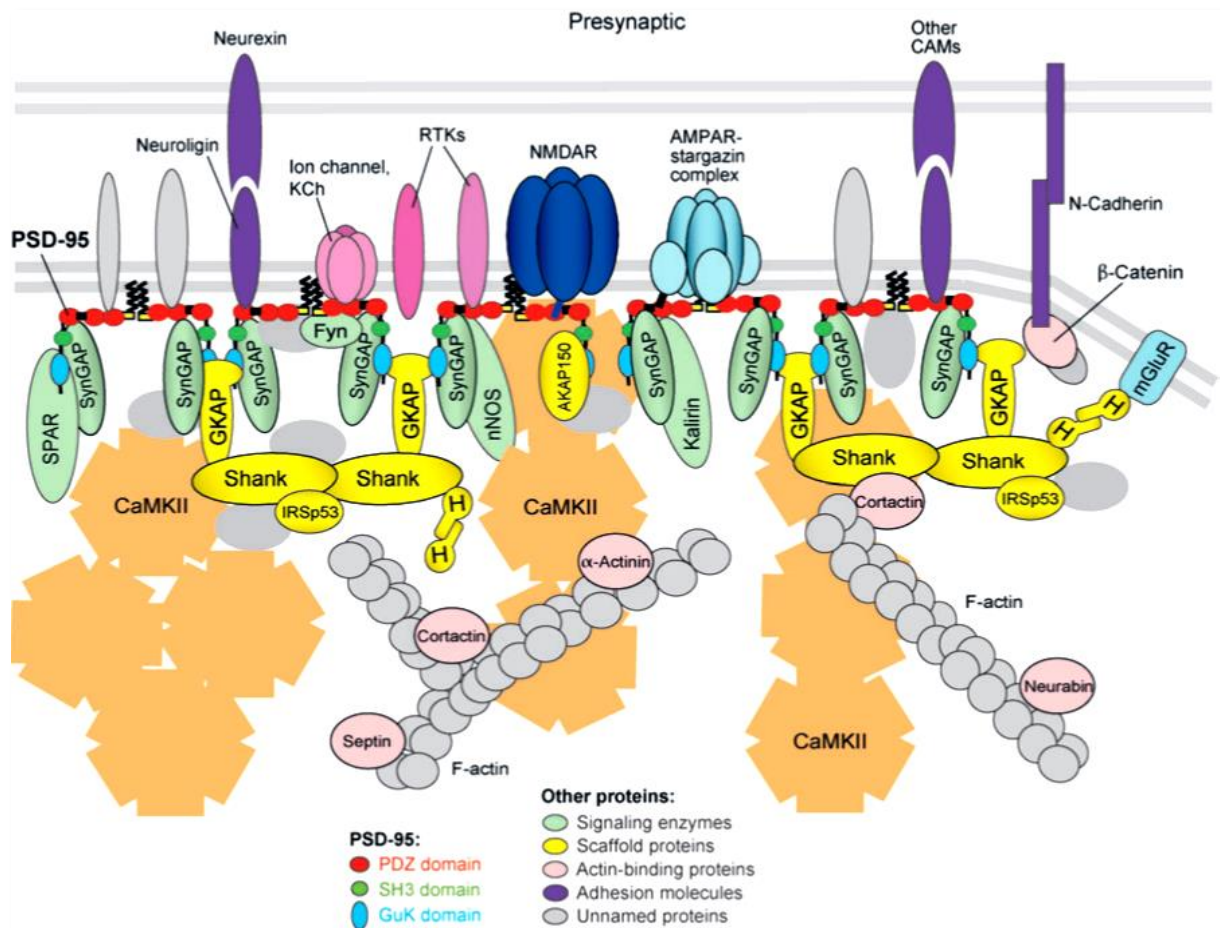


Figure 28: Organisation of proteins and protein-protein interactions in the postsynaptic density (PSD) (adapted from Sheng and Hoogenraad, 2007). Schematic diagram of the network of proteins in the PSD, with edge of PSD depicted at right. Only major families and certain classes of PSD proteins are shown. Contacts between proteins indicate an established interaction between them. Domain structure is shown only for PSD-95 (PDZ domain, SH3 domain, GuK domain). Other scaffold proteins are coloured yellow; signalling enzymes, green; actin binding proteins, pink. CaMKII (calcium/calmodulin-dependent protein kinase II) is depicted as dodecamer. Unnamed proteins signify the many other PSD proteins that are not illustrated in this diagram. Abbreviations: AKAP150, A-kinase anchoring protein 150 kDa; CAM, cell adhesion molecule; Fyn, a Src family tyrosine kinase; GKAP, guanylate kinase-associated protein; H, Homer; IRSp53, insulin receptor substrate 53 kDa; KCh, K^+ channel; mGluR, metabotropic glutamate receptor; nNOS, neuronal nitric oxide synthase; RTK, receptor tyrosine kinases (e.g., ErbB4, TrkB); SPAR, spine-associated RapGAP.

Actin filaments in the spine head are very dynamic and show a high turnover, with a total protein replacement every 2 – 3 minutes (Honkura et al., 2008). Furthermore, previous studies show that the

degree of actin polymerisation affects the various aspects of dendritic spine morphology (Murakoshi and Yasuda, 2012).

One of the most relevant roles of actin cytoskeleton in mature spines is to modulate spine head structure and size in response to synaptic activity (Star et al., 2002). Additionally, it contributes to overall structure of synapses, organising the PSD, anchoring and stabilising postsynaptic receptors, localising the translation machinery (Hotulainen and Hoogenraad, 2010) and is said to serve as a molecular sieve to counteract the free diffusion of unbound signalling molecules, possibly in order to favour their interaction with their substrates (MacGillavry and Hoogenraad, 2015).

Several actin-binding proteins (ABPs) as well as other actin-associated proteins are enriched in dendritic spines and cooperate to regulate actin-based cellular events (**Figure 28**). Some of them play a major role in actin nucleation like the Arp2/3 complex, which orchestrates *de novo* actin polymerisation, and its activators Cortactin, Abp1, N-WASP, WAVE-1 and Abl interactor 2 (Abi2). All of these proteins are essential to spine structure and morphology since the alteration of any of these proteins results in dendritic spine and synaptic structural impairments (Bellot et al., 2014).

Other actin cytoskeleton-interacting proteins participate in F-actin severing (ADF/cofilin and gelsolin), actin bundling (calpoin, dystrophin) or actin polymerisation (profilin). Upon NMDAR activation, calcineurin/PP2B causes a dephosphorylation of cofilin through slingshot protein phosphatase 1 (SSH1) activity, and active cofilin is translocated into dendritic spines for spine remodelling (Pontrello et al., 2012). Thus cofilin is essential in controlling the turnover of F-actin at synapses and a dysregulation F-actin may result in alterations in spine morphology and density, leading to impaired associative learning. Recently, in our research group, cofilin phosphorylation was found to be dysregulated in APP/PS1-21 mice as well as in cultured neurons exposed to A β oligomers leading to the formation of aberrant cofilin-actin rods, which impact spine morphological remodelling, block axonal trafficking and thus may contribute to deficits in synaptic plasticity (Rush et al., 2018).

Together, these data highlight the pivotal role of actin cytoskeleton in the formation and elimination, motility and stability, size and shape of dendritic spines; making the actin cytoskeleton a major actor of synaptic plasticity.

F. Synaptic plasticity

Synaptic plasticity defines the set of molecular mechanisms that take place within the synapse in order to modify its neurotransmission properties depending on its use. Although very few neurons renew themselves within the brain, synaptic connections, however, undergo many changes that can be short-term or longer-term. Indeed, the brain is capable of positively or negatively modulating the efficacy of synaptic connections in response to neuronal activity. This is an essential characteristic for the formation and function of neuronal networks involved in the molecular mechanisms of learning and memory.

The strength of neurotransmission can be increased or decreased for a few minutes, hours or even up to several months. This is called Long-Term Potentiation or Depression of synaptic function (Barnes, 1979). This model is considered as the reference for the study of the molecular and cellular mechanisms of learning and memory consolidation, and has enabled tremendous understanding of the molecular processes underlying cognitive function.

1. Long-term Potentiation (LTP)

Long-term potentiation (LTP) is the experimental paradigm that reproduces one of the types of synaptic plasticity associated to the molecular events at the origin of memory consolidation processes. It can be induced by high-frequency electrical stimulation of the presynaptic neuron.

In 1973, the research group of Bliss and Lomo showed that high frequency stimulation of the perforant path fibres in the hippocampus induced a persistent increase of synaptic transmission efficacy in the dentate gyrus (Bliss and Lomo, 1973). This has also been evidenced in the connections between the Schaeffer collaterals and the dendritic spines of CA3 and CA1 hippocampal areas (Alger and Teyler, 1976) (**Figure 29**). LTP is not inherent to hippocampal excitatory synapses as it has also been demonstrated in synapses of many other brain regions such as the amygdala, the cortex and the cerebellum (Maroun, 2006; Martin et al., 2000; Xin et al., 2006).

In vivo electrical stimulation in animal models can induce an LTP that persists for several days (Douglas and Goddard, 1975). Pharmacological modulation of this LTP can affect the learning capacities of these animals, particularly with the use NMDAr antagonist AP-5 (Morris et al., 1986). Indeed, LTP induction depends mostly on synaptic glutamate release and subsequent postsynaptic Ca^{2+} entry through glutamate-activated NMDAr, this process is coined NMDA-dependent LTP.

However, LTP can also be induced via voltage-gated calcium channels or via mGluRs (mGluR1,5), this is called NMDA-independent LTP (Lanté et al., 2006; Whitlock et al., 2006).

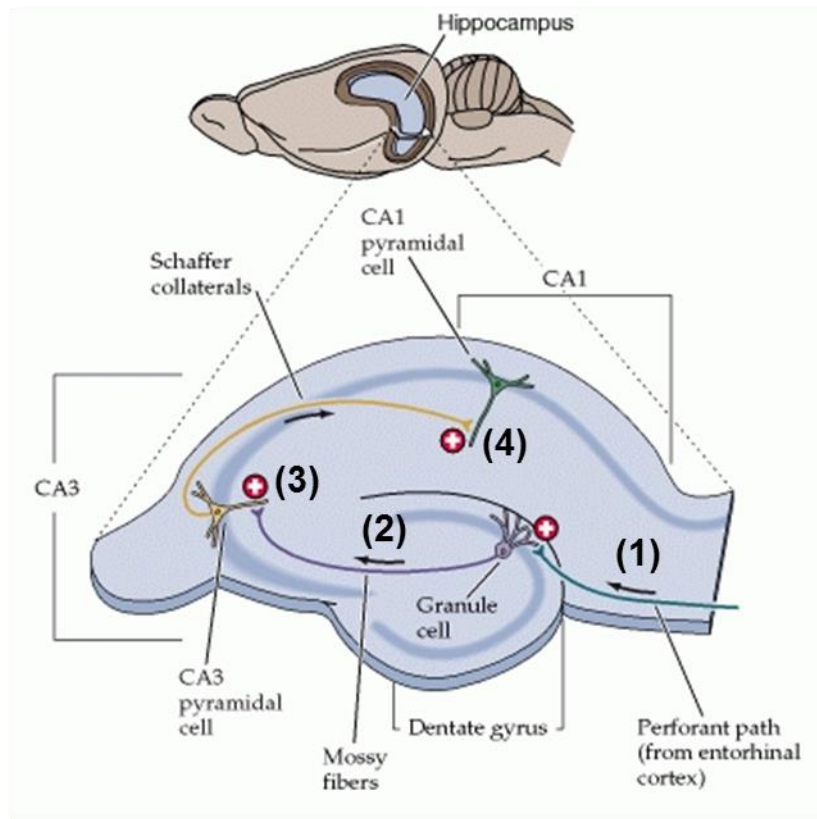


Figure 29: Schematic diagram of a rodent hippocampal slice showing the different regions, excitatory pathways and synaptic connections (adapted from Purves et al., 2001). The excitatory pathways are illustrated with a (+) sign. Influx coming from the entorhinal cortex follows the perforant pathway (1) then goes through the mossy fibres of the dentate gyrus (2) that are connected to the Schaeffer collaterals in CA3 (3) which in turn project in CA1 (4). This pathway enabled to demonstrate LTP, which has been observed in each of the 3 synaptic connections (2, 3 and 4) represented in this diagram.

During a brief low frequency synaptic transmission (**Figure 30**), the action potential coming from the axon terminal induces a Ca^{2+} influx in the presynaptic compartment. This Ca^{2+} influx enables synaptic glutamate-containing vesicle exocytosis and subsequent glutamate release into the synaptic cleft. This glutamate then binds to postsynaptic AMPAR and NMDAr. AMPAR opens and lets Na^+ ions enter the postsynaptic compartment inducing membrane depolarisation. This enables the expulsion of Mg^{2+} blocking NMDAr and this, in turn, induces an ion influx after binding of glutamate and co-agonist to NMDAr. This series of events can induce an EPSP (Excitatory postsynaptic potential) but will not induce the prolonged activation of NMDAr necessary for LTP induction.

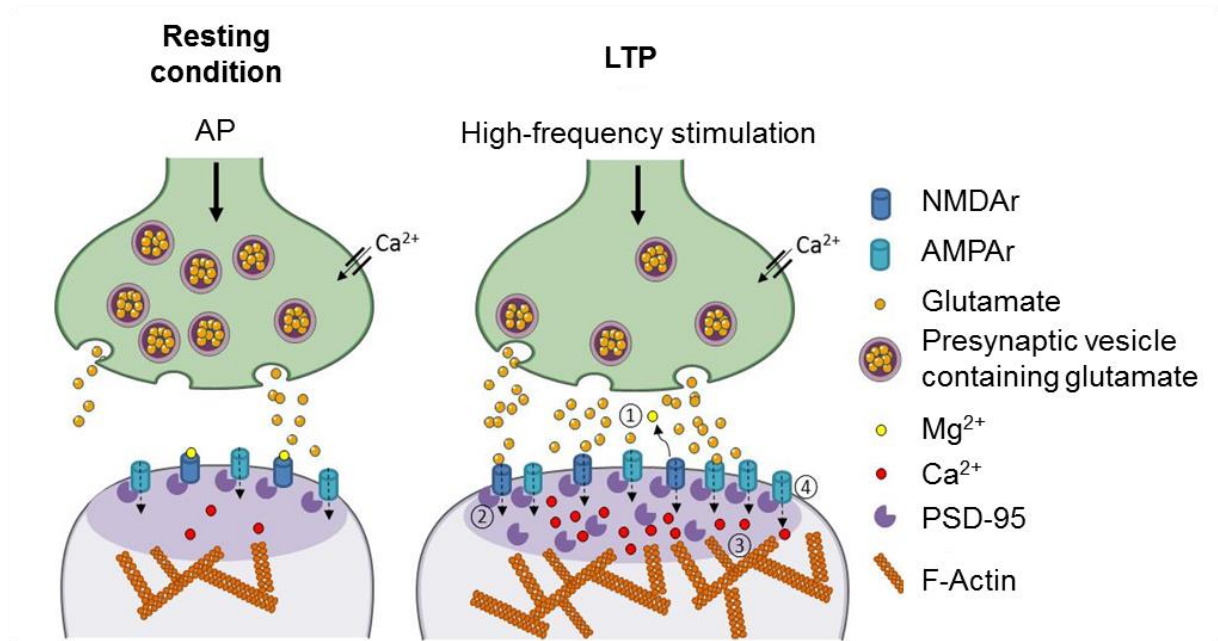


Figure 30: Schematic representation of the main synaptic mechanisms of LTP. During simple neurotransmission, NMDAr are blocked by an Mg^{2+} ion, the synapse is in “resting condition” (left). During high-frequency synaptic activation, the presynaptic compartment releases glutamate that binds to AMPAr and NMDAr. This stimulation induces postsynaptic membrane depolarisation allowing Mg^{2+} ion to be ejected from NMDAr ① and provokes massive Ca^{2+} influx②, which is the major secondary messenger that activates several signalling pathways leading to actin reorganisation to increase spine volume ③ and recruitment of more AMPAr, PSD-95 and NMDAr to the spine ④. This sequence of events, as well as the subsequent structural and functional changes within the synapse, leads to increased synaptic strength via induction and consolidation of long-term potentiation.

LTP has two phases: the induction phase and the consolidation phase. The induction phase can experimentally reproduced by a high-frequency stimulation of the presynaptic neuron like the ones used in LTP protocol (a short stimulation, less than 1 second, at 100 Hz, for example). This type of stimulation induces a sustained depolarisation of the plasma membrane via AMPAr activation following glutamate release into the synaptic cleft. This provokes Mg^{2+} ion expulsion from NMDAr channel (**Figure 30**). This expulsion allows the passage of massive Ca^{2+} ion influx through NMDAr, dramatically rising postsynaptic Ca^{2+} concentration. NMDAr are therefore activated once glutamate release from axon terminal, glutamate binding to receptors and postsynaptic membrane depolarisation occur concomitantly. NMDAr are therefore the main players in Ca^{2+} influx during NMDA-dependent LTP (Collingridge et al., 1983).

Intracellular Ca^{2+} increase is central during the consolidation phase of LTP. It induces transduction pathway activation leading to potentiation of the postsynaptic compartment. LTP induction is associated with activation of transduction pathways involving protein kinases such as protein kinases PKA and PKC (Roberson and Sweatt, 1996), ERKs (Bading and Greenberg, 1991), PI3K (Man et al., 2003) and CAMKs (Malenka et al., 1989). All these protein kinases participate in the increase of

synaptic strength and efficacy. Notably, protein neosynthesis occurs following phosphorylation by the kinases of transcription factors such as CREB (Kandel, 2001). Another example is the activation of CAMKII which induces the phosphorylation of GluA1 at serine 831 of non-synaptic AMPAR and subsequently increases their integration to the PSD; therefore increasing both the number of AMPAR on the postsynaptic membrane and their conductance (for review: Lisman et al., 2012).

The expression level of NMDAR subunits also seems to play a role in the establishment of LTP. Notably, GluN2B subunits give a slow inactivation rate to NMDAR, responsible of longer-lasting current and bestow a longer opening-time of NMDAR, enabling a longer Ca^{2+} influx than GluN1/GluN2A. Moreover, NMDAR GluN1/GluN2B interact more with CAMKII implicated in AMPAR and ERF phosphorylation. Several studies support the hypothesis that GluN1/GluN2B-type NMDAR facilitate LTP induction (Barria and Malinow, 2005). However, others have shown an implication of GluN2A in LTP induction (Bellone and Nicoll, 2007; Matta et al., 2011). Thus, the implication of these different subunits in LTP is still under debate.

2. Long-term Depression (LTD)

It was in the 80s that Long-term Depression (LTD) was discovered. It is, in a way, the “opposite” mechanism of LTP. It is a type of synaptic plasticity present in the synapses of the cerebellum, the cerebral cortex, the hippocampus and the striatum.

LTD is induced by a low-frequency stimulation of the presynaptic neuron (1 – 5 Hz) which provokes a weak to moderate Ca^{2+} influx in the postsynaptic neuron following NMDAR activation (Lüscher and Malenka, 2012). NMDAR are responsible for calcic influx during LTP but also during LTD. However, whilst LTP is induced by a strong depolarisation and a massive calcic influx in the dendritic spine, LTD is induced by a moderate depolarisation and a weak Ca^{2+} influx which translates into phosphatase protein activation (Mulkey et al., 1994) such as calcineurin and PP1 (protein phosphatase 1). These phosphatases will induce endocytosis of AMPAR from the PSD leading to a reduction of the number of AMPAR at the PSD associated to a decrease in dendritic spine volume.

It has been suggested that this insertion/endocytosis of AMPAR during LTP and LTD occur at the perisynaptic level where the receptors reach the postsynaptic density via lateral diffusion (Lüscher and Malenka, 2012).

G. Dendritic spine dynamics, the basis of synaptic plasticity

1. Actin dynamics in dendritic spines

In dendritic spines, the effective remodelling of the actin cytoskeleton is essential for adequate morphological dynamics. In general, actin filaments in spines are exceptionally short and dynamic (Frost et al., 2010; Hotulainen et al., 2009; Korobova and Svitkina, 2010; Koskinen et al., 2014; Star et al., 2002).

In terms of turnover rate, mature spines are proposed to have two distinct pools of actin filaments regulating spine shape and dynamics during maturation and activity (Honkura et al., 2008; Star et al., 2002). The stable pool of F-actin occupies 5–20% of total spine F-actin. These stable pools of F-actin have a turnover rate of 17 minutes and are found in structures generally located at the base of the spine head, where they form the structural backbone of the spine (Honkura et al., 2008). The size of the stable pool in dendritic spines increases during neuronal maturation (Koskinen et al., 2014). Conversely, the dynamic pool of F-actin, which represents 80-95% of total spine F-actin, has a turnover of 40 seconds, approximately 25 times faster than the stable pool. There is also a third pool of filamentous actin with an intermediate turnover rate. This pool appears upon LTP induction and is responsible for spine head growth. Accordingly, this pool was coined the “enlargement pool” (Honkura et al., 2008).

2. The interplay between the actin cytoskeleton and synaptic plasticity

a) *The signalling pathways that regulate F-actin networks*

LTP and LTD correlate with the enlargement and shrinkage of dendritic spines, respectively (Cingolani and Goda, 2008; Matsuzaki et al., 2004; Zhou et al., 2004). Therefore, the organisation and dynamics of the F-actin network requires to be adjusted accordingly to the strength of synaptic transmission (Saneyoshi and Hayashi, 2012). This readjustment is finely tuned by signalling pathways that regulate F-actin networks during synaptic plasticity. These pathways give rise to a sequence of events that lead to morphological remodelling of dendritic spines where the actin cytoskeleton sustains both spine structure and function (**Figure 31**).

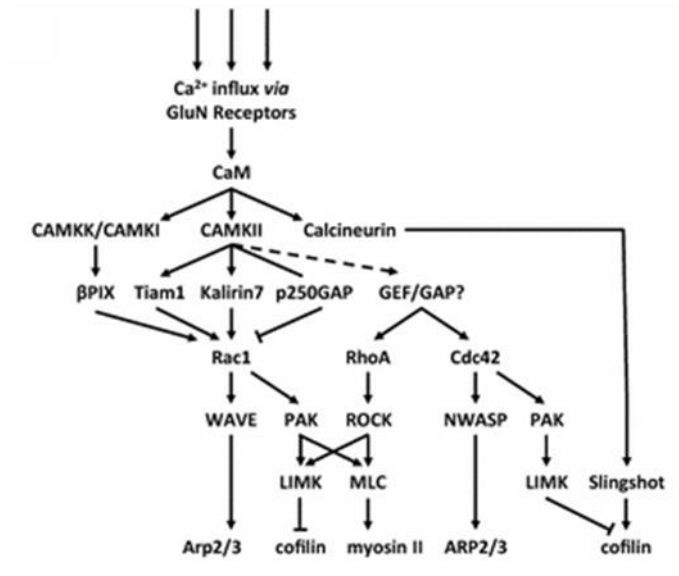


Figure 31: Actin remodelling signalling pathways (adapted from Chazeau and Giannone, 2016). Selected signalling cascades driving F-actin remodelling upon Ca^{2+} influx via GluN receptors. CAMKII is a key triggering protein both involved in functional and structural LTP. CAMKII can activate small RhoGTPases (Rac1, Cdc42, RhoA) by phosphorylating GEFs (Tiam1, Kalirin7) and GAPs (p250GAAP). This will control the spatiotemporal activation of several ABPs (Arp2/3, myosin II, cofilin). (Arrows) indicate positive regulation. (T-shaped bars) indicate negative regulation. (GluN) NMDAr. (CaM) calmodulin. (CaMK) calcium-calmodulin dependent protein kinase. (GEF) guanine nucleotide exchange factor. (GAP) GTPase activating protein. (β PIX) β PAK interacting exchange factor. (Tiam1) T cell lymphoma invasion and metastasis-inducing protein 1. (Rac1) Ras-related C3 botulinum toxin substrate 1. (Cdc42) cell division cycle 42. (RhoA) Ras homologous member A. (ROCK) Rho-associated, coiled-coil containing protein kinase. (PAK) p21-activated kinase. (LIMK) LIM-kinase. (N-WASP) neuronal Wiskott-Aldrich syndrome protein. (WAVE) Wasp-family verprolin homologous protein. (MLC) myosin light chain. (Arp2/3) actin-related protein 2/3.

b) F-actin reorganisation during synaptic plasticity

Early electron-microscopy studies already reported that LTP induction would increase dendritic spine head and PSD (Buchs and Muller, 1996). More recent studies using two-photon glutamate uncaging (Matsuzaki et al., 2004) and time-lapse STED microscopy (Tønnesen et al., 2014) confirmed these findings and also showed that spine enlargement was accompanied by GluA1 recruitment and increased GluA1-mediated current as well as a widening and shortening of the spine neck.

These changes in spine head volume display two distinct phases:

- a transient large volume increase which takes place in the first 5 minutes of LTP and where spine head volume may increase up to 200-400%.
- Followed by long-lasting smaller volume increase for small dendritic spines which takes place within 60 minutes after LTP and where spine volume increases up to 50-150%.

Spine neck changes, however, seem to display only a long-lasting change with an approximate 30% increase in spine neck width and 30% decrease in spine neck length (Tønnesen et al., 2014). These modifications were abrogated by pharmacological treatments that increased F-actin depolymerisation (latrunculin-A) confirming that F-actin supports transient and long-lasting changes of dendritic spine head.

Honkura et al showed in 2008, using two-photon glutamate uncaging, that single spine activation (which triggered spine enlargement) induced the formation of a transient enlargement F-actin pool distributed throughout the spine head, displaying a distinct turnover compared to the stable and dynamic F-actin pools (**Figure 32**). The localisation and dynamics of the enlargement pool often synchronised with spine membrane protrusions, as if F-actin polymerisation drove the enlargement. However, increased volumes triggered by LTP last longer than the life-time of the enlargement pool, suggesting that the long-lasting spine enlargement might be sustained by transfer of F-actin from the enlargement into the stable pool.

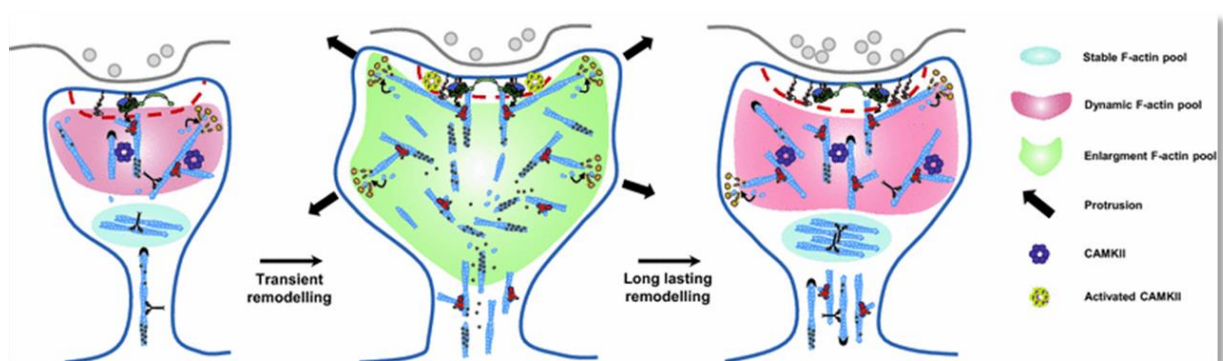


Figure 32: A model for F-actin reorganisation during LTP (adapted from Chazeau and Giannone, 2016). LTP induces a transient and long-lasting increase of the spine head size, a shortening and widening of spine neck and a concomitant ABPs and CaMKII spine recruitment. Activated CaMKII will dissociate from F-actin and phosphorylate multiple proteins leading to a fast F-actin reorganisation and a transient spine head enlargement. This transient reorganisation is characterised by the formation of an enlargement F-actin pool, an increase in the F-actin/G-actin ratio and increased concentration of cofilin and Arp2/3. During the long-lasting spine head enlargement, most ABPs return to their basal concentration, suggesting the formation of a larger dynamic and stable F-actin pool. Those larger dendritic spines most likely provide a “tag” for the capture of newly synthesised synaptic proteins in order to sustain late LTP.

Similar studies, using an adaptation of the two-photon glutamate uncaging to induce LTD named LFU (Low-frequency uncaging), were performed to explore the effects of LTD on dendritic spine remodelling. Results showed an approximate 25% shrinkage of dendritic spines (Oh et al., 2013). Spine shrinkage following LTD was shown to be also F-actin dependent since inhibition of ADF/cofilin, calcineurin or Arp2/3 complex blocked spine shrinkage (Hayama et al., 2013; Nakamura et al., 2011;

Wang et al., 2007). Therefore, as for LTP, regulation of F-actin organisation and dynamics might be critical mechanisms for structural LTD.

Numerous studies have demonstrated that synaptic activity modifies spine morphology, structure and function. In physiological conditions, dendritic spines adapt to the incoming information. However this adaptation is usually the front line target in neurodegenerative diseases, particularly in the case of Alzheimer's disease. Indeed, dendritic spine alterations and eventually loss induced by A β oligomers is one the first steps of AD pathogenesis and may be at the origin of the cognitive impairments in early AD.

IV. A β pathology and excitatory synapses

Extracellular amyloid plaques composed of A β peptides are among the principal pathological characteristics observed during AD development. Many other additional structural and functional alterations are also observed such as inflammatory responses and oxidative stress (McGeer et al., 2006). The combination of all these alterations gives rise to synaptic impairments and eventually loss. Most studies point towards A β accumulation in the brain as the main culprit of these synaptic dysfunctions which are at the basis of disease progression.

Extracellular amyloid plaques were considered as one of the main causative agents of AD and particularly because of cortical and hippocampal amyloid deposits. However, it is now widely accepted that it is in fact the soluble oligomeric A β species that are at the origin of synaptic dysfunction and loss, and AD onset rather than extracellular A β plaques and NFTs (Gong et al., 2003; McLean et al., 1999). Studies using certain mutations of the amyloid peptide found in FADs support this hypothesis, especially the Osaka mutation which causes intraneuronal accumulation of A β and absence of amyloid plaques while still inducing severe cognitive impairments. This is why using FAD mutation-based forms of A β to investigate A β -induced effects on neurons are crucial to understand the role of A β , not only in AD pathogenesis but also to get an insight into the physiological role of A β in the brain.

B. The impact of A β on synaptic transmission

3. Alterations of synaptic activity and cognitive function

At this point in time, the role of the A β peptide within synapses and the effects of synaptic activity on the A β peptide are some of the centre pieces of the unresolved puzzle in the comprehension of AD.

Notably, it has been shown that synaptic activity increases A β secretion in the extracellular space (Cirrito et al., 2005; Kamenetz et al., 2003). It is this extracellular A β which then provokes LTP reduction (Shankar et al., 2008), LTD facilitation and alters the quantity of synaptic proteins (Snyder et al., 2005) as well as the structure of the synapse *in vitro* and *in vivo* (Almeida et al., 2005; Coleman and Yao, 2003; Hsieh et al., 2006; Selkoe, 2002). Therefore, it seems that chronic exposure to extracellular A β induces synaptic alterations which could lead to the accumulation of A β peptides at the origin of extracellular amyloid plaque formation. This suggests that synaptic activity could

contribute to AD development and progression. Furthermore, it has also been shown that, after a traumatic brain injury, there is an extracellular accumulation of A β during the recovery phase of cognitive function (Brody et al., 2008).

Although synaptic activity and cognitive/neuronal activity aren't quite equivalent, studies have shown that cognitive activity stimulation have rather a protective effect against AD progression. Indeed, it seems that a higher education is associated to a reduction in the risk of developing AD (Stern, 2012). Moreover, studies based on APP^{swe}/PS1 Δ E9 transgenic AD mice showed that an enriched, cognitively stimulating environment slows down the formation of amyloid plaques and upregulates genes implicated in learning and memory processes (Lazarov et al., 2005).

