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PhD Thesis

Use of a Cell Permeable Peptide to modulate ADAM10 synaptic localization and activity in a mouse model of Alzheimer's disease

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« On ne fait jamais attention à ce qui a été fait ; on ne voit que ce qui reste à faire. »

Marie Skłodowska-Curie
(1867 - 1934)

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ITALIAN ABSTRACT

La malattia di Alzheimer (AD) è caratterizzata dall'aggregazione del peptide β-amiloide (Aβ). Aβ deriva dalla proteina precursore dell'amiloide (APP), che può subire due vie di taglio proteolitico reciprocamente esclusive. La via amiloidogenica coinvolge l'attività di BACE e γ-secretasi e porta alla formazione di Aβ, mentre la via non amiloidogenica coinvolge ADAM10, una disintegrina e una metalloproteinasi 10, che scinde APP all'interno del dominio corrispondente ad Aβ, precludendone così la produzione. Recentemente, abbiamo identificato un nuovo partner di legame di ADAM10, AP2, che è responsabile dell'internalizzazione di ADAM10, influenzando quindi la sua attività. È interessante notare che la formazione del complesso ADAM10/AP2 è significativamente aumentata nel cervello dei pazienti con AD rispetto a soggetti sani, suggerendo un ruolo di ADAM10/AP2 nella patogenesi di AD. In questo contesto, abbiamo recentemente sviluppato un peptide permeabile alle cellule (denominato PEP3) in grado di interferire con l'associazione ADAM10/AP2. La somministrazione intraperitoneale di PEP3 in un modello murino di AD per due settimane è sicura ed efficace nel ridurre l'endocitosi di ADAM10 e, quindi, nell'incrementare la localizzazione sinaptica di ADAM10. La somministrazione di PEP3 nelle fasi avanzate di malattia è in grato di modificare parametri biochimici, come i livelli di Aβ e la composizione molecolare delle sinapsi, ma senza riuscire a produrre miglioramenti a livello di deficit cognitivo mostrato da questi animali. Invece, i risultati ottenuti nei test comportamentali suggeriscono un recupero della funzione cognitiva nei topi AD in fasi iniziali di malattia dopo la somministrazione di PEP3. Inoltre, ulteriori indagini hanno rivelato che il trattamento con PEP3 aumenta i livelli di una subunità del recettore NMDA e recupera la perdita e le alterazioni delle spine dendritiche osservati nei topi AD. Questi effetti sembrano essere mediati da un aumento dei livelli di sAPPα endogeno. Questi risultati positivi indicano l'internalizzazione di ADAM10 come potenziale bersaglio per lo sviluppo di una terapia efficace per l'AD.

ABSTRACT

Alzheimer's disease (AD) is characterized by the aggregation of amyloid beta peptide (Aβ). Aβ derives from the amyloid precursor protein (APP), which can undergo two mutually exclusive pathways. The amyloidogenic pathway involves BACE and γ-secretase activities and leads to Aβ formation. While, the non-amyloidogenic pathway involves ADAM10, a disintegrin and metalloproteinase 10, which cleaves APP within the domain corresponding to Aβ, thus precluding Aβ production. Recently, we identified a new ADAM10 binding partner, named AP2, which is responsible for ADAM10 internalization, therefore affecting its activity. Interestingly, ADAM10/AP2 interaction is significantly increased in AD patients' brain compared to healthy control subjects, suggesting a role of ADAM10/AP2 in AD pathogenesis. In this framework, we have recently developed a cell permeable peptide (named PEP3) capable of interfering with ADAM10/AP2 association. The intraperitoneal administration of this CPP to a mouse model of AD for two weeks is safe and effective in impairing ADAM10 endocytosis and, thereby, in increasing ADAM10 synaptic localization. At late stages of disease, the PEP3 administration is able to change biochemical parameters, as Aβ levels and the molecular composition of the synapses without ameliorating the cognitive deficits of these mice. On the other hand, at early stage of the pathology the 14-days administration of the PEP3 rescues the cognitive impairment of the AD mice. Further investigations revealed that the synaptic levels of the NMDA receptor subunit GluN2A are increased upon the treatment and that previously observed shrinkage and dendritic spine loss in AD mice were improved after CPP treatment. These results are mediated by an increase in endogenous sAPPα. These positive results point to ADAM10 internalization as a potential target mechanism for the development of an effective AD therapy.

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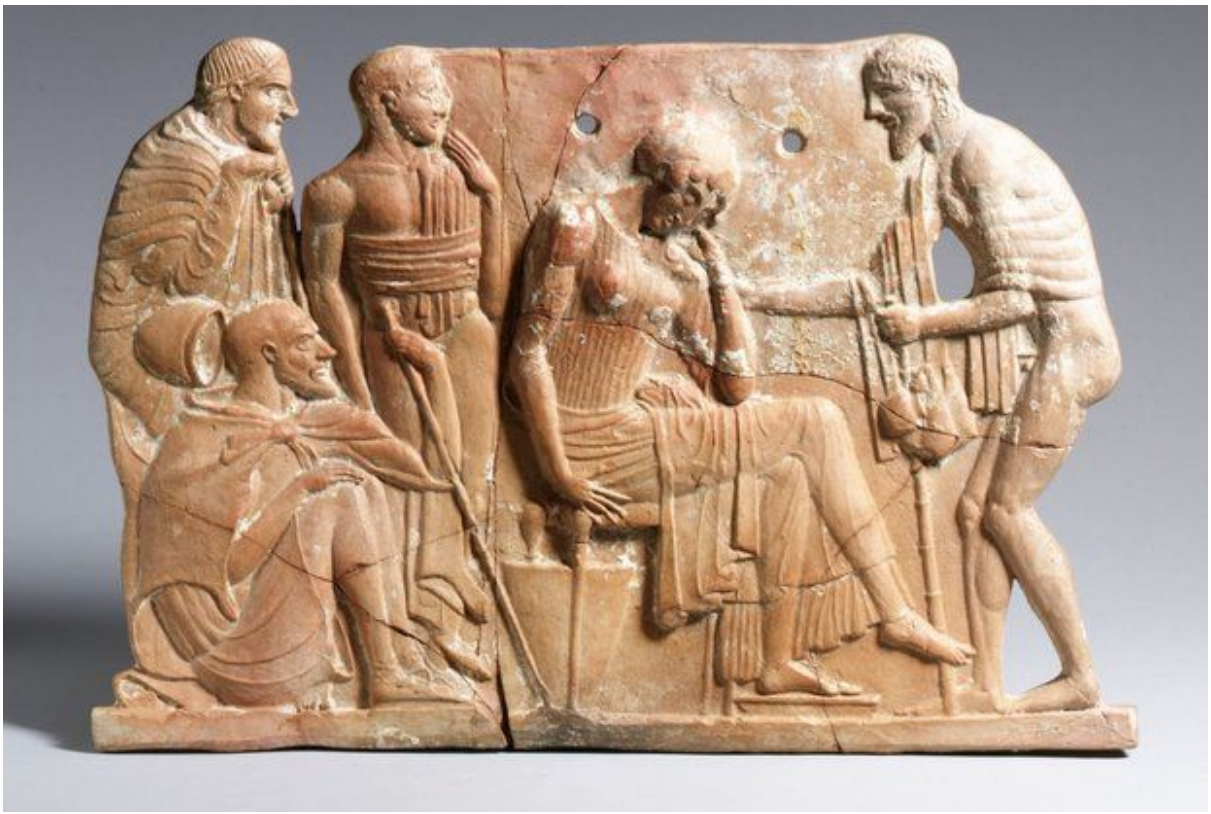
INTRODUCTION

1. Alzheimer's disease

1.1. History of the research

Across time and civilizations, the concept of mind and its perturbations has always been a topic of interest. The fact that aging could be associated with memory impairment was already present in ancient Egypt around 2000 years before our era.[1] The idea that organic lesions could be the cause for cognitive deficits came later thanks to several writers of the Hellenistic Empire (Figure-1). Ancient Greek authors Galen (130–201 AD), Aretaeus of Cappadocia (2nd century AD) and Aulus Cornelius Celsus (1st century AD) wrote about Dementia and its potential causes. Among them, Aretaeus was the first to introduce a distinction between acute and chronic neurological disorders. Chronic disorders, dementia as referred to nowadays, were described by Aretaeus as characterized by irreversible impairment of higher cognitive functions.



(Figure 1) A terracotta plaque, circa 460-450 before our era, depicts Odysseus (right), returning to his native Ithaca. Odysseus is shown approaching his wife Penelope, as members of his house- his father (Seated on the ground), Laertes; his son, Telemachus; and the swineherd Eumaios- look on. "Laertes not being able to recognize his son, could be imagined as an old patient affected by dementia, terribly in need of elements outside his memory, steadily placed in his orchard (his long-term memory), to accomplish the recognition of Odysseus." Interesting depiction of Odysseus's father (Laertes) as potentially suffering from dementia.[2] Plaque in the collection of the Metropolitan Museum of Art, Fletcher Fund, 1930. (Photo credit: The Metropolitan Museum of Art, New York, website: https://www.metmuseum.org/art/collection/search/253053).

No discoveries or evolutions of the conceptualisation of brain diseases were made from that point until the 16th century. Scientific research on the brain and its disfunctions went through a long period of obscurantism during middle age. This medieval interlude was mainly due to the rise of the church and its theological doctrines.[3] Therefore, it's only very recently that technology with the invention of light microscopy, and scientific rationalization allowed scientist like Santiago Ramón y Cajal to get a glimpse of understanding of the brain and its constituents. In 1888, he first provided scientific evidences presenting the brain as made of independent cells.[4] His work referred today as the "Neuron doctrine" lead to new empirical research techniques together with a better comprehension of the brain. Allowing scientists to methodologically bridge potential brain alterations to cognitive dysfunctions.[5]

During beginning of the 20th century, Alois Alzheimer, a German psychiatrist born in 1864, was the first in 1906 to scientifically characterize a unknown pathology affecting memory.[6] In a six-volume study, the 'Histologic and Histopathologic Studies of the Cerebral Cortex,' he described this condition as consisting of aggregation of deposits in the cortex of 51-years-old woman, Auguste Deter, practicing neurology as we know it today by corelating organic brain alterations to cognitive symptoms.[7] Those first histopathological evidences where the first grasped on the pathology later called by its first observer, Alzheimer's disease (AD).

1.2. Situation Nowadays

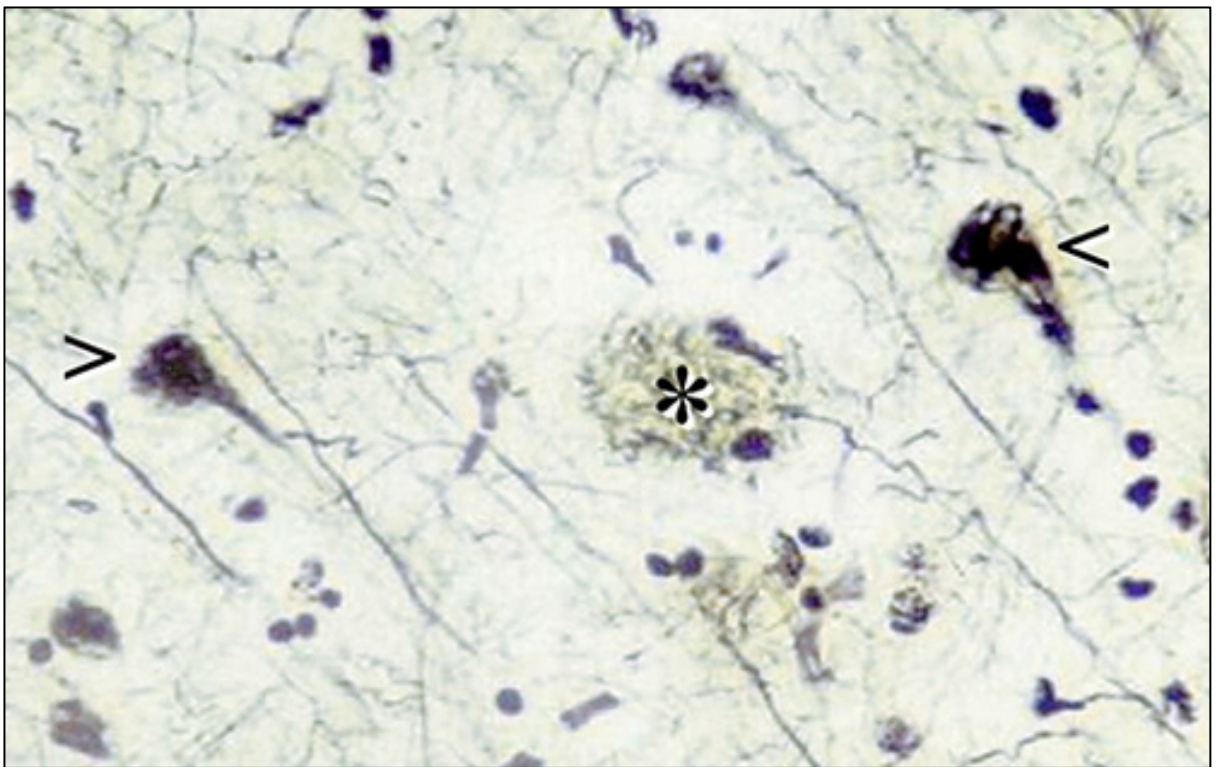
Today, AD is known to be the most common form of dementia however still of unknown etiology. This chronic neurodegenerative disease is characterized by progressive cognitive deficits such as decline in memory, problem-solving, language and other cognitive skills that affects a person's ability to perform simple daily tasks.[8] The severity of the cognitive deficits worsen as the neurodegeneration spread. Starting from the hippocampus, the neuronal death eventually affects other parts of the brain, including those that enable a person to carry out basic functions such as walking and swallowing. In the final stage of the disease, people are strongly incapacitated and require constant healthcare. AD leads ultimately to death due to complications such as pneumonia.[9] The disease affects statistically more women than men in both prevalence and severity.[10] The cause of this unbalance is still not fully understood, even though partly explained by women longer lifespan.

The pathology is affecting an estimated of 14 million people worldwide, with a cost of approximately 105 billions euros per year.[11] This is a global socio-economical health issue that touches not only the patients but also their family from a psychological and economical point of view.[12] These numbers, together with the burden on public health and society, are expected to dramatically aggravate, due to the progressive rise of the life expectancy during the last century.[13] Up to date this tremendous disorder is unfortunately incurable because of the scarce knowledge of the molecular events that drive the onset of the disease. However major advances have been made and science continues to evolve hand in hand with technology promising therapeutics to be developed.