4. Alterations of the number and function of synaptic receptors

Several studies have shown that oligomeric A β peptides exert their neurotoxic effects through alterations of glutamatergic neurotransmission. Although electrophysiological studies have brought to light that A β oligomers cause synaptic dysfunctions, these peptides also alter glutamatergic receptor function and quantity at the synapse. To support this, A β has been found to interact with numerous receptors found at the PSD, such as NMDAr (De Felice et al., 2007; Decker et al., 2010), AMPAr and mGluR5 (Renner et al., 2010), neurotrophin p75^{NTR} (Kraemer et al., 2014), Prion protein (PrP^C) (Laurén et al., 2009), glutamate transporter (Li et al., 2009), ephrinB2 receptor (Cissé et al., 2011) and ephrinA4 (Fu et al., 2014; Vargas et al., 2014) as well as acetylcholine α -7 nicotinic receptor (α 7nAChRs) (Wang et al., 2000). It has also been shown that A β interacts with other proteins of the PSD such as PSD-95 (Pham et al., 2010). In pathological conditions, all of these synaptic receptors and proteins constitute potential binding partners to oligomeric forms of A β and these interactions may be involved in synaptic dysfunctions and synaptic plasticity alterations (for review: Tu et al., 2014).

5. Alterations of synaptic plasticity

It has been brought to light that synaptic plasticity processes of LTP and LTD are significantly altered by oligomeric forms of the A β peptide. Indeed, several studies have shown that LTP was altered/decreased, and LTD was in some cases facilitated, by wild-type oligomeric A β as well as mutated toxic forms of A β (Li et al., 2011, 2009; Selkoe, 2008; Shankar et al., 2007; Tomiyama et al., 2008; Walsh et al., 2002). Furthermore, it has also been shown that the initial spine enlargement

phase of LTP is altered in APP overexpressing/A β overproducing neurons meaning that structural plasticity is also impaired (Wei et al., 2010).

Among the different hypotheses explaining the effects of A β oligomers on the mechanisms of synaptic plasticity, one of them proposes that LTP inhibition may be due to internalisation and endocytosis of NMDAr and AMPAr induced by A β oligomers. Application of A β oligomers on acute hippocampal slices provokes a decrease of AMPAr concentration at the surface of dendritic spines, and more precisely an endocytosis of GluA2 AMPAr, which leads to synaptic transmission depression (Hsieh et al., 2006; Zhao et al., 2010). Other studies have shown that incubating neuronal cultures with A β oligomers increases NMDAr endocytosis via calcineurin and STEP (Striatal enriched phosphatase) activity (Snyder et al., 2005). Taken together, these studies show that A β oligomers induce AMPAr endocytosis, especially GluA1 and GluA2; leading to spine loss and is responsible for reduced NMDAr response (Hsieh et al., 2006; Lacor et al., 2004; Miñano-Molina et al., 2011; Shankar et al., 2007).

Another hypothesis proposes that the changes observed at the level of synaptic receptors could lead to modifications of intracellular signalling pathways which may be responsible for the effects on synaptic plasticity induced by A β oligomers (**Figure 33**). Notably, excessive activation of GluN2B-type NMDAr by A β oligomers could affect the function of certain enzymes, and calcium-dependent protein kinases and phosphatases such as calcineurin, which could eventually lead to synaptic dysfunctions and excitotoxicity due to alterations of certain signalling cascades and mitochondrial homeostasis. According to certain studies, A β oligomers alter different kinases and phosphatases such as, for example, GSK3 β (Glycogen synthase kinase 3 β), Cdk5 (cyclin-dependent kinase 5), members of the MAPK (mitogen-activated protein kinase) family like PAK (p21-activated kinase) or ERF (extracellular-signal-related kinase), AMPK (AMP-activated protein kinase), CaMKII (Dolan and Johnson, 2010; Mairet-Coello et al., 2013; Thornton et al., 2011; Zhu et al., 2007) but also CREB (cAMP response element binding protein) transcription factor. On the other hand, studies based on models which overexpress APP showed that alterations of mitochondrial dynamics, transport and function are associated with synaptic dysfunction and loss (Baliatti et al., 2013). This suggests that A β may affect mitochondrial function thus leading to intracellular calcium concentration dysregulations. However, a recent study showed that oligomeric A β toxicity could be more NMDAr-dependent rather than calcium flux variations alone, since synaptic alterations are attenuated when NMDAr are blocked by AP-5 but not when ion flux are blocked by calcium chelator like BAPTA or when NMDA ion channels are blocked by MK-801 or memantine (Birnbbaum et al., 2015).

Other studies suggest that the effects of A β peptides observed on synaptic plasticity may essentially be due to excessive activation of extrasynaptic NMDAr. Activation of these extrasynaptic receptors is thought to alter calcic regulation processes and oxidative mechanisms due to hindered mitochondria. Furthermore, activation of extrasynaptic NMDAr may be responsible for a preferential shift towards the amyloidogenic pathway (Bordji et al., 2010) leading to increased A β production. More recently, a few studies have showed that A β oligomers alter glutamate reuptake/recycling thus leading to glutamate leakage outside the synaptic cleft (“glutamate spillover” phenomenon) thus activating extrasynaptic NMDAr with subsequent LTP inhibition and activation of proapoptotic signalling cascades (Li et al., 2011; Varga et al., 2015). This extrasynaptic NMDAr recruitment could explain why A β oligomers facilitate LTD. Indeed, it has been shown that LTD induction by A β peptides induces the preferential activation of GluN2B-type NMDAr, a subpopulation of receptors that are more localised at the extrasynaptic level (**Figure 33**) (Kervern et al., 2012).

Other studies have shown that A β peptides are also capable of creating pores in the lipid bilayer of membranes which could contribute to the aberrant calcium influx inside neurons (Demuro et al., 2005; Lin and Arispe, 2015). Nevertheless, according to the literature, it seems unlikely that A β interaction with membrane receptors and the associated neurotoxic phenomena are the only events responsible for A β synaptotoxicity. Indeed, an increasing number of studies carried out on AD animal models as well as *in vitro* studies on neuronal cultures have highlighted a recurrent issue: the existence of intracellular A β inside neurons (Grundke-Iqbal et al., 1989) and their potential role in AD pathogenesis (for review: Gouras et al., 2012).

All of these molecular and cellular mechanisms involved in the synaptotoxicity induced by A β oligomers constitute a large panel of perturbations affecting NMDAr-dependent signalling pathways, calcium homeostasis, proteins involved in various signalling cascades, and mitochondrial function. All of these A β -related defective mechanisms all converge towards a dysfunctional dendritic spine, and as we stated earlier, dendritic spine function and morphology are tightly linked together, meaning that morphological A β -related defects are also observed. These morphological alterations are a readout for the dendritic spine functional defect and are reflected through the state of the actin cytoskeleton in the spine.

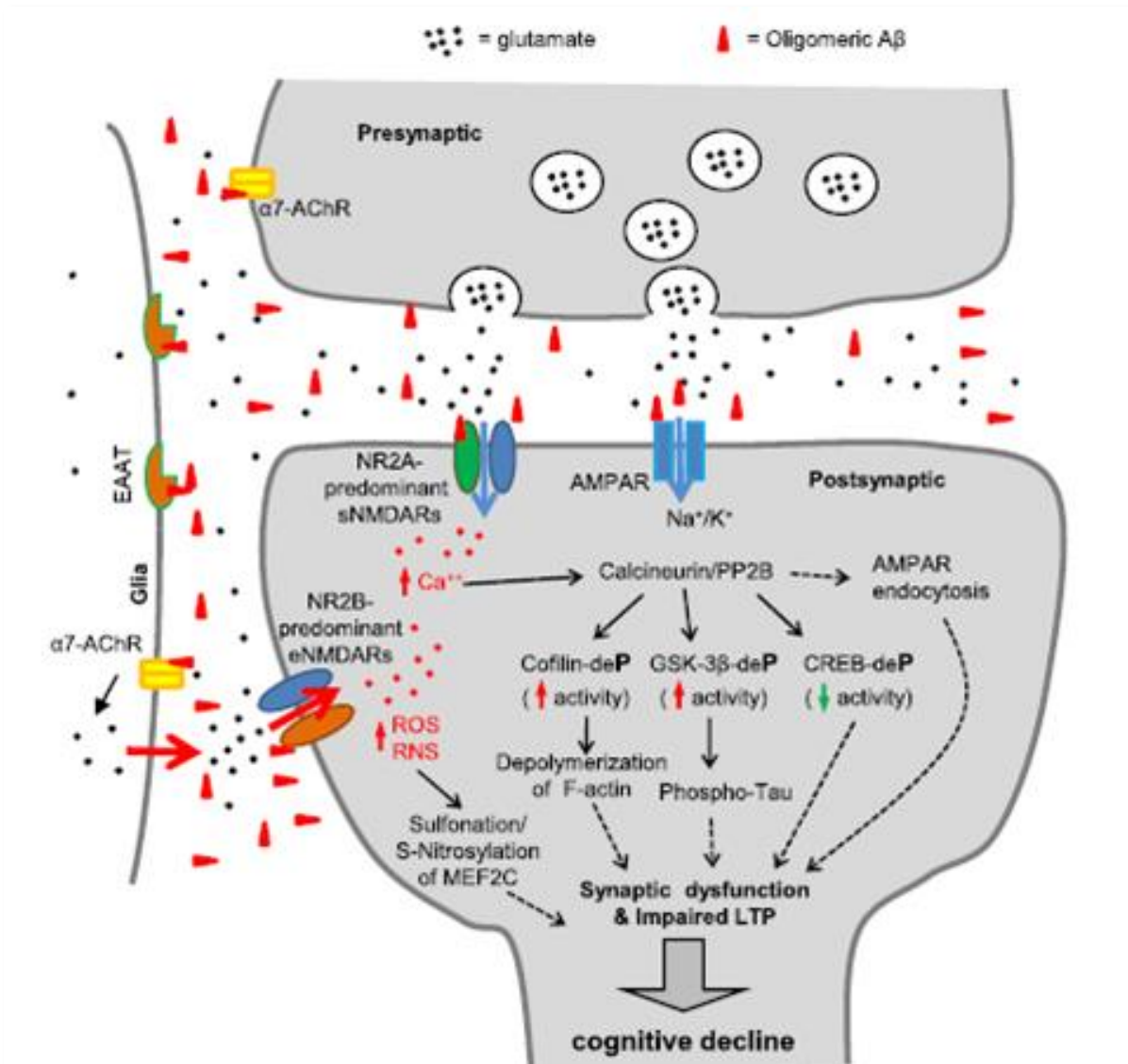


Figure 33: Schematic diagram of the potential mechanisms of oligomeric A β -induced synaptic dysfunction (adapted from Tu et al., 2014). At pathological concentrations, A β oligomers may interact with multiple astrocytic, microglial, and neuronal synaptic proteins, including $\alpha 7$ -AChRs and NMDARs, triggering a series of synaptotoxic events. These events include aberrant activation of NMDARs (especially NR2B-containing extrasynaptic NMDARs), elevated neuronal calcium influx, calcium-dependent activation of calcineurin/PP2B and its downstream signalling pathways, involving cofilin, GSK-3 β , CREB, and MEF2. This results in aberrant redox reactions and severing/depolymerising F-actin, tau-hyperphosphorylation, endocytosis of AMPARs, and eventually leads to synaptic dysfunction and cognitive impairment.

C. The impact of A β on dendritic spine morphology

3. Alterations of the synapse

As well as altering synaptic transmission, A β oligomers drastically impact the density and morphology of dendritic spines, the physical support of synapses (Androuin et al., 2018; Dorostkar et al., 2015). This effect is expected since, as we stated earlier, spine morphology reflects the efficacy of

synaptic transmission. Furthermore, these dendritic spine structural defects correlate much better with the cognitive impairments observed in AD, compared to all the other AD biomarkers, such as NFTs (for review: Masliah et al., 2006; Selkoe, 2002). Numerous studies have established a strong link between functional alterations induced by A β oligomers on synaptic receptors and synaptic transmission, and the resulting morphological alterations of dendritic spines (Androuin et al., 2018; Frandemiche et al., 2014; Miller et al., 2014; Shankar et al., 2007; Talantova et al., 2013; Um et al., 2012). A β oligomers induce this synapse loss whether the oligomers are synthetic, secreted by mutated APP overexpressing cells or extracted from brains of AD patients, whereas monomers and fibrils seem relatively inert (Lacor et al., 2004; Lambert et al., 1998).

In parallel of this spine number reduction there are also alterations of the expression of several proteins of the synaptic compartments such as proteins associated to presynaptic vesicular membranes like synaptophysine or Tau (Arendt, 2009; Frandemiche et al., 2014).

Tau protein, which plays an essential role in the assembly and stability of microtubules, is essentially present in axons and more rarely in dendrites, where Tau interacts with Fyn kinase and regulates postsynaptic NMDAR function (Ittner et al., 2010). A β oligomers induce rapid phosphorylation and translocation of Tau to dendritic spines, similar to what is observed following synaptic activation via GABA inhibition with Bic/4-AP protocol (Frandemiche et al., 2014). However, complementary experiments of protein interactions between Tau and actin suggest that this A β -induced translocation of Tau is unstable as its interaction with actin in the spines is more labile than when the translocation is synaptic-activity-induced (Frandemiche et al., 2014). Furthermore, this spatial translocation of Tau is characterised by a different phosphorylation motif of the protein depending on whether it is in response to synaptic activity or in response to neuronal stimulation via A β oligomers. Indeed, synaptic activation induces phosphorylation of tyrosine 205 whereas in presence of A β oligomers this phosphorylation is reduced and the serine 404 is phosphorylated (Frandemiche et al., 2014). Miller and colleagues confirmed with cultured neurons from AD mouse models harbouring the Swedish mutation that Tau translocation in spines depends on its phosphorylation (Miller et al., 2014). Therefore, these oligomers are responsible for functional alterations of Tau which modifies its interaction with the actin cytoskeleton and therefore modifies the architecture of the spine.

4. Alterations of the actin cytoskeleton

A β oligomers induce important alterations of the dendritic spines which contribute to synapse loss (Penzes and Vanleeuwen, 2011). Studies have highlighted that dendritic spine morphology is tightly linked to its function. Actin filaments, composed of actin molecules and other actin-binding proteins, form the skeletal network inside dendritic spines which plays a crucial role in spine morphogenesis, maintenance and plasticity. Oligomeric forms of A β induce dendritic spine loss (Shankar et al., 2007) which explains the reduction of spine density observed in AD patients' brains as well as in transgenic animal models (Knobloch and Mansuy, 2008).

The mechanisms at the origin of spine loss are still unclear. Nevertheless, one the principal hypothesis of oligomeric A β -induced synaptotoxicity suggests that spine collapse is NMDA-dependent. Indeed, NMDA blockade is sufficient to induce spine loss similar to what is obtained in presence of A β oligomers. Spine loss due to the presence of A β oligomers can be abrogated by treatment with antibodies against A β or treatment with molecules that prevent A β aggregation, but also require a signalling cascade involving NMDAR, calcineurin and cofilin (Shankar et al., 2007). These findings suggest that A β oligomers may activate NMDA-dependent signalling pathways which promote LTD induction. A β oligomers may activate or inhibit NMDAR which induces the activation of a calcium-dependent kinase, calcineurin. This phosphatase may in turn dephosphorylate cofilin (ie: activate this filamentous actin depolymerising protein). Subsequently, cofilin dephosphorylation by calcineurin induces progressive depolymerisation of actin filaments which structure the spine (Cheng et al., 2009; Liu et al., 2005; Rush et al., 2018). These A β oligomers may, therefore, provoke dendritic spine loss by affecting NMDAR which may alter postsynaptic calcium influx and subsequent modifications of downstream signalling pathways ultimately leading to spine weakening, loss and eventually decrease in dendritic spine density (Shankar et al., 2007).

All together, these studies carried out on the mechanistic action of A β seem to indicate that actin disruption is in part responsible for the observed effects, induced by A β , on spine structure, morphology and density. The effect of A β oligomers seems also to occur through NMDAR perturbations and cause downstream signalling pathway modifications. Therefore, there is no consensus on the action of these oligomers neither on dendritic spines or synaptic neurotransmission nor on the mechanism of action depending on the localisation of these A β oligomers, whether there are intracellular or extracellular.

D. Intracellular vs extracellular A β

Quite interestingly, so far, all the described effects of A β oligomers on spine function and morphology depend on the presence of A β oligomers in the extracellular space, whether they are exogenously added or secreted by overproducing cells. However, numerous studies have confirmed the presence of intraneuronal A β (Billings et al., 2005; Gouras et al., 2012, 2010, 2005, 2000; Grundke-Iqbal et al., 1989; LaFerla et al., 2007; Tomiyama et al., 2008).

Intraneuronal A β accumulation constitutes an essential and determining event in AD pathogenicity. More and more studies support the concept that intracellular A β oligomers play a critical role in the development of synaptic impairments at the origin of characteristic AD cognitive deficits (Billings et al., 2005; Gouras et al., 2012, 2010, 2005, 2000; LaFerla et al., 2007; Mohamed and Posse de Chaves, 2011; Mori et al., 2002; Oddo et al., 2003; Sheng et al., 2003). This concept was initially difficultly accepted by the scientific community since the first studies demonstrating the presence of intracellular A β were carried out three decades ago (Grundke-Iqbal et al., 1989) and the antibodies used at the time were not capable of differentiating A β from APP inside the neurons.

3. Intracellular A β accumulation: an early event in AD

It is now more widely accepted that intracellular A β accumulation constitutes an early event in humans and animal models of AD. Studies have brought to light that intracellular A β accumulation precedes NFT and senile plaque apparition (D'Andrea et al., 2001; Gouras et al., 2000). Furthermore, LTP defects and cognitive impairments which appear in the triple transgenic AD mouse model correlate with the presence of intracellular A β , before the apparition of NFTs or senile plaques (Billings et al., 2005; Oddo et al., 2003).

4. Forms of intracellular A β oligomers

Concerning the specific form of A β peptide that accumulates inside neurons, several studies have investigated, by immunocytochemistry, which oligomeric form of A β was mainly present inside the cells. Using antibodies against the C-terminus of A β ₄₀ or A β ₄₂, these studies showed that it is mostly the oligomeric A β ₄₂ peptide which accumulates intracellularly (D'Andrea et al., 2001; Echeverria and Cuellar, 2002; Gouras et al., 2000; Näslund et al., 2000; Ohyagi et al., 2007; Tabira et al., 2002). However, a recent study showed that not only oligomeric A β can be found inside neurons, but also monomeric, aggregated and fibrillary forms can also be found intracellularly (Pickett et al., 2016).

5. Intraneuronal localisation of A β and consequences of its accumulation

A study carried out on an AD transgenic mouse model (APP/PS1) showed via high-resolution imaging techniques that A β accumulates in the synaptic cleft and inside synapses, at the presynaptic terminal but even more so at the postsynaptic terminals close to amyloid plaques (Pickett et al., 2016). These results are in accordance with the general idea that both pre- and postsynaptic compartments are functionally affected by A β oligomers, and that postsynaptic terminals play a particularly important role in intracellular A β -induced synaptic loss. Studies carried out cultured neurons also demonstrate the existence of intracellular A β accumulation and associated synaptic dysfunctions (Greenfield et al., 1999; Skovronsky et al., 1998; Takahashi et al., 2004). Particularly, data show that oligomeric A β , A β_{40} and A β_{42} can accumulate in several intracellular compartments (such as the endoplasmic reticulum, Golgi complex, late endosomes, mitochondria and exocytosis vesicles) but also in the cytosol (in this case A β is rather found as “clusters”) (Zheng et al., 2013). This intracellular accumulation of A β may be responsible for synaptic dysfunction (Meyer-Luehmann et al., 2006; Takahashi et al., 2004, 2002) leading to possible more severe cellular pathologies.

Indeed, it is now known that A β accumulation inside neurons precedes degenerative events in all animal models presenting neuronal loss and synaptic dysfunction (Wirhth and Bayer, 2010). Several studies have shown that intracellular A β accumulation coincided with electrophysiological and behavioural perturbations characteristic of AD animal models (Billings et al., 2005; Knobloch and Mansuy, 2008). Furthermore, electrophysiological studies based on primary cultured neurons and acute hippocampal brain slices of AD mouse models, harbouring an APP mutation, show that synaptic activity increases the production/release of extracellular A β but reduces intracellular A β (Tampellini et al., 2009). Also, Tampellini and collaborators show that despite intra- and extracellular A β toxicity, under synaptic activation PSD-95 protein expression in transgenic mice is restored to control levels. These data, therefore, suggest a protective role of synaptic activity against synaptic defects induced by A β . To conclude, the effect of synaptic activity on the intracellular pool of A β is a key element in understanding AD pathogenesis.

E. The relationship between intra- and extracellular A β

Whether a focus is made on the possible mechanisms explaining the origin of an intracellular and extracellular pool of A β or on the effects of these two pools at the neuronal and synaptic level, any studies converge towards the importance of the particularly evident interconnection between the intracellular and extracellular A β pools.

3. Origin of intracellular A β

The intraneuronal A β pool may originate from an APP cleavage within the neurons on one hand, or from an internalisation of extracellular A β on the other. Although both of these mechanisms are distinct and regulated differently, understanding which of these mechanisms is most important or contributes the most to AD pathogenicity may highlight crucial information in order to identify new potential therapeutic targets for treating AD.

On one hand, several studies have described an *in situ* intraneuronal production of A β_{1-42} (Greenfield et al., 1999; Nathalie and Jean-Noël, 2008) and its intracellular accumulation, which have been reviewed by several (Bayer and Wirths, 2010; LaFerla et al., 2007; Mohamed and Posse de Chaves, 2011).

On the other hand, other studies converge towards a mechanism involving an internalisation of A β from the extracellular pool (D'Andrea et al., 2001; LaFerla et al., 2007; Ohyagi et al., 2007). Notably, it has been shown that cells treated with synthetic A β peptides exhibited an intracellular accumulation of A β_{42} and this accumulation was prohibited when endocytosis was blocked (Knauer et al., 1992). However, another possible mechanism of A β internalisation has been suggested such as the passive diffusion of extracellular A β through the plasma membrane (Li et al., 2007). Other studies showed that the mechanism of A β internalisation could occur through A β binding to $\alpha 7$ nACh nicotinic receptors followed by endocytosis of the complex resulting in inhibition of this receptor function and consequently leading to cognitive and memory defects (Dziewczapolski et al., 2009; Wang et al., 2000). It has also been suggested that A β may be internalised in the cells of CA1 region of the hippocampus but not the other hippocampal regions and this intracellular accumulation of A β may be responsible for a reduction of PSD-95 and GluR1 synaptic expression, resulting in synaptic activity perturbations (Almeida et al., 2005; Bahr et al., 1998). Others have brought to light the role of integrins and NMDAR in A β internalisation by showing a facilitation of A β internalisation with the use of integrin antagonists but an inhibition of internalisation with NMDA antagonists (Bi et al., 2002; De Felice et al., 2007).

Together these data bring to light the dynamic relationship that seems to exist between these two pools of A β and the potential detrimental role of this relationship.

4. Functional relationship between the intra- and extracellular A β pools

A β internalisation from the extracellular medium and intraneuronal A β production from APP cleavage seem to be linked by what we could call an “autocatalytic vicious circle”, meaning the intracellular accumulation of A β_{42} induces an overproduction of neosynthesised A β_{42} inside neurons (for review: Mohamed and Posse de Chaves, 2011).

Accumulating evidence supports the hypothesis of this mechanism. Indeed, it has been shown that extracellular application of A β_{42} on cultures of human embryonic kidney cells (HEK) increases the intracellular production of neosynthesised A β_{42} (Yang et al., 1999).

Another study carried out on hippocampal neurons has brought to light, through electrophysiological and confocal imaging methods, the major role of A β_{42} intracellular accumulation in the alterations of synaptic glutamatergic transmission (Ripoli et al., 2014). This study showed that A β_{42} synaptotoxicity occurs independently from an interaction with membrane receptors and brings forward the hypothesis of an internalisation from the extracellular space and intracellular accumulation which play a significant role in synaptic perturbations. Notably, via a fluorescent marking of A β_{42} perfused in the extracellular medium of neuronal cultures, it has been shown that this fluorescent marking was found inside the somatic and dendritic compartments of the cultured neurons within 20 minutes. When a variant of A β_{42} (A β_{42}^{M0}), oxidised on methionine 35 residue preventing it from crossing the plasma membrane (Ripoli et al., 2013), was applied on the cultured neurons, it was still located in the extracellular space after 20 minutes and did not affect synaptic neurotransmission or plasticity. However, when this variant was directly applied inside the neurons, via the electrophysiological recording pipette, it induced synaptic depression and inhibited LTP in a similar fashion than intracellular A β_{42} . This study suggests that the synaptotoxic effects of intracellular A β requires its internalisation and depends on its interaction with intracellular partners (Ripoli et al., 2014).

Regarding familial mutations of AD, a study recently evidenced an important intracellular increase of A β_{42} /A β_{40} ratio associated to a PS2 mutation. This study showed the existence of a specificity of action of the γ -secretase complex depending on its localisation in the cell and its PS1/PS2 subunit composition. The PS1/ γ -secretase complex is ubiquitously distributed throughout the cell and notably at the plasma membrane. However, the PS2/ γ -secretase complex is preferentially located in late endosomes/lysosomes where it will target its substrate in these compartments then generate an intracellular pool of A β peptides, especially of the A β_{42} isoforms (Sannerud et al., 2016). Consequently, this data suggests that it is the restricted localisation of PS2 in late

endosomes/lysosomes which contributes to the generation of the intracellular A β pool associated to AD development (Bayer and Wirths, 2010; Gouras et al., 2010).

Furthermore, this discovery of the Osaka mutation (APP- Δ E693), a mutation found in a Japanese pedigree about a decade ago, reinforce the importance of the role of intracellular A β in the mechanisms of synaptotoxicity observed in AD (Tomiya et al., 2008). This mutation induces early disease onset (45 \pm 4 years of age). Animal models harbouring this mutation have been generated and enable the analysis of the role of intracellular A β . According to the studies carried out on these transgenic animals, the synaptic perturbations are mainly caused by intracellular soluble A β oligomers.

Together these data highlight the importance of the interplay between intra- and extracellular pools of A β in AD pathogenicity and especially bring forward the underrated intracellular A β pool as a key player in synaptotoxicity setup. When many deleterious consequences of extracellular A β on glutamatergic neurotransmission have already been identified; one more repercussion of this A β secretion outside the neuron still needs to be outlined and that is its effect on surrounding neurons.

5. A β secretion and spreading of the disease in the brain

Many studies have focused on the effects and consequences of A β treatment/overproduction on neurons as a cell-autonomous mechanism. Indeed, in most cases, observations and data were acquired from what we would call “pathological neurons”, the effects are observed on the cell that is overexpressing/overproducing A β . However, one very important part of the story of AD pathogenesis, especially in the cases of sporadic AD, still needs to be clarified; that is how the disease spreads throughout the brain. Some have investigated the effect of A β secretion of one neuron on a neighbouring “healthy neuron” (that does not overproduce A β). Indeed, it has been shown in organotypic hippocampal mouse brain slices that one “healthy neuron” surrounded by “infected neurons” (neurons overexpressing APP^{swe}) has a significant decrease of NMDA and AMPA transmission compared to a “healthy neuron” surrounded by “non-infected neurons” (Kamenetz et al., 2003). Meaning that A β may act intercellularly, where an infected neuron induces synaptic depression onto a healthy nearby neuron and this seems to occur in a concentration-dependent manner of A β (Kamenetz et al., 2003).

Other studies have shown that A β that is secreted from one neuron, whether it is dendritic or axonal A β , decreases spine density of nearby healthy neurons and alters the structural synaptic plasticity of the healthy neuron’s remaining spines by hindering LTP-induced spine enlargement (Wei

et al., 2010). It has also been shown, as previously discussed, that extracellular A β may be internalised by a cell and cause an increase of intracellular neosynthesised A β (for review: Mohamed and Posse de Chaves, 2011; Yang et al., 1999).

Interestingly, the evidence accumulated of the effects of “pathological neurons” on “healthy neurons” is very similar to what is observed on the pathological neuron itself. Together these studies bring to light a potential neuron-to-neuron spreading mechanism of the disease whereby: one A β overproducing neuron alters a healthy nearby neuron’s spine density, structural plasticity, neurotransmission and induces an intracellular increase of neosynthesised A β within that healthy nearby neuron. The healthy nearby neuron subsequently becomes pathological and, in turn, may affect other healthy nearby neurons. This initial mechanism could be, in physiological conditions where A β levels are stable, a negative feedback loop to keep neuronal activity in check (Kamenetz et al., 2003) but in conditions of excess A β this mechanism could become substantially problematic, ultimately leading to synaptic defects and cognitive impairments. This spreading of the disease is reminiscent of a “prion-like” mechanism.

Together these studies point at the hypothesis that the synaptotoxic phenomena induced by A β oligomers are due to a set of events involving i) the intra- and extracellular localisation of A β oligomers as well as ii) the concentration, sequence thus conformation, of these oligomers. It is, therefore, crucial to identify the events that are at the origin of A β_{42} internalisation and/or overproduction, and the intracellular interacting partners responsible for the synaptotoxic effects observed in AD as well as the mechanisms of spreading of the pathology in the brain. More detailed studies are needed to identify the intracellular molecular targets in order to bring to light new therapeutic strategies which would prevent and/or alleviate the synaptic and cognitive alterations of AD.

V. The research project

In view of the bibliographical data aforementioned, several problematics subside, particularly in the case of the toxic species of A β since various studies show conflicting results depending on the species and/or concentration of A β oligomers used. Among the different subjects that still need to be addressed, the extra- and/or intracellular origin of A β and the pathway by which A β induces its synaptotoxic effects are some of the most controversial subjects to date. Many studies have highlighted spine loss as one of the major defects observed in consequence of excess and/or toxic A β , and most likely explain AD onset and the subsequent cognitive impairments. However, less focus has been made on the state of the remaining spines of A β -burdened neurons and the way this synaptotoxicity is sustained as it propagates throughout the brain. It is well accepted that A β disrupts the synaptic actin cytoskeleton organisation, ultimately leading to spine degeneration; however the mechanistic behind this phenomenon is still unclear. Also, most studies converge towards the hypothesis that propagation of the disease occurs via extracellular A β which can be internalised then re-exocytosed and re-internalised by other neurons. Some data suggests that extracellular A β causes an increase of neosynthesised intracellular A β which could affect neighbouring neurons in a “prion-like” mechanism by which A β oligomers induce A β overproduction which propagates from neuron to neuron.

Indeed, several steps within this “auto-catalytic vicious circle” of A β internalisation from the extracellular space and intraneuronal A β production need a more in-depth analysis. Shedding some light on the driving force of amyloidogenic APP processing and the subsequent impact of its proteolytic derivatives on excitatory synapses, in pathological but also possibly in physiological conditions, therefore embodies a critical and essential step in order to improve our comprehension of the molecular mechanisms leading to synaptic dysfunction in AD.

Furthermore, accumulating evidence show that A β may take various conformations, and aggregation properties depending on its mutation, and therefore takes on a more or less toxic role depending on this sequence. Investigating the common denominator that is affected by all these different variants of A β , which all lead to synaptic defects and subsequent cognitive impairments, is crucial. Indeed, with the rising notion that one individual with AD may in fact have several strains of misfolded A β peptides within the brain and the recent failings of β -secretase inhibition and immunotherapies in clinical trials; it is critical, at this point, to shed some light on this potential common denominator in order to better understand AD pathogenesis and find new therapeutic targets.

In this thesis project, we investigated the synaptotoxic effects induced by intra- and/or extracellular A β and the possible mechanism behind disease propagation in the brain, using different variants of APP. The reasoning is that most studies on AD have used synthetic human A β oligomers at very variable concentrations, often leading to debatable results. In our approach we bypass these uncertainties by using different mutants of APP which give rise to A β peptides with unique molecular signatures. This strategy enables us to study the impact of intracellular and secreted (extracellular) A β on the morphology and function of dendritic spines in conditions closer to the physiopathology of AD.

- [The different mutants of APP used](#)

In our study we focused on 3 mutants of Human neuronal APP₆₉₅ that generate different pools of A β but all induce a very aggressive form of AD and one mutant which, allegedly, protects against AD. Firstly, there is APP₆₉₅ wild-type which itself induces synaptic alterations such as loss of synapses as well as long term plasticity defects when it is overexpressed. Secondly, we selected the well-studied Swedish mutation (K670N/M671L) which leads to an increased production of A β secreted into the extracellular space, favouring the formation amyloid plaques. The third pathologic variant we have chosen is the Osaka mutation (E693 Δ). This particular mutation leads to an intracellular accumulation of A β and an absence of amyloid plaques in brains of patients as well as in animal models harbouring this mutation. Lastly, the final variant used carries the Icelandic mutation which, unlike the other mutants, produces an A β peptide which allegedly protects against AD and promotes better cognitive aging.

- [Investigating A \$\beta\$ -induced dendritic spine structural plasticity alterations](#)

As the excitatory synapse is one of the frontline targets of A β peptides and dendritic spine morphology and function are tightly related, we looked into the impact of intra- and/or extracellular and non-toxic A β on spine morphology.

To do so, we studied spine density and spine volume. First this analysis was carried out in an AD transgenic mouse model harbouring the Swedish mutation in order to highlight the defects observed. Then we assessed whether these defects were transposable to cultured cortical neurons overproducing the various A β mutants. This was carried out in both resting conditions as well and conditions of synaptic activity.

- [A look into activity-dependent actin dynamics and the presence of A \$\beta\$](#)

Since the actin cytoskeleton frames the spine and reorganises itself following synaptic activation, and it has been reported that this mechanism is impaired in AD, we analysed dendritic spine actin dynamics in cultured cortical neurons overexpressing the various mutants of APP, in resting conditions and following synaptic activation.

This allowed us to evaluate the impact of our different variants of A β on structural plasticity of the remaining spines, and provide a potential synaptotoxic target of intracellular A β : the actin cytoskeleton.

- [Synaptic activity as a modulator of both spine plasticity and APP processing](#)

As synaptic transmission shapes the dendritic spine depending on its activity and this mechanism is influenced by the presence of A β . We next assessed whether synaptic activity may also modulate APP processing and subsequent A β production in neurons overexpressing the various mutants of APP, using a β -secretase inhibitor. This would shed some light on the possible mechanistic behind the “auto-catalytic vicious circle” of A β .

- [Exploring a possible direct functional link between Actin and A \$\beta\$](#)

Since activity-dependent actin dynamics was disrupted in spines due to the presence of A β we questioned a possible interaction between these two partners. After identification of a potential actin-binding sequence on A β , a subsequent mutant of the peptide was generated which, allegedly, could no longer interact with actin.

Spine morphology and activity-dependent structural plasticity and APP processing was also investigated using this new variant of A β coined A β 3M.

- [A \$\beta\$ synaptotoxicity: sequence vs concentration](#)

It has been reported that the synaptotoxic effects of A β are due to excess A β production and conversely, that the neuroprotective effect of some A β mutants (Icelandic) are due to a decrease in A β production. Here we analysed the effects of equal “pathological” concentrations of our various toxic and non-toxic A β s on dendritic spine morphology and plasticity to assess A β sequence vs A β concentration.

- [An APP-dependent propagation of the pathology throughout the brain](#)

To get an insight into how the disease propagates throughout the brain at AD onset, we used an innovative system whereby one neuron overexpresses one of the APP mutants and a nearby neuron does not. This technique enables to investigate the effect of a “pathological neuron” on a nearby “healthy” neuron. This experiment was carried out in both wild-type and APP knock-out (APPKO) mouse cultured cortical neurons in order to assess the importance of APP in disease progression from neuron to neuron.

VI. Results

A. Introduction

The results of this thesis are presented in the attached document, currently the subject of a research article to be submitted for publication:

B. Research article

APP mutations unveil distinct roles for A β : while intracellular A β modulates synaptic plasticity, extracellular A β participates in Alzheimer's disease propagation

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Abstract

Alzheimer's disease (AD) is defined as a neurodegenerative disorder where synaptic defects lead to neuronal loss and concurrent memory impairments. It is now well-established that synaptic dysfunction in AD is initiated by oligomeric forms of the amyloid- β peptide ($A\beta$), a proteolytic derivative of Amyloid Precursor Protein (APP). However, the pathway by which $A\beta$ induces its deleterious effects, whether it is due to intra- and/or extracellular $A\beta$ pools, and how these effects are sustained and propagated throughout the brain, are still unclear. In this study, we used several mutated forms of APP which give rise to $A\beta$ peptides with unique molecular signatures, such as: the Swedish mutation (K670M/N671L) (APP_{swe}) which increases secreted (extracellular) $A\beta$; the Osaka mutation (E693 Δ) (APP_{osa}) which causes intraneuronal (intracellular) accumulation of $A\beta$; and the Icelandic mutation (A673T) (APP_{ice}) which has been reported to decrease $A\beta$ production and protect against AD. These mutated forms of APP were overexpressed in cultured mouse cortical neurons in order to study spine morphology and function by confocal microscopy, get a better insight into pathology propagation and identify a novel interacting partner bringing to light the possible physiologic role of $A\beta$ in neurons. We report that pathological intracellular $A\beta$ accumulation, due to APP_{wt} , APP_{swe} and APP_{osa} overexpression but not APP_{ice} overexpression induces a significant decrease in spine density especially mushroom spines, accompanied by a significantly increased volume of the remaining mushroom spines. These enlarged mushroom spines have impaired structural plasticity seemingly as a result of defective activity-dependent actin dynamics in the spines. These synaptic alterations seem to be due to a newly-identified interaction between actin and $A\beta$, hinting a possible physiological role for $A\beta$ in activity-dependent synaptic plasticity. This synaptic activity modulates amyloidogenic APP processing which would explain synaptic dysfunction in pathological conditions. Furthermore, we show that $A\beta$ sequence is as important as $A\beta$ concentration in inducing synaptic alterations. Lastly, we bring to light that neurons secreting $A\beta$ also affect nearby neurons in an APP-dependent manner, reminiscent of a prion-like mechanism. Together these results demonstrate that APP processing is a finely tuned equilibrium involved in actin-remodelling during activity-dependent synaptic plasticity and opens a new route for AD therapeutic strategies.

Introduction

Alzheimer's disease (AD) is defined as a neurodegenerative disorder where neuronal defects lead to neuronal loss and concurrent memory impairments. As first described in the 1900s, two histopathological signatures can be found in the brain, namely intracellular neurofibrillary tangles and extracellular senile plaques composed of β -amyloid ($A\beta$) peptides².

$A\beta$ is generated by the amyloidogenic proteolytic cleavage of type I trans-membrane Amyloid Precursor Protein (APP) by β -secretase (BACE) and γ -secretase sequentially³. Although APP mainly matures through the canonical secretory pathway, beginning in the endoplasmic reticulum (ER) to the Golgi apparatus, where it undergoes post-translational modifications before vesicular transport to the plasma membrane; it may also be directly processed in the ER/intermediate compartment. $A\beta$ generated in the ER is essentially $A\beta_{42}$ and is not appointed for secretion⁴, suggesting the existence of several pools of $A\beta$ produced by neurons: a secreted extracellular pool and another pool which accumulates intracellularly⁵. Furthermore, $A\beta$ peptides have the capacity to aggregate into multiple forms of oligomers, and eventually into protofibrils and fibrils⁵. It is now well-established that $A\beta$ toxicity and subsequent cognitive impairments are due to oligomeric forms of $A\beta$ ⁶. However, the pathway by which they induce their deleterious effects, whether it is initiated by intra- and/or extracellular $A\beta$ pools, is still unclear. Indeed, several studies have shown that extracellular $A\beta$ oligomers hinder learning and memory processes on many levels such as impairing long-term potentiation, decreasing glutamatergic synaptic transmission and altering synapse morphology⁷⁻¹². In contrast, other studies have demonstrated that similar memory defects in various transgenic AD models precede, if not exclude, extracellular $A\beta$ accumulation into plaques¹³⁻¹⁵. Intracellular $A\beta$ accumulation and associated cognitive deficits have also been observed in human AD brains^{16,17} suggesting that this could be a main feature in AD memory and learning impairments.

Dendritic spines are the highly heterogeneous postsynaptic compartments of neurons, varying in size and number, and their architecture depends mainly on their dynamic actin cytoskeleton¹⁸. Spine shape has been broadly categorised as "mushroom", "thin" or "stubby". Although electron-microscopy studies tend to show more of a continuum between these categories, there is growing evidence that different spine morphologies reflect different developmental stages and/or altered strength of synapses whereby thin and stubby spines are the least - and mushroom spines are the most - mature and functional^{19,20}. Dendritic spine morphology and function are therefore intimately linked²¹ and this relationship has been shown to be dysfunctional in AD, ultimately leading to synapse and, eventually, neuronal loss^{22,23}. It has been proposed that AD is in fact a synaptopathy²⁴ and

studying dendritic spine morphological and functional alterations could shed some light on the underlying mechanisms of AD pathogenesis.

Despite familial AD (FAD) only representing less than 1% of all AD cases, these rare hereditary forms, mostly due to mutations on APP and γ -secretase's PS1/PS2 genes, provide crucial insight into the mechanistic of sporadic AD onset and progression²⁵.

Thus, in this study, to gain more insight on A β synaptotoxicity, we used several mutated forms of APP which give rise to A β peptides with unique molecular signatures, such as the well-studied Swedish mutation (K670M/N671L) (APP_{swe}) which increases secreted (extracellular) A β ¹; the Osaka mutation (E693 Δ) (APP_{osa}) which causes intraneuronal (intracellular) accumulation of A β ¹⁷; and the Icelandic mutation (A673T) (APP_{ice}) which has been reported to decrease A β production and protect against AD²⁶. These mutated forms of APP were overexpressed in cultured mouse cortical neurons in order to study spine morphology and function. Our results will allow a deeper understanding of the mechanisms leading to synaptotoxicity and disease propagation throughout the brain and bring to light that A β sequence is as important in inducing synaptotoxicity as A β concentration.

Materials and Methods

Transgenic animals

Thy1-YFP-H (The Jackson Laboratory, Ellsworth, Maine, USA, B6 Cg-Tgn 2Jrs) mice were crossed with heterozygote APP/PS1-21 (APP/PS1) mice to generate *C57BL/6Thy1-eYFP APP/PS1-21* mouse colony. These mice were used at 3 months of age for spine density and mushroom spine volume analysis in the hippocampus and cortex. All experiments involving animals were conducted in accordance with the policy of Institut des Neurosciences de Grenoble and French legislation, in compliance with the European Community Council Directive of November 24, 1986 (86/609/EEC). The research involving animals was authorised by the Direction Départementale de la protection des populations–Préfecture de l'Isère France and by the ethics committee of Institut des Neurosciences de Grenoble accredited by the French Ministry of Research.

Primary cultures of cortical neurons

Mouse cortical neurons were cultured from 14- to 15-d-old OF1 embryos (Janvier, Lyon, France) as described previously²⁷. After extraction of the embryonic brains, the cerebral membranes were removed and the cortices were dissected, mechanically dissociated and cultured in Dulbecco's Modified Eagle's Medium supplemented with 5% horse serum, 5% foetal bovine serum and 1mM

glutamine (all from Sigma, Lyon, France) on 24-well plates (Falcon, Corning, N.Y., USA; Becton Dickinson, Le Pont de Claix, France) for biochemical experiments.

Neurons were seeded on 35 mm glass-bottom dishes (MatTek, Ashland, MA, USA) at a final concentration of two cortical hemispheres per dish for confocal experiments. All plates, dishes, and coverslips were coated with 0.1 mg/mL poly-D-lysine and 0.02 mg/mL laminin (Sigma, Lyon, France). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂/95% air. After 3–4 days *in vitro* (DIV), cytosine arabinoside (AraC, 10 μM; Sigma, Lyon, France) was added to inhibit proliferation of non-neuronal cells in cultures used for biochemistry experiments; 98% of the cells were considered as neuronal. The day before the experiments, cells were washed in DMEM. Treatments were performed on neuronal cultures at 14–15 DIV.

Lentivirus production

Lentiviruses were produced in HEK293T cells from the following plasmids: pLenti-APP_{wt}-mCherry, pLenti-APP_{wt3M}-mCherry, pLenti-APP_{swe}-mCherry, pLenti-APP_{swe3M}-mCherry, pLenti-APP_{osa}-mCherry and pLenti-APP_{ice}-mCherry. To generate infectious lentiviral particles, sequences of various human APP₆₉₅ mutants were cloned into pLenti-C-mCherry vector. pLenti-C mCherry vector was a gift from Dr Christophe Bosc (Grenoble Institute of Neuroscience) and was derived from pLenti-C-mGFP (Snapgene, Chicago, IL, USA). psPAX2 is a packaging plasmid encoding HIV-1 gag/pol sequences under the control of a SV40 promoter (Addgene plasmid # 12259). pCMV-VSV-G is an envelope-expressing plasmid encoding for VSV-G glycoprotein under the control of a CMV promoter (Addgene plasmid # 8454).

For the virus production, cells were transfected using Ca²⁺-phosphate in cell culture dishes (100 x 15 mm) with a given lentiviral plasmid and the two helper plasmids psPAX2 and pCMV-VSV-G. Six hours after transfection, the medium was changed to remove transfection reagent in the conditioned medium to which the virus is secreted. 48 h after transfection conditioned medium was spun at 250g for 5 min at 4°C before being collected and filtered using a 0.45 μm sterile filter (Sarstedt, Nuembrecht, Germany). Then virus particles were pelleted by ultra-centrifugation for 2 h at 4 °C and 20,000 rpm in a Beckman SW32Ti swinging bucket rotor. Supernatant was discarded and virus was suspended in PBS (X100 concentrated according to the initial volume of supernatant) and aliquots of the viral solution were snap-frozen in liquid nitrogen before storage at -80°C until use. Lentivirus titration was performed by FACS analysis after viral transduction of HEK293T and was estimated around 2 10⁸ UI/mL.

For viral transduction, lentiviral solutions were diluted (1:10) in complete culture medium and 50 μ L of the diluted preparation were added to the culture medium of a 24-well plate (for human neuronal cultures). Cell culture supernatant or lysates were harvested 48 h to 72 h post-infection.

A β measurements by ELISA assay

To assess the level of « total » A β secreted into the medium or in cell lysate, after 72 h infection of cortical neurons with lentivirus producing various APP mutants we performed an ELISA assay. For this assay, samples (200 μ L of cell culture medium or lysates corresponding to 150 μ g of proteins) and standards were incubated overnight at 4°C in a maxisorb 96-well plate (Thermo Fisher, Waltham, MA, USA). The A β (1–42 aa) standards were prepared using synthetic A β ranging from 0.1 to 2 μ g/mL. The plates were then aspirated and blocked with 3% bovine serum albumin (BSA) in PBS buffer for at least 1 h at 37°C. The samples and standards were added to the plates and incubated at RT for 1 h. The 6E10 antibodies diluted to 0.2 μ g/mL in blocking buffer was incubated in the wells for 1 h at RT. The plates were washed three times with wash buffer (0.05% Tween 20 in PBS buffer containing 1% BSA). Horseradish peroxidase conjugated anti mouse antibodies (Jackson Laboratories, Cambridgeshire, UK), diluted 1:5000 in blocking buffer, was added to the wells for 1 h at 37°C. Then the plates were washed three times with wash buffer and once with PBS. The colorimetric substrate, Ultra TMB-ELISA (Thermo Fisher, Waltham, MA, USA), was added and allowed to react for 15 min, after which the enzymatic reaction was stopped with addition of 1 M H₂SO₄. Reaction product was quantified using a Molecular Devices Vmax spectrophotometer measuring the difference in absorbance at 405 nm and 650 nm. The low end sensitivity of this assay is 50 ng/mL (14 nM; data not shown).

Plasmids

cDNAs of WT human APP₆₉₅ and the Swedish mutant (APP_{swe}) were cloned into pmcherry-N1 vector (Snapgene, Chicago, IL, USA) using the BamHI and AgeI restriction sites. Then, using overlapping PCR and the Infusion Kit (Thermo Scientific, Waltham, MA, USA) we generated the various APP mutant-mCherry plasmids as described by manufacturer's instructions. These plasmids included: APP_{osa}-mCherry corresponding to the deletion of E22 in the A β ₁₋₄₂ sequence; APP_{ice}-mCherry (A598T); APP_{wt3M}-mCherry (G625A/L630A/G633A); APP_{osa3M}-mCherry and APP_{swe3M}-mCherry. All constructions in pmCherry vector were verified by sequencing.

Neuronal transfection

Transfections were performed on cortical neuron cultures after 12 DIV with calcium phosphate precipitation. Growth medium (DMEM and sera) was removed and kept at 37°C until the last step of transfection. Cells were washed in DMEM and incubated for 30 min in DMKY buffer containing the

following: 1 mM kynurenic acid, 0.9 mM NaOH, 0.5 mM HEPES, 10 mM MgCl₂ and phenol red 0.05%, pH 7.4. Then, 3 µg of the plasmids containing the APP₆₉₅ mutants APP_x-mCherry (human neuronal Amyloid Precursor Protein) and Actin-GFP (for FRAP experiments) or LifeAct-GFP, a peptide which specifically binds filamentous actin²⁸, were mixed with CaCl₂ (120 mM) in HBS containing the following: 25 mM HEPES, 140 mM NaCl, and 0.750 mM Na₂HPO₄, pH7.06) and left for 15 min to precipitate the DNA.

Two types of transfections were performed as follows:

Co-transfection: 3 µg of the plasmids containing the APP₆₉₅ (full-length human neuronal Amyloid Precursor Protein) mutants fused to an mCherry tag (APP_{695x}-mCherry) and Actin-GFP (for FRAP experiments) or LifeActin-GFP, a peptide which specifically binds to filamentous actin, were mixed with 1 M CaCl₂ and HBS buffer (20 mM HEPES, 150 mM NaCl, 1.5 mM Na₂HPO₄, pH 7.4) and left for 15 min to precipitate the DNA. Plasmids were then applied to cells for 60 min.

Two-step transfection: 3 µg of APP_{695x}-mCherry plasmid (mixed with 1 M CaCl₂ and HBS buffer) was first applied to cells for 40 min. Then, 3 µg of LifeActin-GFP plasmid (mixed with 1M CaCl₂ and HBS buffer) was added to the cells for 40 min.

Transfection medium was replaced with conditioned growth medium and cultures were returned to the incubator until use at DIV 14-15.

Confocal imaging

Transfected neurons were placed in HBBSS solution containing the following (in mM): 110 NaCl, 5 KCl, 2 CaCl₂, 0.8 MgSO₄, 1 NaH₂PO₄, 12 HEPES, 5 D-glucose, 25 NaHCO₃, and 10 glycine (all from Sigma, Lyon France) 1.5–2 h before experiments. Neurons were visualised using a Nikon Ti C2 confocal microscope with a Nikon 60X water-immersion objective and NIS-Elements software (Nikon, Melville, NY, USA). Excitation of GFP and mCherry fluorophores was performed with an argon laser excited at 488nm (emission filtered at 504-541 nm) and at 543 nm (emission filtered at 585-610 nm) respectively. Images were acquired as Z-stacks (tridimensional section) with 0.3 µM per step immediately before and 15 min after treatment. The acquired images were then deconvoluted using AutoQuantX3 software (Media Cybernetics, Abingdon, Oxon, UK).

Spine density and volume was assessed using NeuronStudio software (CNIC – Mount Sinai School of Medicine).

Fluorescence recovery after photobleaching experiments

Fluorescence recovery after photobleaching (FRAP) was performed on cultured neurons 48 h after transfection. Images were acquired with an inverted Nikon Eclipse Ti C2 confocal microscope with a Nikon 60x water objective with a 1.33 numerical aperture. Actin-GFP in the spine head was bleached

at 405 nm and the fluorescence recovery was measured for 80 s (at 1 s/frame). Fluorescent signal analysis was performed with the Nikon software Nis-Elements.

Production of recombinant HIS-tagged A β proteins

To make the plasmids for the fusion protein A β (His) of various mutants of human β -amyloid 1-42 protein (A β _{wt}, A β _{osa}: Δ E22, A β _{ice}: A2T, A β _{wt3M}: G29A/L34A/G37A), the cDNA containing the sequence for these human A β ₁₋₄₂ were obtained from synthetic oligonucleotides (Sigma, Lyon, France) (containing a Nde1 restriction site as forward primers and a PspXI restriction site as reverse primers) using overlapping PCR. PCR products were then cloned into a pet28a-vector (Novagen, Paris, France) and subsequently constructed as various mutant HIS-A β ₁₋₄₂ expressing plasmid (pet28a-A β His pet28a-A β (His)osa: Δ E22, pet28a-A β (His)ice: A2T and pet28a-A β (His)wt3M: G29A/L34A/G37A). The resulting plasmids were verified by sequencing. *Escherichia Coli* BL21 (DE3) was transformed with the fusion protein plasmids and a single colony was chosen to grow in a 250 mL starter culture in Luria broth (LB medium) overnight at 37°C. The next day, 10 mL of culture was diluted in 1L LB culture medium. When the culture reached an OD_{600nm} of 0.8, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to 1 mM for induction. The culture was grown for an additional 4 h and the cells harvested by centrifugation at 4000g for 20 min. The pellet was re-suspended in 10 mL ice-cold PBS and lysed by sonication at ice-cold temperature. The cell extract was then centrifuged at 20,000g for 15 min at 4°C. The pellet was re-suspended in 10 mL of 8 M urea in PBS and sonicated as previously described before centrifugation at 20,000g for 15 min at 4°C. The supernatant (5 mL) was diluted with 15 mL of binding buffer (PBS with 10 mM imidazole at pH 8.0). Before affinity purification using nickel-nitriloacetic acid (NTA) column purification, samples were filtered on 0.45 μ m. The Ni-NTA column (3 mL of protino Ni-NTA Agarose from Macherey Nagel) was equilibrated with binding buffer prior to loading the sample on the column. Then the column was washed with the washing buffer (PBS with 30 mM imidazole at pH 8.0) with 5-10 column volumes. The protein was then eluted with the elution buffer (PBS with 500 mM imidazole at pH 7.4). The absorbance at 280 nm was used to monitor the elution but the concentration of the fusion proteins was estimated by comparing the intensity of the band of the protein on SDS-PAGE with that of known quantity of BSA (Sigma, Lyon, France). A final concentration of 100 μ M was obtained and aliquots were stored at -80°C. Aliquots from all subsequent purification steps were analysed by SDS-PAGE (Laemmli, 1970), and the identity of A β ₁₋₄₂ and mutants was verified by western blots using 4G8 monoclonal antibodies against A β sequence (4G8).

A β binding assay

This assay was adapted from²⁹. Briefly, cortical neuron cultures were lysed by Dounce homogenisation in PBS and then centrifuged at 100,000 \times g for 1 h at 4°C. The pellet was

resuspended in 1% Triton X-100 in PBS and centrifuged at $16,000 \times g$ for 30 min. The supernatant was kept and used as detergent-soluble membrane proteins in farwestern experiments. For this purpose, various A β mutants (4 μ g) or the vehicle control (PBS) was applied to nitro-cellulose membrane, and the membrane was blocked with 5% BSA for 2 h at room temperature. The membrane was then incubated with detergent-extracted membrane proteins for 12 h at 4°C, followed by incubation with the APP monoclonal antibody 22C11 and development by ECL.

Actin depolymerisation assay

Pyrene-labeled muscle actin (20%) was mixed with dark actin at 2 μ M in general actin buffer (5 mM Tris-HCl pH 8, 0.2 mM CaCl₂) following the manufacturer's instructions (Cytoskeleton, Inc., Denver, CO, USA). Into wells of a black assay 96 well plate, 2 μ M of pyrene actin were mixed along with 1 mM dithiothreitol (DTT) and 0.2 mM adenosine-tri-phosphate (ATP). After 3 min, 20 μ L of 10x MKEI polymerisation buffer (20 mM MgCl₂, 500 mM KCl, 10 mM EGTA, 200 mM imidazole, pH7) were added to each well and mixed. Actin polymerisation was monitored by pyrene fluorescence (exc.: 360 nm; em.: 407 nm) every 30 s for 1 h (microplate reader PHERAstar Plus, BMG LABTECH, Champigny-sur-Marne, France). Depolymerisation was induced by diluting polymerised actin preincubated or not with 5 μ M A β_x mutant at 1:10 in general actin buffer. Pyrene fluorescence was monitored every 10 s for 12 min.

Brain slices preparation

Horizontal brain slices containing the somatosensory cortex were prepared from 20 to 30 day-old SWISS mice. Mice were cervically dislocated and immediately decapitated. Their cortices were dissected out and 300 μ m thick transverse slices were cut in ice-cold cutting solution (in mM: KCl 2.5, NaH₂PO₄ 1.25, MgSO₄ 10, CaCl₂ 0.5, NaHCO₃ 26, Sucrose 234, and Glucose 11, saturated with 95% O₂ and 5% CO₂) with a Leica VT1200 blade microtome. After the dissection, slices were kept in oxygenated ACSF at 37 \pm 1 °C for at least 1 h.

Electrophysiology recordings

Slices were visualised in a chamber on an upright microscope with transmitted illumination and continuously perfused at 2 ml/min with oxygenated Artificial Cerebro-Spinal Fluid (ACSF in mM: 119 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 1.3 MgSO₄, 2.5 CaCl₂, 26 NaHCO₃, and 11 Glucose) at room temperature. Stimulating electrodes (bipolar microelectrodes) were placed in the stratum radiatum to stimulate the Schaffer collaterals pathway. Field EPSPs (fEPSPs) were recorded in the stratum radiatum using a recording glass pipette filled with ACSF and were amplified with an EPC 10 Amplifier Patchmaster Multi-channel (HEKA Elektronik Dr. Schulze GmbH, Wiesenstrasse, Germany). Recordings were

filtered at 1 kHz using the Patchmaster Multi-channel data acquisition software (HEKA Elektronik Dr. Schulze GmbH, Wiesenstrasse, Germany). The initial slope of the fEPSPs was measured to avoid population spike contamination. For LTP experiments, test stimuli (0.2 ms pulse width) were delivered once every 15 s and the stimulus intensity was set to give baseline fEPSP slopes that were 50% of maximal evoked slopes.

Slices that showed maximal fEPSP sizes < 1mV were rejected. LTP was induced by applying 2 trains of 100 stimuli at 100 Hz with an interval of 20 s.

Statistical analysis

Statistical analyses were performed with Graphpad 7.0 Software (La Jolla, CA, USA) using non-parametric test: Mann-Whitney or Wilcoxon Signed Rank Test or Kruskal-Wallis followed by Dunn's multiple comparison test or RM two-way ANOVA followed by Tukey's multiple comparison test. Significance was set at 0.05. Results are expressed as the mean \pm SEM from independent experiments.

Results

AD transgenic mouse model displays impaired dendritic spine density and mushroom spine volume in the hippocampus and cortex

We first looked at the morphology of dendritic spines of our transgenic AD mouse model, *C57BL/6Thy1-eYFP APP/PS1-21 (APP/PS1 Thy1-eYFP)*. *This mouse is an APP/PS1-21 with an added eYFP under the Thy1 neuronal promoter which allows us to visualise neurons by confocal microscopy without having to infect, transfect or label them. Images were acquired with 350 μ m brain slices of 3-month-old animals by confocal microscopy and isolated dendrites from the hippocampus were analysed (fig1.A). Our results show that APP/PS1 Thy1-eYFP mice have a significant $22.79 \pm 5.04\%$ ($p < 0.0001$) decreased total spine density (fig1.B, comparing the whole bars) compared to their *C57BL/6Thy1-eYFP (Wild-type Thy1-YFP) littermates*. When we looked in more detail at the spine subpopulation (thin, stubby and mushroom spines) of these mice we found that there was a $138.64 \pm 13.65\%$ ($p < 0.0001$) increase in thin spines (fig1.B, comparing the grey segments of the bar graphs). Yet, most remarkably, the major spine loss was due to a strong $44.50 \pm 4.35\%$ ($p < 0.0001$) decrease in mushroom spine density (fig1.B, comparing the black segments of the bar graph) in the AD mouse hippocampus compared to wild-type Thy1-YFP littermates. We next measured the volume of the remaining mushroom spines in the AD mice and found a significant $114.64 \pm 5.51\%$ ($p = 0.0003$) increase in volume of these spines compared to wild-type Thy1-YFP littermates (fig1.C). We carried out the same set of analyses for the cortices of these AD mice (fig1.*

D) and found a similar decrease of $27.43 \pm 5.18\%$ ($p=0.0099$) in total spine density (fig1.E, comparing the whole bars) as well as a strong decrease of $40.59 \pm 6.43\%$ ($p=0.0002$) of mushroom spine density (fig1.E, comparing the black segments of the bar graph) compared to their wild-type Thy1-YFP littermates. The volume of the remaining mushroom spines tended also to be increased compared to wild-type Thy1-YFP littermates (fig1.F) though non-significant ($p=0.07871$).

Taken together these data show a synaptic morphological alteration of these AD mouse brains at 3-months-old, shifting from a functional to a less functional set of spines with enlarged mushroom spines.

Primary cortical cell cultures transfected with pathogenic forms of APP have similar impaired spine density and mushroom spine volume

Given the results we found with the AD mouse brains, which highly express the human form of APP transgene bearing the Swedish mutation (KM670/671NL), we next wanted to assess whether similar results could be obtained by overexpressing mutated human neuronal forms of APP in primary cortical cell cultures. To do so, we selected various mutants of APP that are pro-AD: APP_{wt}, APP_{swe}, APP_{osa} and one protective mutation against AD: APP_{ice} which were fused to mCherry and co-transfected with LifeActin-GFP (LA-GFP), a small peptide which specifically binds to filamentous actin without disrupting actin stoichiometry (fig2.A Top and middle row), which enables the visualisation of the dendritic arbour and spines (fig2.A, Bottom row). Spine density analysis showed a marked decrease in total spine density of neurons overexpressing APP_{wt}, APP_{swe} and APP_{osa}-mCh with $20.86 \pm 3.07\%$ ($p=0.0002$), $25.75 \pm 1.29\%$ ($p<0.0001$) and $24.49 \pm 0.71\%$ ($p<0.0001$) reduction respectively, compared to control neurons which only overexpress LA-GFP (fig2.B, comparing the whole bars of bar graph). However, overexpression of APP_{ice}-mCh had no effect on total spine density (fig2.B). When we looked at spine subpopulation we also found a strong decrease of mushroom spine density when APP_{wt}, APP_{swe} or APP_{osa}-mCh was overexpressed with $39.20 \pm 2.58\%$ ($p=0.0456$), $63.80 \pm 1.17\%$ ($p=0.0002$) and $67.62 \pm 1.34\%$ ($p<0.0001$) reduction respectively, compared to control (fig2.B, comparing the black segments of bar graph). As seen in the AD mouse brain (fig1.), there was also an increase in thin spine density for neurons overexpressing APP_{wt}, APP_{swe} and APP_{osa}-mCh (fig2.B, $p=0.0063$; $p=0.0084$ and $p=0.0003$ respectively, comparing they grey segments of bar graph). Once again APP_{ice}-mCh had no effect on mushroom spine density and distribution (fig2.B). Moreover, we found that APP_{wt}, APP_{swe} and APP_{osa}-mCh expressing neurons also had enlarged mushroom spines, with an increase of $121.81 \pm 6.43\%$

(* $p=0.025$); $151.38\pm 5.40\%$ (** $p=0.0012$) and $141.75\pm 12.14\%$ (** $p=0.0058$) respectively, compared to control (fig2.C).

Together these data show that our study design replicates what is observed with AD transgenic mouse brains *in vivo*, namely an impaired spine density and enlarged mushroom spines.