Thanks to the advent of genetic studies, AD can be divided in two groups now: the early-onset AD (EOAD) and the late-onset AD (LOAD). The EOAD is diagnosed before the age of 65 and represents only 2% of all the cases therefore it is considered extremely rare. The EOAD is a familiar form of AD triggered by genomic alteration through autosomal dominant mutations. Such mutations occur in three specific genes: Presenilin1 (PS1), Presenilin2 (PS2) and amyloid precursor protein (APP).[14] On the contrary, the LOAD is the most common form of the disease. It is characterized by genetic predisposition that involves several genes polymorphisms (for instance Apolipoprotein E (APOE)),[15] that are associated with an increased risk for AD, but are not sufficient to cause the pathology.[16] Other risk-factors have been also identified as increasing risk of developing AD such as head injury in males, diabetes mellitus, smoking, and also a lower social engagement.[17] Although carrying at least one APOE ε4 allele increases the most the probability to develop the pathology.[18]

1.3. Hallmarks of the pathology

Studies of the pathological mechanisms of the disease have identified two markers as to be involved in the Disease and required for its diagnostic.[19] The composition of those signature markers have been discovered during the 80s. Discovered in 1986, the neurofibrillary tangles (NFT) are mainly intracellular deposits due to the disassembling of tau proteins that then get hyperphosphorylated.[20] Discovered in 1984, the Aβ plaques are mainly extra cellular and due to the processing of the APP by the β- and then γ-secretase (Figure-2).[21]

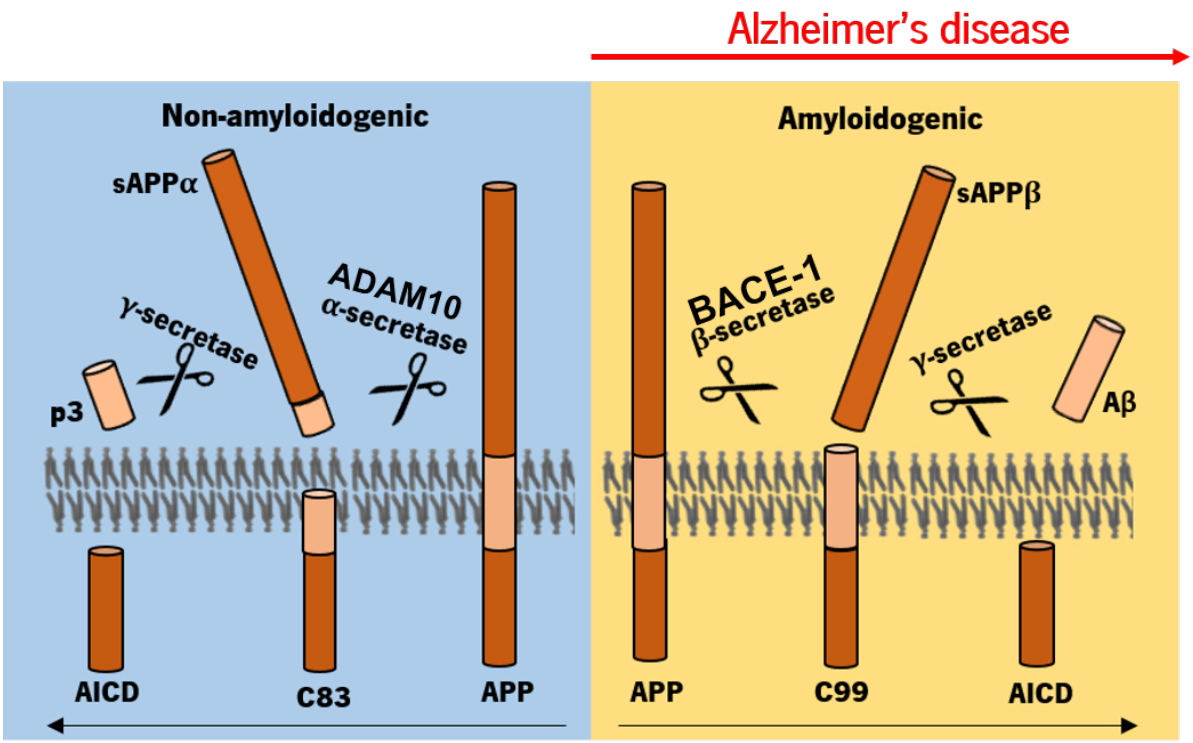


(Figure 2) Neurofibrillary tangles (pointed with arrow) and amyloid plaque (marked with star) in cortex of famous patient Auguste Deter. Amyloid plaques are due to aggregation of amyloid-β peptide produced by β-secretase activity on APP. NFT are composed of aggregated hyperphosphorylated tau proteins. (Image credit: "Alzheimer A, Uber eine eigenartige Erkankung der Hirnrinde. Allg. Zschr. F Psychiatr. Psychisch- Gerichtl Mediz, 1907 64:p. 146-8.")

Research has enabled detailed understanding of the molecular pathogenesis of the hallmarks of the disease. It is known that the Amyloid plaques are formed of aggregated Aβ peptides. The formation of the Aβ peptide is the result of the cleavage of APP by the β-secretase BACE1 in the N-terminus domain. This cleavage leads to release of the soluble APPβ and formation of membrane embedded CTF99 fragment. The CTF99 fragment is then cleaved by the γ-secretase which is a multi-subunit complex composed of Presenilin-1 (PS1), Presenilin-2 (PS2), PEN2, Nicastrin, and APH1. This final cleavage by the γ-secretase is leading to the production of Amyloid Intracellular Domain (AICD) and the formation of Aβ peptide (figure-3). In pathological conditions, the overproduction of Aβ peptide is then leading to a succession of negative events consisting in the accumulation and aggregation of the peptide in neurotoxic amyloid plaques, the aggregation of hyperphosphorylated tau protein in NFTs and a serious neuronal loss together with a strong inflammatory response. Aβ peptide ending at residue 42 being more prone to aggregation compared to Aβ peptide ending at residue 40.[22] Some studies suggest that Aβ40 could antagonize Aβ42 tendency to aggregates putting the increase of Aβ42 or Aβ42/Aβ40 ratio as a more relevant marker of the pathology.[23]

Moreover average plasma levels of Aβ42 or Aβ42/Aβ40 ratio is known to be higher in prodromal stage of AD.[24]

The Amyloid cascade has been proposed since now 25 years to be the major cause of cognitive dysfunction in AD. A second processing pathway of APP complex that counteracts the activity of the β-secretase BACE1 involves a different secretase whose activity do not lead to the production of the Aβ peptide.[25] This non-amyloidogenic pathway has for main protagonist the major α-secretase called A Disintregrin And Metalloprotease 10 (ADAM10). This sheddase is a member of the disintegrin and metalloprotease family and cleaves APP within the Aβ domain therefore precluding the production of the Aβ peptide.[26] The ADAM10-mediated cleavage of APP protein leads to the production of neuroprotective soluble APPα fragment and membrane-embedded fragment CTF83. This CTF83 fragment is then further cleaved by the γ-secretase complex. This complex activity produces the P3 fragment together with the AICD (figure 3).



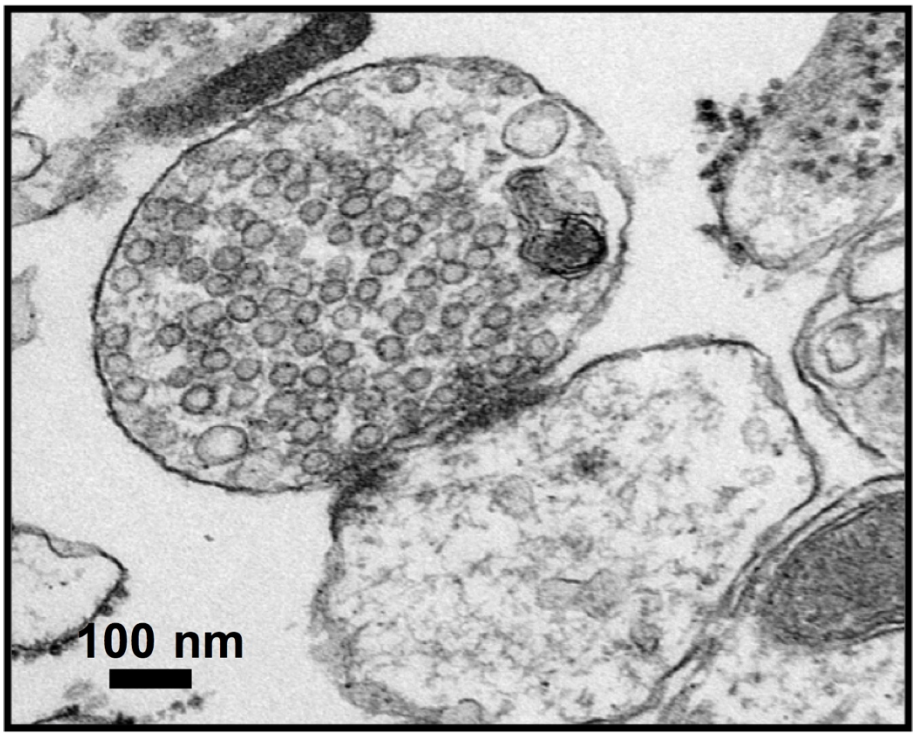
(Figure 3) Schematic diagram of exclusive APP processing pathways (not drawn in proportion). On left, APP non-amyloidogenic pathway involving first the major α-secretase ADAM10, cutting APP within Aβ domain and producing sAPPα fragment and C83, which is cleaved further by γ-secretase producing P3 and AICD fragments. On the right, APP Amyloidogenic pathway involves the β-secretase BACE1, cutting APP in N-terminus domain and therefore, producing sAPPβ and C99 fragement, which is further cleaved by γ-secretase producing AICD peptide and Amyloid-β peptide. (Scheme credit: A. Ribeiro).

The unbalance between those two exclusive pathways is pointed as potential cause of the development of the disease.[27] Many scientific efforts are focusing on unravelling the molecular mechanisms triggering this unbalance yet no tangible elements have been brought to light. The unbalance of APP processing and the excessive formation of the Aβ peptide could lead to dramatic repercussion on synaptic transmission and therefore cause the deficits AD.

2. Alzheimer's disease as synaptopathy

2.1. Synaptic transmission

With an average mass of 1,3 kilogram able to fit in hands, the brain represents the most complex system in the universe. Composed of a billion neurons, with each of them forming up to 100.000 synaptic connections, there are more synapses in an average human brain than stars in the milky way. Those synapses are close but not continuous connection between neuronal cells allowing them to exchange information thanks to substile changes in ion gradients that drives the release of chemical neurotransmitters.[28] This notion of synaptic transmission has been proposed as taking place in this tight assembling that consist of a pre-synaptic part, a synaptic cleft were neurotransmitters are released and a post-synaptic density (PSD). The presynaptic active zone is filled with neurotransmitters-filled synaptic vesicles ready to fusion with the presynaptic membrane to release in the synaptic cleft (figure 4).[29] The synaptic cleft is a space of 23,8nm width containing a extracellular matrix mainly composed of cell adhesion molecules to hold pre and post-synaptic compartment together and keep proper distance of separation.[30]



(Figure 4) Image acquired via Electron microscopy. Visualization of a synapse Cross section with dendrite synapsing with axon containing synaptic vesicles. The plasma membrane of the pre-synaptic cell is specialized in the release of vesicles. These vesicles filled with neurotransmitter, are grouped in these areas as ready to be released. The post-synaptic density facing the presynaptic cell, contains receptors that can be activated by neurotransmitter binding. (Credit Image: the Okinawa Institute of Science and Technology, website: https://oist-prod-www.s3-ap-northeast-1.amazonaws.com/s3fs-public/photos/synaptosome.png).

The PSD facing the presynaptic active zone appears to be thickened and electron dense. This dense post synaptic membrane contains many proteins embedded in the membrane such as receptors, adhesion molecules, signaling molecules. This assembly together with all its molecular actors are sustaining synaptic transmission. This biological process allows neurons to communicates with a target cell across the synapse. Another type of Electrical synapse transmission involves the transfer of electrical signals through gap junctions. This type of synaptic transmission is found in nerves were fastest response possible is needed as in defensive reflexes. In contrary, the chemical synaptic transmission involves the release of a neurotransmitter from the pre-synaptic neuron, and neurotransmitter binding to specific post-synaptic receptors as described above. Chemical synapses are crucial for the biological computation supporting basic regulation of body functions but also high cognitive processes. It is known that the hippocampus is dependent on glutamate signaling to a greater extent than other neocortical tissue, a feature that underlies cognitive functions such as learning and memory.

2.2. Glutamatergic transmission

The Glutamate system is the principal excitatory neurotransmitter in the CNS.[31] The principal mediator of this system is the amino acid glutamate that can bind on different glutamate receptors (GluRs) such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), N-methyl-D-aspartate receptors (NMDARs) and metabotropic Glutamatergic receptors (mGluRs), which are specifically targeted and clustered at the PSD.[32] Those receptors are assembling of proteins complexes tightly associated to regulate signal transduction but also membrane removal, local expression and clustering.[31]

Two major GluRs are known: Ionotropic receptors favorized the positively charged ions flux through their pores when activated by glutamate. They serve as ion channels. Metabotropic receptors do not conduct ion flux but rather when activated by glutamate trigger intracellular cascade via G protein. Thanks to crystallography studies different types of ionotropic glutamatergic receptors (iGluR) have been identified: AMPA, NMDA and Kainate rectors named after their agonists. After studies using cloning, they have been reported to have different functions depending on their subunit composition. The iGluR have four large subunits of more than 900 residues. All iGluR have three transmembrane domains (M1, M3 and M4) and a cytoplasmic membrane loop (M2) with the N-terminus located outside the cell and the C-terminus inside the cell. Agonist binding on the iGluR forces a conformational change leading to an increase probability of the opening of the ion channel. The different glutamate receptors have different affinity. The EC50 (half maximal effective concentration) of glutamate at NMDA receptors is approximately 1 μmol/l, while at AMPA receptors it is approximately 400 μmol/l.[33]

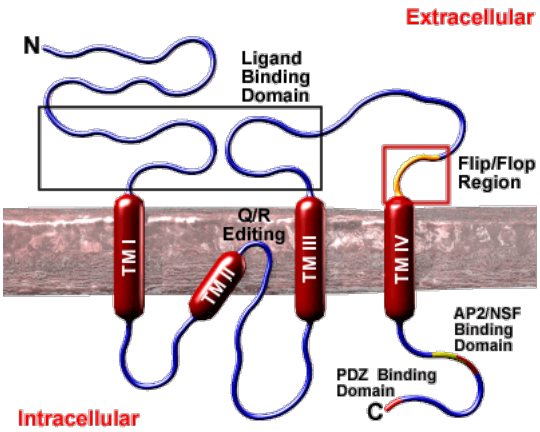
The number of surface receptors is regulated by insertion and removal from the membrane allowing glutamatergic transmission events to be subject to precise use-dependent changes.
The two main paradigms by which synaptic plasticity modulate excitatory synapses are the Long-Term potentiation (LTP) and Long-Term Depression (LTD). These two paradigms are primary models for investigating the synaptic basis of learning and memory in vertebrates.[34]
The induction of LTP leads to an increase of the strength of the synapses and is triggered by activation of NMDARs. This activation induces the insertion of AMPARs into the postsynaptic membrane via a signaling cascade. The induction of LTD leads to a decrease of

synapse efficacy and is mediated by the endocytosis of AMPARs.[35] Blocking the activation of NMDARs using antagonist DL-2-amino-5-phosphonovalerate (APV) prevents LTP and LTD induction in the hippocampus.[36/37]

2.2.1. AMPA Receptors

AMPA receptors are known to support fast synaptic excitatory transmission. They are heterotetrameric as assembles of four subunits in dimer-of-dimers manner (Glua1-Glua4).[38] With predominant Glua1/Glua2 heteromers (comprising for around 80% of all synaptic AMPARs) together with Glua2/Glua3.[39] The Glua4 subunit being essentially present during development and mostly absent in mature adult synaptic neurons.[40] The AMPARs can be activated by its agonist glutamate and blocked by antagonists such as 6-ciano-7-nitroquinoxaline-2,3-dione (CNQX) and 2,3- dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX). The subunits of AMPAR have two splice variants occurring in the flip and flop region. They are found at the C-terminal end of the loop between third and fourth transmembrane sites. The small change in amino acids composition can result in altered desensitization kinetics (Figure-5).

The different subunits composing the AMPARs modulate their permeability to Calcium (Ca^{2+}), sodium (Na^{+}) and potassium (K^{+}).[41] The Glua2 subunit dictating the permeability to calcium and thus guarding against excitotoxicity.[42]



(Figure 5) Scheme showing structure of the GluN2A subunit of AMPARs. The subunit is composed of 4 transmembrane domains (TMI-TMIV). The C-terminus domain being intracellular and the N-terminus which is the binding domain for ligands is extracellular. Splice variation have been identified in the Flip/Flop region, leading to two possible variants for each gene sequence. (Representation credit: Bristol University Center for synaptic plasticity, website: http://www.bristol.ac.uk/synaptic/receptors/ampar/).