Spine density and volume impairments observed in pathogenic APP-transfected neurons are driven by A β production

To decipher whether the effects observed in our APP-transfected neurons were due to an overproduction of A β and not just an effect induced by the mutated APPs alone, we treated the neurons which had the strongest impairments, namely APP_{swe}- and APP_{osa}-mCh transfected cells, with BACE1 Inhibitor IV [$1\mu\text{M}$] (βSec1) just after transfection until day of confocal imaging. This treatment shuts down the amyloid pathway of APP. We evaluated dendritic spine density and volume of the treated neurons (fig3.A). Our data show that inhibition of the amyloid pathway restored spine density close to control values (fig3.B), especially for mushroom spine density (fig3.B, comparing the black segments on bar graph). Furthermore, the volume of mushroom spines was also restored to control values (fig3.C). These results confirm that the observed effects are indeed A β -induced and that intracellular A β is sufficient, since restoration was also observed in APP_{osa}-mCh transfected cells.

Processing of APP_{wt}, APP_{swe}, APP_{osa} and APP_{ice} in infected neurons generates different localisations of A β pools

Since our different mutants of APP are said to yield A β in different ways (sequence, quantity, localisation), we evaluated the amount of A β found in cell lysate (fig4.A) and extracellular medium (fig4.B) of neurons expressing APP_{wt}, APP_{swe}, APP_{osa} or APP_{ice}. For this, we infected cultured cortical neurons with the different APPs (APP_x) and performed ELISA assays to analyse the production of the different A β s (A β_x) using 6E10 antibody. Our results showed that APP_{wt} and APP_{ice} produced A β_{wt} and A β_{ice} respectively and these peptides were detected in both cell lysate and extracellular medium. APP_{osa} yielded mostly intracellular A β_{osa} . APP_{swe} produced the most A β which could be found in both cell lysate and extracellular medium (fig4.A and B). Neurons overexpressed similar levels of APP as confirmed by western blot (fig4.C) that is processed into C-terminal fragments (CTF) and A β peptides.

These data show that overexpression of our different mutants of APP in neurons resulted in similar levels of APP with an increased production of A β and differences in the localisation of the peptide.

APP_{wt}, APP_{ice} produce A β peptides that are both intracellular and secreted; APP_{osa} produces mostly intracellular A β peptides; and APP_{swe} produced the most A β intracellularly and secreted.

Synaptic activity modulates APP processing

Several studies have shown that synaptic activity modulates APP processing and A β production³⁰⁻³². We wanted to see if we could observe this effect in our study model by assessing the red marking induced by APP_x-mCh expression in neurons before and after synaptic activity. Here we quantified and compared the red APP marking in the soma of APP_{wt}- (fig5.A), APP_{swe}- (fig5.B), APP_{osa}- (fig5.C) and APP_{ice}-mCh (fig5.D) in neurons cotransfected with LA-GFP, before synaptic activation (Before BIC15) *versus* after induction of synaptic activity (After BIC15: incubation with bicuculline methiodide [50 μ M] and 4-aminopyridine [2,5 mM] for 15 min). Our results show that red APP_{wt}, APP_{osa} and APP_{ice} marking area decreased by 23.80 \pm 1.16%; 20.46 \pm 3.37% and 23.07 \pm 3.96% respectively (fig5.G). Interestingly, APP_{swe}-mCh had a very marked 51.09 \pm 2.99% decrease of red marking after BIC15 (fig5.G).

In order to confirm that these observed effects are indeed due to the processing of APP following synaptic activation, we carried out the same set of experiments looking with APP_{swe}- and APP_{osa}-mCh transfected neurons using β SecI pretreatment (fig5.E and F). Interestingly, we noticed an approximate 2-fold increased marking of APP_{swe} and APP_{osa} when β SecI was present, before BIC15 (data not shown). This seems to point towards an accumulation of APP, most likely in vesicular compartments (given the dot shaped appearance of the marking), when the amyloid pathway is inhibited (fig5.E and F, middle and right panels). After synaptic activity was induced by BIC15, there was no significant decrease of neither APP_{swe}- nor APP_{osa}-mCh marking (fig5.H, 1.96 \pm 2.12% and 3.75 \pm 1.03% decrease respectively), compared to without β SecI.

Together these data show that our study model allows visualisation of APP processing, mostly the amyloidogenic pathway, and that it is indeed modulated by synaptic activity.

Spine morphology modulation mediated by synaptic activity is impaired in toxic A β overproducing neurons

One of the main characteristics of dendritic spines is their ability to reshape via different modulators such as synaptic activity³³⁻³⁷ and it is reported that this plasticity is altered in AD, prior to neuronal and memory loss³⁸⁻⁴⁰. Synaptic activation is known to induce dendritic spine

enlargement^{34,41,42}. Here we wanted to evaluate this phenomenon in our APP_x-mCh transfected neurons by measuring mushroom spine volume before and after synaptic activation by BIC15, using LA-GFP marking as readout (fig6.A). Consistently with literature^{34,43}, we found a 117.64±2.18% (**p*=0.0156) increase in mushroom spine volume in control neurons (expressing LA-GFP only) after BIC15 (fig6.B). Similarly, APP_{ice}-mCh transfected neurons, overproducing Aβ_{ice} (see fig3.), showed a 116.3±3.34% (**p*=0.0156) increase in mushroom spine volume after BIC15 (fig6.B). However, neurons transfected with APP_{wt}- or APP_{swe}-mCh, which overproduce the wild-type form of Aβ (see fig3.) and display enlarged mushroom spines (see fig2.E), failed to increase the volume of these spines after BIC15 (fig6.B). APP_{osa}-mCh (fig6.B) transfected neurons which overproduce intraneuronal Aβ_{osa} (see fig3.) and displayed enlarged mushroom spines (see fig2.E) also failed to increase spine volume after BIC15, suggesting that Aβ overproduction leads to no spine volume variation. We carried out the same experiment using βSec1 pretreatment. It is to note that this inhibition of the amyloid pathway not only inhibits the overproduction of Aβ coming from the APP_x-mCh overexpression but also shuts down the endogenous amyloid processing of APP. Meaning there is hardly any Aβ in these cells on the day of imaging. Interestingly, the neurons pretreated with βSec1 all failed to increase mushroom spine volume after BIC15, even the control and APP_{ice}-mCh neurons (fig6.C) bringing to light the necessity of the presence of Aβ to induce activity-dependent remodelling of the dendritic spine.

Taken together, these results show that: on one hand, overproduction of toxic forms of Aβ (wt and osa) lead to enlarged mushroom spines which fail to respond to synaptic activity but not the overproduction Aβ_{ice}; and on the other hand, absence of Aβ also causes mushroom spines to not respond to synaptic activity, suggesting that Aβ is important for mushroom spine formation, structure and regulation. As dendritic spine remodelling depends on its actin cytoskeleton dynamics²¹ and this remodelling seems hindered in an Aβ-dependent manner, we next questioned the possible link between actin and Aβ.

Activity-dependent actin dynamics in mushroom spines is altered in toxic Aβ overproducing neurons, but also in absence of Aβ

To further investigate the Aβ-dependent spine morphology and remodelling impairments observed previously, we next evaluated the actin dynamics within dendritic spines in resting conditions and in the context of synaptic activity. For this, we performed Fluorescence Recovery After Photobleaching (FRAP) experiments on individual mushroom spines of neurons co-expressing Actin-GFP (ActGFP) with either APP_{wt}-, APP_{swe}-, APP_{osa}- or APP_{ice}-mCh, in resting conditions and after BIC15 (fig7.A and B). Briefly, an ROI was placed on individual mushroom spines and ActGFP was

photobleached (only spines with >80% loss of fluorescence were kept for analysis), fluorescence recovery was monitored for 2 min following photobleaching (fig7.A and B). By normalising the fluorescence to prebleached intensity of the spine of ActGFP we obtained fluorescence recovery curves expressed as % of prebleached fluorescence in spine (fig7.C and E). In control neurons only overexpressing ActGFP, and in resting conditions, we obtained 80% fluorescence recovery after 80 s before reaching a plateau (fig7.A, C and D), which is consistent with what is found in literature⁴⁴. This was also the case for all the other conditions where the different APP_x-mCh were overexpressed, including the condition with β Sec1 pretreatment (fig7.D). This result shows that in absence of synaptic activity, A β overproduction or even absence of A β does not affect actin dynamics in spines. However, when we looked at what happened after BIC15 results were very different. In control neurons, synaptic activation resulted in 49.04 \pm 2.63% FRAP (fig7.B top row, E and F) which means synaptic activity induces actin stabilisation in spines (50% S). In neurons overproducing toxic A β _{wt} and A β _{osa}, such as APP_{wt}-mCh, APP_{swe}-mCh and APP_{osa}-mCh overexpressing neurons, synaptic activation resulted in 31.40 \pm 2.70% (**p*=0.0362); 31.56 \pm 1.76% (**p*=0.0380) and 30.78 \pm 2.22% (**p*=0.0457) FRAP respectively (fig7.B bottom row, E and F) which means there is an over stabilisation of actin in spines (Over S). Interestingly, in neurons overproducing A β _{ice} or neurons that have no A β (APP_{swe} + β Sec1 pretreatment), synaptic activity did not induce actin stabilisation in spines with 68.08 \pm 2.40% (**p*=0.0115) and 80.30 \pm 1.82% (*****p*<0.0001) respectively (fig7.B middle row, E and F) which means there is no activity-dependent stabilisation of actin in spines (No S).

Taken together these results demonstrate that: 1) A β is required for activity-dependent stabilisation in spines, 2) excess or toxic form of A β increases this activity-dependent stabilisation and 3) absence of A β prevents activity-dependent stabilisation. This brings to light a possible direct interaction between A β and actin.

A β _{wt} and A β _{osa} decrease *in vitro* depolymerisation of actin

Since A β seems to modulate actin cytoskeleton stabilisation in the spines, we next wanted to assess whether there is a possible interaction between actin and A β . To do so, we carried out an *in vitro* actin depolymerisation assay. In control conditions, in absence of A β , actin depolymerises, translated as a decrease of fluorescence (fig8.A). This Δ fluorescence (from starting point to plateau) is of 58.4 \pm 0.94% (fig8.B). This Δ fluorescence is significantly decreased when actin is in presence of A β _{wt} and A β _{osa} to 41.56 \pm 0.22% (**p*=0.0382) and 41.64 \pm 0.71% (**p*=0.0276) respectively, whereas in presence of A β _{ice} there is no statistical difference (fig8.B.).

These first results indicate that toxic $A\beta_{wt}$ and $A\beta_{osa}$ seem to interact with actin and hinder its depolymerisation rate, whereas non-toxic $A\beta_{ice}$ does not.

A β has a common sequence with other actin binding proteins and mutating this sequence within APP causes loss of generated A β synaptotoxicity without modifying APP processing

As we hypothesised a possible direct interaction of A β with the actin cytoskeleton, we compared with A β sequence with other actin binding proteins (ABPs). This brought to light a sequence of 3 common amino-acids GXXXXLXXG (fig9.A) between A β and ABPs involved in actin bundling (namely: calponin, L-plastin, dystrophin and ABP280). We mutated these 3 amino-acids into 3 Alanine within the A β sequence and generated the 3M mutation in order to lose the potential interaction of A β with actin. This mutation was applied to APP_{wt}⁻, APP_{swe}⁻ and APP_{osa}-mCh, giving rise to APP_{wt3M}⁻mCh, APP_{swe3M}⁻mCh and APP_{osa3M}⁻mCh. Preliminary *in vitro* bi-complementation assays with actin and A β_{wt3M} showed an absence of fluorescence hence a loss of interaction between these 2 proteins, compared to actin with A β (data not shown).

We first analysed the spine morphology of cortical neurons transfected with APP_{wt3M}⁻, APP_{swe3M}⁻ or APP_{osa3M}⁻mCh and LA-GFP (fig9.B). Unlike APP_{wt}⁻, APP_{swe}⁻ or APP_{osa}-mCh overexpressing neurons, spine density (total as well as the different spine subtypes) was not affected when APP_{wt3M}⁻, APP_{swe3M}⁻ or APP_{osa3M}⁻mCh was overexpressed (fig9.C), neither was mushroom spine volume (fig9.D) compared to control neurons.

We next assessed spine morphology modulation by synaptic activity in APP_{wt3M}, APP_{swe3M} and APP_{osa3M} expressing neurons (fig10.A). Unlike APP_{wt}, APP_{swe} or APP_{osa} overexpressing neurons, where mushroom spines were enlarged and failed to increase in size after BIC15, APP_{wt3M}, APP_{swe3M} and APP_{osa3M} overexpressing ones had mushroom spines with the same volume as control neurons (only overexpressing LA-GFP); and these spines were able to significantly increase their volume by 119.05±2.65% (* p=0.0156); 118.87±5.33% (*p=0.0313) and 116.69±3.73% (*p=0.0469) respectively after BIC15 (fig10.A and B).

These first results indicate that APP_{wt3M}, APP_{swe3M} and APP_{osa3M} have no effect on spine morphology and do not disrupt synaptic plasticity, pointing towards a loss of synaptotoxicity compared to their non-3M counterparts.

As these APP_{x3M}⁻mCh seemingly lost their toxicity, we next questioned whether this effect could be due to a modification of the processing of these APPs which would subsequently modify A β

production. We first evaluated the red marking in APP_{wt3M}⁻, APP_{swe3M}⁻ and APP_{osa3M}⁻mCh expressing neurons before and after BIC15 (fig10.C, D and E) and compared them to their respective non-3M counterparts (fig10.F). Consistent with the results obtained previously (fig5.) BIC15 induced a decrease in red marking of APP_{wt3M}⁻, APP_{swe3M}⁻ and APP_{osa3M}⁻mCh (fig10.C, D and E, right panels). This decrease was of 26.80±3.92% for APP_{wt3M}⁻mCh and of 21.19±2.41% for APP_{osa3M}⁻mCh, similar to what was obtained with APP_{wt}-mCh and APP_{osa}-mCh (fig10.F). APP_{swe3M}⁻mCh also displayed a similar strong 47.23±1.08% decrease in red marking as APP_{swe}-mCh (fig10.F).

Taken together, these results show that with APP_{wt3M}, APP_{swe3M} and APP_{osa3M} we are in the same context of A β overproduction as with APP_{wt}, APP_{swe} and APP_{osa}; only we do not have any signs of synaptotoxicity.

The 3M mutation abrogates the effect of toxic A β on actin *in vitro* and in cortical neuron dendritic spines

Since the 3M mutation caused loss of synaptotoxicity when inserted in APP_{wt}, APP_{swe} and APP_{osa}, we wanted to validate that this loss of toxicity could be due to a loss of interaction of A β with actin. To do so, we carried out the *in vitro* actin depolymerisation assay (fig7.) using A β _{wt3M} (fig11A. and B.) As expected, A β _{wt3M}, compared to A β _{wt}, no longer decreased the depolymerisation rate of actin (fig11.B, $p=0.5391$), but rather behaved like A β _{ice} (fig11.A). These results validate that the 3M mutation causes loss of interaction of A β with actin.

We next examined actin dynamics in the context of synaptic plasticity using FRAP on mushroom spines of neurons overexpressing either APP_{wt3M}⁻, APP_{swe3M}⁻ or APP_{osa3M}⁻mCh and ActGFP. Like previously seen in fig9., in control conditions (before BIC15), there was no difference in actin dynamics across the different mutants; they all had 80% FRAP (fig11.C, D and E). However, after BIC15, synaptic activation resulted in no actin stabilisation with 69.39±2.98%; 67.39±1.31% and 73.31±3.34% FRAP in spines for APP_{swe3M}⁻, APP_{osa3M}⁻ and APP_{wt3M}⁻mCh overexpressing neurons respectively (fig11.F and G), similar to what was obtained with A β _{ice} overproducing neurons or in absence of A β with β Sec1 pretreatment (see fig8.E and F). This indicates that, when A β _{wt3M} or A β _{osa3M} is overproduced in neurons, activity-dependent actin stabilisation in spines no longer occurs.

Exogenous application of toxic A β _{wt} and A β _{osa} on neurons causes mushroom spine loss and enlarged remaining ones but not A β _{wt3M} nor A β _{ice}

So far, all the effects observed seemed to be mainly due to the intracellular pool of A β , since A β_{osa} , which is mostly intracellular, induced the same toxic effects as excessive wild-type A β . We next wanted to test the effects of extracellular A β_{wt} , A β_{osa} , A β_{ice} and A β_{wt3M} at equal pathological concentrations on spine morphology. For this, we first imaged LA-GFP expressing cultured cortical neurons (fig12.A). Then we incubated these neurons for 24 h with 100 nM of either: A β_{wt} , A β_{osa} , A β_{ice} or A β_{wt3M} , and then imaged them again (fig12.B). We then analysed spine density (fig12.C to F) and volume of mushroom spines (fig12.G). Our results showed that total spine density was affected by 24 h of A β_{wt} and A β_{osa} with a $15.23 \pm 3.36\%$ ($****p < 0.0001$) and $28.67 \pm 3.12\%$ ($****p < 0.0001$) decrease respectively (fig12.A, B: 2nd and 3rd column, and C). This loss of spines was mainly due to a strong decrease in mushroom spine density, other spine subtypes were not significantly affected (fig12.D, E and F). Furthermore, the volume of the remaining mushroom spines was increased by $112.66 \pm 2.34\%$ ($****p < 0.0001$) and $114.14 \pm 1.84\%$ ($****p < 0.0001$) after A β_{wt} and A β_{osa} application respectively.

These results show that, at equivalent concentrations, toxic A β_{wt} and A β_{osa} induce synaptotoxic effects whereas non-toxic A β_{ice} and A β_{wt3M} do not. This demonstrates that the 3M mutation induces A β loss of toxicity, since A β_{wt} is toxic and A β_{wt3M} is not (fig12.C, D and G, comparing A β_{wt} vs A β_{wt3M} : $##p = 0.0034$ for total spine density; $###p = 0.0004$ for mushroom spine density and $####p < 0.0001$ for mushroom spine volume), and A β_{ice} seems to behave in the same way as A β_{wt3M} .

Long-term potentiation is impaired by exogenous application of A β_{wt} and A β_{osa} but not with A β_{ice} or A β_{wt3M}

To go further into the investigation of A β 's synaptotoxicity depending on its sequence, we next carried out functional tests by evaluating synaptic plasticity via electrophysiological recordings. To do so, we performed long-term potentiation experiments (LTP) on wild-type mouse acute hippocampal slices incubating in 100 nM of A β_{wt} , A β_{osa} , A β_{ice} or A β_{wt3M} peptides. We observed an impaired LTP for slices incubating in A β_{wt} ($**p = 0.0017$, compared to Ctrl) and even more so for slices incubating in A β_{osa} ($****p < 0.0001$, compared to Ctrl). A β_{ice} and A β_{wt3M} , however, had no effect on LTP (fig13.). Here also we noted A β 's loss of toxicity when it bears the 3M mutation, since A β_{wt} induced LTP impairment whilst A β_{wt3M} did not ($\dagger p = 0.0128$, A β_{wt} vs A β_{wt3M}).

Neurons overproducing secreted toxic A β affect neighbouring neurons through APP, whereas neurons overproducing toxic intracellular A β or non-toxic A β do not

Several studies have shown that A β overproduction in one cell affects the neighbouring healthy cells and could explain the spreading of the pathology across the brain^{11,45}. Here we examined the effects of an APP_{wt}-, APP_{swe}-, APP_{osa}- or APP_{ice}-mCh overexpressing neuron on a healthy nearby neuron. To do this, we first transfected either of the APP_x-mCh in cultured cortical neurons then, 30 min later, we added LA-GFP. This allowed us to have one neuron expressing both LA-GFP and one of the APP_x-mCh (APP neuron) next to another neuron which only expresses LA-GFP (healthy neuron) (fig14.A). We then examined the spine density of the dendrites of the healthy neuron depending on their distance from the APP neuron. Results showed that neurons overproducing secreted A β , namely APP_{wt}- and APP_{swe}-mCh overexpressing neurons, decrease the spine density of the nearby healthy neuron. Indeed, APP_{wt} neuron significantly decreased spine density of healthy neuron from 0 (dendrites from both neurons are overlapping) to 20 μ m (distance from APP neuron). APP_{swe} neuron had an even stronger impact on healthy neuron by decreasing its spine density from a range of 0 to 40 μ m. However, APP_{osa} neuron had no effect on healthy neuron, neither did APP_{ice} neuron. These results show that, in order for the pathology to propagate from cell to cell, A β has to be of a toxic sequence and secreted (fig14.B).

Furthermore, we wanted to assess the role of APP in this spreading of toxic effects. We carried out the same set of experiments using APP knock-out cultured cortical neurons. Here, one APPKO neuron is overexpressing both APP_{swe} and LA-GFP and the neighbouring healthy APPKO neuron is only overexpressing LA-GFP. Interestingly, when the healthy neuron has no APP, the toxic effects induced by the APPKO(+ APP_{swe}) neuron do not occur, demonstrating the implication of APP in the spreading of the pathology (fig14.B). To verify that the absence of effect of A β _{osa} and A β _{ice} was not attributed to an impaired interaction with APP, we carried out an A β binding assay (fig14.C) where the different A β _x were loaded on a nitrocellulose membrane and neuronal plasma membrane extracted proteins were added. The A β -APP interaction was revealed with 22C11. Our results show that all A β _{wt}, A β _{osa}, A β _{ice} and synthetic A β (A β _{synth}) are capable of interacting with APP at the plasma membrane further supporting our hypothesis that amyloid pathology might spread from neuron to neuron via APP.

Discussion

In the last decade, A β peptides have been identified as the main conductor driving synaptotoxicity and have emerged as the pivot in the pathophysiological development of AD. Although it is now well-established that A β accumulation is the trigger of synaptic deficits, and has multiple effects on neurons²⁴, the pathway by which the A β peptides exert and sustain their synaptotoxicity is still under

debate. In this study, we get a deeper insight into the way A β initially impairs synapse morphology and function, and how APP processing sustains and propagates these effects in a synaptic activity-dependent manner. Here we show that A β accumulation induces a decrease in spine density especially mushroom spines, accompanied by an increased volume of the remaining mushroom spines, and that intracellular A β is sufficient to induce these effects. These enlarged mushroom spines have impaired structural plasticity as they did not increase in volume following synaptic activation and this seems to be due to defective activity-dependent actin dynamics in the spines. This alteration of synaptic morphology, structure and plasticity seems to be due to a newly-identified interaction between actin and A β , hinting a possible physiological role for A β in activity-dependent synaptic plasticity. We also show that synaptic activity modulates amyloidogenic APP processing, further exacerbating these synaptic defects. Furthermore, we show that A β sequence is as important as A β concentration in inducing synaptic alterations. Lastly, we bring to light that secreted A β , not only affects the A β -secreting neuron itself, but also affects nearby neurons in an APP-dependent manner, reminiscent of a prion-like mechanism.

One of the first issues we wanted to address was the pathway which A β follows to induce its synaptotoxic effects. Is it extracellular A β that causes synaptotoxicity? Or rather could it be intracellular A β and/or internalised A β from the extracellular space? In our study we show that intracellular A β is sufficient to induce synaptic alterations, since APP_{osa} overexpression led to intraneuronal accumulation of A β , no A β secretion and spine density decrease along with abnormal spine head enlargement. Several have brought to light that intracellular A β accumulation precedes NFT and senile plaque apparition^{16,46}. Our results support this hypothesis and point towards intracellular A β as the initiating factor of synaptic distress leading to the early cognitive impairments in AD. Our results also show that overexpression of APP_{swe} and APP_{wt} lead to an increase in intracellular A β , as well as an increase in secreted A β which could be uptaken by neighbouring neurons.

Dendritic spines adapt their shape thus function to match the incoming synaptic activity²¹. Long-term potentiation (LTP) leads to spine enlargement, whereas Long-term depression (LTD) leads to spine shrinkage^{35,47,48}. In our study, we used BIC15 protocol which mimics a chemical LTP-type synaptic activation. We confirmed spine enlargement following synaptic activation. However in pathological neurons, where a form of toxic APP is overexpressed, mushroom spines are already enlarged and fail to undergo activity-dependent spine enlargement, indicating synaptic plasticity defects in these neurons, presumably due to intracellular A β . Interestingly, although an excess amount of intracellular A β leads to impaired synaptic plasticity, an absence of A β also seems adverse. Indeed, upon β Sec1 treatment, which was carried out just after transfection and inhibition of β -

secretase was maintained until the end of the experiments, effectively all amyloidogenic processing was blocked in the cells. Although this inhibition prevented toxic A β -induced spine density decrease, our results show that absence of A β still leads to failed spine enlargement following synaptic activation and brings to light a possible physiological role for A β in activity-dependent spine stabilisation. Our FRAP data further confirmed this possible role as excess intracellular toxic A β led to actin overstabilisation in spines whereas absence of A β , as well as protective A β_{ice} , led to no activity-dependent stabilisation. Interestingly, these results were confirmed in cultured cortical neurons from APPKO mice (data not shown) where activity-dependent actin remodelling and subsequent spine enlargement did not occur. However, this model is not ideal to study the effects of absence of A β as APPKO neurons had overall decreased spine density, pointing out a possible implication of APP or its proteolytic derivatives in spine structure and maintenance. This decrease in spine density in APPKO neurons has already been reported by others and, according to these studies, this could be either due to the lack of sAPP α , the N-terminal fragment of APP generated in the non-amyloidogenic pathway after cleavage by α -secretase⁴⁹ or due to the lack of interaction of cell-surface APP with proteins from the extracellular matrix⁵⁰. More recently, it has also been evidenced that dendritic spines from APPKO neurons also have altered pre- and post-synaptic protein and receptor content, further supporting the role of APP in spine structure and function⁵¹.

The interplay between intracellular A β and actin which had been hinted by others^{52,53}, led us to question a potential interaction between them. We identified a common 3 amino-acid actin-binding sequence between A β and other actin-binding proteins involved in actin bundling, and so we generated the 3M mutation within the A β sequence. After confirming that this mutation did not hinder APP processing or A β production we reported a loss of synaptotoxicity of A β (wt, osa) when it harboured the 3M mutation as well as a loss of activity-dependent actin overstabilisation in the spines. In 2003, Kamenetz and collaborators⁴⁵ proposed a physiological activity-dependent negative feed-back system, where A β production modulates synaptic transmission, and synaptic activity modulates A β production, to keep neural hyperactivity in check. In this light, excess A β would drive synaptic depression and eventually spine loss. Our findings support this hypothesis and add that: i) this mechanism might occur via excess A β preventing proper activity-dependent actin remodelling in the spines and ii) absence of A β also leads to improper activity-dependent actin-remodelling in the spines. This suggests that the pathway to synaptotoxicity depends not only on a balance of the quantity of A β produced but also on the sequence of A β and its ability to interact with the actin cytoskeleton in spines.

Several have reported that synaptic activity modulates APP processing^{31,45}. Our results support these findings and bring to light that activity-dependent amyloidogenic processing of APP seems

predominant at the intracellular level. Indeed, our results show that the mCherry signal inside our cultured cortical neurons from transfected APP_x-mCh constructs decreased following synaptic activity, especially APP_{swe}, and this decrease was largely blocked by β -secretase inhibition. Additionally, according to several studies^{16,46,54-57} it is preferably the toxic A β ₄₂ isoform that is produced intracellularly. Our findings shed some light on the possible purpose of activity-dependent processing of APP, where generated A β regulates activity-dependent actin remodelling and subsequent synaptic transmission. Excess A β or conversely, lack of A β , both ultimately lead to defective activity-dependent synaptic plasticity and transmission. This indicates that activity-dependent amyloidogenic processing of APP is likely to be a finely tuned equilibrium and misbalancing this mechanism would lead to the early cognitive impairments of AD. In the bigger picture, this provides possible answers about the recent failings of β -secretase inhibition as treatment for AD in clinical trials⁵⁸. In addition to the fact that BACE1 has other substrates involved in various synaptic processes, blocking this enzyme and subsequent A β production would still lead to synaptic defects, ultimately worsening cognitive impairments.

It is now well established that A β toxicity arises from pathological/excessive amounts of A β . Indeed, nowadays the most used AD transgenic animal models carry the FAD Swedish mutation on APP, which increases A β production⁵⁹. Conversely, it has been proposed that less A β would be protective against AD. This has been suggested following the discovery of the protective Icelandic mutation which, allegedly, leads to a 30 to 40% reduction in A β generation²⁶. Although this might seem quite significant, it is to note that the amyloidogenic processing accounts for only 10% of total APP processing⁶⁰. Furthermore, other studies based on FAD mutations such as the Arctic mutation, showed that toxicity may occur from an imbalance of A β ₄₂/A β ₄₀ ratio when total A β is unchanged⁶¹, highlighting that A β sequence may be as important as A β concentration in exerting synaptic dysfunction. In our study we used equal “pathological” concentrations of A β _{wt}, A β _{osa}, A β _{ice} and A β _{wt3M} (100 nM) on cultured cortical neurons to assess spine morphology, as well as on acute mouse hippocampal slices for LTP electrophysiological experiments. As expected, we observed the toxic effects of A β _{wt} and A β _{osa} in both models. Interestingly, however, neither A β _{ice} nor A β _{wt3M} induced any alterations on spine morphology or LTP. This puts forward the importance of the sequence of A β as well as its concentration, and that the Icelandic mutation may in fact be protective against AD via its sequence along with its decreased concentration. Interestingly, although A β _{ice} has no common mutated amino-acids with A β _{wt3M}, throughout our study they both induced very similar effects regarding actin depolymerisation rate, spine morphology and activity-dependent spine actin stabilisation. This led us to question whether their sequence may induce similar three-dimensional folding of the peptide, preventing their interaction with actin. Poduslo and Howell highlighted the

importance of A β sequence in A β aggregation/fibril formation in the development of neuronal degeneration²⁵. Taken together, with our findings, we show that A β sequence may lead to different peptide behaviour in the cells: where it preferentially localises or how it aggregates/misfolds, and it is capital that this aspect is taken into account when developing new therapeutic strategies.

The last question we wanted to address regarding the way A β exerted and sustained its synaptotoxicity in early stages of AD was how the disease propagated throughout the brain, particularly relevant for sporadic AD cases. Our results showed that mutant APP overexpression, which leads to increased secreted A β in the extracellular space (wt and swe), not only did it more than likely influence the A β overproducing neuron itself, but also affected nearby “healthy” neurons which did not overexpress APP. This effect seemed to be proportional to the amount of secreted A β . Indeed, APP_{swe} overexpressing neurons, which secreted the most A β , decreased spine density of “healthy” neuron at an effective distance of 40 μ m; whereas APP_{wt} overexpressing neurons, which also secreted A β but less than with APP_{swe}, decreased “healthy” neuron’s spine density at an effective distance of 20 μ m. It would seem the more A β is secreted into the extracellular space, the more the “healthy” neuron’s spine density is affected. These results outline that the rate of A β production and secretion into the extracellular space, which can be in part modulated by the sequence of A β , might dictate the severity of sporadic AD pathology development. Interestingly, when we carried out the same experiments using APPKO cultured mouse cortical neurons, the “healthy” APPKO neuron was not affected by the nearby A β secreting neuron. This suggests that the effects observed on the “healthy” wild-type neuron are due to APP. It has been proposed that A β internalisation may occur via its binding to plasma membrane constituents⁶². Here we propose that A β internalisation from the extracellular space may occur via cell-surface APP, which in turn affects synaptic function in the recipient “healthy” neuron, reminiscent of a prion-like mechanism where the disease would progress from neuron to neuron.

To conclude, our study has brought to light a novel interaction between A β and the actin cytoskeleton revealing a potential physiological function for A β in activity-dependent spine stabilisation and a possible new route for therapeutic development. This function in structural plasticity is seemingly dependent of an equilibrium of the quantity and sequence of A β , and off-balancing this quantity and sequence would lead to synaptic dysfunction which propagates from one neuron to the neighbouring neuron if A β is secreted into the extracellular space. In the light of our findings and recent evidence showing that patients with sporadic AD may, in fact, have several strains of A β within the brain⁶³, we show that unravelling AD development entails an extra level of complexity where A β quantities and sequence have to both be taken into account in order to find

new therapeutic strategies. Our findings support the idea that there is no one-size-fits-all for AD treatment and that it should rather be made-to-measure for each individual AD patient.

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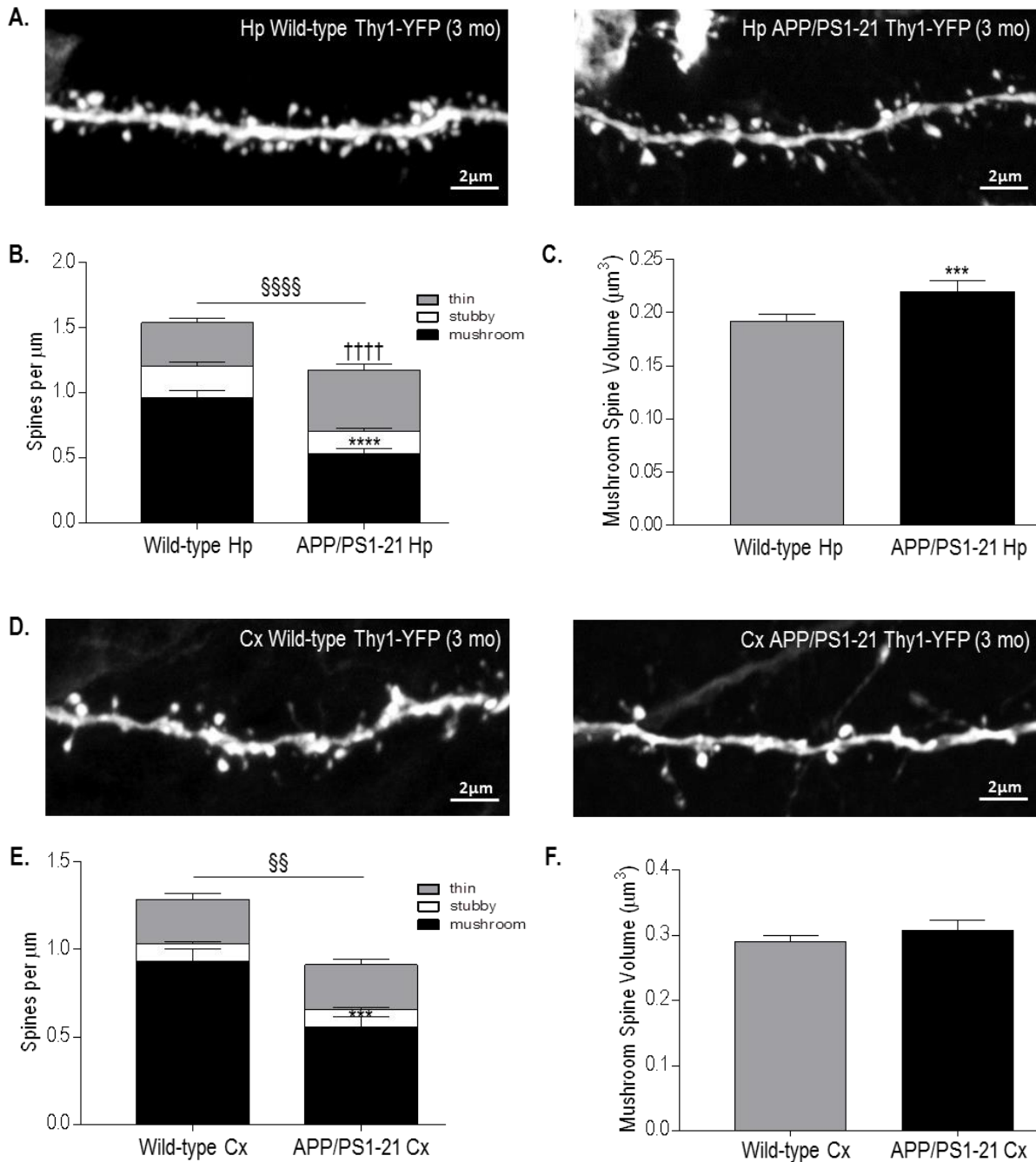


Figure 1. APP/PS1-21 Thy1-YFP mice have a decreased spine density and increased mushroom spine volume, in the hippocampus and cortex, at 3 months old. (A) Representative confocal images of dendrites with spines from acute hippocampal (Hp) slices of (Left panel) wild-type Thy1-YFP and (Right panel) APP/PS1-21 Thy1-YFP mice at 3 mo (scale bar = 2 μm). (B) Bar graphs (mean \pm SEM) show the distribution of dendritic spine subtypes (spine type and density per μm of dendrite) per condition. §§§§ $p < 0.0001$ when compared to total spine density (whole bar) of wild-type Thy1-YFP Hp; **** $p < 0.0001$ when compared to mushroom spine density (black segment in bar) of wild-type Thy1-YFP Hp; †††† $p < 0.0001$ when compared to thin spine density (grey segment in bar) of wild-type Thy1-YFP Hp. (C) Bar graphs (mean \pm SEM) show mushroom spine volume (in μm^3) per condition. *** $p < 0.001$ when compared to wild-type Thy1-YFP Hp. (D) to (F) are the same as (A) to (C) but for cortical (Cx) slices. §§ $p < 0.01$ when compared to total spine density (whole bar) of wild-type Thy1-YFP Cx; *** $p < 0.001$ when compared to mushroom spine density (black segment in bar) of wild-type Thy1-YFP Cx; † $p < 0.05$ when compared to thin spine density (grey segment in bar) of wild-type Thy1-YFP Cx. Mann-Whitney test. N=3 animals per strain; n=19 to 23 neurons per condition.

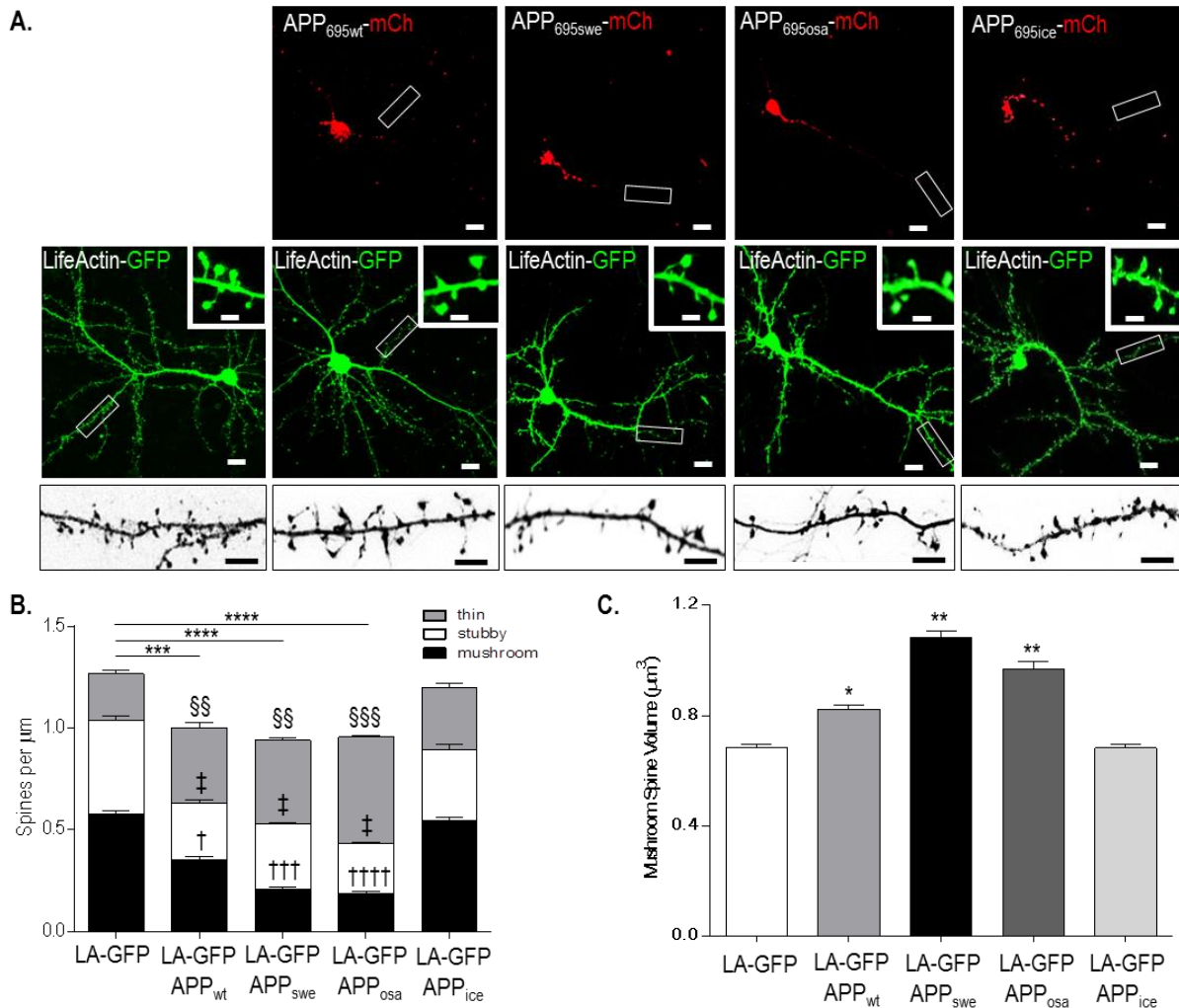


Figure 2. Overexpression of APP_{wt}, APP_{swe} and APP_{osa} in cortical cell cultures decrease spine density and increase mushroom spine volume whereas APP_{ice} overexpression does not. (A, Top row) Representative confocal images of cultured cortical neurons overexpressing APP_{wt}-mCh, APP_{swe}-mCh, APP_{osa}-mCh, APP_{ice}-mCh and **(A, middle row)** LifeActin-GFP (LA-GFP) (scale bar = 10 μm). Small square inlay show representative dendritic spines (scale bar = 2 μm). **(A, bottom row)** Representative dendrite portions (scale bar = 5 μm). **(B)** Bar graphs (mean \pm SEM) show the distribution of dendritic spine subtypes (spine type and density per μm of dendrite) per condition. *** p <0.001; **** p <0.0001 when compared to total spine density (whole bar) of control neurons (neurons only expressing LA-GFP); † p <0.05; ††† p <0.001; †††† p <0.0001 when compared to mushroom spine density (black segment in bar) of control neurons; ‡ p <0.05 when compared to stubby spine density (white segment in bar) of control neurons; §§ p <0.01; §§§ p <0.001 when compared to thin spine density (grey segment in bar) of control neurons. **(C)** Bar graphs (mean \pm SEM) show mushroom spine volume (in μm^3) per condition. * p <0.05; ** p <0.01 when compared to control neurons (expressing only LA-GFP). Kruskal-Wallis followed by Dunn's test. N=7 neurons per condition from at least 3 different cultures.

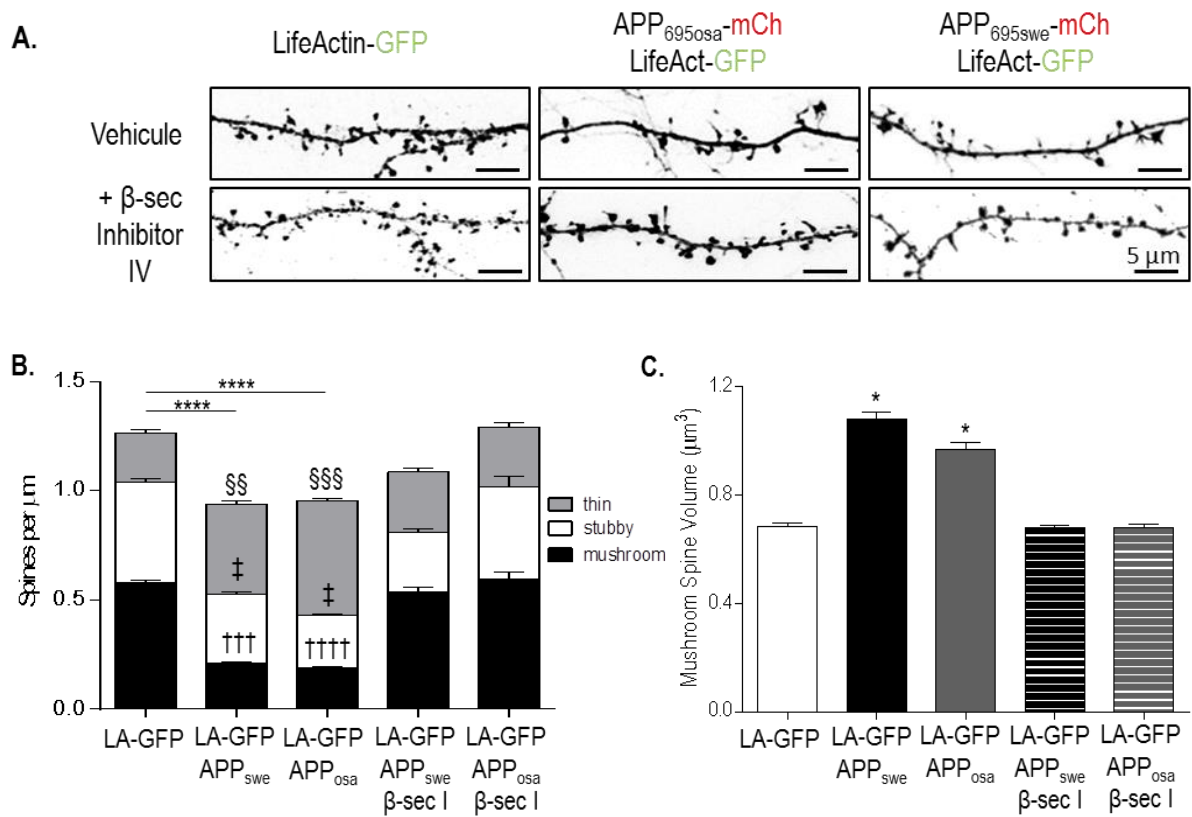


Figure 3. The decrease in spine density and increase in mushroom spine volume induced by APP_{swe} and APP_{osa} overexpression is abolished when β -secretase (BACE-1) is inhibited. (A, Top panels) Representative confocal images of portions of dendrites of cortical neurons overexpressing either APP_{swe} or APP_{osa} and LA-GFP, in control conditions (Vehicle). **(A, Bottom panels)** Representative confocal images of portions of dendrites of neurons overexpressing either APP_{swe} or APP_{osa}, with LA-GFP, and β -secretase Inhibitor IV [1 μ M] (β SecI) was added post-transfection until cultures were imaged (48 h later). Scale bar = 5 μ m. **(B)** Bar graphs (mean \pm SEM) show the distribution of dendritic spine subtypes (spine type and density per μ m of dendrite) per condition. * p <0.05 when compared to total spine density (whole bar) of control neurons (neurons only expressing LA-GFP); † p <0.05 when compared to mushroom spine density (black segment in bar) of control neurons; ‡ p <0.05 when compared to stubby spine density (white segment in bar) of control neurons; § p <0.05 when compared to thin spine density (grey segment in bar) of control neurons. **(C)** Bar graphs (mean \pm SEM) show mushroom spine volume (in μm^3) per condition. * p <0.05 when compared to control neurons (expressing only LA-GFP). Mann-Whitney test. N=7 neurons per condition from at least 3 different cultures.

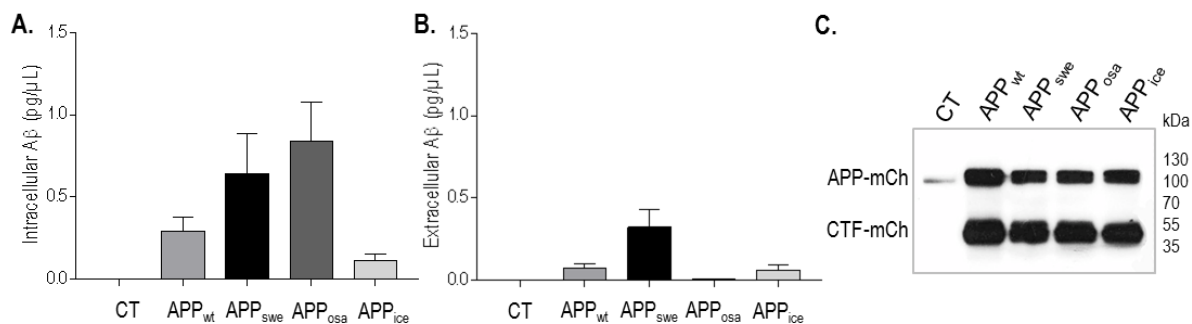


Figure 4. Quantification of intracellular and extracellular Aβ_x from neurons infected with APP_{wt}, APP_{swe}, APP_{osa} or APP_{ice}. (A) Bar graphs (mean ± SEM) show that cortical neurons infected with APP_{wt}, APP_{swe}, APP_{osa} or APP_{ice} produce intracellular Aβ_x, especially APP_{swe} and APP_{osa}. (B) Bar graphs (mean ± SEM) show that cortical neurons infected with APP_{wt}, APP_{swe}, APP_{osa} or APP_{ice} produce extracellular Aβ_x especially APP_{swe}. (C) Westernblot of infected neuron lysate showing no difference in overall APP levels using Y188 antibody. N=4 different cortical neuron cultures.

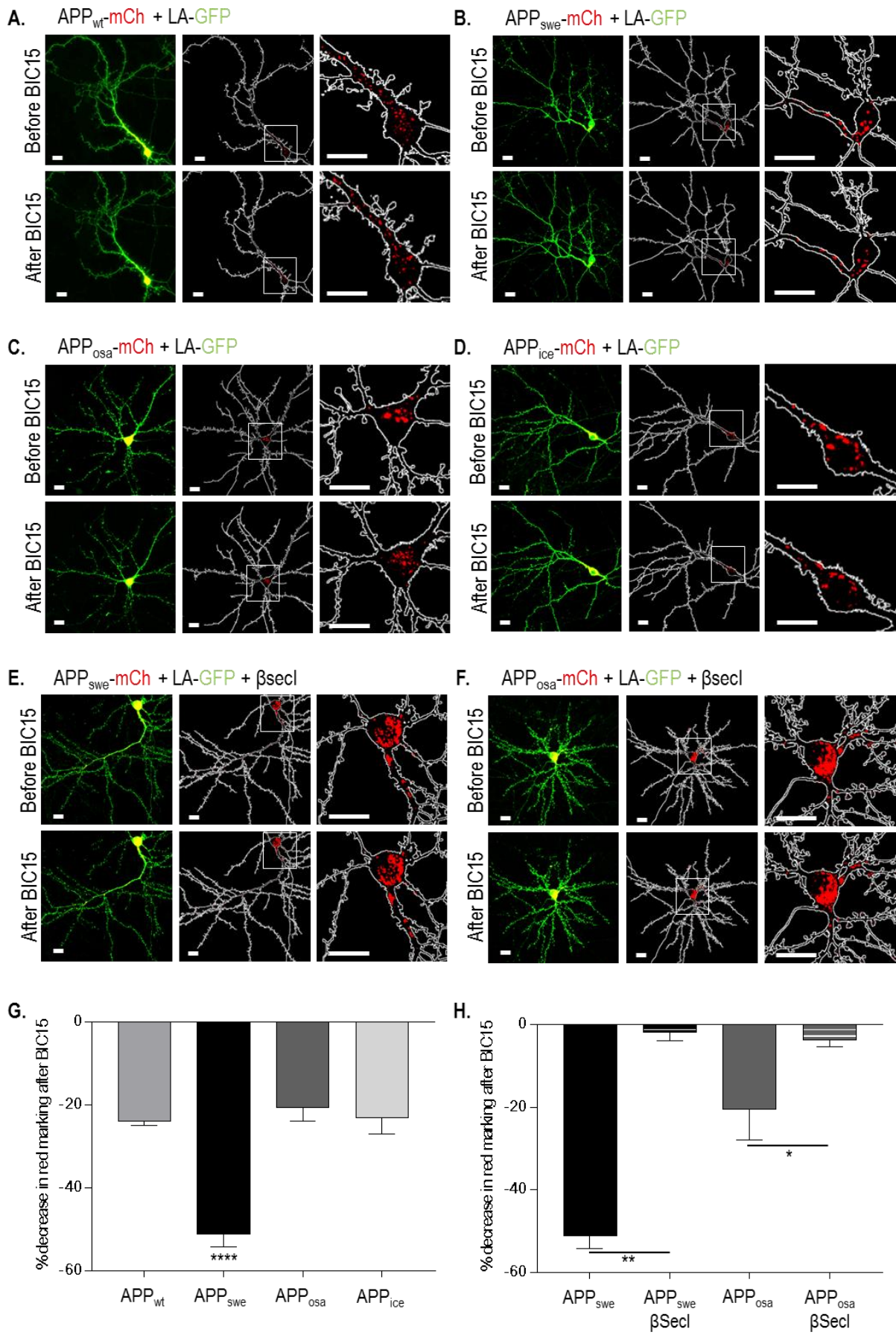


Figure 5. Synaptic activity decreases red marking in APP_x-mCh, especially APP_{swe}-mCh, expressing cortical neurons and is abrogated by βsecl pretreatment. (A, Top row) Representative confocal images of cortical neuron overexpressing APP_{wt}-mCh and LA-GFP, before synaptic activation. **(Top left panel)** overlay of APP_{wt}-mCh and LA-GFP. **(Top middle panel)** outline of neuron with APP_{wt}-mCh marking. **(Top right panel)** cell body of neuron. **(Bottom row)** same neuron with decreased red marking after synaptic activation with BIC15 protocol (BIC15: incubation with bicuculline methiodide [50 μM] and 4-aminopyridine [2,5 mM] for 15 min). **(B)** Same as **(A)** with cortical neuron overexpressing APP_{swe}-mCh and LA-GFP before and after BIC15. **(C)** Same as **(A)** with cortical neuron overexpressing APP_{osa}-mCh and LA-GFP before and after BIC15. **(D)** Same as **(A)** with cortical neuron overexpressing APP_{ice}-mCh and LA-GFP before and after BIC15. **(E, Top row)** Representative confocal images of cortical neuron pretreated with βsecl (1 μM post-transfection) and overexpressing APP_{swe}-mCh + LA-GFP before BIC15. **(Top left panel)** overlay of APP_{swe}-mCh and LA-GFP. **(Top middle panel)** outline of neuron with increased APP_{swe}-mCh fluorescence. **(Top right panel)** cell body of neuron. **(Bottom row)** same neuron with no decreased red marking after BIC15 protocol. **(F)** Same as **(E)** with cortical neuron pretreated with βsecl (1 μM post-transfection) and overexpressing APP_{osa}-mCh + LA-GFP before and after BIC15, showing no decrease in red marking. **(G)** Bar graphs (mean ± SEM) show a similar decrease of APP_x-mCh red marking as APP_{wt}-mCh after BIC15 protocol (expressed as % variation of APP_x-mCh fluorescence normalised to APP_x-mCh fluorescence before BIC15) except APP_{swe}-mCh which displays a significant decrease. *****p*<0.0001 compared to APP_{wt}-mCh. Kruskal-Wallis followed by Dunn's test. **(H)** Bar graphs (mean ± SEM) show an inhibition of red marking decrease induced by BIC15 protocol when neurons are pretreated with βsecl. ***p*<0.01 APP_{swe} vs APP_{swe}+βsecl; **p*<0.05 APP_{osa} vs APP_{osa}+βsecl. Mann-Whitney test. All scale bars = 10 μm. N=5 to 6 neurons per condition from at least 3 different cultures.

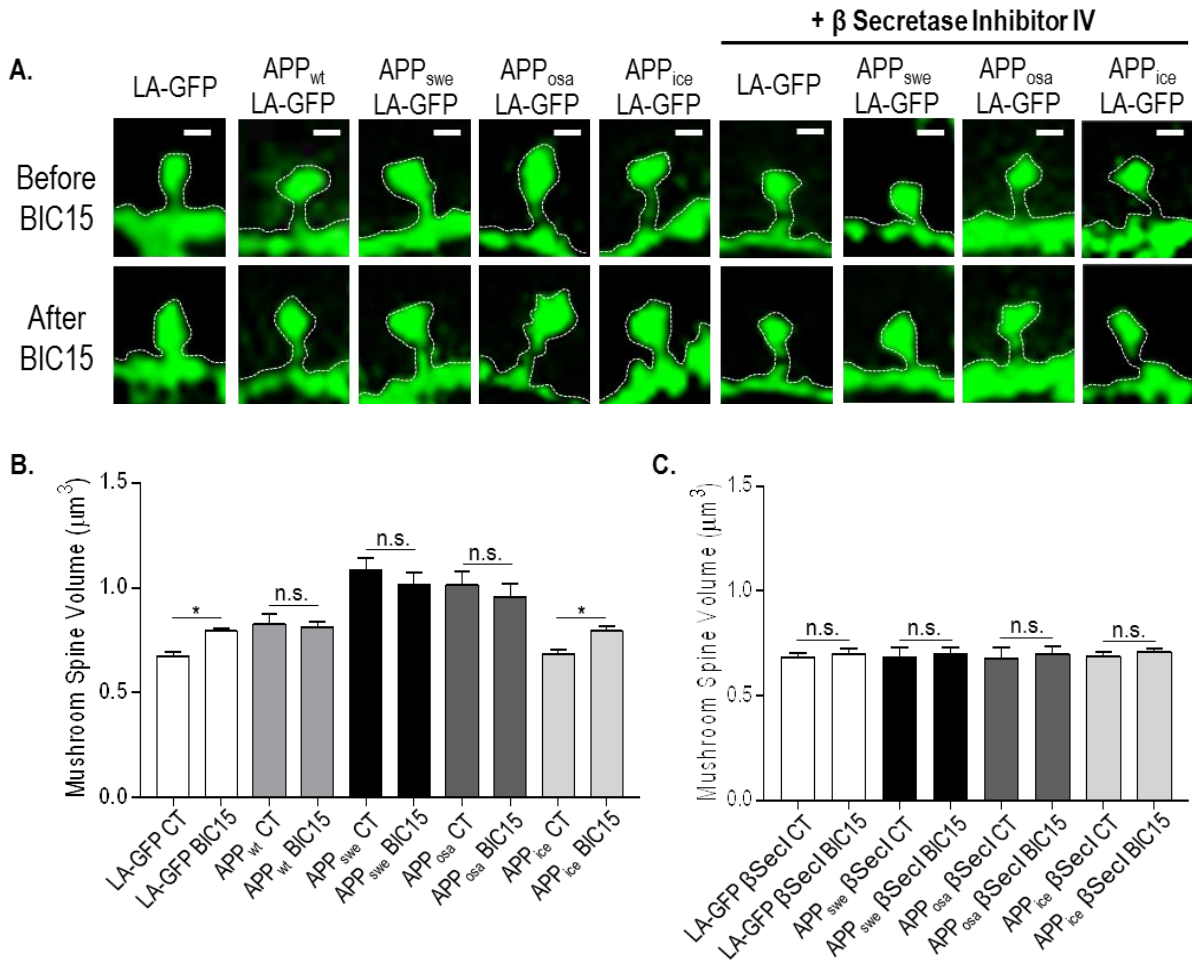


Figure 6. Enlarged mushroom spines, induced by APP_{wt}, APP_{swe} or APP_{osa} overexpression, lose their ability to increase their volume after synaptic activation protocol (BIC15). Mushroom spines analysed by confocal microscopy after neurons transfected with APP_x-mCh and LA-GFP. **(A, Top panels)** Representative confocal images of a mushroom spine per condition before synaptic activation (scale bar = 1 μ m). **(Bottom panels)** the same mushroom spines per condition after synaptic activation (BIC15: incubation with bicuculline methiodide 50 μ M and 4-aminopyridine 2,5 mM for 15 min). **(B)** Bar graphs (mean \pm SEM) show mushroom spine volume before (CT) and after BIC15 per condition. * p <0.05; n.s. p >0.05 when compared to mushroom spine volume before BIC15. **(C)** Bar graphs (mean \pm SEM) show mushroom spine volume before and after BIC15 per condition when there is no A β present. n.s. p >0.05 when compared to mushroom spine volume before BIC15. Wilcoxon Signed Rank test. N=7 neurons per condition from at least 3 different cultures.