GLUA1 and GLUA2 containing AMPA receptors are principally found in the forebrain. The modulation of synaptic plasticity and its impact on learning can also be the result of AMPAR subunits phosphorylation. This modulation via phosphorylation can impact general function of the AMPARs such as conductance, channel localization and opening probability.
The GluA1 subunit is mainly present in hippocampal and neocortical neurons.[43] Its phosphorylation occurs on serine, threonine and tyrosine sites residues on intracellular C-terminal domain.[44] Threonine and Serine being the major residues on which phosphorylation occurs.[45] Synaptic plasticity studies have shown implication of Ser831 and Ser845 phosphorylation in LTP and LTD expression.[46] Phosphorylation on Ser831 is due to action of the PKC and CamKII, while Ser845 is phosphorylated by Protein Kinase A (PKA).[47] The phosphorylation of Ser831 helps insertion of GluA1-containing AMPAR into the synapse while the phosphorylation of Ser845 regulates its opening probability. Those mechanisms of phosphorylation can also increase synaptic strength by enhancing channel conductance of the AMPARs.[48]

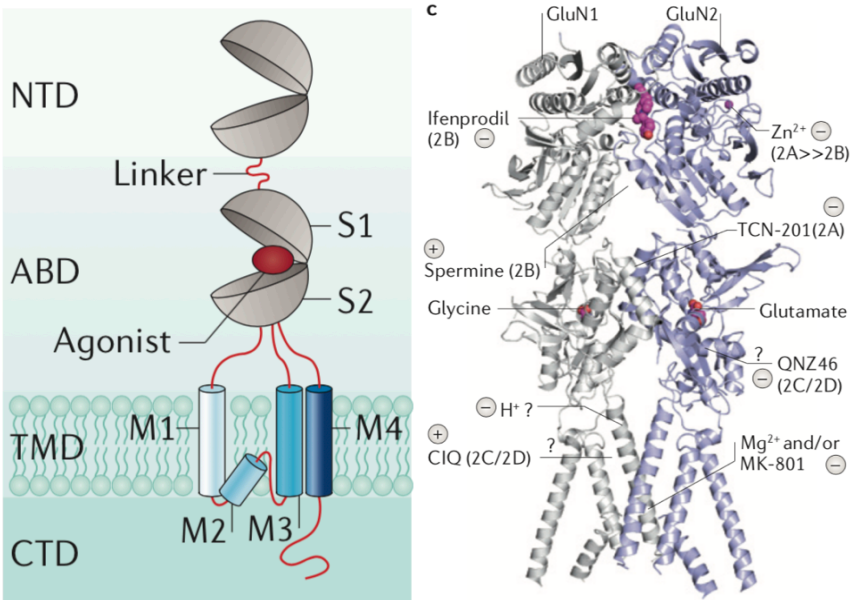
The GluA2 subunit is essential to confer AMPARs impermeability to Ca2+.[49] Phosphorylation sites have been observed on GluA2 intracellular C-terminal domain. Among those, Ser863 and Ser880 are the most common. PKC Phosphorylation of Ser880 residue has been shown to be implicated in synaptic plasticity.[50]

The GluA3 and GluA4 subunits are strongly present in forebrain structures during adult stage in comparison to GluA1 and GluA2 subunits.[51] Similar phosphorylation sites implicated in synaptic plasticity mechanisms are found on GluA1, GluA2 subunits and on GluA3, GluA4 subunits. GluA3-containing AMPARs might contribute to synaptic potentiation to form hippocampal LTP. This potentiation doesn't require AMPARs trafficking but instead would require an increase of the channel opening probability of GluA3-containing receptors at synapse via cAMP activity.[52]

Spontaneous activity has been observed to trigger an increase of GluA4-containing AMPARs in the synapse. Their expression is confined to early post-natal development period and is specific to pyramidal neurons of the hippocampus. This may have unique plasticity rules relevant for synapse maturation. However, GluA4 implication in synaptic plasticity is still unclear.[53]

2.2.2. NMDA Receptors

NMDA receptors play a critical role in synaptic plasticity and the learning process. Functional NMDA receptors are heteromeric, assembled from seven identified subunits to date, falling in three different subfamilies: GluN1 subunit , Four GluN2 subunits A–D, and two GluN3 subunits A–B (Figure-6).[54]



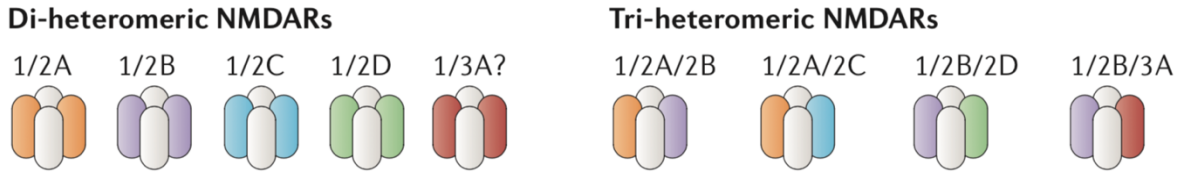
(Figure 6) Scheme of NMDA receptor subunits composition. Effect and binding sites location of agonists (Glutamate), co-agonist (Glycine), inhibitors (Ifenprodil). (Representation credit: Review 2013, Paoletti et al).

The number of residues per subunit ranges from 900 to over 1,480. Each subunit is alternatively spliced, making the number of potential NMDA receptor subunit combinations very large. Most commonly GluN1 being associated to GluN2 or GluN2 associated with GluN3.[55] GluN1 is ubiquitously expressed in the CNS from E14 to adulthood.[56] The GluN2A subunit is responsible for the diversity of composition of NMDARs and is known to have drastically

different spatiotemporal expression.[57] This subunit is fully expressed in all CNS at adult stage and inversely GluN2D subunit decrease along the development to be only sparsely expressed in low levels in the diencephalon and mesencephalon at adult stage. The GluN2B subunit is expressed in high level post-natal and reach a peak around a week old to then lower to be restricted to forebrain. The GluN2C subunit expression appears around postnatal day 10 and is mainly restricted to the olfactory bulb and the cerebellum. The GluN3A subunit expression peaks in early postnatal life and then declines progressively. Conversely, GluN3B expression increases throughout development, and is expressed at high levels in motor neurons at adult stage. The implication of GluN2B, GluN2D and GluN3A during early development suggest important function of these subunits in plasticity.

Receptors containing GluN1 subunit coupled to different types of GluN2 subunits have been described and divided in two categories. The Di-heteromeric NMDARs containing only one type of GluN2 subunit (e.g. GluN1/GluN2B or GluN1/GluN2A). And the Tri- heteromeric

NMDARs containing more than one type of GluN2 subunit (e.g. GluN1/GluN2A/GluN2B) that are present importantly in the hippocampus and cortex where they represent from 15% to 50% of total receptors (figure-7).[58]



(Figure 7) Scheme representing potential Di- and Tri-heteromeric NMDA receptor composition. Di-heterotrimeric NMDARs composed of only one type of GluN2 subunit. Tri-heterotrimeric NMDARs containing more than one type of GluN2A subunit. (Representation credit: Review 2013, Paoletti et al).

NMDARs are cationic channels permeable to sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) ions. In particular, Ca^{2+} influx is the critical factor mediating many of the NMDAR-specific physiological and pathological events. Characteristically all NMDARs are voltage dependent-Mg^{2+} blocked and are permeable to Ca^{2+} conferring them slow kinetic type of

responses. Glutamate binding but also a co-agonist such as Glycine or D-serine is needed for them to be activated. They have also modulatory sites that confer them a fine sensibility to their environment. Those binding sites act as positive or negative allosteric modulators in response to numerous substance such as protons, polyamine and Zinc.[59] NMDARs are

expressed on astrocytes and have been observed in peri- and pre-synaptic site in the CNS.[60] However they are mainly expressed on the post synaptic site of the synapse.[61]

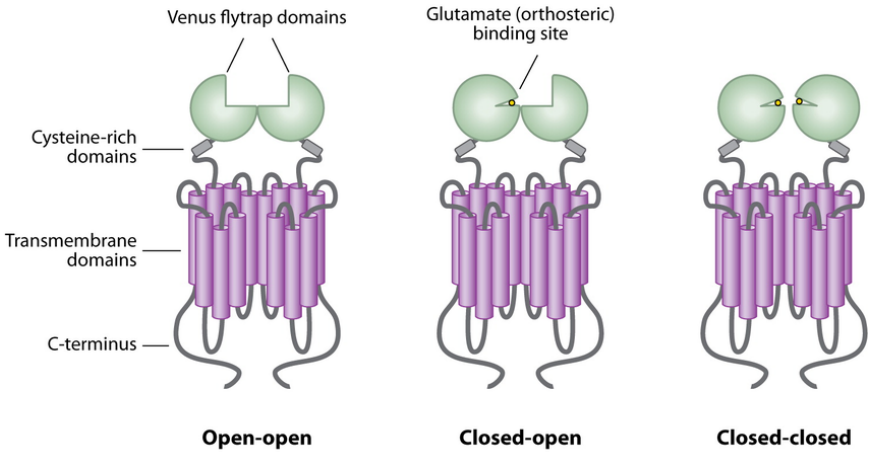
2.2.3. Metabotropic Glutamate Receptors

Glutamate acts not only ionotropic channels but also on metabotropic receptors and their signaling pathways. Metabotropic glutamate receptors (mGlus) comes in eight subtypes mGlu1-mGlu8. Among them seven are express in the CNS exerting neuromodulatory role. They have been categorized in three functional classes considering their amino-acid sequence homology, their agonist pharmacology and the signal transduction pathways they are associated with. The following categorization in currently accepted and used by the scientific community: Group I (mGluR1 and mGluR5), Group II (mGluR2 and mGluR3), and Group III (mGluR4, mGluR6, mGluR7, and mGluR8)). All mGluRs are G protein-coupled receptors (CGRPs) therefore, link to G trimeric cytoplasmic enzymes that can activate a wide range of

intracellular signaling pathways. Protein G activation is dependent on a disulfide bridge between cysteine residues in the extracellular loop and the third transmembrane domain. Most of these identified receptors display a large extracellular domain essential for ligand recognition. This extracellular domain called a Venus Flytrap module is structurally similar to bacterial periplasmic proteins involved in the transport of small molecules (Figure-8).[62] The classification of mGlu receptors into three groups is further supported by a consideration of their signal transduction mechanisms.

G protein-coupled receptors Group I includes mGluR1 and mGluR5, that are coupled to G_q-proteins activating Phospholipase C (PLC) to produce inositol 1,4,5-trisphosphate (IP3). Signaling molecule, IP3 is known to trigger opening of Ca2+ channels in the endoplasmic reticulum increasing in this way the intracellular calcium concentrations. Activation of phospholipase C leads not only to the formation of IP3 but also diacylglycerol (DAG). DAG remains in the membrane as a co-factor for PKC activation. Splice variants have been described for both mGlu1 and mGlu5. The main mGluR1 variants are 1b and 1d, for which the last 318 C-terminal amino acid residues of the original mGluR1a variant are replaced by 20 and 26 (22 in human) residues in 1b and 1d, respectively.[63] For mGlu5R, the 5b variant differs from the first identified 5a receptor by the insertion of a cassette of 32 amino acid

residues 49 residues after the 7TM.[64] Agonist 3,5-dihydroxyphenylglycine (DHPG) is specifically selective for group I.[65]



(Figure 8) Scheme of the mGluR dimer in different activity states. mGluR dimers contain two large extracellular domains called Venus flytrap domains (VFDs), which bind glutamate and other orthosteric ligands. The cysteine-rich domain links the VFDs to seven transmembrane-spanning domains. The intracellular C-terminal domain is subject to alternative splicing leading to generation of different C-terminal protein tails. The open-open state (left) is the inactive state and can be stabilized by different antagonists. Either one or two VFDs can then bind glutamate, resulting in active receptor conformations. (Representation credit: Niswender Cm, Conn PJ. 2010. Annu. Rev. Pharmacol. Toxicol. 50 :295-322.).

G protein-coupled receptors Group II includes mGluR2 and mGluR3 that are coupled to G_i-proteins and are able to inhibit the adenylyl cyclase activity and different types of Ca2+ channels. Agonist 2R,4R-4-aminopyrrolidine- 2–4-dicarboxylate (APDC) is strongly selective and mildly potent (400 nmol/l) for G protein-coupled receptors group II. G protein-coupled receptors Group III includes the four others mGluR (4,6,7,8) and are coupled to $G_{i/o}$ proteins. Like group II, mGluRs group III are able to inhibit the adenylyl cyclase activity together with Ca2+ and K+ channels activity. Agonist l-amino-4-phosphonobutyrate (l-AP4) is selective of group III mGluRs.

An important number of ligand-gated Na^+ and K^+ channels are also modulated by mGlu receptor activation including NMDA and kainate receptors. Activation of mGlu receptors acts to inhibit or potentiate ionotropic receptor depending on the signal transduction mechanism. Action of mGlu receptors can be excitatory by increasing conductance and therefore the release of glutamate from the presynaptic cell. Or else their activation can inhibits the release of glutamate and therefore modulate voltage-dependent calcium channels.[62] In hippocampal pyramidal cells, group I mGlu receptor activation potentiates currents through NMDA receptors activity. This effect is reduced by inhibitors of either protein kinase C or Src tyrosine kinase, and may proceed through dual signaling pathways.

2.3. Dysfunction of the synapse

The synaptic transmission is highly efficient but a fragile mechanism. Dendritic spines go through activity-dependent morphological and density changes allowing the structural plasticity of the spines to be closely modulated by synaptic function. Studies observed that an enlargement of the spines accompanies LTP. In contrary, LTD was observed to be associated with a shrinkage of the dendritic spines. These subtle morphological changes affect connectivity in neuronal circuits and the overall synaptic function.[66] Because synaptic transmission is the base for cognitive processes such as memory. The morphology and density of synapse have been studied has a potential driving force for AD. Extensive postmortem pathological studies of AD patients showed a low level of spines density throughout the cortex and hippocampus of affected brains.[67] Interestingly, a stronger correlation has been observed between cognitive decline and synapse/dendrite loss rather than to neuronal loss or neurofibrillary tangles. This synaptic loss could appear early in AD as it has been observed in Mild cognitive impairment (MCI) and has been also observed in greater extent in AD. Indicating that AD synaptic loss could worsen together along the cognitive deficit.[68] Moreover, the synaptic loss has been observed to be higher than expected in regard of the neuronal loss. This underlying synaptic defects as an important pathological mechanism of AD thereby, as a strong drive for the cognitive deficits rather than a exclusive consequence of neuronal death.[69] Some studies interestingly observed synaptic compensatory mechanisms in AD such as an increase of the size of the remaining dendritic spines. Further investigation of these compensatory mechanisms is important as representing future potential disease modifying strategies.

The overproduction of the Aβ peptide in AD is known to impact negatively synaptic transmission in the absence of significant neurodegeneration. Human Aβ can exist in diverse species as its process of aggregation goes on. These different species of Aβ include monomers, dimers, trimers, tetramers, dodecamers and after further aggregation protofibrils that can lead to mature fibrils. These mature fibrils can ultimately form amyloid plaques in brain tissues. These Amyloid plaques created by aggregation of small peptides of not more than 50 amino acids are finally of detectable size via microscopy.[70] The soluble Amyloid-β forms are the perfect candidates that could first trigger synaptic dysfunctions prior to the

heavily network disruptions caused by neurotoxic Amyloid plaques load. Studies show, that soluble species of Amyloid-β produced at early stage of the pathology are implicated in synaptic failure.[71] Small soluble Amyloid-β oligomers were observed to alter synaptic plasticity as leading to rapid inhibition of long term potentiation.[72] That effect on synapses precede neuronal death and leads first to synaptic failure causing the memory loss. The association of Amyloid-β peptide with memory impairment started from a study on transgenic mice Tg2576 which express human APP695 containing the Swedish mutation (K670N/M671L).[73] These transgenic mice have high levels of $A\beta_{1–42}$ in the brain and display cognitive deficits when performing spatial memory task.[74] Also, a correlation between severity of the cognitive deficits and spine density has been shown in many other studies. Furthermore, a decrease in synaptic density is observed in hippocampus of AD patients.[75] This decrease is synaptic density is disproportionate to the actual loss of neurons supporting again the fact that synaptic failure precede neuronal loss.[76] Moreover, several electrophysiological studies observed a significant synaptic deficit in human APP transgenic mice well before the development of Amyloid-β deposits detectable via microscope.[77] The post synaptic compartment of excitatory synapse could be the early target of Amyloid-β as it has been observed to bind PSD-95-containing post synaptic sites.[78] Together with post synaptic density complexes containing NMDA receptors.[79] Experiments also showed that Amyloid-β induces a reduction of PSD95 levels in a time- and dose-dependent manner.[80] Furthermore, NMDAR internalization occurs via high affinity binding of the Amyloid-β_{1-42} to the a7-nicotinic acetylcholine receptor. The binding of Amyloid-β_{1-42} enhanced the a7-mediated Ca2+ influx and activation of the serine-threonine protein phosphatase 2B, a Ca2+ sensitive enzyme that regulates NMDA transmission and synaptic plasticity. PP2B action dephosphorylate and activates striatal-enriched tyrosine phosphatase, which will dephosphorylate the NMDA receptors subunit NR2B on tyr1472. This dephosphorylation result in the internalization of NR2B containing NMDAR.[81] Amyloid-β has undoubtedly many effect on the synaptic plasticity.