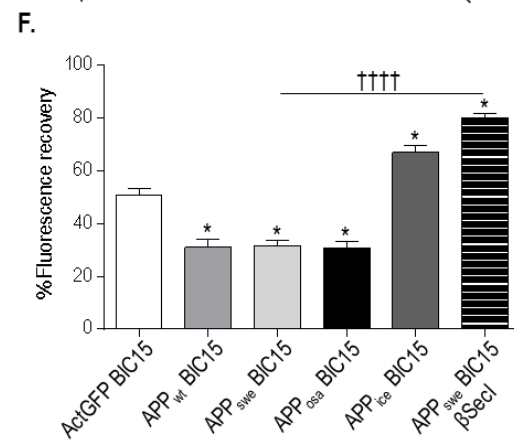
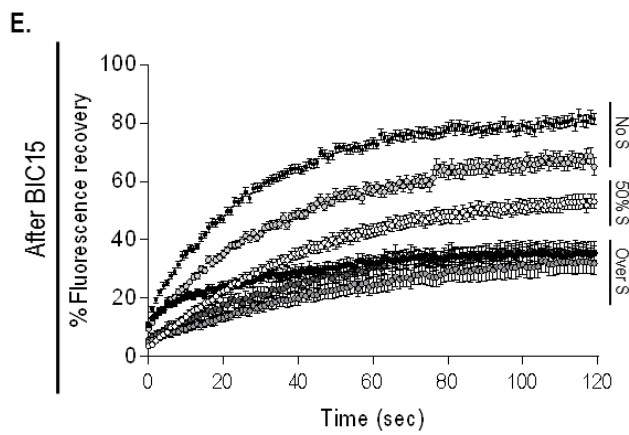
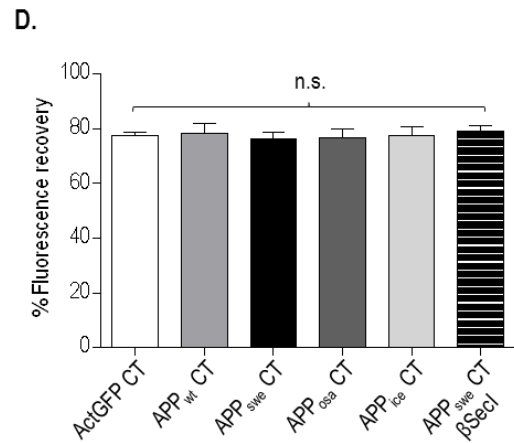
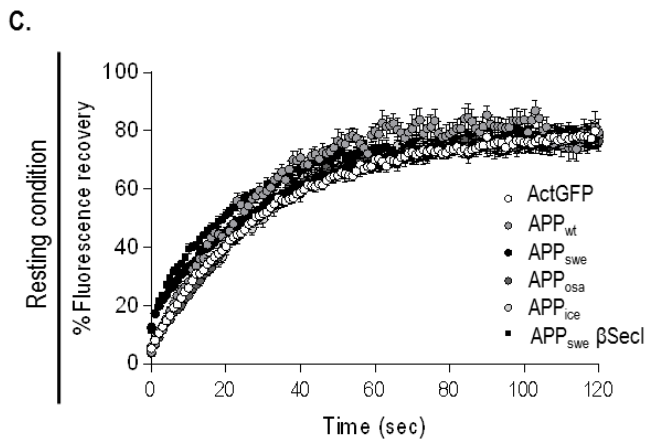
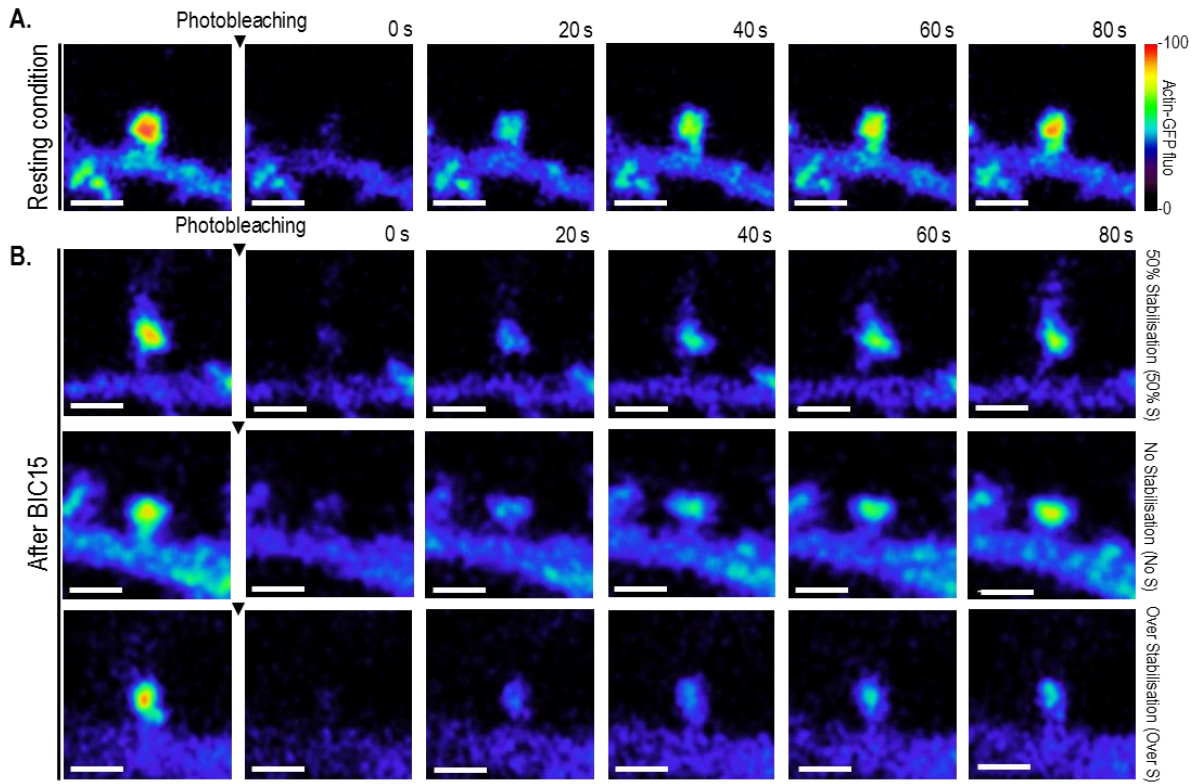


Figure 7. Activity-dependent actin dynamics in mushroom spines is altered by APP_{wt}, APP_{swe}, APP_{osa} but also APP_{ice} and β secl treatment. **(A)** Representative FRAP timelapse of a mushroom spine in resting conditions (no synaptic activation) showing 80% fluorescence recovery. **(B)** Representative FRAP timelapses of mushroom spines pretreated with BIC15. **(Top row)** representative FRAP timelapse of mushroom spine pretreated with BIC15 showing activity-dependent stabilisation of actin cytoskeleton in spine (Act-GFP only) with 50% fluorescence recovery. **(Middle row)** representative FRAP timelapse of mushroom spine pretreated with BIC15 when there is no activity-dependent stabilisation of actin cytoskeleton in spine (>60% fluorescence recovery). **(Bottom row)** representative FRAP timelapse of mushroom spine pretreated with BIC15 when there is activity-dependent over-stabilisation of actin cytoskeleton in spine (<50% fluorescence recovery). **(C)** Representative FRAP curves (% FRAP normalised to prebleached intensity of the spine of Actin-GFP +/- APP_x-mCh transfected neurons +/- β secl) of Actin-GFP in resting conditions (before BIC15 protocol). **(D)** Bar graphs show the plateau values (mean \pm SEM of the last 10 points for each FRAP curve) for each condition before BIC15 protocol. No statistical differences across the different conditions. **(E)** Representative FRAP curves of Actin-GFP (+/- APP_x-mCh +/- β secl) after BIC15 protocol showing either activity-dependent 50% stabilisation (Actin-GFP only) or no activity-dependent stabilisation (APP_{ice}-mCh, APP_{swe}+ β secl) or an over-stabilisation (APP_{wt}-mCh, APP_{swe}-mCh, APP_{osa}-mCh) of actin cytoskeleton in spines. **(F)** Bar graphs (mean \pm SEM) of plateau values for each condition after BIC15 protocol showing an activity-dependent stabilisation of actin cytoskeleton in spines of Actin-GFP only expressing neurons (50% fluorescence recovery); no activity-dependent stabilisation (>60% fluorescence recovery) of actin (for neurons expressing Actin-GFP and APP_{ice}-mCh or APP_{swe}-mCh+ β secl); and an activity-dependent over-stabilisation (<50% fluorescence recovery) of actin in neurons expressing Actin-GFP and APP_{wt}-mCh or APP_{swe}-mCh or APP_{osa}-mCh. * p <0.05 when compared to Act-GFP only BIC15; +++ p <0.0001 APP_{swe} BIC15 vs APP_{swe}+ β secl BIC15. Kruskal-Wallis followed by Dunn's test. N=at least 25 spines per condition from at least 3 different cultures.

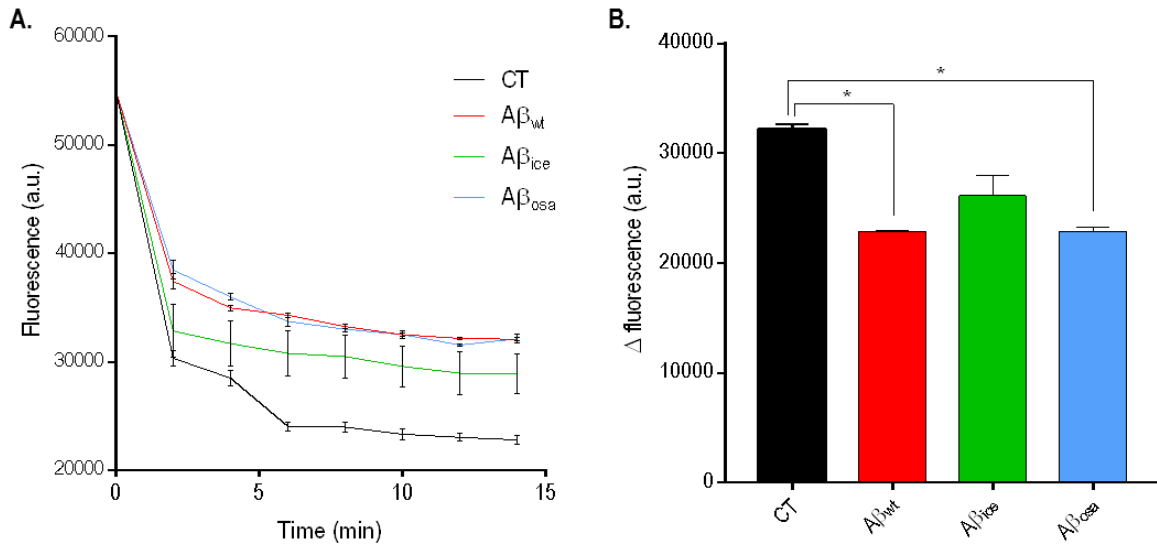


Figure 8. A β_{wt} and A β_{osa} , but not A β_{ice} significantly decreases depolymerisation rate of actin in vitro. (A) Depolymerisation curve of actin in presence of 100 nM of either A β_{wt} , A β_{osa} and A β_{ice} . **(B)** Bar graph (mean \pm SEM) shows a significant decrease of the depolymerisation rate of actin (expressed as Δ fluorescence from beginning to mean plateau value) in presence of A β_{wt} and A β_{osa} but not A β_{ice} . * $p=0.0382$ for CT vs A β_{wt} ; n.s. $p=0.9245$ for CT vs A β_{ice} ; * $p=0.0276$ for CT vs A β_{osa} . Kruskal-Wallis followed by Dunn's test. N=3 independent experiments.

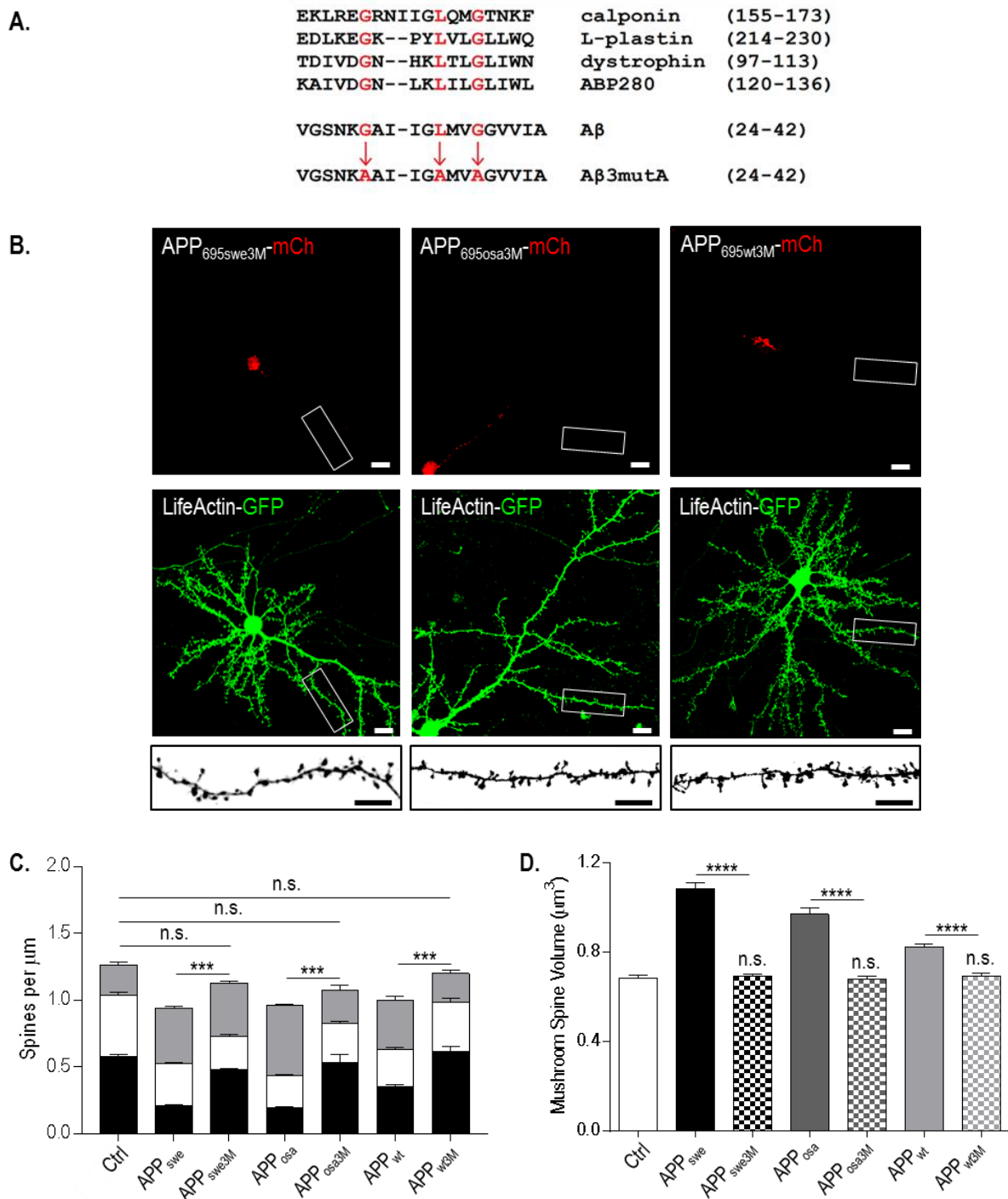


Figure 9. Overexpression of APP_{swe3M} and APP_{osa3M} and APP_{wt3M} in cortical cell cultures did not induce a decrease in spine density or an increase in mushroom spine volume. (A) Comparison of A β _{wt} with other actin binding proteins involved in actin bundling showing a common sequence and design of A β _{3M} mutation. **(B, Top row)** Representative confocal images of cultured cortical neurons overexpressing APP_{swe3M}-mCh, APP_{osa3M}-mCh, APP_{wt3M}-mCh and **(B, Middle row)** LA-GFP (scale bar = 10 μ m). **(B, Bottom row)** Representative dendrite portions (scale bar = 5 μ m). **(C)** Bar graphs (mean \pm SEM) show the distribution of dendritic spine subtypes (spine type and density per μ m of dendrite) per condition. n.s. $p > 0.05$ when compared to Ctrl (LA-GFP only) total spine density (whole bar); *** $p < 0.001$ APP_{swe} vs APP_{swe3M}, APP_{osa} vs APP_{osa3M} and APP_{wt} vs APP_{wt3M}. **(D)** Bar graphs (mean \pm SEM) show mushroom spine volume (in μ m³) per condition. n.s. $p > 0.05$ when compared to Ctrl (LA-GFP only); **** $p < 0.0001$ APP_{swe} vs APP_{swe3M}, APP_{osa} vs APP_{osa3M} and APP_{wt} vs APP_{wt3M}. Kruskal-Wallis followed by Dunn's test. N=7 neurons per condition from at least 3 different cultures.

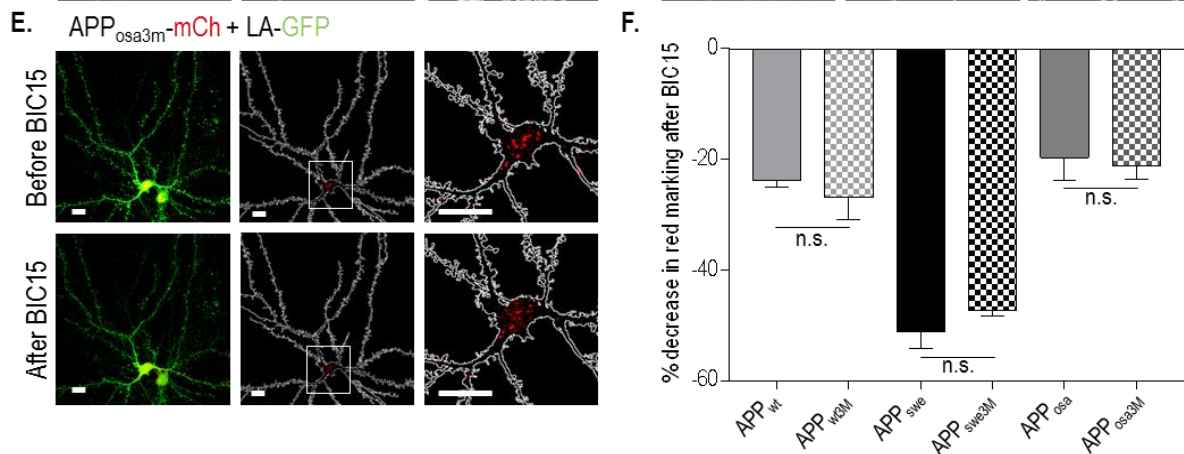
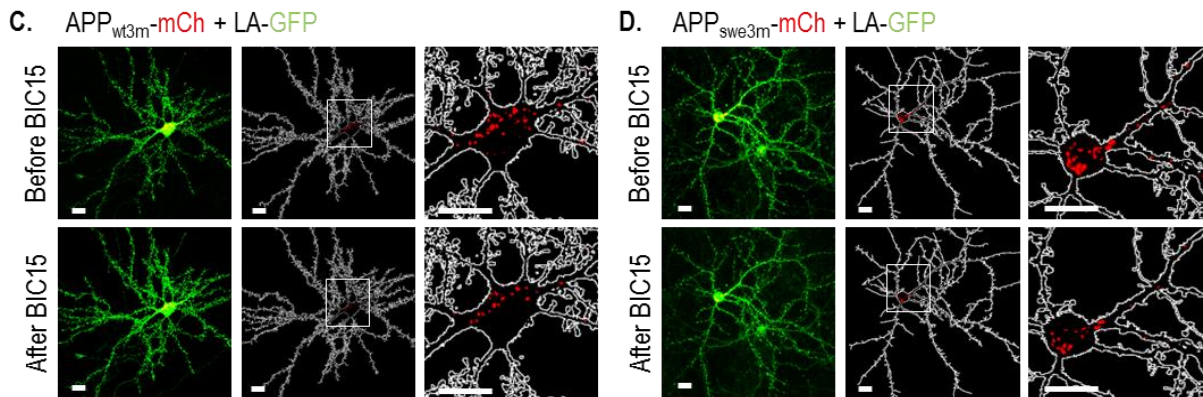
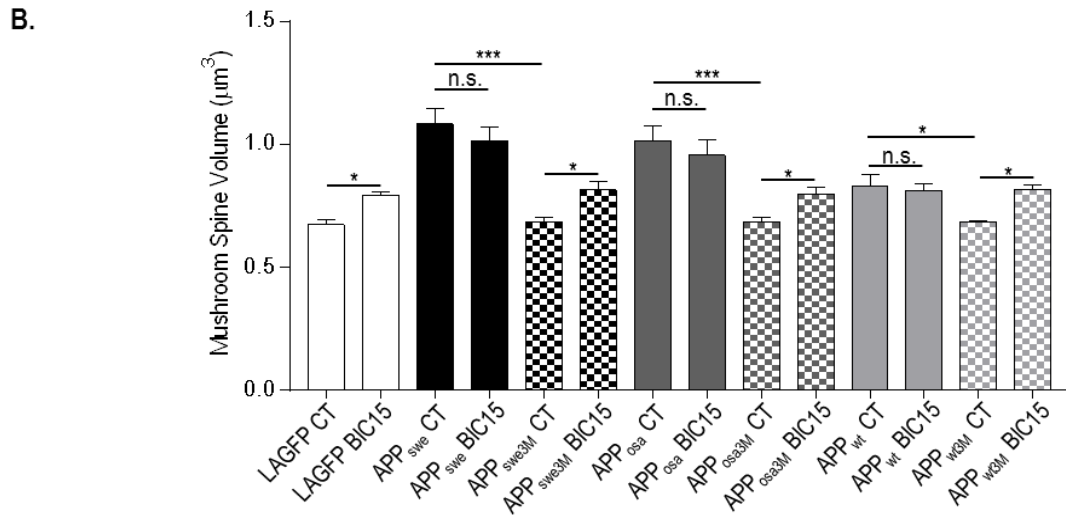
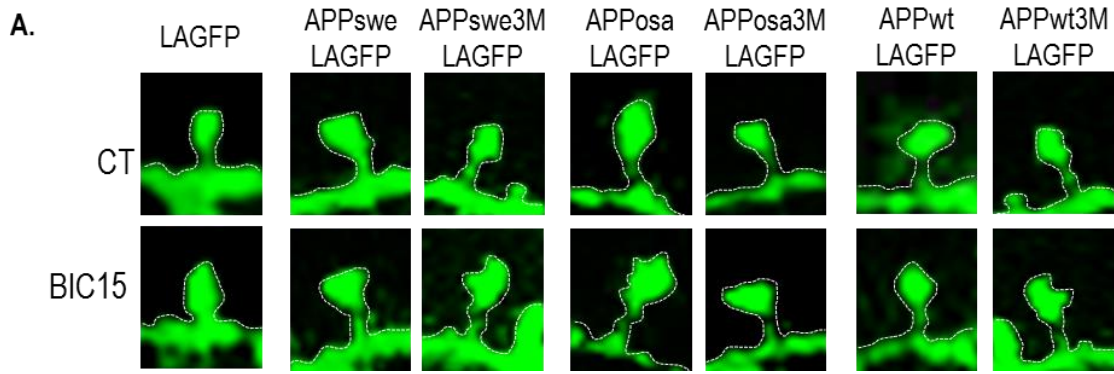


Figure 10. APP_{swe3M}, APP_{osa3M} and APP_{wt3M} overexpression in cortical neurons does not induce spine enlargement impairment after BIC15 protocol although APP_x processing is unchanged compared to their non-3M counterparts. (A, Top panels) Representative confocal images of a mushroom spine, per condition, before synaptic activation (Before BIC15). **(Bottom panels)** the same mushroom spines per condition after BIC15 (scale bar = 1 μ m). **(B)** Bar graphs (mean \pm SEM) show mushroom spine volume before (CT) and after BIC15 per condition. * p <0.05; n.s. p >0.05 when compared to mushroom spine volume before BIC15. *** p <0.001; * p <0.05 APP_{swe} CT vs APP_{swe3M} CT, APP_{osa} CT vs APP_{osa3M} CT, APP_{wt} CT vs APP_{wt3M} CT. Wilcoxon Signed Rank test. N=7 neurons per condition from at least 3 different cultures. **(C, Top row)** Representative confocal images of cortical neuron overexpressing APP_{wt3M}-mCh and LA-GFP before BIC15. **(Top left panel)** overlay of APP_{wt3M}-mCh and LA-GFP. **(Top middle panel)** outline of neuron with APP_{wt3M}-mCh fluorescence. **(Top right panel)** cell body of neuron. **(Bottom row)** same neuron with decreased red fluorescence after BIC15 protocol. **(D, Top row)** Representative confocal images of cortical neuron overexpressing APP_{swe3M}-mCh + LA-GFP before BIC15. **(Top left panel)** overlay of APP_{swe3M}-mCh and LA-GFP. **(Top middle panel)** outline of neuron with APP_{swe3M}-mCh fluorescence. **(Top right panel)** cell body of neuron. **(Bottom row)** same neuron with a marked decrease in red fluorescence after BIC15 protocol. **(E, Top row)** Representative confocal images of cortical neuron overexpressing APP_{osa3M}-mCh + LA-GFP before BIC15. **(Top left panel)** overlay of APP_{osa3M}-mCh and LA-GFP. **(Top middle panel)** outline of neuron with APP_{osa3M}-mCh fluorescence. **(Top right panel)** cell body of neuron. **(Bottom row)** same neuron with decreased red fluorescence after BIC15 protocol. **(F)** Bar graphs (mean \pm SEM) show a similar reduction of red fluorescence after BIC15 of APP_{wt3M}-mCh, APP_{swe3M}-mCh and APP_{osa3M}-mCh as their non-3M counterpart. n.s. p >0.05 APP_{wt} vs APP_{wt3M}; APP_{swe} vs APP_{swe3M}; APP_{osa} vs APP_{osa3M}. All scale bars = 10 μ m. Mann-Whitney test. N=6 neurons per condition from at least 3 different cultures.

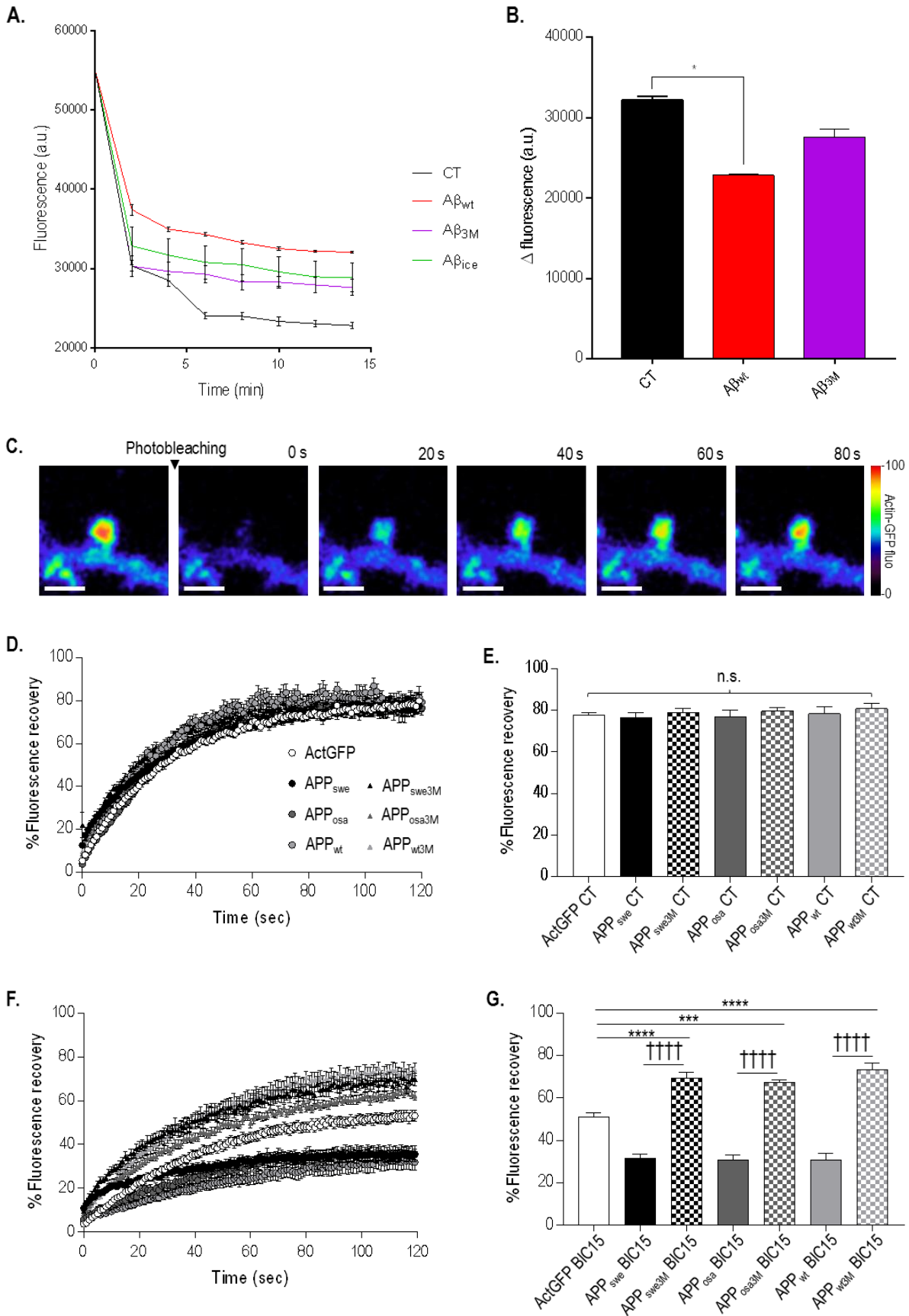


Figure 11. The 3M mutation abrogates the effect of toxic A β on actin *in vitro* and in cortical neuron dendritic spines. (A) Depolymerisation curve of actin in presence of 100nM of either A β_{wt} , A β_{wt3M} (and A β_{ice} to show similar behaviour with A β_{wt3M}). **(B)** Bar graph (mean \pm SEM) showing a significant decrease of the depolymerisation rate of actin (expressed as Δ fluorescence from beginning to mean plateau value) in presence of A β_{wt} but not A β_{wt3M} . * $p=0.0219$ for CT vs A β_{wt} . Kruskal-Wallis followed by Dunn's test. N=3 independent experiments. **(C)** Representative FRAP timelapse of a mushroom spine showing no actin stabilisation (80% fluorescence recovery). **(D)** Representative FRAP curves (% FRAP normalised to prebleached intensity of the spine of Actin-GFP +/- APP $_x$ -mCh transfected neurons) of Actin-GFP in control conditions (before BIC15 protocol). **(E)** Bar graphs show the plateau values (mean \pm SEM of the last 10 points for each FRAP curve) for each condition before BIC15 protocol. No statistical differences across the different conditions. **(F)** Representative FRAP curves of Actin-GFP (+/- APP $_x$ -mCh) after BIC15 protocol showing either activity-dependent 50% stabilisation (Actin-GFP only) or no activity-dependent stabilisation (APP $_{swe3M}$ -mCh, APP $_{osa3M}$ -mCh, APP $_{wt3M}$ -mCh) or an over-stabilisation (APP $_{wt}$ -mCh, APP $_{swe}$ -mCh, APP $_{osa}$ -mCh) of actin cytoskeleton in spines. **(G)** Bar graphs (mean \pm SEM) of plateau values for each condition after BIC15 protocol showing an activity-dependent stabilisation of actin cytoskeleton in spines of Actin-GFP only expressing neurons (50% fluorescence recovery); no activity-dependent stabilisation (>60% fluorescence recovery) of actin (for neurons expressing Actin-GFP and APP $_{swe3M}$ -mCh or APP $_{osa3M}$ -mCh, or APP $_{wt3M}$ -mCh); and an activity-dependent over-stabilisation (<50% fluorescence recovery) of actin in neurons expressing Actin-GFP and APP $_{wt}$ -mCh or APP $_{swe}$ -mCh or APP $_{osa}$ -mCh. *** $p<0.001$; **** $p<0.0001$ when compared to Act-GFP only BIC15; +++ $p<0.0001$ APP $_{swe}$ BIC15 vs APP $_{swe3M}$ BIC15, APP $_{osa}$ BIC15 vs APP $_{osa3M}$ BIC15, APP $_{wt}$ BIC15 vs APP $_{wt3M}$ BIC15. Kruskal-Wallis followed by Dunn's test. N=at least 19 spines per condition from at least 3 different cultures.

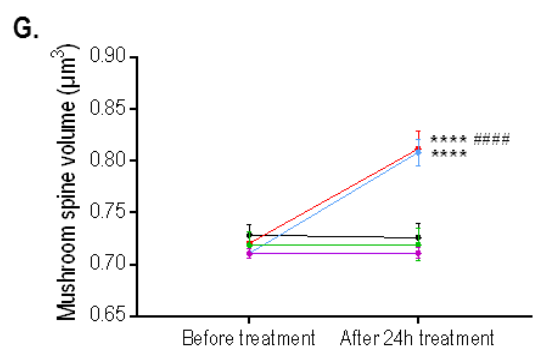
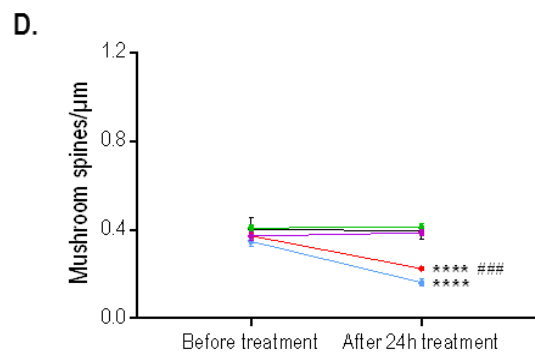
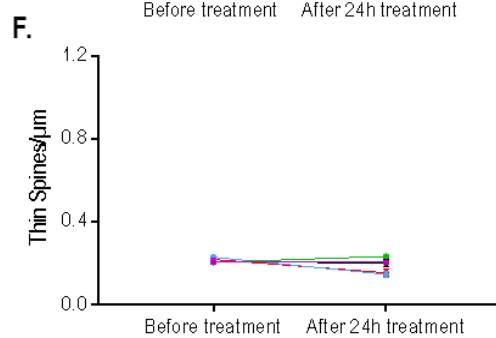
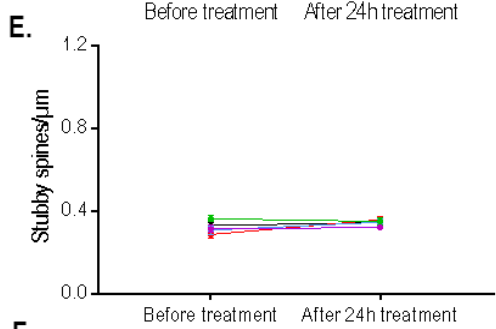
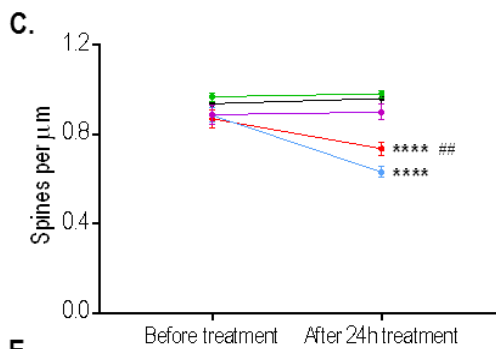
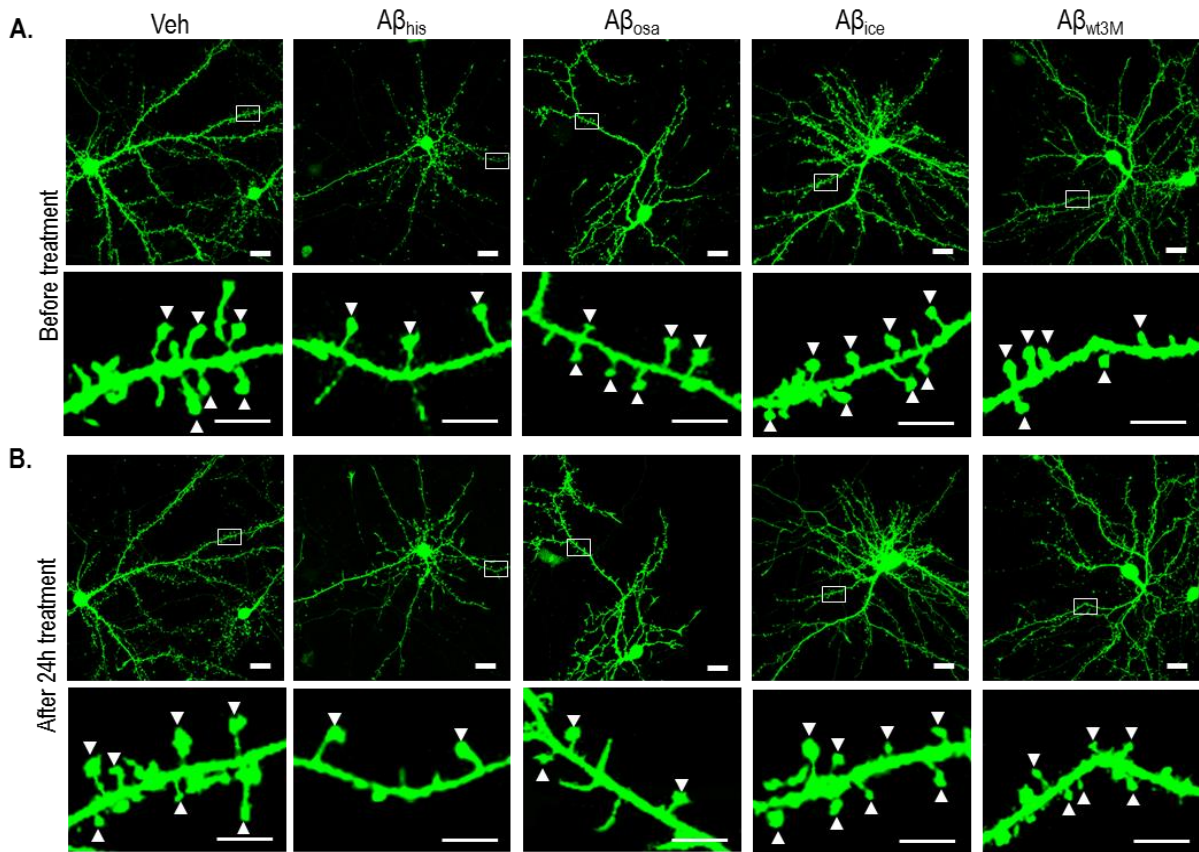


Figure 12. Exogenous application of $A\beta_{wt}$ and $A\beta_{osa}$ induces mushroom spine density reduction and mushroom spine enlargement but not $A\beta_{ice}$ nor $A\beta_{wt3M}$. **(A, Top row)** Representative confocal images of cultured cortical neurons expressing LA-GFP before treatment with exogenous application of $A\beta_{wt}$ (scale bar = 10 μ m). **(Bottom row)** Dendrite portions with mushroom spines (white arrows) before treatment with different $A\beta$ (scale bar = 5 μ m). **(B, Top row)** Representative confocal images of the same neurons after 24 h incubation with 100 nM of different $A\beta$ (scale bar = 10 μ m). **(Bottom row)** Dendrite portions with less mushroom spines (white arrows) after 24 h incubation with $A\beta_{wt}$ and $A\beta_{osa}$ but not with $A\beta_{ice}$ or $A\beta_{wt3M}$ (scale bar = 5 μ m). **(C)** Quantification of total spine density (mean \pm SEM) shows a reduction of total number of spines after treatment with $A\beta_{wt}$ and $A\beta_{osa}$. $****p < 0.0001$ when compared to treatment with Veh; $##p < 0.01$ $A\beta_{wt}$ vs $A\beta_{wt3M}$. **(D)** Quantification of mushroom spine density (mean \pm SEM) show a reduction of number of mushroom spines after treatment with $A\beta_{wt}$ and $A\beta_{osa}$. $****p < 0.0001$ when compared to treatment with Veh; $###p < 0.001$ $A\beta_{wt}$ vs $A\beta_{wt3M}$. Quantification of **(E)** stubby spine and **(F)** thin spine density (mean \pm SEM) show no changes after treatment with the different $A\beta$. **(G)** Quantification of mushroom spine volume (mean \pm SEM) shows an increase after treatment with $A\beta_{wt}$ and $A\beta_{osa}$ but not with $A\beta_{ice}$ or $A\beta_{wt3M}$. $****p < 0.0001$ when compared to treatment with Veh; $####p < 0.0001$ $A\beta_{wt}$ vs $A\beta_{wt3M}$. RM two-way ANOVA followed by Tukey's multiple comparison test. N=6 neurons from at least 3 different cultures.

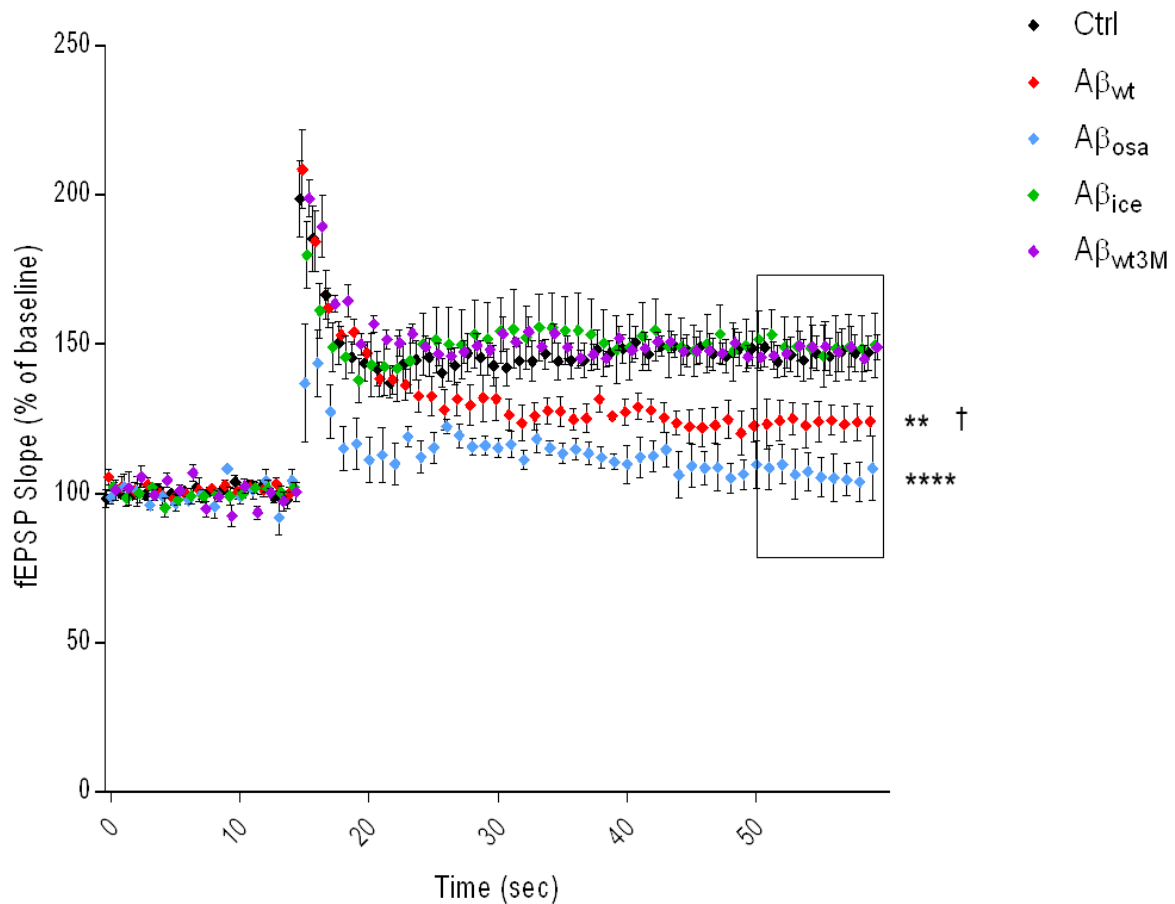


Figure 13. Long-term potentiation in acute mouse hippocampal slices is impaired by exposure to $A\beta_{wt}$ and $A\beta_{osa}$ but not by $A\beta_{wt3M}$ or $A\beta_{ice}$. Long-term potentiation (LTP) in the hippocampal CA1 region was induced by delivering two 100 Hz protocols (2 x 100 Hz) with an interval of 20 sec to the Schaffer collateral/commissural pathway. $A\beta_x$ peptides were added to the ACSF bath (final concentration of $A\beta_x$: 100 nM) 15 min prior to recording. 2 x 100 Hz was delivered after 15 min of stable baseline. Each point on the graph represents the mean \pm SEM. ** $p=0.0017$; **** $p<0.0001$ when comparing the last 10 time points of fEPSP Slope (% of baseline) to Control conditions (Ctrl). † $p=0.0128$ $A\beta_{wt}$ vs $A\beta_{wt3M}$. Kruskal-Wallis followed by Dunn's test. N=at least 5 slices per condition.

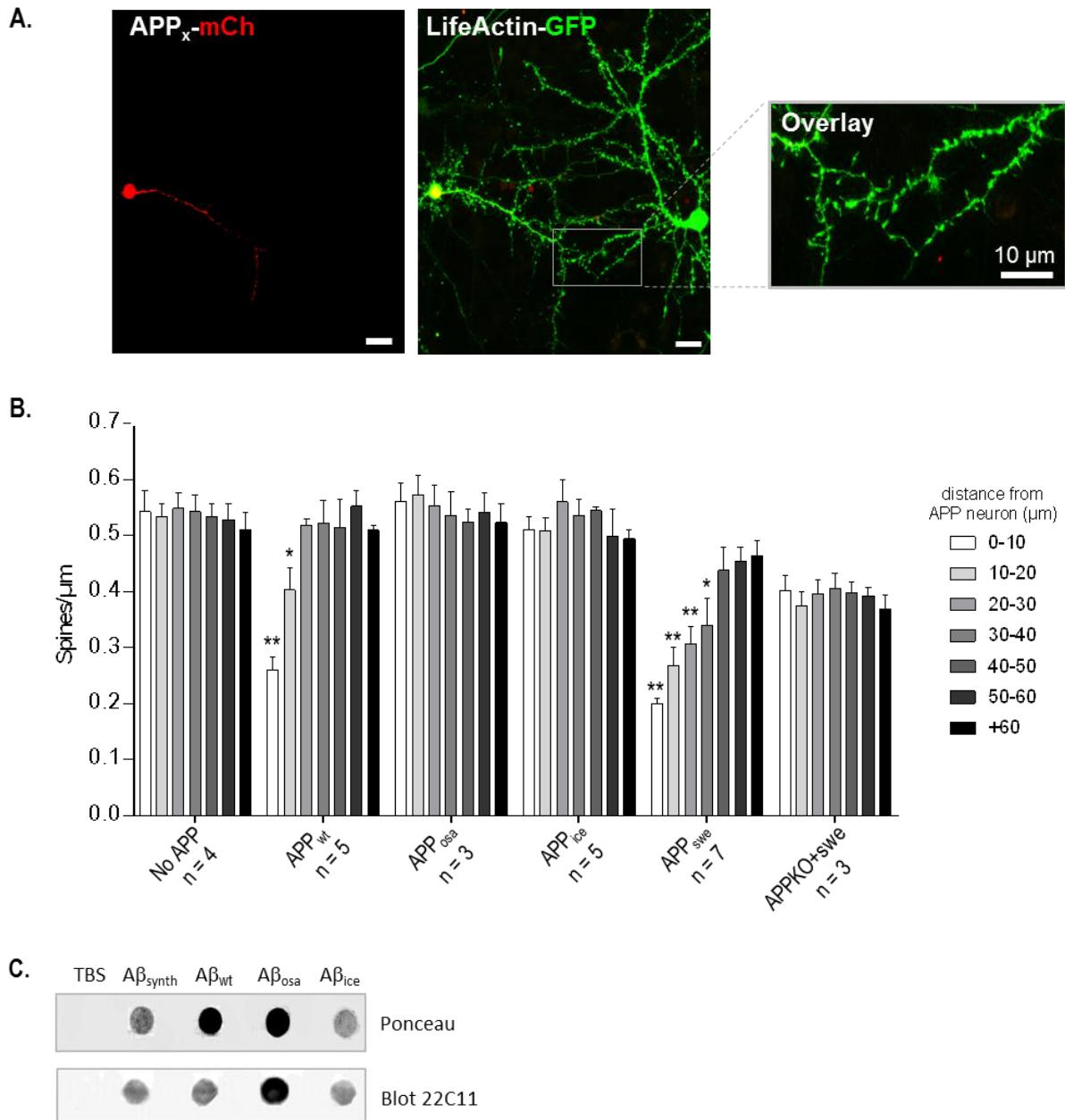


Figure 14. Overexpression of APP_{wt}, APP_{swe} in cortical cell cultures decrease spine density of neighbouring healthy wild-type neuron, in an APP-dependent manner, whereas APP_{ice} and APP_{osa} overexpression does not. (A) Representative confocal images of cultured cortical neurons where the neuron on the left is overexpressing either APP_{wt}-mCh, APP_{swe}-mCh or APP_{osa}-mCh or APP_{ice}-mCh and LA-GFP (APP neuron); and the neuron on the right is only overexpressing LA-GFP (healthy neuron) (scale bar = 10 μm). (B) Bar graphs (mean ± SEM) show spine density of healthy neuron depending on the distance from APP neuron. **p*<0.05; *p*<0.01 when compared to control condition (both neurons only overexpress LA-GFP) at equivalent distance. RM two-way ANOVA followed by Tukey's multiple comparison test. N=at least 3 neurons per condition from 3 different cultures. (C) Dotblot of Aβ_{synth}, Aβ_{wt}, Aβ_{osa} or Aβ_{ice} with added wild-type neuronal membrane extracted protein revealed with Ponceau red (to show total protein load before membrane extracted protein added) and 22C11 (to reveal APP from membrane extracted protein bound to Aβ_x) showing an interaction of the different Aβ_x with APP at the neuronal plasma membrane.**

VII. Discussion & Perspectives

In the last decade, A β peptides have been identified as the main conductor driving synaptotoxicity and have emerged as the pivot in the pathophysiological development of AD. Indeed, AD has been coined as a synaptopathy, meaning that synaptic defects are the initiating factor leading to memory and learning impairments. Although it is now well-established that A β accumulation is the trigger of these synaptic deficits, and has multiple effects on neurons (Selkoe, 2002), the pathway by which the A β peptides exert and sustain their synaptotoxicity is still under debate. In this study, we get a deeper insight into the way A β initially impairs synapse morphology and function, and how APP processing sustains and propagates these effects in a synaptic activity-dependent manner, using different mutants of APP with unique molecular signatures. Here, we show that A β accumulation induces a decrease in spine density especially mushroom spines, accompanied by an increased volume of the remaining mushroom spines, and that intracellular A β is sufficient to induce these effects. These enlarged mushroom spines have impaired structural plasticity as they did not increase in volume following synaptic activation and this seems to be due to defective activity-dependent actin dynamics in the spines. This alteration of synaptic morphology, structure and plasticity seems to be due to a newly-identified interaction between actin and A β , hinting a possible physiological role for A β in activity-dependent synaptic plasticity. We also show that synaptic activity modulates APP processing, ostensibly the amyloidogenic pathway, which further exacerbates these synaptic defects when A β is already abounding. Furthermore, we show that A β sequence is as important as A β concentration in inducing deleterious effects, as a pathological concentration of a non-toxic mutant of A β (A β_{ice} , A β_{wt3M}) does not induce synaptotoxicity. Lastly, we bring to light that secreted A β , not only affects the A β -secreting neuron itself, but also affects nearby neurons in an APP-dependent manner, reminiscent of a prion-like mechanism.

A. Intracellular A β : the instigator of the early cognitive alterations in AD?

Intracellular A β was initially difficultly accepted within the scientific community, yet more and more evidence have confirmed their presence and subsequent toxicity inside neurons (Gouras et al., 2012, 2010, 2005, 2000; Grundke-Iqbal et al., 1989; LaFerla et al., 2007; Tomiyama et al., 2010). Although many studies have identified the detrimental effects of A β through extracellular application or its presence in the extracellular space (Selkoe, 2008; Shankar et al., 2008, 2007; Walsh et al., 2002), the question of the pathway which A β follows to induce these effects is still unclear. Is it

extracellular A β that causes synaptotoxic effects? Or rather could it be intracellular A β and/or internalised A β from the extracellular space? In our study we show that intracellular A β is sufficient to induce synaptic alterations, since APP_{osa} overexpression led to intraneuronal accumulation of A β , no A β secretion and spine density decrease along with abnormal spine head enlargement. Our ELISA assay results also show that overexpression of APP_{swe} and APP_{wt} led to an increase in intracellular A β , as well as an increase in secreted A β . Furthermore, unpublished data from our team show that LTP inhibition by extracellular A β application requires β -secretase cleavage of APP, entailing an intracellular generation of A β that disrupts synaptic transmission. Several have brought to light that intracellular A β accumulation precedes NFT and senile plaque apparition (D'Andrea et al., 2001; Gouras et al., 2000) and correlates much better with AD cognitive decline. Together, our data support this hypothesis point out intracellular A β as the instigator of synaptic distress leading to the early cognitive impairments in AD.

B. Regulation of dendritic spine actin dynamics: a physiological role for A β ?

Dendritic spines adapt their shape thus function to match the incoming synaptic activity (Hotulainen and Hoogenraad, 2010). Long-term potentiation (LTP) leads to spine enlargement, whereas Long-term depression (LTD) leads to spine shrinkage (Cingolani and Goda, 2008; Matsuzaki et al., 2004; Zhou et al., 2004). In our study, we used a Bic/4-AP (BIC15) protocol which mimics LTP-type synaptic activation. We confirmed spine enlargement following synaptic activation. However in pathological neurons, where a form of toxic A β is in excess, mushroom spines are already enlarged and fail to undergo activity-dependent spine enlargement, indicating synaptic plasticity defects in these neurons, presumably due to intracellular A β . To support these findings, an earlier study carried out by our team also showed aberrant actin-F stabilisation in spines due to application of synthetic oligomeric A β peptides (Rush et al., 2018). Interestingly, although an excessive amount of intracellular A β leads to impaired synaptic plasticity, an absence of A β also seems adverse. Indeed, upon β Sec1 treatment, which was carried out just after transfection and inhibition of β -secretase was maintained until the end of the experiments, effectively all amyloidogenic processing was blocked in the cells. Although this inhibition prevented toxic A β -induced spine density decrease, our results show that absence of A β still leads to failed spine enlargement following synaptic activation and brings to light a possible physiological role for A β in activity-dependent spine stabilisation. Our FRAP data further confirmed this possible role as excess intracellular toxic A β lead to actin overstabilisation

in spines whereas absence of A β , as well as protective A β_{ice} , lead to no activity-dependent stabilisation.

It is noteworthy that these results were confirmed in cultured cortical neurons from APPKO mice (see **Supplementary data**) where activity-dependent actin remodelling (**Supplementary Data, FRAP experiments, figures C. and D.**) and subsequent spine enlargement (**Supplementary Data, spine volume analysis before/after BIC15, figure B.**) did not occur. However, this model is not ideal to study the effects of absence of A β as APPKO neurons had overall decreased spine density, including decreased mushroom spines (**Supplementary Data, Spine density analysis, figure A.**), pointing out a possible implication of APP or its proteolytic derivatives in spine formation, structure and maintenance. This decrease in spine density in APPKO neurons has already been reported by others with different explanations as to why this is (Lee et al., 2010; Weyer et al., 2014). On one hand, according to Lee and collaborators this is likely due to a lacking interaction of cell surface APP with proteins from the extracellular matrix (Lee et al., 2010). On the other hand, more recent data from Weyer and coworkers suggested that this decreased spine density in APPKO neurons is due to the lack of sAPP α , the soluble N-terminal fragment of APP generated in the non-amyloidogenic pathway after cleavage by α -secretase (Weyer et al., 2014b). Furthermore, a very recent study by Martinsson and collaborators also showed that dendritic spines from APPKO neurons have altered pre- and post-synaptic proteins and receptors (Martinsson et al., 2019). Moreover, these data also show that there is no compensation by APP-like proteins (APLPs), which doesn't contain the A β sequence. Together these studies support the hypothesis that APP and its cleavage products are important for spine formation and maintenance. Our data also support this hypothesis whilst highlighting that A β is more involved in activity-dependent actin remodelling during synaptic plasticity.

This interplay between intracellular A β and actin which had been hinted by others (Kommaddi et al., 2018; Maloney et al., 2005), led us to question a potential interaction between them. We identified a common 3 amino-acid actin-binding sequence between A β and other actin-binding proteins and lead us to generate the 3M mutation within the A β_{ice} sequence. After confirming that this mutation did not hinder APP processing or A β production, we reported a loss of synaptotoxicity of A β (wt, osa) when it harboured the 3M mutation as well as a loss of activity-dependent actin overstabilisation in the spines. In 2003, Kamenetz and collaborators, proposed a physiological activity-dependent negative feed-back system, where A β production modulates synaptic transmission, and synaptic activity modulates A β production, to keep neural hyperactivity in control. In this light, excess A β would drive synaptic depression and eventually spine loss. Our findings support this hypothesis and add that: i) this mechanism might occur via excess A β preventing proper activity-dependent actin remodelling in the spines and ii) absence of A β also leads to improper

activity-dependent actin-remodelling in the spines. This suggests that the pathway to synaptotoxicity depends not only on a balance of the quantity of A β produced and where it is produced, but also on the sequence of A β and its ability to interact with the actin cytoskeleton in spines since A β_{osa} and A β_{wt} seems to interact with actin, whereas A β_{ice} and A β_{wt3M} do not.

Although our results may have identified a new physiological role for A β it is important to highlight that there are some caveats that will need to be addressed. Indeed, a major question which is still under debate is the exact localisation of intraneuronal A β . Since our data show that A β is able to interact with the actin cytoskeleton this entails that some A β is localised in the cytoplasm. This is still debated within the scientific community although there are some data to support this. Indeed, some studies have showed that, at sub-lethal concentrations, A β_{42} can be translocated to the nucleus of neuroblastoma cells and act as a repressor of transcription of the genes *LRP1* and *KAI1* and also regulates the gene expression of growth factors such as insulin-like growth factor binding proteins 3 and 5 (IGFBP3/5) (Barucker et al., 2015, 2014). Although these studies did not specifically look for intracytoplasmic A β peptides they did, however, detect the presence of A β in the nucleus implying that A β has to, at some point, be in the cytoplasm in order to travel to the nucleus. As it is known that A β is generated within endosomes with an acidic milieu, since BACE1 is active at pH 6, the question of how A β can get into the cytoplasm still remains to be answered. The presence of intracellular A β has been shown in human AD and Down syndrome brains as well as in numerous transgenic rodent models of AD (Gouras et al., 2010; Kobre-Flatmoen et al., 2016; Welikovitch et al., 2018). Although we were able to detect intracellular A β within the neurons, our data did not allow us to identify the exact localisation of intracellular A β . However, a dynamic relationship exists between pools of intracellular and extracellular A β (Oddo et al., 2006). Thus, we speculate that, in physiological conditions, cytoplasmic A β would originate from two possible pathways: i) a processing of APP inside the neurons and/or ii) via uptake of A β from the extracellular space.

The first involves the maturation pathway of APP. Newly biosynthesised APP travels through the TGN via the secretory pathway before reaching the plasma membrane. In this section of the pathway, APP is within TGN vesicles with its N-terminal domain on the outside and its C-terminal domain on the inside of the vesicle. Then, when APP reaches the plasma membrane it is rapidly re-endocytosed. Now in these endosomes APP is orientated the opposite way with its N-terminal domain on the inside and its C-terminal domain on the outside. At both of these stages, APP can be cleaved by BACE-1, generating two pools of A β : one destined to remain inside the neuron (processing of newly biosynthesised APP) and one destined to be secreted (processing of endocytosed APP). Accordingly, we speculate that β -cleaved newly biosynthesised APP generates A β into the cytosol

whereas β -cleaved endocytosed APP generates $A\beta$ which remains in the vesicles in order to be secreted.

The second pathway to intracytoplasmic $A\beta$ involves secretion of $A\beta$ into the extracellular space and re-internalisation into the cytoplasm either by simple diffusion of the peptide through the plasma membrane or via a partner that, in the light of our findings, could be APP.

It has been shown that intracellular $A\beta$ accumulation in AD vulnerable neurons leads to more prominent endosomal damage (Willén et al., 2017; Yang et al., 1998). In this light, we further hypothesise that, in pathological conditions, these endosomal alterations lead to an $A\beta$ leakage from the endosomes into the cytoplasm, further exacerbating the deleterious synaptotoxic effects. Due to the aggregating nature of the peptide and the multitude of cleavage products of APP of which their sequences overlap with one another; it makes it quite complex to target and track $A\beta$ specifically. We have to bear in mind that this is a recurring issue and one of the main difficulties in the field of $A\beta$ research. Nevertheless, our results confirm the presence of an intracellular pool of $A\beta$ that is sufficient to induce synaptic alterations and bring to light that this pool may have a role in synaptic plasticity when in homeostatic concentrations.

In order to get a better understanding of the physiological role of $A\beta$ in actin interaction and remodelling following synaptic activity, it would be of interest to generate a transgenic mouse harbouring the 3M mutation within its endogenous mouse APP gene. This transgenic APP_{wt3M} model would allow: i) behavioural assessments ii) *In vivo* electrophysiological recordings as well as on acute hippocampal slices iii) *ex vivo* neuron architecture evaluation (spine density, spine volume, dendritic arbour length and branching). We speculate that this transgenic animal would be similar to the APPKO mouse (in the sense that they both lack $A\beta$ to interact with actin) but without the synaptic defects linked to the absence of APP. The stream of data that would be provided by this model would give a greater insight into the neurotransmission state of neuronal networks and unravel this potential novel physiological role for $A\beta$.

C. Activity-dependent amyloidogenic processing of APP: a finely tuned equilibrium?

As higher levels of education have been associated with decreased risks of developing AD, one could easily envisage that higher levels of synaptic activity would be neuroprotective. However, the role of synaptic activity in the context of AD is complex. AD brain dysfunction is initiated particularly

in areas known to be more chronically active, known as the “default network”. It has also been shown in AD mouse models that excessive brain activity from stress or seizures worsens brain β -amyloidosis, while reduced brain activity or excessive sleep also damages synapses in the AD models but not wild-type models (Mohajeri et al., 2002; Tampellini et al., 2010). Moreover, our team has shown that APP/PS1 mice have hyperactive hippocampal neurons at 1 month of age but become hypoactive at 3 months (unpublished data). This raises the question of the role of synaptic activity in AD development. Several have reported that synaptic activity modulates APP processing (Kamenetz et al., 2003; Tampellini and Gouras, 2010). Our results support these findings and bring to light that activity-dependent amyloidogenic processing of APP seems predominant at the intracellular level. Indeed, our results show that the mCherry fluorescence inside our cultured cortical neurons from transfected APP_x-mCh constructs decreased following synaptic activity, especially APP_{swe}, and this decrease was largely blocked by β -secretase inhibition. Additionally, according to several studies (D’Andrea et al., 2001; Echeverria and Cuello, 2002; Gouras et al., 2000; Näslund et al., 2000; Ohya et al., 2007; Tabira et al., 2002) it is preferably the toxic A β ₄₂ isoform that is produced intracellularly. Our findings shed some light on the possible purpose of activity-dependent processing of APP, where generated A β regulates activity-dependent actin remodelling and subsequent synaptic transmission. In this context, excess A β or conversely lack of A β , both ultimately lead to defective activity-dependent synaptic plasticity and transmission. In the bigger picture, this raises concerns, and provides possible answers, about the recent failings of β -secretase inhibition as treatment for AD in clinical trials (Zhu et al., 2018a). Indeed, in addition to the fact that BACE1 has other substrates involved in various synaptic processes, long term inhibition of this enzyme and subsequent A β production would still lead to synaptic defects, ultimately worsening cognitive impairments. Taken all together, our data indicate that activity-dependent amyloidogenic processing of APP is likely to be a finely tuned equilibrium and misbalancing this mechanism would lead to the early cognitive impairments of AD.

So what is the factor that tips the scale in favour of excess A β and AD development? One of the possibilities, and one of the major risk factors for AD development, is aging. Aging impacts all organs and biological pathways, and is a risk factor for numerous diseases. What is more, although everybody and every living organism ages, the aging process in each individual is essentially unique as it is tainted by events one may have had to endure and lifestyle choices one may have made throughout their life, such as the amount of physical activity, the amount of brain activity (higher education, stress...), sickness, diet and so one and so forth. Therefore, the molecular and cellular mechanisms critical for how aging impacts AD remain unclear. Nevertheless, these mechanisms of aging are crucial for AD pathogenesis, as it has been shown in many rodent models of AD and even

APPKO animals. Indeed, in the case of AD rodents, although these animals are genetically programmed to develop the pathology, the symptoms do not appear until adulthood, at several months of age. Even in the case of APPKO mice, these animals do not present any particular deficits at a young age despite a reduction in spine density; however they do present defective LTP at older age, further demonstrating the importance of the process of aging in neurodegenerative pathology development. Common themes shared among various age-related neurodegenerative diseases such as Parkinson's disease and AD are aberrant protein aggregations and early synapse dysfunction. As we now know, synapses are early targets of damage in AD and reduced brain activity is a well-known outcome of the disease. The brain uses a large proportion of energy, with synaptic transmission being its main source of energy use (Harris et al., 2012), and energy metabolism in the aging brain is affected by numerous factors. Altered energy metabolism with aging can influence several mechanisms such as early protein accumulation/aggregation due to altered protein degradation/clearance, and protein aggregation/accumulation in turn can impact brain energy metabolism. In this light, it is comprehensible that the homeostatic state of A β and the dynamics of actin cytoskeleton could be shifted due to these age-related altered mechanisms, thus becoming a burden to vulnerable neurons overtime, ultimately causing synaptic dysfunction then loss. This intricate interplay between altered energy metabolism in the aging brain and protein sorting mechanisms requires further investigation and could open new routes for AD therapy and probably several other neurodegenerative diseases.

To get a deeper insight into this activity-dependent processing of APP, it would be interesting to look more closely at the localisation of this processing within neurons. If A β has a role in actin remodelling in spines, then one could envisage that APP would be cleaved by β -secretase at the spines. To test this hypothesis, it would be of interest to study the potential colocalisation of APP and β -secretase at the spines. This could be done either by i) co-transfection of APP and BACE1, each protein fused to fluorescent tag of different colours, or ii) by bi-complementation assays whereby APP and BACE1 are each fused to one half of a fluorescent tag and if the proteins interact, a fluorescence will emit (Das et al., 2016, 2013). Also, to go further into the regulation of amyloidogenic processing of APP in neurons, we could investigate the two pathways (secretory/endocytic) of β -cleavage of APP using double fluorescently tagged APP construct (mcherry-APP_{swe}-EYFP). In this construction, mcherry fluorescent protein was inserted into the ectodomain and YFP fused to the C-terminal domain of APP695_{swe}. While unprocessed APP appeared in yellow, the presence of red or green puncta revealed the predominant processing pathway involved. Red puncta correspond to the accumulation of the shredded ectodomain in endocytic vesicles and reveal a predominant endocytic pathway for APP processing. Whereas green puncta

correspond to the accumulation of C-terminal fragments and reveal a processing of APP during the secretory pathway. Unpublished data from our team already show that in presence of extracellular A β , green puncta is mainly observed in neurons due to the C-terminal fragments sequestered in vesicles whereas shredded soluble APP ectodomain fragments were released in the cytosol. These results were further confirmed by subcellular fractionation and Western-Blot analysis of APP full-length and APP fragments in endosome-, lysosome-, endoplasmic reticulum- and cytosol-enriched fractions obtained from primary cortical neurons exposed to A β for 30 min. After A β application, APP localised in all fractions, but sAPP was also found in the cytosol. This result confirms the processing of APP during the secretory pathway. Remarkably, we also observed an increase of A β oligomers in the cytosol of A β -treated neurons compared to control, further confirming the presence of intracytoplasmic A β pool. It would now be of crucial importance to carry out the same set of experiments using BIC15 protocol, which would demonstrate and confirm activity-dependent amyloidogenic processing of APP and intracytoplasmic A β generation. Nevertheless, this stream of data already point out an involvement of A β in APP processing, potentially revealing a vicious circle where amyloidogenic processing of APP is modulated by both synaptic activity and A β , highlighting how this mechanism may get out of hand in pathological conditions, ultimately leading to excess A β production and subsequent AD cognitive impairments.