Amyloid-β negatively impact number of postsynaptic NMDAR creating an unbalance of activity between synaptic and extrasynaptic NMDAR. Applied Amyloid-β also enhances NMDAR endocytosis and decrease synaptic expression of cultured cortical neurons.[81] In accord with these finding the calcium influx after NMDA activation via glutamate uncaging at single spines is significantly altered after treatment with Amyloid-β.[82] Hippocampal slices treated with Amyloid-β containing media show reduced expression of PSD-95 and NR2B in

synaptic but not extrasynaptic fraction.[83] In the same manner, Amyloid-β peptide in hippocampal cultures has been observed to decrease synaptic currents from glutamatergic neurons.[84] Together these results reveal that Aβ specifically triggers NMDAR internalization of at the synapse but not in extra synaptic. However, it is well known that Aβ promotes tonic glutamate build up in the extracellular space therefore activating extrasynaptic NMDARs. Reduction in Glutamate transporters expression is observed in tissue from AD patients.[85] Studies have also observed in hippocampal neurons an enhancement of presynaptic glutamate release, an inhibition of glutamate uptake, and a general increase in extracellular glutamate concentration provoked by Aβ application.[86] It was also recently shown to reduce the expression of glial glutamate transporter GLT-1.[87] When using MK-801 to preblock synaptic NMDAR, Aβ from brain extract, cell culture or synthetic Aβ have been observed to still induce NMDA dependent current.[84] Using Ro25-6981 this effect is blocked suggesting that Aβ leads to increased activation of NR2B-containing NMDAR extrasynaptic. Adding to the reduction of glutamate uptake, Aβ can also provoke extrasynaptic NMDAR activation by glutamate release from glia cells. Whole cell current from cultured microglia demonstrated that the outward current composed of glutamate and sodium in response to potassium stimulation are significantly higher in cultures treated with Aβ.[88] Furthermore, Aβ was also observed to directly impact glutamate release from glia cells, thus increasing the probability of activation of extra-synaptic NMDAR.

Synaptic transmission together with exocytosis dictates the extracellular release of Aβ.[89] Bath application of NMDA to cultured cortical neurons leads to a shift in α-secretase to β-secretase processing of APP, increasing the production and release of Aβ and reducing sAPPα levels. This effect via NMDA application was underlined by upregulating expression of APP containing kunits protease inhibitor domain.[90] Synaptic NMDA activity increase alpha-secretase mediated processing of APP.[91] In contrary, extrasynaptic NMDA mediated production of Aβ creates a vicious circle of toxicity in which Aβ promotes extrasynaptic NMDAR activity, which lead to further production and secretion of the peptide. Many signaling pathways are implicated in these extrasynaptic NMDAR mecanisms and AD synaptic disfunctions. CREB mediated gene expression is implicated in cell survival together with synaptic plasticity as well as memory. Interestingly decreased levels of phosphorylation of CREB serine-133 was observed in AD patients.[92] This decreased was also observed after extrasynaptic NMDAR stimulation.[93] Using treatment to increase cAMP signaling pathways

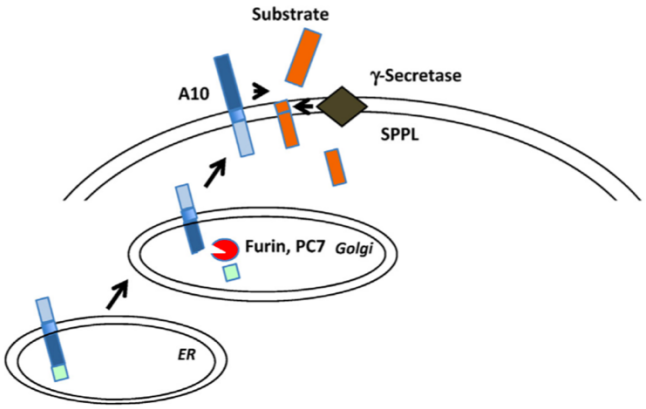
such as rolipram and forskolin reverts CREB phosphorylation together with the synapse loss and LTP deficits observed in AD.[94] Aβ treatment translocates Jacob to the nucleus in the non-phosphorylated form.[95] After synaptic NMDAR stimulation Phosphorylation of Jacob is associated BDNF, CREB and Arc signaling as neuroprotection.[96] At contrary the non-phosphorylated form of Jacob is transported directly after excitation of the extra-synaptic NMDAR. This effect on Jacob lead to a decrease of the activity of CREB, a decrease of the complexity of dendritic ramifications and a decrease in synaptic density. Moreover this effect on Jacob is blocked by the NR2B-specific antagonist ifenprodil, demonstrating that Jacob translocation induced by Aβ is dependent on NR2B-containing NMDAR.[97] BDNF can promote Serine-133 CREB phosphorylation through CamKIV or ERK1/2 activation, furthermore the BDNF gene is a CREB target.[98] BDNF levels decreases with CREB activity levels in the brain, serum and CSF of AD mouse models.[99] Interestingly this was also observed in of AD patients.[100] Moreover BDNF induction is blocked by extrasynaptic NMDAR activity.[93] Supporting the use of stem cell as therapeutic against neurodegenerative diseases, studies mouse model of AD showed that stem cell implantation can restore cognitive deficits. Very interestingly this cognitive improvement was independent to Aβ and role of BDNF downstream Aβ and/or tau pathogenic effects.[101] In the same fashion, improvements of cognitive deficits via BDNF increase in transgenic mice ,was also achieved via viral delivery of CREB binding protein independently to tau or Aβ pathology.[102] These results show that tau or Aβ pathology rely in some extent on negative effects of CREB and BDNF signaling.

High numbers of studies have shown that Aβ can impair NMDA dependent LTP at hippocampal CA1 synaptic plasticity in AD.[103] This deficit in synaptic plasticity could be due to overstimulation of extrasynaptic NMDAR. Aβ-induced LTP inhibition can be prevented by decreasing extrasynaptic glutamate levels. Inhibition of calpain or p38MAPK was also observed to prevent the LTP impairment. Aβ wide impact on synaptic transmission mechanisms could lead to dramatic events. To some extent the aggregation of the peptide could also lead to phosphatase dysfunctions provoking the scrambling down of cell's cytoskeleton caused by tau hyperphosphorylation and ultimately followed by neuronal death. Among those pathological mechanisms, Amyloid-β could also interferer with the physiological function of receptors, signaling pathways and general maintenance of the cells responsible for affecting the synaptic transmission, leading to disruption in information and cognition.

3. The alpha secretase ADAM10

3.1. ADAM10 as member of the synapse

Members of the "A Desintegrin and Metalloproteases" (ADAM) family are composed of inactive and active proteases such as ADAM9 and ADAM17. Among them, ADAM10 is a type 1 membrane glycoprotein composed of 748 amino acids. It is implicated in many important processes in the CNS such as synaptic plasticity, neurodevelopment and regulation of spine morphology. ADAM10 is membrane-anchored protease that has for role to cleave ectodomain of transmembrane or simply membrane bound proteins. Over ten years ago, it has been described as the major alpha secretase acting on the Amyloid precursor protein (APP). Because of this, interest kept growing since ADAM10 could represent a new therapeutic target in AD. ADAM10 is found inactive in the Golgi as the proenzyme needs a post-processing to be functional. After removal of the signal sequence, the inactive form of the sheddase is translocated to the secretory pathway where its activated by the proprotein furin or PC7 (Figure-9).[104] The Furin cleavage of ADAM10 takes place basic amino acid sequences R-X-K/R-R and R-X-X-R of the C-terminus domain.



(Figure 9) Furin cleavage within the Golgi removes ADAM10 prodomain and leads to the mature and fully active form of the sheddase. ADAM10-mediated ectodomain shedding of substrates triggers intra-membrane proteolysis by γ-secretase or by signal peptide peptidase-like proteases (SPPLs).[105] (Representation credit: Review 2015, Lichtenthaler et al).

The furin endoprotease acting in the secretory pathway is present mainly in the Trans Golgi Network (TGN), however it can be found also in clathrin-coated vesicles, on the plasma membrane and in the cytosol of the cell.[106]

By coexpressing a prodomain-deleted ADAM10 mutant together with its prodomain *in trans,* ADAM10 prodomain was shown to exhibit a dual function: The prodomain can inactivate endogenous ADAM10 in cell culture conditions while overexpressing ADAM10 lacking its prodomain was observed to be inactive. At contrary, the prodomain co-expressed in trans restors the proteases role of ADAM10 mutant lacking intracellular prodomain.[104] In further in vitro experiments, application of purified prodomain was observed to inhibits potently and selectively the enzyme.[107] Put together, these data show that ADAM10 prodomain acts transiently as an inhibitor and also as a modulator of the enzyme maturation. Mutation of ADAM10 active site zinc-binding motif leads to a decrease production of sAPPα fragment.[108] Although the deletion of ADAM10 disintegrin domain was shown to not affect strongly the shedding of APP protein in cell cultures, the processing of some other substrates of ADAM10 is supposedly thought to be impacted also by non-active sites.[109] Supporting experiments using cells knockout for ADAM10 that were overexpressing a mutant of ADAM10 lacking the cytoplasmic domain showed a partial impairment of epidermal growth factor cleavage.[110] Translocation of ADAM10 through the secretory pathway leads to its active protease form. ADAM10 was observed to display proteolysis activity in secretory pathway and mainly at the cellular membrane. Experiments using cell surface biotinylation showed that most active ADAM10 is found embedded in the plasma menbrane.[111]

Apart from APP, ADAM10 has numerous substrates among the most important: Prion protein (PrP), neuronal (N)-cadherin, ephrins, Notch and many others reaching at least a total number of 40 substrates.[112] PrP processing involves ADAM10 and ADAM17 mediated cleavage. An increase of ADAM10 and ADAM17 activity could represent a therapeutic strategy as increased shedding of PrP could reduce its toxic effect. [113] N-cadherin cleavage by ADAM10 downregulates neuronal cell adhesion. Moreover, further N-cadherin shedding at plasma membrane is realized by γ-secretase. Cleavage of N-cadherin by γ-secretase releases its adaptor protein β-catenin promoting the induction of β-catenin target genes. Shedding by ADAM10, therefore, turns the adhesive function of N-cadherin at the cell surface into a signaling function which is critical for N-cadherin mediated induction of gene expression.[114]

ADAM10 can cleave ephrin A5 bound to EphA3 and thus terminate binding via ephrin. Notably, the ephrin receptor EphB2 also undergoes calcium-influx and *n*-methyl-d-aspartate (NMDA)-induced cleavage that is sensitive to ADAM10 inhibition. Thus, by cleavage of ephrins as well as their receptors, ADAM10 can be regarded as promoter of axon guidance and extension in the CNS.[115]

The receptor Notch and its ligand Delta 1 take part in many neurogenesis-related functions as implicated in embryogenesis via neuroepithelial development, but also in neuronal stem cell sustainment and their self-renewal in the adult CNS. Both sheddase ADAM10 and ADAM17 cleave Notch extracellular domain. The remaining cell-associated Notch is then directly followed by γ-secretase intramembranous cleavage. This results in the generation of a cytoplasmic cleavage fragment which can translocate into the nucleus and function as transcription factor. Thus, ADAM10 and potentially ADAM17 are critically involved in the transcriptional signaling pathway of Notch and are required for its functions in neurogenesis even in the adult CNS.[116]

Through its action towards APP and its other substrates, ADAM10 is implicated in many molecular mechanisms responsible for the formation, maturation and stabilization of dendritic spines.[117] It's an important and dynamic actor of the of PSD as sensitive to changes in synaptic plasticity. Changes in synaptic activity strength affects its interaction with binding partners and modulate synaptic localization. As in the case of glutamate receptors, ADAM10 insertion in the synaptic membrane is controlled by LTP and LTD. [118]

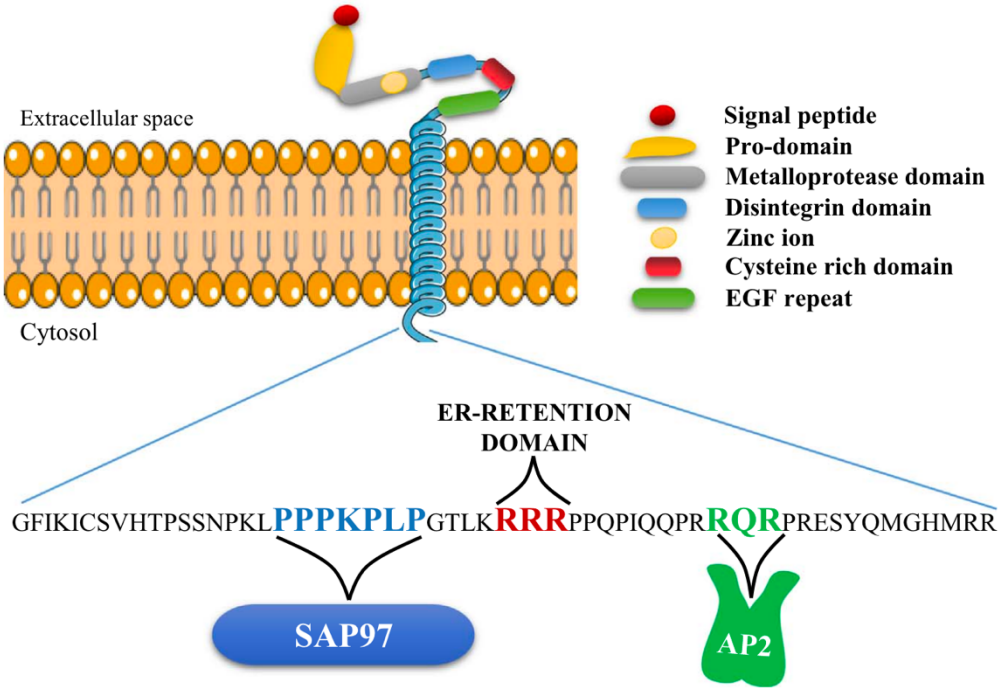
3.2. Trafficking of ADAM10

Regulation of ADAM10 activity is mediated at different levels, i.e. modulation of promoter activity, the degree of prodomain release, cellular trafficking, cellular signaling and lipid and protein interactions. ADAM10 activity in neurons is first of all modulated by its intracellular trafficking. The interaction with the synapse-associated protein 97 (SAP97), which modulate the trafficking of glutamate receptors, has been observed to directly bind ADAM10 and positively influence the sheddase's activity.[118]

The SH3 domain (PPPKPLP) of SAP97 binds to the proline-rich domain of the cytoplasmic tail of ADAM10, therefore driving the insertion of the sheddase in the postsynaptic membrane, *in fine* increasing α-secretase activity. This mechanism was observed to be promoted by short-term NMDA receptors activation during in vitro experiments on primary neurons.[119] NMDAR activation can also trigger an increase expression of genes encoding for ADAM10 and β-catenin proteins. This upregulation of ADAM10 was blocked using inhibitors of Wnt/β-catenin signaling pathway. On the other hand, the activation of the

Wnt/β-catenin signaling pathway has been observed to increase ADAM10 expression. Furthermore, ERK inhibitors blocked both the NMDAR and Wnt3A-mediated increase expression of ADAM10. All these observations point to a control of ADAM10 expression by the NMDAR via the Wnt/MAPK signaling pathway.[120]

ADAM10 C-terminal was shown to contain a motif for Endoplasmic Reticulum (ER) retention. Deletion by mutagenesis of a sequence rich in arginine (^{723}RRR) of this motif was leading to the confinement of ADAM10 in the ER.[121] ADAM10 internalization is mediated by clathrin-mediated endocytosis. The clathrin adaptor 2 (AP2), a heterotetrametric complex, bind to the sheddase C-terminal and initiates the internalization.[122]



(Figure 10) SAP97 and AP2 binding site on ADAM10 cytoplasmic tail. Promoting membrane insertion, SAP97 binds prolin rich domain of ADAM10 cytoplasmic tail. Promoting chlatrin-mediated endocytosis, AP2 binds RQR sequence of ADAM10 cytoplasmic tail. This sequence is composed of 2 positively charged residues, R^{735} and R^{737}, and a hydrophilic amino acid, Q^{736}. The two R residues have been observed to be crucial for the binding. (Representation credit: Review 2017, Marcello and Musardo).

Using different experimental methodology, the details of ADAM10/AP2 association have been characterized. An AP2-binding sequence (RQR) has been identified on the cytoplasmic tail of ADAM10. This sequence is essential for AP2 binding on ADAM10 and therefore for its internalization.[118]

Studies have shown, that ADAM10 synaptic availability is significantly affected by its internalization with AP2. This demonstrating the relevance of clathrin-dependent

internalization in the regulation of ADAM10 cell surface expression. Furthermore, both SAP97 and AP2 directly bind the Cytoplasmic domain of the sheddase in a non-overlapping fashion (Figure-10).