D. A β sequence over A β concentration?

Many studies have established that A β toxicity arises from pathological/excessive amounts of A β . Indeed, nowadays the most used AD transgenic animal models carry the FAD Swedish mutation on APP, which increases A β production (Citron et al., 1992). Conversely, it has been proposed that less A β would be protective against AD. This has been suggested following the discovery of the protective Icelandic mutation which, allegedly, leads to a 30 to 40% reduction in A β generation (Maloney et al., 2014). Although this might seem quite significant, it is to note that the amyloidogenic processing accounts for only 10% of total APP processing (Sinha and Lieberburg, 1999). Furthermore, other studies based on FAD mutations such as the Arctic mutation (E693G), showed that toxicity may occur from an imbalance of A β ₄₂/A β ₄₀ ratio when total A β levels are unchanged (Nilsberth et al., 2001), highlighting that A β sequence may be as important as A β concentration in exerting synaptic dysfunction. In our study we used equal “pathological” concentrations of A β _{wt}, A β _{osa}, A β _{ice} and A β _{3M} (100 nM) on cultured cortical neurons to assess spine morphology, as well as on acute mouse hippocampal slices for LTP electrophysiological experiments. As expected, we observed the toxic effects of A β _{wt} and A β _{osa} in both models. Interestingly, however, neither A β _{ice} nor A β _{3M} induced any

alterations on spine morphology or LTP. This puts forward the importance of the sequence of A β as well as its concentration since its sequence may lead to different behaviour of the peptides in the cells, whether it is where it localised or how it aggregates/misfolds. A study by Poduslo and Howell in 2015 showed the immediate transition to fibrils of the Osaka (E693 Δ) A β mutant and the rapid transition from aggregates to fibrils of the Arctic (E693G), Dutch (E693Q) and Icelandic (A673T) mutants highlighting the importance of aggregates/fibrils in AD pathogenesis. This study also brings to light that the reproducibility of the footprints of the mutated A β peptides and their uniqueness for each mutation suggest an individuality that was not recognised up until these findings and which likely contribute to differences in the clinical and pathological features of AD. Further supporting these findings, it has recently been evidenced that patients with sporadic AD may, in fact, have several strains of A β within the brain, creating an extra level of complexity in elaborating therapeutic strategies as each strain implies an individuality that could potentially modulate the clinical and neuropathological expression of AD (Condello and Stöehr, 2018).

E. AD pathology propagation in the brain: a prion-like APP-dependent mechanism?

The last question we wanted to address regarding the way A β exerted and sustained its synaptotoxicity in early stages of AD was how the disease propagated throughout the brain. Indeed, this is especially relevant for sporadic AD cases. Our results showed that mutant APP overexpression, which leads to increased secreted A β in the extracellular space (wild-type and swedish), not only did it more than likely influence the A β overproducing neuron itself, but also affected nearby “healthy” neurons which did not overexpress APP. This effect seemed to be proportional to the amount of secreted A β , the more A β is secreted into the extracellular space, and the farthest the “healthy” neuron’s spine density was affected. Interestingly, when we carried out the same experiments using APPKO cultured mouse cortical neurons, the “healthy” APPKO neuron was not affected by the nearby A β secreting neuron. This suggests that the effects observed on the “healthy” wild-type neuron are dependent of APP expression. It has been proposed that A β internalisation may occur via its binding to plasma membrane constituents (Bharadwaj et al., 2018). Indeed, A β entry into cells could potentially occur through ligand-receptor type interactions such as: direct or indirect interaction with integrins, receptor for advanced glycation end products (RAGE), and APP itself (Verdier et al., 2004). Other interactions with TrkA, p75NTR, some G-proteins, NMDA, and AMPA receptors (Benilova et al., 2012), and/or interactions via the prion protein (Laurén et al., 2009; Resenberger et al., 2011) have also been reported. Here we propose that A β internalisation from the extracellular space mainly

occurs via APP on the plasma membrane, which in turn affects synaptic function in the recipient neuron. In the light of our findings and some of our unpublished data mentioned above, we can further speculate that the healthy recipient neuron also undergoes modulations of its processing of APP due to A β internalisation. This would further increase intracellular A β levels and subsequent synaptic deficits, reminiscent of a prion-like mechanism where the disease would progress from neuron to neuron that are either interconnected (Gary et al., 2019) or just simply in physical proximity (Wei et al., 2010).

To go further into this investigation, it would now be of great interest to study in further detail the state of the “healthy” nearby neuron that is “contaminated” by A β . Does this recipient neuron have an increased APP processing? Also, to give further support to the “prion-like” mechanism of disease propagation it would be worthy to take a look at the state of the remaining spines, of the “healthy” nearby neuron, that are in the vicinity of the pathological neuron. Are these spines affected in the same way as the pathological neuron? To do so, it would be relevant to carry out FRAP experiments on these remaining spines. Moreover, to further support the idea that APP is necessary for disease propagation from neuron to neuron it would be of interest to carry out the same experiment of neuron treatment with 24 h of pathological concentrations of oligomeric A β but with APPKO cultured cortical neurons and assess spine density, spine sub-population distribution and spine volume. These data would provide a deeper understanding of the mechanistic behind disease propagation from neuron to neuron.

Taking all our results together, our study has brought to light a novel interaction between A β and the actin cytoskeleton revealing a potential physiological function for A β in activity-dependent spine stabilisation (**Figure 34**) and a possible new route for therapeutic development. This function in structural plasticity is seemingly dependent of an equilibrium of the quantity and sequence of A β , and off-balancing this quantity and sequence would lead to synaptic dysfunction which propagates from one neuron to the neighbouring neuron if A β is secreted into the extracellular space (**Figure 35 and 36**). In the light of our findings and recent evidence showing that patients with sporadic AD may, in fact, have several strains of A β within the brain (Condello and Stöehr, 2018), we show that unravelling AD development entails an extra level of complexity where A β quantities and sequence have to be taken into account in order to find new therapeutic strategies. Our findings support the idea that there is no one-size-fits-all for AD treatment and that it should rather be made-to-measure for each individual AD patient.

F. Take-home messages

In this section I sum up the main messages and ideas to take home from this thesis, outlining the effects of the balance of A β on activity-dependent actin remodelling in dendritic spine plasticity, when there is a sufficient amount (Physiologic condition, **Figure 34**), an excessive amount (Pathologic condition: excess A β , **Figure 35**) or a lack/absence of the peptide (“Pathologic” condition: absence A β , **Figure 36**).

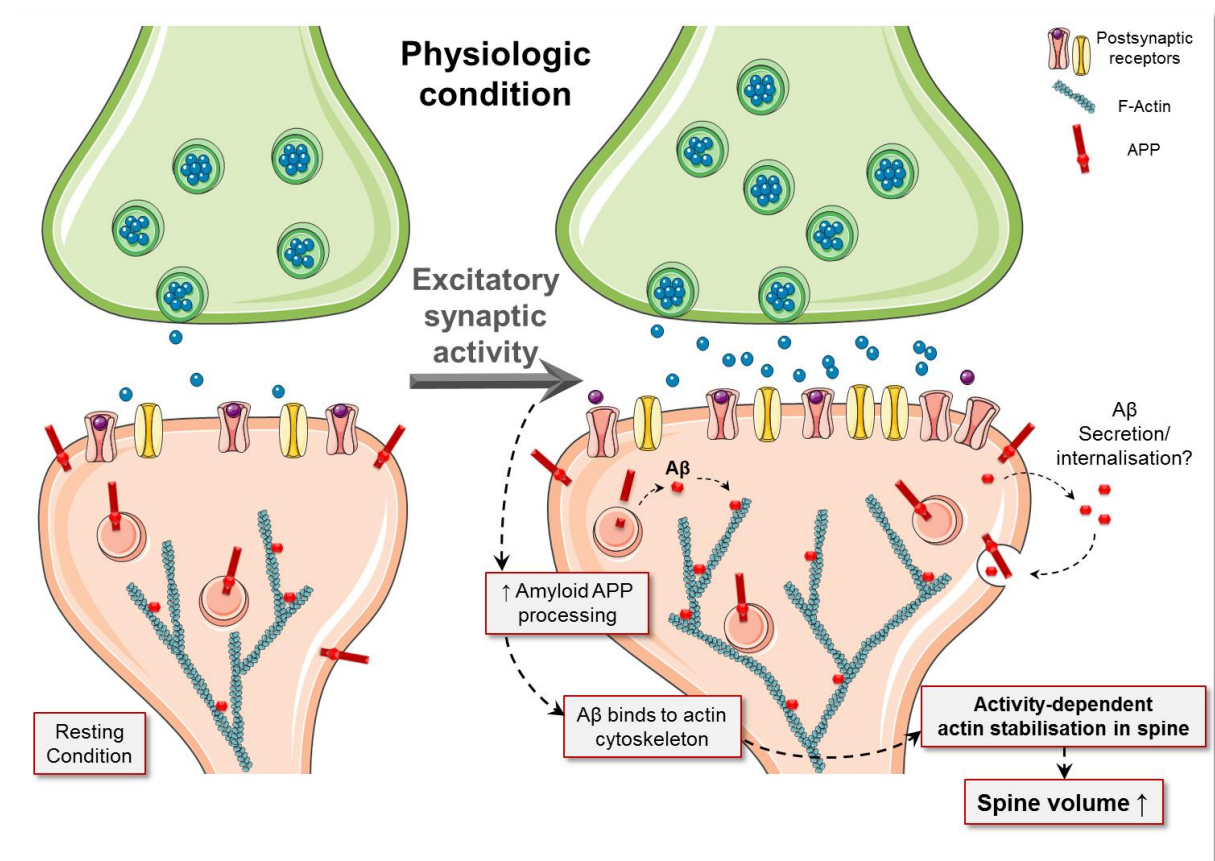


Figure 34: Potential novel physiological role for A β in activity-dependent actin stabilisation in dendritic spines. (Left side) An excitatory glutamatergic synapse in resting conditions. **(Right side)** Upon synaptic activation, amyloidogenic processing of APP is increased, generating intracellular A β peptides (that can be secreted and reinternalised depending on its sequence?) which bind to the actin cytoskeleton in spines; decreasing the depolymerisation rate of, and ultimately stabilising, actin cytoskeleton. This stream of events could contribute to the activity-dependent increase in volume of mushroom spines, revealing a physiological role for A β in synaptic plasticity.

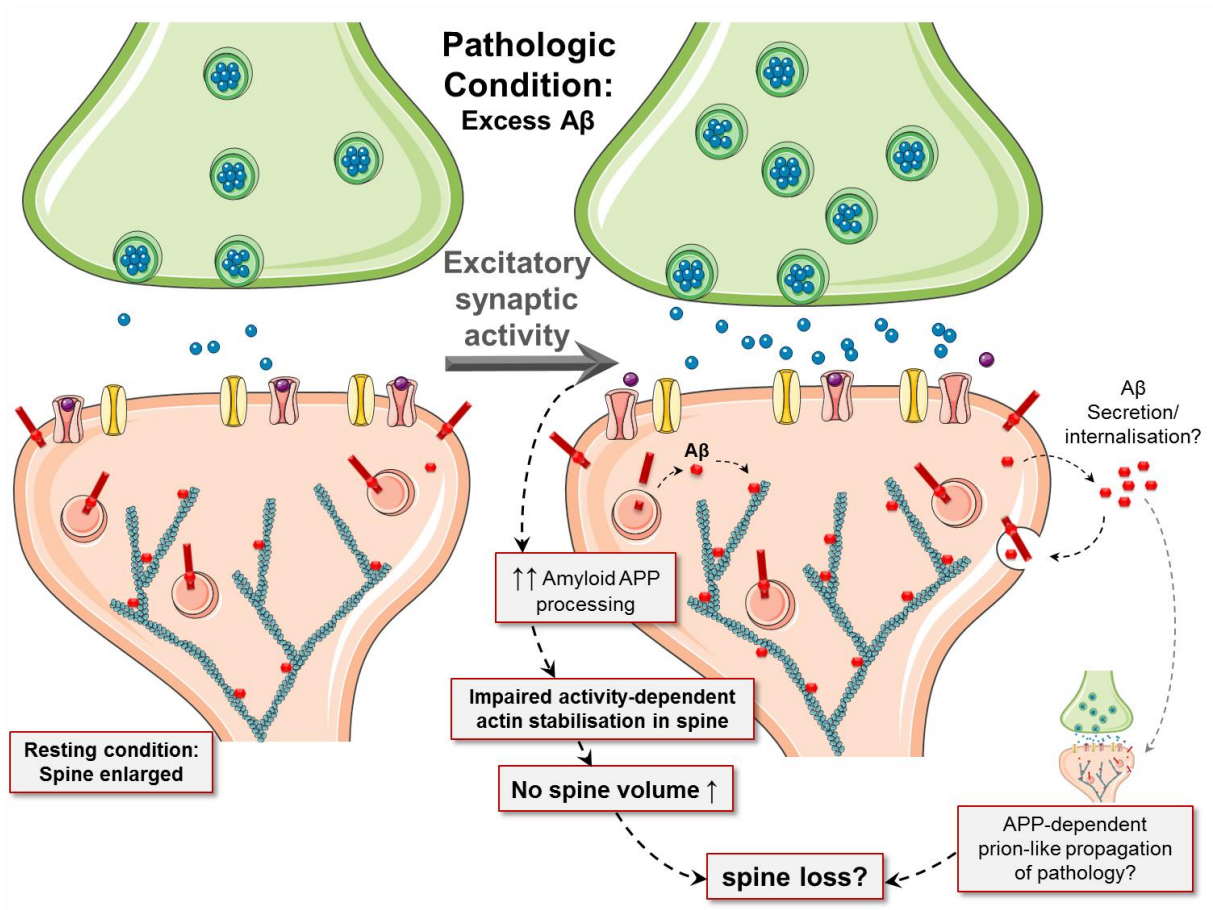


Figure 35: When the physiological role becomes pathological with excess Aβ and leads to synaptic dysfunction and eventually loss. (Left side) An excitatory glutamatergic synapse in resting conditions where there is excess intracellular Aβ. This Aβ, which could be interacting with the actin cytoskeleton, could in part explain the increased spine volume in resting conditions. **(Right side)** Upon synaptic activation, as APP is more abundant, amyloidogenic processing of APP is even more increased, generating excessive amounts of intracellular Aβ peptides which bind to the actin cytoskeleton in spines; further decreasing the depolymerisation rate of, and ultimately overstabilising actin cytoskeleton, which results in no increase in spine volume. If the Aβ sequence allows the peptide to be secreted into the extracellular space, it can then “contaminate” neighbouring neurons via an APP-dependent mechanism. This stream of events could in part explain synaptic dysfunction and eventual spine loss, observed in AD.

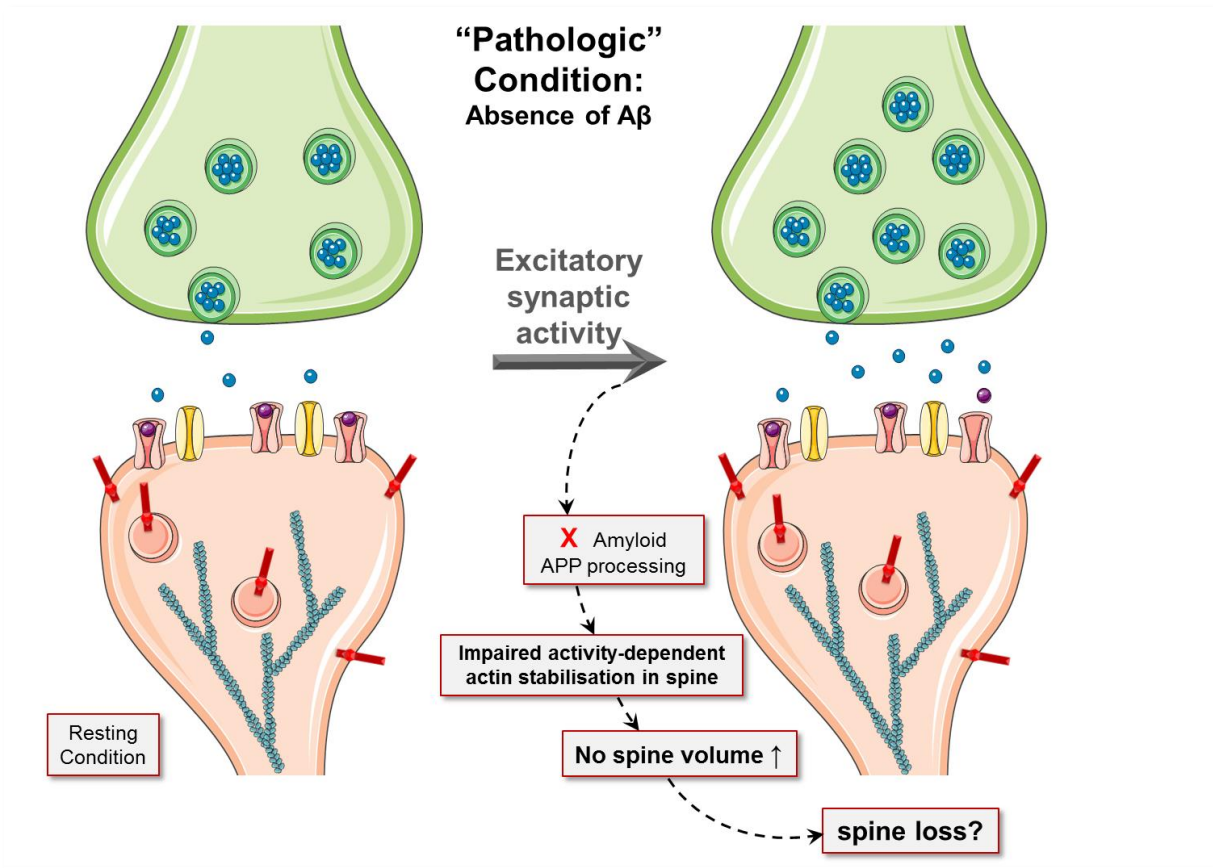
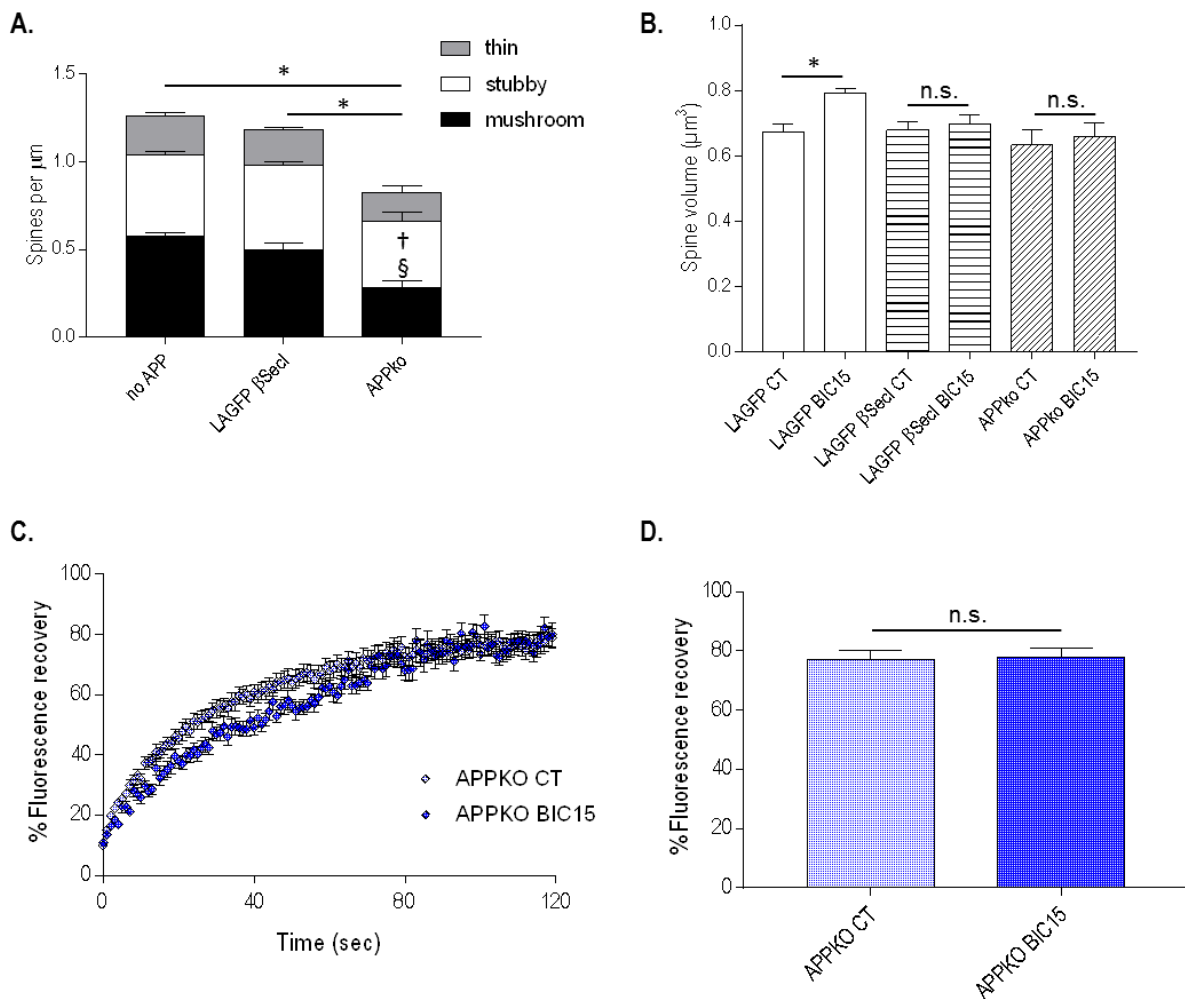


Figure 36: Absence of A β could also lead to synaptic dysfunction and eventual loss. (Left side) An excitatory glutamatergic synapse in resting conditions where there is no intracellular A β . **(Right side)** Upon synaptic activation, as amyloidogenic processing of APP no longer occurs (either by absence of APP or by inhibition of β -secretase), no A β is generated thus there is no A β to potentially interact with the actin cytoskeleton in spine. Therefore there is no decrease of the depolymerisation rate of actin cytoskeleton, which results in an impaired activity-dependent stabilisation of actin in spines ultimately leading to no increase in spine volume. This stream of events could also lead to synaptic dysfunction and eventual spine loss.

VIII. Supplementary data



Supplementary Figure: Cultured cortical neurons from APPKO mice are not the best model for studying the effects of the absence of $A\beta$, as absence of APP causes altered spine density.

(A) Bar graph (mean \pm SEM) showing the distribution of dendritic spine subtypes (spine type and density per μm of dendrite) in wild-type (wt) neurons only overexpressing LA-GFP (no APP), wt neurons overexpressing LA-GFP and pretreated with β -secretase inhibitor [$1 \mu\text{M}$] (LAGFP β SecI) and APPKO neurons overexpressing LA-GFP (APPko). * $p < 0.05$ when comparing total spine density (whole bar) of APPKO neurons vs wt neurons (no APP) or vs wt neurons pretreated with β SecI (LAGFP β SecI); † $p < 0.05$ when comparing mushroom spine density (black segment in bar) of APPKO neurons vs wt neurons (no APP); § $p < 0.05$ when comparing mushroom spine density (black segment in bar) of APPKO neurons vs wt neurons pretreated with β SecI (LAGFP β SecI). Mann-Whitney test. $N =$ at least 4 neurons per condition from at least 3 different cultures.

(B), (C), and (D) show that activity-dependent actin remodelling in spines of cultured cortical neurons from APPKO mice is similar to wt neurons pretreated with β SecI, demonstrating that $A\beta$ is involved in this process, not APP.

IX. List of publications

Published:

Ducarouge B, Pelissier-Rota M, Powell R, Buisson A, Bonaz B, Jacquier-Sarlin M. **Involvement of CRF2 signaling in enterocyte differentiation.** *World J Gastroenterol* 2017; 23(28): 5127-5145

Submitted for publication:

Interaction of A β oligomers with neuronal APP triggers a vicious cycle leading to the propagation of synaptic plasticity alterations to healthy neurons

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APP mutations unveil distinct roles for A β : while intracellular A β modulates synaptic plasticity, extracellular A β participates in Alzheimer's disease propagation

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In preparation:

Amyloid beta oligomers: a novel actin binding complex that influence synapse structure and function in Alzheimer's disease

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Synaptotoxicité dans la maladie d'Alzheimer : Influence du processing de l'APP sur les synapses excitatrices

La maladie d'Alzheimer (MA) est définie comme une maladie neurodégénérative où des altérations synaptiques mènent à la perte neuronale parallèlement à des défauts de mémoire et d'apprentissage. Il est établi que les dysfonctions synaptiques observées dans la MA sont initiées par les formes oligomériques du peptide β -amyloïde ($A\beta$), un dérivé protéolytique de l'Amyloid Precursor Protein (APP). Cependant, le chemin qu'empreinte $A\beta$, selon son origine intra- ou extracellulaire, afin d'induire ces effets délétères et la façon dont ses effets sont maintenus et se propagent dans le cerveau restent encore à définir.

Dans cette étude, nous avons utilisé plusieurs formes mutées de l'APP qui conduisent à des peptides $A\beta$ avec des signatures moléculaires uniques, tel que : la mutation Swedish (K670M/N671L) (APP_{swe}) qui augmentent la sécrétion (extracellulaire) d' $A\beta$; la mutation Osaka (E693 Δ) (APP_{osa}) qui cause une accumulation intraneuronale (intracellulaire) d' $A\beta$; ainsi que la mutation Icelandic (A673T) (APP_{ice}) qui a été établi comme diminuant la production d' $A\beta$ et protégeant contre la MA. Ces formes mutées d'APP ont été surexprimées dans des cultures de neurones corticaux murins et on permit : i) d'étudier la morphologie et fonction des épines dendritiques, l'élément post-synaptique, par microscopie confocale; ii) de tenter de mieux comprendre comment la pathologie se développe et se propage dans le cerveau et iii) d'identifier un nouveau partenaire d'interaction avec l' $A\beta$ faisant la lumière sur un possible rôle physiologique de ce peptide dans les neurones.

Nous montrons qu'une accumulation pathologique d' $A\beta$, due à la surexpression d' APP_{wt} , APP_{swe} et APP_{osa} mais pas APP_{ice} , induit une diminution significative de la densité synaptique particulièrement celle des épines les plus fonctionnelles, dites « mushroom ». Ses épines mushroom restantes présentent également une augmentation significative de leur volume et il semblerait que l' $A\beta$ intracellulaire soit suffisant pour induire ses effets. Ses épines mushroom élargies présentent également une plasticité structurale altérée puisqu'elles n'ont pas augmenté d'avantage de volume à la suite d'une activation synaptique. Il semblerait que ceci soit la résultante d'un défaut de la dynamique activité-dépendante du cytosquelette d'actine dans les épines. Ces altérations de la morphologie, structure et plasticité synaptique serait dû à une interaction, nouvellement identifiée, de l' $A\beta$ avec l'actine et pourrait faire lumière sur un possible rôle physiologique de l' $A\beta$ dans la plasticité synaptique activité-dépendante. De plus, nous montrons que le clivage amyloïde de l'APP est aussi activité-dépendant et que la séquence du peptide $A\beta$ généré est aussi importante, dans l'induction de la synaptotoxicité, que sa concentration. En effet, car nous montrons que des concentrations pathologiques du peptide $A\beta_{ice}$ n'engendrent pas de perte ou de gonflement des épines mushroom. Enfin, nous mettons en lumière que l' $A\beta$ sécrété dans le milieu extracellulaire affecte, non seulement le neurone sécrétant lui-même, mais aussi la densité synaptique des neurones sains avoisinant (qui ne surexpriment pas d'APP) d'une manière APP-dépendante, rappelant un mécanisme de propagation du type prion. L'ensemble de ces données démontrent que le clivage protéolytique de l'APP et la production d' $A\beta$ qui en découle est un processus finement accordé, impliqué dans le remodelage de l'actine dans la plasticité synaptique activité-dépendante et ouvre de nouvelles voies pour le développement de stratégies thérapeutiques contre la MA.

Synaptotoxicity in Alzheimer's disease: Influence of APP processing on excitatory synapses

Alzheimer's disease (AD) is defined as a neurodegenerative disorder where synaptic defects lead to neuronal loss and concurrent memory impairments. It is now well-established that synaptic dysfunction in AD is initiated by oligomeric forms of the amyloid- β peptide ($A\beta$), a proteolytic derivative of Amyloid Precursor Protein (APP). However, the pathway by which $A\beta$ induces its deleterious effects, whether it is due to intra- and/or extracellular $A\beta$ pools, and how these effects are sustained and propagated throughout the brain, are still unclear.

In this study, we used several mutated forms of APP which give rise to $A\beta$ peptides with unique molecular signatures, such as: the Swedish mutation (K670M/N671L) (APP_{swe}) which increases secreted (extracellular) $A\beta$; the Osaka mutation (E693 Δ) (APP_{osa}) which causes intraneuronal (intracellular) accumulation of $A\beta$; and the Icelandic mutation (A673T) (APP_{ice}) which has been reported to decrease $A\beta$ production and protect against AD. These mutated forms of APP were overexpressed in cultured mouse cortical neurons in order to: i) study the morphology and function of dendritic spines, the post-synaptic element of synapses, by confocal microscopy, ii) get a better insight into pathology development and propagation and iii) identify a novel interacting partner bringing to light the possible physiologic role of $A\beta$ in neurons.

We report that pathological $A\beta$ accumulation, due to APP_{wt} , APP_{swe} and APP_{osa} overexpression but not APP_{ice} overexpression induces a significant decrease in spine density especially mushroom spines, accompanied by a significantly increased volume of the remaining mushroom spines, and that intracellular $A\beta$ is sufficient to induce these effects. These enlarged mushroom spines have impaired structural plasticity as they did not increase in volume following synaptic activation seemingly as a result of defective activity-dependent actin dynamics in the spines. This alteration of synaptic morphology, structure and plasticity seems to be due to a newly-identified interaction between actin and $A\beta$, hinting a possible physiological role for $A\beta$ in activity-dependent synaptic plasticity. We also show that synaptic activity modulates amyloidogenic APP processing which, in pathological conditions, further exacerbates these synaptic defects. Furthermore, we show that $A\beta$ sequence is as important as $A\beta$ concentration in inducing synaptic alterations since pathological concentrations of $A\beta$ harbouring the Icelandic mutation had no effect on spine density or volume. Lastly, we bring to light that secreted $A\beta$, not only affects the $A\beta$ -secreting neuron itself, but also affects spine density of nearby neurons in an APP-dependent manner, reminiscent of a prion-like mechanism. Together these results demonstrate that APP processing is a finely tuned equilibrium involved in actin-remodelling during activity-dependent synaptic plasticity and opens a new route for AD therapeutic strategies.