Thus, synaptic plasticity has been observed to affect the binding of SAP97 or AP2 on ADAM10. In particular, LTD was found to upregulate the sheddase insertion in the plasma membrane as promoting its SAP97-dependent forward trafficking. On the contrary, LTP was found to reduce the synaptic availability of the sheddase by promoting AP2 clathrin-mediated endocytosis.[118]

3.3. ADAM10 in AD

The alpha-secretase role is deeply intricated in synaptic physiology and also observed to be modulated in an activity-dependent manner by synaptic activity. ADAM10 has many further functions through the cleavage of its substrates such as promoting hippocampal neurogenesis, the homeostasis of neuronal networks and axonal guidance. AD pathological mechanisms act on neurons by first degradation the synaptic transmission.[26] Evidences show that synaptic dysfunction plays a central role in AD, since it drives the cognitive decline and it is not just a consequence of cell death.[69] AD patients, cognitive decline has a stronger correlation to synapse loss rather than to neurofibrillary tangles or neuronal loss.[67] The production of the Aβ oligomer act on synaptic transmission leading to dysfunction of the synapse. Indeed, pathological Aβ levels and Aβ oligomers may indirectly cause a partial block of NMDA receptors and shift the activation of NMDA receptors-dependent signaling cascades toward pathways involved in the induction of LTD and synaptic loss.[82] This effect on NMDAR and synaptic transmission would affect ADAM10 well function through diverse mechanisms.

Recent studies, showed that ADAM10 trafficking mechanism and ADAM10/SAP97 association are involved in AD pathogenesis. Interestingly, ADAM10 synaptic levels and ADAM10/SAP97 association are reduced in the hippocampus of AD patients at an early stage of disease.[123] Furthermore, interfering with the ADAM10/SAP97 complex for 2 weeks by means of a cell-permeable peptide strategy in mice is sufficient to increase amyloid levels and leads to the reproduction of initial phases of sporadic AD.[124] Together with the exocytosis, ADAM10 internalization triggered by ADAM10/AP2 association has also

pathological relevance. In fact parallel to a defect in ADAM10 binding to SAP97 a concomitant increase in ADAM10 association to AP2 in the hippocampus of AD patients has been reported.[123] Results suggest that in early stages of the disease, the reduction of α-secretase synaptic localization and activity is due to a defect in ADAM10 exocytosis/endocytosis processes rather than to an alteration of its expression.[118]

Regulation of the interaction between the sheddase ADAM10 and its binding partners SAP97 and AP2, represents a clear physiological mechanism by which the activity of ADAM10 at the synapses can be modulated. This potential action on the sheddase could upregulate ADAM10 synaptic levels and thereby, its activity at the synapse. The balance between these two partners association is impaired in AD patient's hippocampus at early stages of disease, leading to a reduction of ADAM10 levels at the postsynaptic compartment.[123] This loss could affect also activity-dependent synaptic plasticity in AD. In light of the role of ADAM10 in Aβ production and synapse function, this loss of balance could affect both APP processing and lead to formation of a downward spiral towards production of more Aβ leading to more unbalance. In this frame 2 mutations have been identified as leading to attenuation of a-secretase activity and shifting APP processing towards β-secretase mediated cleavage increasing Aβ plaque load. On the contrary, ADAM10 expression has been shown to potentiates hippocampal neurogenesis.[125] All those indicators lead to ADAM10 as having major implications in AD synaptic pathology. Among those implications, ADAM10 activity on N-cadherin at synaptic sites could contribute to spine remodeling and availability/stabilization of active iGluRs. Inhibition of ADAM10 activity on N-cadherin induced a significant increase in size of dendritic spines and a modification of the number and the currents of synaptic AMPA receptors. In this perspective, ADAM10 represents a clear new therapeutical target.

4. Drug development

4.1. Available therapeutics

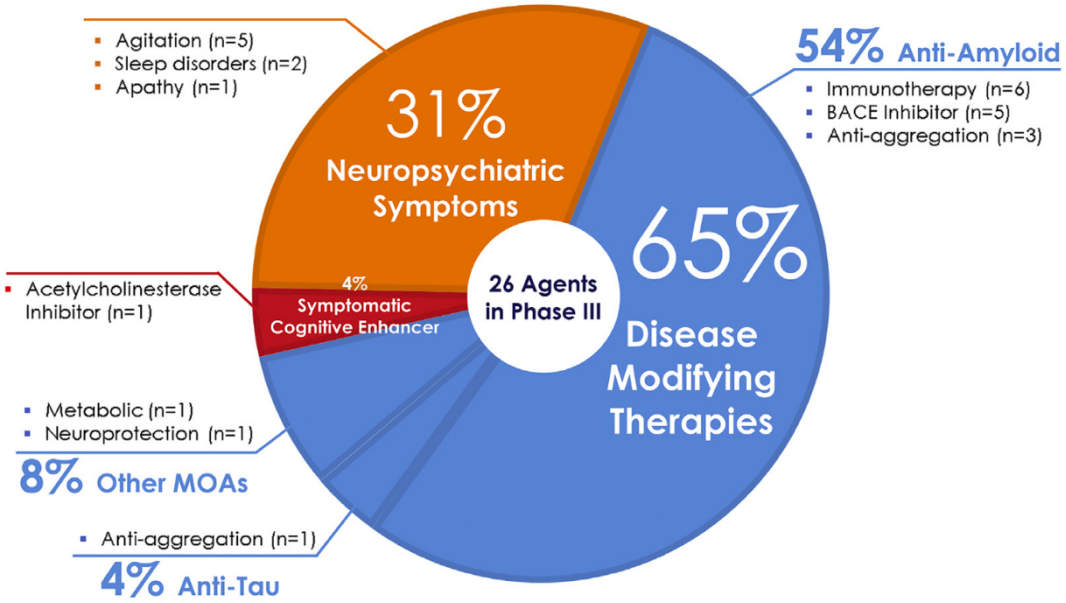
AD drug development has been very active during the last decade, with at least 25 amyloid related drugs tested. This intense research effort has not yet provided with a real cure for the disease nevertheless research efforts have already produced effective drugs capable of improving symptoms. Among currently available drugs for treatment of the pathology are

acetylcholinesterase inhibitors. Early research has shown that the process of neurodegeneration in AD affects the basal forebrain by decreasing the number of cholinergic neurons and the synthese of acetylcholine.[126] Since the cholinergic system was known to be implicated in cognitive processes such as learning and memory, a strategy to increase the cholinergic transmission was developed. This strategy using Cholinesterase Inhibitors (CI) slow down the rate by which acetylcholine present in the synaptic cleft is degraded. Three FDA-approved CIs are currently on the market to treat patients suffering from mild to moderate AD: Donepezil (Pfizer, New York, USA), Rivastigmine (Novartis, Basel, Switzerland) and Galantamine (Janssen Pharmaceutica, Beerse, Belgium).[127] CI drugs are the standard treatment first administrated to AD patients, however they are not disease modifying agents. After later research, a N-methyl d-aspartate receptor antagonist was developed as new treatment option for moderate to severe AD. The drug named memantine (Lundbeck, Valby, Denmark) acts as an uncompetitive NMDAR antagonist with moderate affinity. This compound protects neurons against excitotoxicity.[128] Clinical trial studies of 6 months treating with memantine have shown improvement of cognitive functions and overall behaviors in patients suffering from moderate to severe AD. Supporting these results, a review of six randomize controlled trial showed that NMDAR antagonist memantine can help decrease the psychological and cognitive symptoms of the pathology. The most frequent side effect observed in memantine trials were dizziness, headache and confusion. This compound is also known to potentially develop agitation in a small group of patients.[129] In most cases, a combination therapy of CIs and Memantine is prescribed to help patients. Randomized Clinical Trial studies on patients suffering from moderate to severe AD demonstrated a significant improvement of the cognitive function of the patients when treated with a combination of memantine and donepezil compared to a group treated with memantine and placebo.[130] Unfortunately no improvement was observed in patients suffering from mild to of therapeutics contribute minimal impact on the disease and target late aspects of the disease. They slow the evolution of the pathology and provide symptomatic relief but fail to achieve a definite cure that would stop the progression.[131]

4.2. Current Clinical trials

Drugs targeting different actors of the pathology are still under extensive research with some of them already reaching clinical trials. Currently in the AD treatment pipeline, there are 112

compounds being tested. Phase III counts 26 compounds that are in 35 different trials. Phase II counts 63 compounds in 75 different trials. Phase I counts 23 compounds in 25 different trials. Among agents in phase III clinical trials, 65% are disease-modifying strategies addressing amyloid, tau or using other mechanisms of action (MOAs), 4% are cognitive enhancers acting as acetylcholinesterase inhibitors, and 31% are compounds addressing the neuropsychiatric and behavioral symptoms such as agitation and apathy (figure-11). [131]



(Figure 11) percentage representation of the different mechanisms of actions of agents currently in development phase III. 65% of those agents are disease modifying therapies. Among these drugs 8% focus on neuroprotection and metabolic issues, 4% are ant-tau based on anti-aggregation mechanism of action, 54% are considered as anti-amyloid using immunotherapy, anti-aggregation as mechanisms of action, also BACE inhibitors. The rest is composed at 31% of neuropsychiatric agents focusing on symptoms such as agitation, sleep disorders or apathy. Last 4% are cognitive enhancers acting as acetylcholinesterase inhibitors to improve symptoms. (Scheme credit: Zhong et al, 2018)

Disease modifying compound represents most of the research efforts now as strong the need for such a drug able to stop the disease increases by the day. Those treatment targets the main elements of the disease trying to block or counteract the pathological mechanisms.

Several compounds that could inhibits the activity of the γ-secretase in the brain have been developed. Such strategy could reduce the activity of the sheddase and therefore decrease the Aβ production. However, γ-secretase has many essential biological roles through its action on its substrates such as the signaling protein Notch known to be implicated in the proliferation and differentiation of progenitor cells. Experimentation on transgenic mice demonstrated that inhibitors of the γ-secretase at sufficient doses to decrease Aβ production prevented the differentiation of lymphocytes representing a clear safety issue for this type of agent.[132]

Scientific research on agents able to block the β-secretase enzyme (BACE) are also in the preclinical development phase. Derivative compounds from 11 *N*-phenyl-1-arylamide, *N*-phenylbenzenesulfonamide were developed and experimentally tested for their β-secretase inhibitory activities.[133] Neocorylin compound with a potential β-secretase inhibitory effect was recently isolated from extract of *Psoralea corylifolia* L. (Fabaceae). In vitro this agent exhibits significant inhibitory effects on purified baculovirus-expressed BACE-1 in a dose-dependent manner.[134] In large Randomized clinical trials inhibitors of BACE1 blocked dose-dependently the formation of Aβ peptide in CSF of AD patients. However, these agents did not show any improvement in terms of cognition and psychology of the patients. BACE inhibition may be not sufficient to decrease brain Aβ plaques in advanced stage of the disease. Evidences suggest that the optimal timing for treatment with BACE1 inhibitors should be before appearance of the first cognitive symptoms. The limitation of such preventive treatment still represents a crucial issue.[135] finally, the location of BACE1 in the brain and in the lumen of endosomes makes the development of effective BACE1 inhibitors challenging as inhibitors need to cross the blood-brain barrier and neuronal membranes to access the target.

Immunotherapy targeting tau proteins or amyloid plaques are extensively studied. Approaches using vaccination against tau is considered complicated because of the intracellular localization of tau protein. Regarding plaque load the promising antibody Gantenerumab, developed by Chugai Pharmaceuticals and Hoffmann-La Roche, is being investigated in two Phase III studies to assess the safety and efficacy of subcutaneous administration for the treatment of early AD patients. This IgG1 monoclonal antibody designed to bind to aggregated forms of Aβ has been previously demonstrated to lower amyloid plaque levels in AD patients. The ongoing clinical trial is enrolling more than 1,500 patients in more than 30 countries worldwide and represents the only anti-amyloid program being developed with subcutaneous administration. The possibility to use this route of administration could enable home administration for patients affected by this disease. The promising data readout of this anti-amyloid clinical trial is expected in 2022.

Recently drug starting to target the alpha-secretase have been also studied. French company ExonHit therapeutics recently developed Etazolate (EHT 0202, ExonHit Therapeutics) that is described as an enhancer of the α-secretase activity pathway and via its action on the non-

amyloidogenic pathway inhibits Aβ-induced neuronal death, thus leading to symptomatic and effect.

4.3. The hope for future drugs

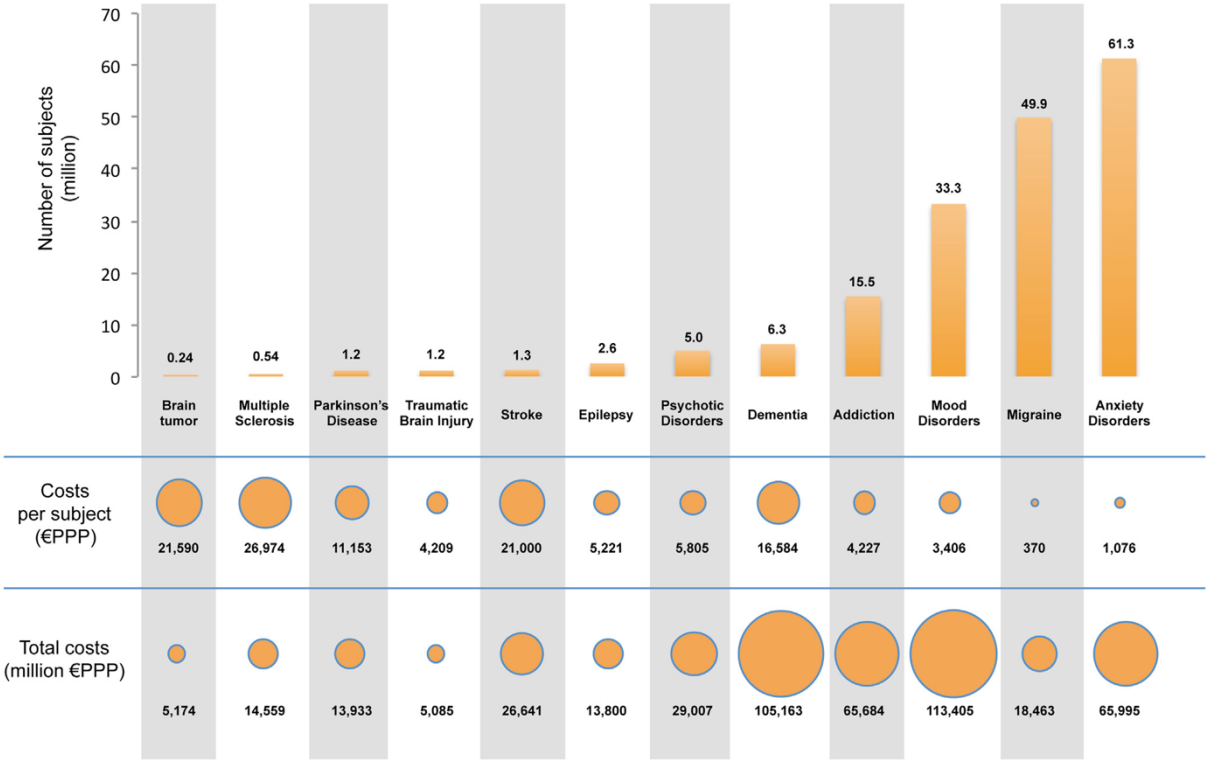
Increasing evidence points towards the major alpha-secretase ADAM10 as a good therapeutic target for development of future drugs. The sheddase is known to reduce the generation of Aβ but may also affect the AD pathology through potential mechanisms including maintaining normal synaptic functions, promoting hippocampal neurogenesis and the maintaining homeostasis of neuronal networks. The sheddase ADAM10 modulates these functions by processing its brain substrates such as postsynaptic cell receptors and adhesion molecules. In light of these considerations a new agent has been developed.

The drug Etazolate known as compound EHT0202 (exon hit therapeutics, Paris, France) is an alpha secretase potentiator that stimulates the a-secretase neurotrophic activity in the non-amyloidogenic pathway and inhibits β-secretase related neuronal death. *In vitro*, the compound is neuroprotective against toxic Aβ42 as associated with sAPPα induction effect. After a phase I study in healthy volunteers, a phase II clinical trial has been recently completed. This clinical trial assessed safety, tolerability and preliminary efficacy on cognition and behaviour in AD patients, as well as quantification of sAPPα in blood.[136] Etazolate clinical trial, conducted in 159 patients with mild to moderate AD pathology, was observed to be safe and well tolerated by the patients. Now trying to test the peptide on a longer period of time and on a larger cohort of patients to confirm its efficacy and tolerability. A reduction of the symptoms of 30% was observed in patients treated with the drug compared to placebo.[137]

This promising compound that could have both symptomatic effect and disease modifying effect by increasing the production of the alpha-secretase together as well as modulating GABA-A receptor and PDE-4 inhibitor could represent a way to fight AD. In this frame, our study tries to develop an alpha secretase enhancer based on ADAM10 local trafficking in AD patients.

AIM

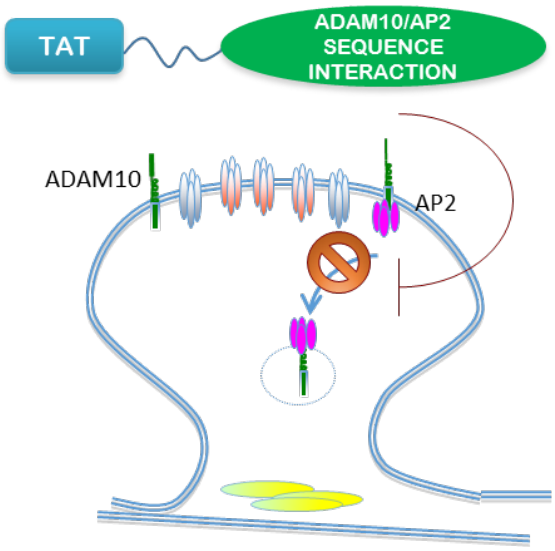
AD is still representing a major burden for society. In Europe the cost per year of dementia is in fact estimated to account for 105 billion euros. These numbers are set to growth due to the increase in life expectancy, thus bringing the problem to the level of unsustainability. Therefore, finding a cure for AD represents an imperative for modern medicine.



(Figure 12) The cost of dementia reaches an estimated 105 billion euros in Europe. (Representation credit: DiLuca and Olesen, Neuron 2014).

This thesis challenged the hypotheses that ADAM10 trafficking at the synapse is a relevant therapeutic target in AD. The increase of the ADAM10 activity could (I) counteract the action of BACE thus reducing Aβ formation (II) increase the production of the neuroprotective sAPPα fragment. In light of these considerations, the main goal was to stabilize ADAM10 levels and activity at the synaptic membrane by disrupting ADAM10/AP2 complex, thereby interfering with the internalization process of the sheddase. To this, we made use of cell permeable peptides already validated in the lab able to disrupt ADAM10/AP2 complex.

These cell permeable peptides are composed of a TAT sequence and of the sequence responsible for AP2/ADAM10 association (Figure 13). Four CPPs were developed and two of them (PEP2 and PEP3) were able to disrupt ADAM10/AP2 complex. Preliminary data have demonstrated that the most efficient CPP (PEP3) is capable to specifically interfere with ADAM10/AP2 association and to increase ADAM10 synaptic localization in vitro. PEP3 was selected to perform further in vivo tests.



(Figure 13) Representative image of the CPP and the therapeutic strategy used. We aim at blocking ADAM10 endocytosis by using a peptide that comprises ADAM10/AP2 sequence interaction. This peptide interacts with AP2 and therefore avoid its binding on the cytoplasmic tail of the secretase and its internalization.

The specific aim of the thesis was to verify cell peptide candidate efficacy in a mouse model of AD (APP/PS1 mice) at different time points and diseases stages, assessing specific molecular, structural and behavioral outcomes.

Furthermore, we aimed at improving the CPPs pharmacokinetics profile by developing and testing the peptidomimetics in collaboration with Pr. Belvisi (University of Milano) and Dr. Di Marino (University of Lugano).

MATERIALS & METHODS

1. ANIMALS

Animals that have been used in the frame of this project for *in-vivo* testing of the peptide were male/female C57/BL6 mice and AD model APP/PS1 mice of 6- 9- and 12-month-old. For *in-vitro* experiments, rat embryos at embryonic day 18 (E18) from Sprague-Dawley rats, have been used for the purpose of primary hippocampal neuron cultures. The handling of all these animals and the potential surgical procedures were performed with great care taken with the ultimate goal to attenuate as much as possible pain and discomfort. The Institutional Animal Care and Use Committee of University of Milan and the Italian Ministry of Health approved all the experiments involving primary neuronal cultures preparation (#326/2015) and mice treatments (#497/2015). All experiments using animal models were performed following the ethical guidelines and regulations of the European Parliament and of the Council on protection of animals used for scientific purposes (Directive of 22 September 2010, 2010/63/EU).

2. PRIMARY HIPPOCAMPAL NEURONS

In-vitro experiments carried out in the frame of this project were performed on neuronal culture. The primary hippocampal neuron cultures were prepared using rat hippocampi at embryonic day 18 (E18) and following protocol (Piccoli et al., 2007) as previously described in the literature. The cultured neurons at DIV15 were then either treated with active or inactive CPP (PEP3) and lysed for further biochemical experiments or transfected for imaging.

3. TREATMENTS WITH CELL-PERMEABLE PEPTIDES

The CPP were developed by coupling the active peptides sequence with HIV-1 TAT (Trans-activating transcriptional activator) derived peptide. The developed peptide is secured under patent No.102017000149130. This short basic peptide derived from HIV-1 virus is able to deliver successfully a large variety of cargoes such as peptides, proteins and nucleic acids by

overcoming the lipophilic barrier of the cellular membranes. The active domain of the CPP is comprised in a short (11 amino acids) organization of basic amino acids with the sequence YGRKKRRQRRR (Ruben et al 1989). After studies on the interaction domain between the sheddase ADAM10 and heterotetrametric complex AP2 we have developed and used an active peptide containing the sequence RQR and the inactive alternative peptide EQE containing a modified sequence in which arginine residues have been replaced by glutamic acids. Adult male and female wild-type mice and transgenic mice have been treated with active CPP or its inactive alternative using concentration of 3nmol/g in sterile saline. Peptides were administered for 14 days with a daily intraperitoneal injection respecting a precise interval of 24 hours between each daily injection. Following the treatment of the animals, brains were dissected and rapidly frozen using dry ice prior to be stored at -80°C or pre-fixed in paraformaldehyde prior to be used for further experiments.

4. TREATMENTS OF PRIMARY HIPPOCAMPAL NEURONS

For in-vitro treatments, primary hippocampal neuron plated in 60mm petri dish (750,000 cells/Petri dish) were incubated at DIV14 at 37°C, 5% CO_2 with the respective CPP at a concentration of 1µM in 2 mL of Neurobasal medium supplemented with B27 for a duration of 30 minutes prior to be lysed and/or purified to triton postsynaptic fraction for further experiments.

5. HOMOGENIZATION AND PURIFICATION OF POSTSYNAPTIC FRACTIONS

The Triton Insoluble Fraction (TIF) is a subcellular fraction dense in synaptic proteins present in the PSD (Receptor subunits, signaling molecule, scaffolding proteins, and cytoskeletal elements) and absent of any presynaptic markers. Subcellular purification was performed and TIF was isolated from mouse half brains and hippocampi after CPP treatment. The animals were sacrificed and half brains together with hippocampi were rapidly dissected. In order to purify the TIF fractions from half brain, homogenization of the samples was

performed at 4°C using 2,5 mL of ice-cold buffer containing protease inhibitors (Complete™, GE Healthcare, Mannheim, Germany), phosphatase inhibitors (PhosSTOP™, Roche Diagnostics GmbH, Mannheim, Germany), 0.32 M Sucrose, 1 mM Hepes, 1 mM NaF, 0.1 mM PMSF, 1 mM $MgCl_2$, 1mM $NaHCO_3$ using a hand-held glass-teflon tissue homogenizer. An aliquot of homogenate was kept for further analysis of protein levels via Western Blotting. In order to further purify the fraction, the centrifugation of the homogenates was performed at 1,000g for a duration of 5 minutes at 4°C in order to remove white matter and potential nuclear contamination. The supernatant (S1) obtained was further centrifuged at 13,000g for a duration of 15 minutes at 4°C. The obtained pellet (P2 crude membrane) was then resuspended using hypotonic buffer (1 mM Hepes containing protease inhibitors (Complete™, GE Healthcare)) and then further centrifuged at 100,000g for 1 hour at 4°C. Resuspension of the obtained pellet was performed using glass-glass tissue homogenizer prior to let rest the solution at 4°C for 15 minutes in the extraction buffer (1% Triton-X-100, 75 mM KCl and protease inhibitors (Complete™, GE Healthcare)). After extraction, further centrifugation of the samples was performed at 100,000g for 1 hour at 4°C. The resulting TIFs were resuspended in 20 mM HEPES buffer containing protease inhibitors (Complete™, GE Healthcare) using glass-glass homogenizer.

In order to purify the TIF fractions from hippocampi a shorter protocol has been used as volumes processed were smaller. Homogenization of the samples were performed at 4°C using 800 uL of ice-cold buffer containing protease inhibitors (Complete™, GE Healthcare, Mannheim, Germany), phosphatase inhibitors (PhosSTOP™, Roche Diagnostics GmbH, Mannheim, Germany), 0.32 M Sucrose, 1 mM Hepes, 1 mM NaF, 0.1 mM PMSF, 1 mM $MgCl_2$, 1mM $NaHCO_3$ using a hand-held glass-glass tissue homogenizer. An aliquot of homogenate was kept for further analysis of protein levels via Western Blotting. In order to further purify the fraction, the centrifugation of the homogenates was performed at 13,000 g for a duration of 15 minutes at 4°C. Resuspension of the obtained pellet was performed using glass-glass tissue homogenizer prior to let rest the solution at 4°C for 15 minutes in the extraction buffer (1% Triton-X-100, 75 mM KCl and protease inhibitors (Complete™, GE Healthcare)). After extraction, further centrifugation of the samples was performed at 100,000g for 1 hour at 4°C. The resulting TIFs were resuspended in 20 mM HEPES buffer containing protease inhibitors (Complete™, GE Healthcare) using glass-glass homogenizer.

In order to purify the TIF fractions from primary hippocampal neurons, cells were scraped in ice-cold lysis buffer prepared with 0.32 M sucrose, 1 mM Hepes, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 1 mM NaF, 0.1 mM PMSF pH 7.4 and homogenized using glass-glass homogenizer. Homogenates from cells were then centrifuged at 1,000g for 15 minutes at 4°C. The obtained pellet was then resuspended in 1 mM Hepes buffer containing Complete™ using glass-glass homogenizer. In order to purify the TIF, further centrifugation was performed at 13,000g for 15 minutes at 4°C. The pellet obtained was resuspended in 150mM KCl, 0.5% Triton and centrifuged at 100,000g for 1 hour at 4°C. The TIF obtained was then resuspended in 20mM Hepes buffer containing protease inhibitors (Complete™, GE Healthcare) using a glass-glass homogenizer. Finally, all protein samples obtained from these protocols of purification have been assessed using Bio-Rad protein assay (Hercules, CA, USA).

6. PURIFICATION OF SOLUBLE FRACTION

In order to quantify the production of soluble APPα fragment generated by ADAM10 activity, the Cortex of the mice was homogenized, in an ice-cold lysis buffer prepared with EDTA (2mM), EGTA (1mM), PMSF (0,1mM), Hepes (25mM), protease inhibitors Complete™ (1X) and phosphatase inhibitors PhosSTOP™ (1X), using a teflon-glass tissue homogenizer at 4°C. The homogenates obtained were then centrifuged for a duration of 10 minutes at 10,000g in 4°C cold room. The resulting supernatant (S1) was then further centrifuged at 4°C for a duration of 1 hour at 100,000g in order to obtain the soluble fraction (S2). The resulting soluble fraction (S2) obtained was then used for further ELISA experiments.

7. ELISA ASSAYS

To assess soluble Aβ and soluble APPα levels in treated animals we have used different ELISA assays for human or mouse/rat metabolites. At early stage of the disease, assessment of humanized sAPPα and sAβ was performed from cortex together with an assessment of mouse/rat sAPPα from hippocampus. At late stage of the disease, assessment of only humanized sAPPα and sAβ from cortex was performed. This experiment was performed using respectively soluble fraction (2S) of the cortex of the animals or Homogenates of the hippocampus. 100 µL of prepared standard and test samples were added to the wells. Plate was Covered and incubated at 4°c overnight. The next day, liquid was discarded and wells were wash 4 times. 100 µL of diluted detection antibody were added to the wells and incubated at room temperature for 1 hour. liquid was discarded and wells were wash 5 times. 100 µL of diluted HRP conjugate were added to each well and incubated at room temperature for 30 minutes. After a last set of 5 washes, 100 µL of chromogenic substrate were added to each well. Plate was then developed at room temperature in the dark for 30 minutes. After this time, 100 µL of stop solution was added to each well. The plate was then evaluated within 30 minutes of stopping the reaction. The absorbance of each well was read at 450 nm and. A curve-fitting statistical software was used to plot a four-parameter logistic curve fit to the standards and then calculate concentrations for the test samples. The concentration of the protein of interest was normalized on the total protein concentration and adjusted according to the dilution factor used during preparation of the test samples.

8. ELECTROPHYSIOLOGY ASSAY

Electrophysiological screening of a potential effect of the peptide was performed in JANSSEN Pharmaceuticals in Belgium during a secondment. Recordings were performed using four setups run simultaneously of 64-channels Micro electrode array system (12µm ø; 200 µm gap) (Multi Channel System company, Reutlingen, Germany). Acute Sagittal hippocampal slices from treated 6-month-old APP/PS1 mice (54 mice), with CA3 region cut out to avoid epileptic loops, were placed on the electrodes of the microchips. A stimulation of 75% of I/O maximum response was set. Stimulation was applied in CA2 Schaffer collaterals

and field-Excitatory Postsynaptic Potential were recorded from CA1 pyramidal neurons. Using different blockers, the components of the responses were isolated and recorded. The AMPA components were recorded during diffusion of nACSF containing bath. The NMDA components were recorded during diffusion of nACSF 0,5µM Mg2+, NBQX 10µM (Sigma-aldrich company, USA) and PTX 10µM (Sigma-aldrich company, USA) containing bath. The GluN2A containing NMDA components of the responses were recorded during diffusion of nACSF + 0,5µM Mg2+ (Sigma-aldrich company, USA), NBQX 10µM (Sigma-aldrich company), PTX 10µM (Sigma-aldrich company, USA), and Ro25 10µM (Hello Bio company, UK) containing bath. Signals were then all blocked using APV 50µM (Sigma-aldrich company, USA) to make sure responses obtained were not composed of artefacts. Analysis were performed considering peak response or area of the response. Effect of Input Output protocols with incrementation of 5µA from 5 to 100 µA were also recorded. Recording software MC Rack was used (Multi Channel System Company, Reutlingen). Analysis were performed using Igor Pro 6.37 software (Wavemetrics company).

9. DENDRITIC SPINES LABELING

Carbocyanine dye DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate ('dii'; $diic_{18}(3)$)) (Invitrogen) is a lipophilic membrane stain that diffuses laterally to stain neurons. It is weakly fluorescent until incorporated into membranes. The powder form mixed into an inert, water-resistant gel was used to stain the spines of the treated mice. The following protocol used for spines labeling was previously published (Vicini & Bregman 2007). First, the intra-cardiac perfusion of the animals through the right atrium was performed with PBS 0.1M (room temperature) for about 10 minutes and then with cold 1.5% PFA in PB 0.1M. The brain was post-fixed during 40 min in 1.5% PFA in PB 0.1M at 4°C and then washed twice with PB 0.1M. The brain was cut coronally around the hippocampal region making slices of around 2-3mm in order to have the beginning and end of the hippocampus visible. Application of the DiL crystals was performed by touching delicately the region of interest on both sides of the coronal slice with needle. The Dil was left in PB 0.1M covered from the light and at room temperature to diffuse for at least 12 hours. The Slice was then fixed in 4%PFA in PB 0.1 M for 45 minutes at 4°C. Coronal slices of 100-150 um were prepared in PB 0.1M on ice using a vibratome. Slices were finally mount on glass

slides with Fluoromount^{TM} mounting medium (sigma-aldrich) and seal with nail polish. Fluorescence images from slides were then obtained using a Confocal microscope Zeiss LSM510 Meta system with an objective 63X performing sequential acquisition at a resolution of 1024x1024 pixels.

10. CO-IMMUNOPRECIPITATION ASSAYS

To immunoprecipitate ADAM10/AP2 protein complex of interest, samples were first prepared in a RIA buffer containing 50mM Tris HCl (pH 7.2), 150mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulphate for a volume final of 150µl. Added to the samples, 1µl of ADAM10 antibody to precipitate ADAM10 complex. Samples were then incubated overnight at 4°C. A control sample was also prepared by incubating in absence of the ADAM10 antibody for protein of interest. After the first phase of incubation, a well-mixed volume of 20µl of A/G beads (Santa-Cruz, Protein A/G Sepharose beads) were added to the samples. The samples were then further incubated at room temperature for 2 hours on a rotating wheel. Three sets of centrifugations at 12,000g for 5 minutes, together with washes with 200ul of RIPA buffer were performed in order to clear beads from unbonded proteins. Finally, stop-mix (Containing β-mercaptoethanol) for SDS-PAGE was added to the samples and boiled for 10 minutes at 100°C. Prior to load samples, beads were pull-down by short centrifugation, and the supernatant was then loaded into 7% SDS-PAGE gels and developed via western blot.

11. WESTERN BLOTTING

Protein samples from homogenates or TIF fractions were prepared and loaded into 7% SDS-PAGE gels to be separated by electrophoresis at 20mA. Following the electrophoresis, the proteins spread on acrylamide gels have been transferred to a 0.45 mm nitrocellulose membrane by running them at 240mA for 2 hours in a buffer containing 20% Methanol and

1X Blotting buffer (Tris 0,025 M, glicina 0,192 M, MeOH 20%, pH 8.3). After transfer, the nitrocellulose membranes have been incubated in iBlock-TBS (Invitrogen, T2015) for at least 30 minutes. After blocking phase, membranes were subsequently incubated overnight at 4°C in primary antibody prepared in iBlock-TBS. After overnight incubation the membranes were washed at room temperature three times for a duration of 10 minutes in Tris-Buffered Saline/Tween20 (TBS-T). After washes, membranes have been incubated for 1 hour at room temperature in horseradish peroxidase-coupled (HRP) secondary antibody (BioRad Laboratories) prior to be washed again three times in TBS-T for 10 minutes at room temperature. For bands detection, Clarity™ ECL Solution (BioRad Laboratories) was applied for 5 minutes on membranes prior to exposition with chemiluminescence trans-UV (302nm) using Chemidoc™ gel imaging System (Bio-Rad Laboratories).

12. CONFOCAL STUDIES

To acquire images of co-localization to test membrane crossing, hippocampal neurons were first transfected to express DsRed and imaged live at room temperature. neurons were plated on Polylysine-coated Matek dishes at 75,000 cells/well. Transfection was realized between 7 and 10 DIV and was performed using $Ca2^{+}$-phosphate DNA transfection protocol. Previously present Gibco™ Neurobasal™ medium in Matek dishes was replaced with Gibco™ Mem, Glutamax™ (thermos fisher scientific). DNA precipitate was prepared by adding DNA (for 2μg/well) drop by drop in HeBS (Hepes Buffer Saline solution) pH 7.05 containing: 274 mM NaCl, 10 mM KCl, 1.4 mM $Na2HPO4$, 15mM D-Glucose and 42 mM Hepes; while being constantly vortex and let rest in the dark 30 minutes at room temperature. Solution was then transfer to the Matek dishes and let to rest 15 minutes at 37°C.

Microscope was then used to assess presence of residual precipitate. At least 2 washes were performed and repeated until complete removal of the precipitate. Matek dishes were placed in live imaging chamber and CO_2 (5%) together with temperature (37.5°C) was constantly computer-monitored. The PEP3 expressing fluorescein was added to Matek dishes at a concentration of 1μM and Fluorescence images from cells were then obtained using a Confocal microscope Zeiss LSM510 Meta system with an objective 63X performing sequential acquisition at a resolution of 1024x1024 pixels. Each image contained up to four

0.5µm sections and Z stack projection was performed (Malinverno et al., 2010). The SP5 CLSM system (Leica-microsystems, Germany) equipped with diode (405nm), argon (488nm) and diode pumped solid state (561nm) lasers was used for live imaging of PEP3 membrane crossing test. Images were acquired using l20x/1.00w objective (Leica, Germany).

13. ANTIBODIES

Primary and secondary antibodies used for western-blot, Co-immunoprecipitation or confocal studies in the frame of the project are the following: Polyclonal antibody (pAb) anti-ADAM10 (rabbit) and mAb anti-GluA1 p845 (rabbit) were purchased from Abcam Company; anti-ADAM10 (rat) was purchased from R&D; mAb anti-α-adaptin, mAb anti-β2-adaptin, mAb anti-µ2 and mAb anti-N-Cadherin CTF (C-Terminal Fragment) were purchased from BD Science Company; mAb anti-Notch1 was purchased from Cell Signaling Company; mAb anti-tubulin and pAb anti-GluN2A were purchased from Sigma Company; mAb anti-GluR1, mAb anti-GluN2B, mAb anti-GluN1, mAb anti-GluA1 total and mAb anti-PSD-95 were purchased from Neuromab Company. Peroxidase-conjugated secondary anti-mouse Ab were purchased from the Pierce Company. The Peroxidase-conjugated secondary anti-rat Ab were purchased from the Abcam Company. Peroxidase-conjugated secondary anti-rabbit Ab were purchased from the BioRad company.

14. Y-MAZE TEST

The Y-maze test assesses the ability of the rodents to discriminate a novel environment. This test involving the hippocampus does not contain any reinforcements as its methodology evaluates the natural propensity of mice to prefer and thereby explore more novelty. The task procedure of the test consists of two phases: Sample trial, and test trial. During the sample trial, the mouse is placed in the Y-shaped apparatus with 2 arms open for a duration of 5 minutes. After a retention delay of 30 minutes, the animal is returned for a duration of 5 minutes in the same Y-maze for the test trial, phase during which a third arm has been open. The process of exploration of the mice is captured live during the test using a computer-

assisted recording system. Only the exploration time of the Novel and familiar arms were considered as the statistical analysis was realised calculating the Index of preference for either the Familiar arm (*time familiar/(time familiar + time novel*)) or Novel arm (*time novel/(time familiar + time novel*)). Prior to include mice in the analysis, well performance in terms of total exploration time and freezing time were checked to be in accordance with preset threshold.

15. NOVEL OBJECT RECOGNITION TEST

The novel object recognition test (NORT) assesses the ability of the rodents to discriminate a novel object. As in the Y-maze test, the NORT involves the hippocampus and does not contain positive or negative reinforcers. This test evaluates the natural propensity of the mice to prefer and thereby explore more a Novel object compared to a Familiar object. Prior to performing the test, mice were handled for few minutes for at least 3 days for habituation of the mice to the experimenter. The protocol of the test consists of two phases: A first phase of familiarization and the actual test phase. During the familiarization phase, the animal was exposed to two identical sample objects for 10 minutes directly in its cage. After a retention delay of 24 hours, the mice were exposed for a duration of 10 minutes to one of the Familiar objects and introduced to a Novel object. During the different phases of the test, the objects were placed in the opposite sides of the cage, alternating the position of the respective objects. The process of exploration of the mice is captured live during the test using a computer-assisted recording system. Only exploration Time of Novel and familiar objects were taken in consideration as the analysis was performed by calculating the Index of preference for either the Familiar object (*time familiar/(time familiar + time novel*)) or Novel object (*time novel/(time familiar + time novel*)). Prior to include mice in the analysis, well performance in terms of total exploration time and freezing time were checked to be in accordance with preset threshold.

16. STATISTICAL ANALYSIS AND QUANTIFICATION

The quantification of western blotting analysis was performed using a Chemi-fluorescence image software (ImageLab, Biorad Company). The different protein levels were quantified as optical density (OD) read-outs and were further normalized on tubulin protein OD. However, the OD of the C-terminal fragment of N-cadherin and the Phosphorylated p845 Glua1 subunit were respectively normalized to the full-length levels and on non-phosphorylated form levels. In order to quantify the co-immunoprecipitation of ADAM10/AP2 complex, the different subunits of AP2 were normalized on the OD of ADAM10 total levels. The different values have been expressed as mean ± Standard Error of the Mean (SEM). Annotated N numbers represent the number of animals used for the *in vivo* experiments while when testing *in vitro* N numbers represent the replicates of the experiments. Spines morphology analysis was performed using Image-j software. Statistical tests for the significance of the data were performed as appropriate, by Student t-test or by One-way or Two-way ANOVA followed by Bonferroni Post-hoc test (Two-way ANOVA using data from four experimental groups: Tg PEP3, Tg inPEP3, WT PEP3, WT inPEP3). Finally, graphic representations have been realized using Prism software.

RESULTS

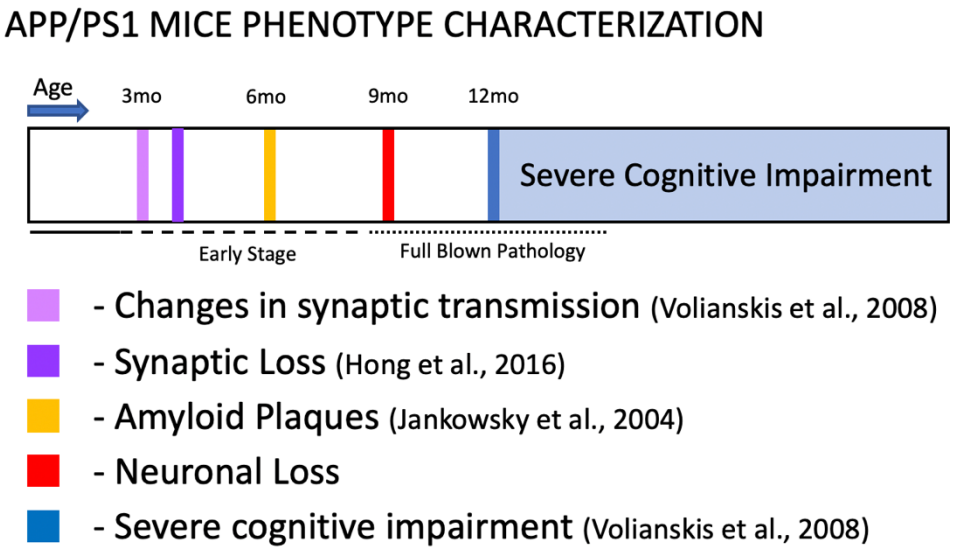
1. Testing PEP3 treatment at full-blown stage of Alzheimer's disease in APP/PS1 mice.

1.1. Alzheimer's disease mouse model: APP/PS1 mice

Previous *in-vitro* experiments performed testing 4 different CPPs in our laboratory, leaded to the conclusive observation concerning PEP3 as representing a promising agent for further *in-vivo* testing (data not shown). As first, the efficacy *in-vivo* of the selected active peptide (PEP3) was assessed in wild-type mice. The agent PEP3 was observed to cross the blood-brain barrier (BBB) as, after an acute intraperitoneal injection, the peptide was detected *via* immunohistochemistry in the brain parenchyma of the animals (data not shown). Toxicology study revealed that PEP3 did not have any toxic effect on the wild-type mice and did not lead to any systemic or organ-specific failure or damages (data not shown).

Following these tests, the present study focuses on the test of PEP3 in a mouse model of AD. The APP/PS1 mouse model of AD was used as to test PEP3 at different stages of the pathology in these animals. These transgenic mice have been generated by co-injecting two vectors encoding for a mutant APP and mutant PS1.[138] The APP sequence encodes for a chimeric mouse/human form of APP (Mo/HuAPP695swe) carrying the Swedish mutation under the mouse prion protein promoter which leads to overexpression of humanized APP protein in the CNS of these mice. The PS1 sequence encodes human presenilin-1 lacking exon 9 (dE9) under the mouse prion protein promoter leading to increased cleavage of APP and overproduction of $A\beta_{1-42}$ peptide. Therefore, these mice represent a good model of amyloid-related pathology as they develop plaques and show synaptic loss around the age of 6-month-old, which is to be considered as an early stage of the disease. At full-blown pathology, considered to initiate around 9- to 12-month-old these mice start to present strong neuronal loss associated with severe cognitive deficits. These cognitive deficits have been observed at 12-month-old by use of Morris water maze behavioural tests, however cognitive impairments are assumed to be present before this age as due to synaptic loss. (Figure-1).

Fig.1



(Figure 1) Scheme describing time-related phenotype of APP/PS1 mice used to test PEP3 in-vivo. These mice develop Amyloid Plaques and Gliosis associated with changes in synaptic transmission and synaptic loss by 6 months of age which represents the early stage of the disease. At full-blown pathology, considered to start around 9 to 12-months-old, the mice show important neuronal loss associated with severe cognitive impairment. (Scheme credit: Modified from Alzheimer forum: https://www.alzforum.org/research-models/appswepsen1de9-0)

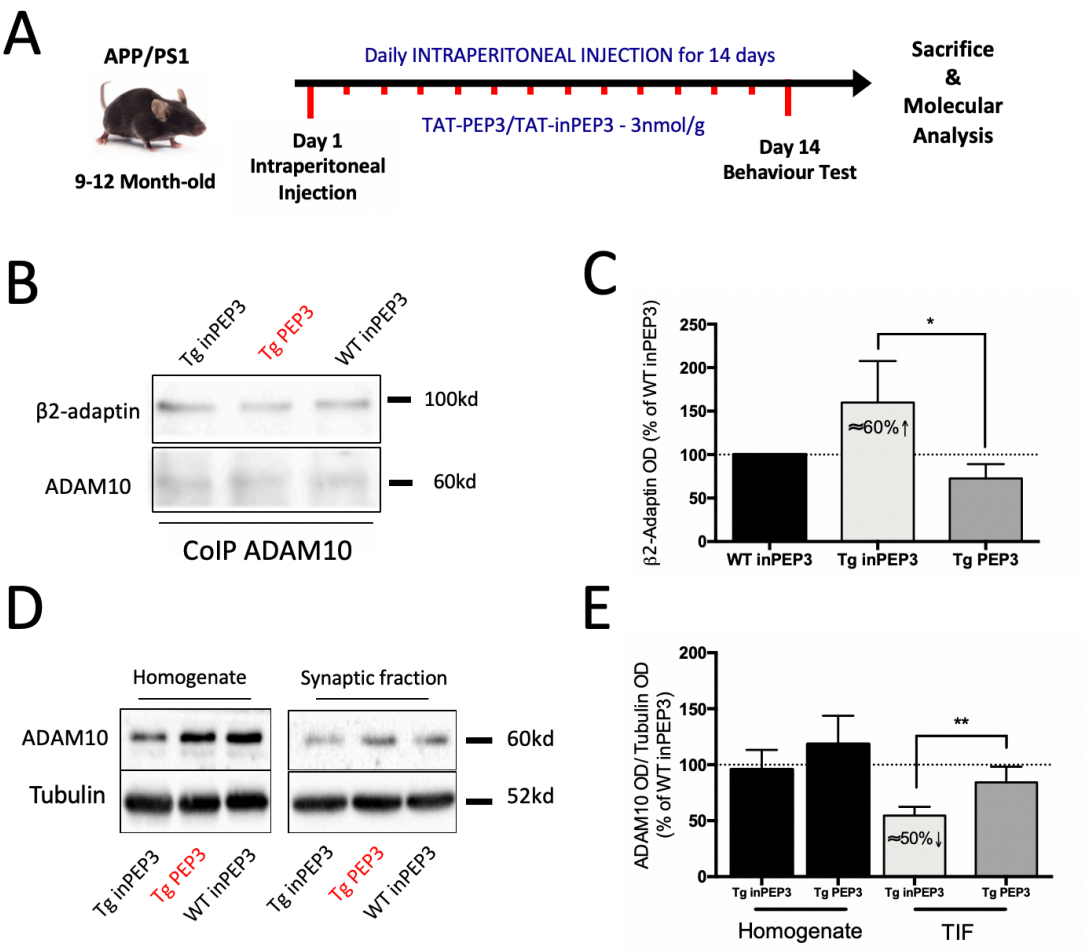
1.2. Treatment with PEP3 efficiently increases ADAM10 synaptic localization

In order to study the efficacy of the peptide to interfere with the interaction between ADAM10 and AP2 subunit β2-adaptin in this mouse model of AD, we performed a 14-days treatment of 9-month-old APP/PS1 mice, consisting of a daily intraperitoneal injection of PEP3 at 3nmol/g (Figure-2 A). An inactive peptide appropriately designed was used at the same dose as negative control in all experiments (inPEP3).

The forebrain of the animals was lysated and the homogenate was used to assess ADAM10/AP2 interaction. Co-immunoprecipitation assay was performed in order to quantify the effect of the peptide on the interaction between these two respective proteins. We precipitated the protein complex using anti-ADAM10 antibody and observed an increased association of around 60% between ADAM10 and the β2-adaptin subunit of AP2 in the transgenic mice treated with control peptide inPEP3 compared to wild-type animals. Interestingly, the treatment with PEP3 reduces the ADAM10/AP2 interaction in APP/PS1

mice to a level comparable to one of wild-type mice (Figure-2 B and C). The statistical analysis showed that the treatment of the transgenic mice with PEP3 leaded to significantly lower ADAM10/AP2 association levels (β2-adaptin Tg PEP3: 72.53% ±16.46%, N=9) compared to transgenic mice treated with inPEP3 (β2-adaptin Tg inPEP3: 159.9% ±47.78%, N=9), which presented increased ADAM10/AP2 association levels and in same manner comparable to the increased association observable in AD patients.[118]

Fig.2



*(Figure 2) Assessment of PEP3 effect on 9-month-old APP/PS1 and wild-type mice. (A) Experimental paradigm scheme of the 14-days treatment with a daily intraperitoneal injection with either PEP3 or inPEP3 at a concentration of 3nmol/g. (B) Brain homogenates were immunoprecipitated using anti-ADAM10 antibody and β2-adaptin co-precipitation was evaluated considering Optical Density (OD). (C) OD quantification of co-immunoprecipitation experiment in B. Significantly higher ADAM10/AP2 interaction was observed in the transgenic mice treated with inPEP3 (β2-adaptin Tg inPEP3: 159.9 ±47.78, N=9; Two-way ANOVA: ***p=0.0006) compared to transgenic mice treated with PEP3 (β2-adaptin Tg PEP3: 72.53 ±16.46, N=9), which presented ADAM10/AP2 interaction levels comparable to wild-type animals. (D) Representative immunoblot of ADAM10 Western blotting (WB) analysis of homogenate and TIF fraction. (E) OD quantification of WB experiment in D. No significant differences were observed in the homogenate. In the TIF fraction, OD ratio between ADAM10 and tubulin revealed a significantly increased ADAM10 synaptic availability in APP/PS1 mice treated with PEP3 (ADAM10 Tg PEP3: 84.19 ±14.07, N=9) compared to the transgenic animals treated with inPEP3 (ADAM10 Tg inPEP3: 54.58 ±7.90, N=9; Two-way ANOVA: **p=0.0081).*

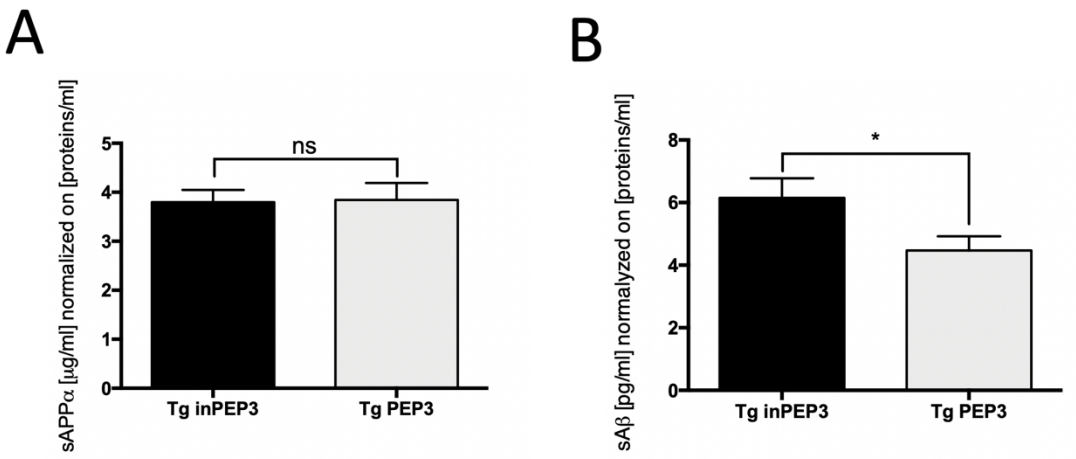
Thus, confirming that the unbalance of ADAM10 endocytosis is also present in this mouse model of AD. This unbalance in ADAM10 local trafficking can be considered as a "synaptic trait" of the pathology. Indeed, the impairment of ADAM10 synaptic availability could lead to a decrease of α-secretase activity on APP with a concomitant increase of β-secretase activity. In light of these consideration, the rescue of this impairment of the synaptic trafficking of ADAM10 could enhance ADAM10 activity towards APP; therefore, representing a promising therapeutic approach for the development of a disease modifying agent.

To assess whether the disruption of the ADAM10/AP2 protein complex by PEP3 treatment could affect the synaptic localization of ADAM10, we measured ADAM10 synaptic levels in triton-insoluble fraction (TIF), that is enriched in postsynaptic proteins, purified from the forebrains collected from the same pool of previously treated APP/PS1 mice. Western blot analysis of ADAM10 in total homogenate and TIF, revealed a decrease by around 50% of ADAM10 synaptic levels in the transgenic mice treated with inPEP3 compared to the wild-type animals. These data confirm that the aberrant increase in ADAM10/AP2 association is accompanied to a decrease in ADAM10 synaptic availability. Interestingly, in the APP/PS1 mice treated with PEP3 (ADAM10 Tg PEP3: 84.19% ±14.07%, N=9), we observed a significant increase of ADAM10 synaptic levels when compared to inPEP3-treated APP/PS1 mice (ADAM10 Tg inPEP3: 54.58% ±7.90%, N=9). No significant differences were observed in the homogenate when comparing the experimental groups, suggesting that the expression of the protein is not affected. In the APP/PS1 mice treated with PEP3, ADAM10 synaptic levels have been observed comparable to ADAM10 synaptic levels in wild-type mice (Figure-2 D and E). Thus, showing that active PEP3 was able to rescue the drop of ADAM10 synaptic levels and reverse it to the wild-type level. The effect of the active peptide on ADAM10/AP2 complex is positively affecting ADAM10 synaptic levels and could increase the α-secretase activity of the sheddase on APP; therefore, counteracting the activity of the β-secretase BACE1. This increase in the α-secretase activity is a key mechanism by which the peptide could have strong disease modifying features.

1.3. Treatment with PEP3 decreases Aβ levels without changing sAPPα release in APP/PS1 mice

After observing the disruption of ADAM10/AP2 interaction and the enhancement of ADAM10 synaptic localization, we decided to further investigate the repercussion of the treatment on the APP protein processing via assessment of resulting metabolites. The assessment of metabolites of the APP protein processing is essential to screen ADAM10 α-secretase activity. The purification of soluble fraction from the cortex of the animals was performed. We used ELISA kits to measure human sAPPα and $A\beta_{1-42}$ levels in order to analyse PEP3 effect. The treatment with PEP3 in the transgenic mice did not affect the human soluble APPα release since the levels of this metabolite were comparable with transgenic mice treated with inPEP3 (Figure-3 A). These results suggest that an increase in ADAM10 synaptic availability cannot counteract the effect of the Swedish mutation of APP transgene that promotes β-cleavage of APP. The levels of $A\beta_{1-42}$ in the cortex of APP/PS1 mice treated with PEP3 were significantly lower (Tg sAβ PEP3: 4.471 ±0.4509, N=9) compared to the transgenic mice treated with inPEP3 (Tg sAβ inPEP3: 6.149 ±0.6307, N=8) (Figure-3 B). We can hypothesize that the decrease in human $A\beta_{1-42}$ could be ascribed to an increased degradation of the peptide rather than to a shift of APP metabolism towards the non-amyloidogenic pathway.

Fig.3



(Figure 3) ELISA assays were performed to assess metabolites levels in the cortex of the treated *9-month-old APP/PS1 mice* with either inPEP3 or PEP3. *(A) ELISA assays were performed to assess human sAPPα levels in the cortex of the transgenic mice. No significant difference was observed in the APP/PS1 mice (Tg sAPPα inPEP3: 3.796 ± 0.2520, N=9; Tg sAPPα PEP3: 3.843 ±0.3455, N=9). (B) ELISA assays were performed to assess sAβ levels in the cortex of transgenic mice treated either with PEP3 or inPEP3. A significant decrease in sAβ levels was observed in the transgenic mice treated with PEP3 (Tg sAβ PEP3: 4.471 ±0.4509, N=9) compared to mice treated with inPEP3 (Tg sAβ inPEP3: 6.149 ±0.6307, N=8; t-test: *p<0.0438).*

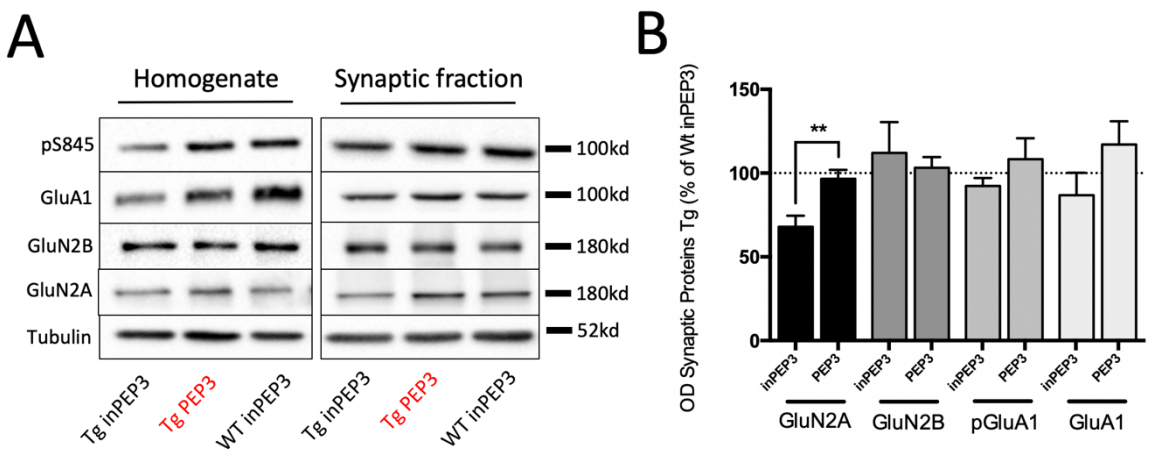
1.4. Treatment with PEP3 affects synaptic levels of NMDAR subunits

To understand the impact of the cell-permeable peptide on different potential mechanisms implicated in the synaptic transmission, different synaptic proteins were screened. The levels of the main subunits of NMDAR and AMPAR, that are crucial for synaptic transmission and plasticity mechanisms, were screened in TIF and homogenate of the same pool of treated 9-month-old APP/PS1 mice. No significant differences among the experimental groups were observed in the homogenate of the forebrain of the treated mice, suggesting that the treatment does not affect the expression of relevant synaptic proteins.

In the TIF, no significant differences were observed in levels of GluA1 subunit of the AMPAR. In addition, we measured the phosphorylation levels at the Ser845 that is relevant for the AMPAR regulation, but no significant differences were detected.[139] No differences were observed in synaptic levels of the GluN2B subunit of the NMDAR. Interestingly, a decrease of GluN2A subunit synaptic levels of around 30% was observed in the transgenic mice treated with the inPEP3 compared to wild-type animals.

Statistical analysis showed that the transgenic mice treated with PEP3 present a significant increase of GluN2A synaptic levels (GluN2A Tg PEP3: 96.54 ±5, N=9) compared to transgenic mice treated with inPEP3 (GluN2A Tg inPEP3: 67.93 ±6, N=9) (Figure-4 A and B). Thus, showing that active PEP3 was able to rescue the drop of GluN2A subunit synaptic levels and reverse it to the wild-type level. This effect of PEP3 on the synaptic levels of a subunit of NMDAR clearly represent a mechanism by which synaptic transmission could be improved in the transgenic animals.

Fig.4



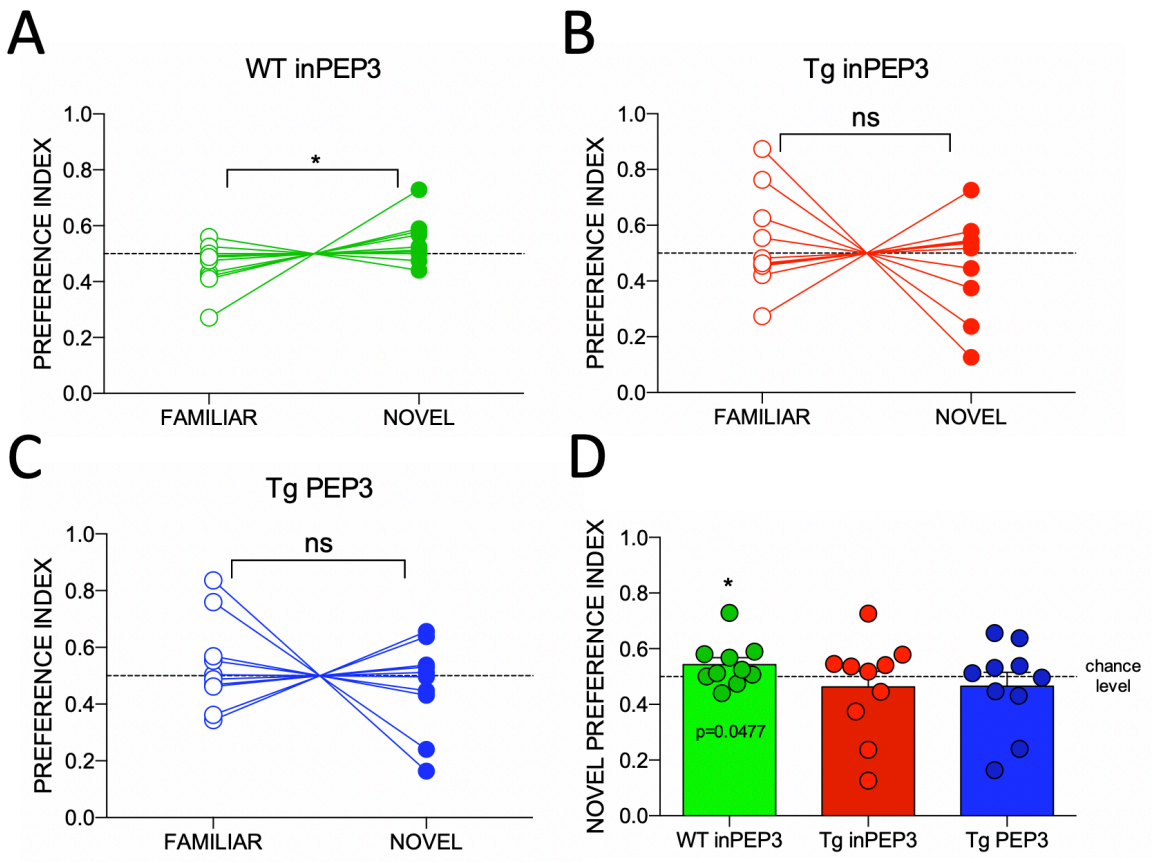
*(Figure 4) Synaptic proteins assessment after 14-days treatment of 9-month-old APP/PS1 and wild-type mice with either inPEP3 or PEP3. (A) Representative immunoblot of synaptic proteins WB analysis using homogenate and TIF fraction of treated transgenic mice (B) OD quantification of WB experiments (only TIF) in A. Significantly higher GluN2A synaptic levels were observed in the transgenic mice treated with PEP3 (GluN2A Tg PEP3: 96.54 ±5, N=9; t-test: **p<0.0040, inPEP3 Tg vs PEP3 Tg) compared to the transgenic mice treated with inPEP3 (GluN2A Tg inPEP3: 67.93 ±6, N=9). No significant differences were observed in GluN2B levels, p845 GluA1 phosphorylation levels nor in total GluA1 levels.*

1.5. Treatment with PEP3 does not improve cognition in APP/PS1 mice at full-blown pathology

Investigation of the effect of PEP3 treatment on the behaviour was performed in order to understand to which extent these changes in NMDAR subunits at the synapse could improve the synaptic transmission underlying the cognitive processes, and thereby rescue a potential cognitive deficit in these mice. A commonly used NORT (Novel Object Recognition Test) was performed at the end of a 14-days treatment, of 12-month-old transgenic and wild-type mice, with either the inPEP3 or PEP3. The NORT, based on animals' preference to explore novelty, is involving the hippocampus through spatial recognition and memory processes, giving a robust readout on potential cognitive impairment. Mice were exposed to two identical objects for 10 minutes and after 24 hours were re-exposed to one identical object (FAMILIAR) and one completely different object (NOVEL). Analysis of the videos captured during the test, showed that the wild-type mice prefer to explore the novel object (WT preference index Novel: 0.5425 ±0.0254, N=10) compare to Familiar object (WT preference index Familiar: 0.4575 ±0.0254, N=10). The results of this test have confirmed the conserved cognitive function of the wild-type animals even at this advanced age (Figure-5 A and D). However, no differences of preference index were observed in transgenic mice

(Figure-5 B and C). The transgenic mice either treated with the inPEP3 or PEP3 were cognitively impaired. These data showing no effect of the peptide on the rescue of the behaviour of these mice at this stage of the pathology. This result might be due to the far too advanced stage of the disease in these transgenic mice. Previous studies at full-blown pathology have already observed abundant plaques in the hippocampus of these mice together with neuronal loss adjacent to these plaques.[140] This neuronal loss could irreversibly affect cognition of the transgenic mice, thus preventing the synaptic effect of PEP3 to be sufficient in order to reverse the cognitive impact of neuronal death.

Fig.5



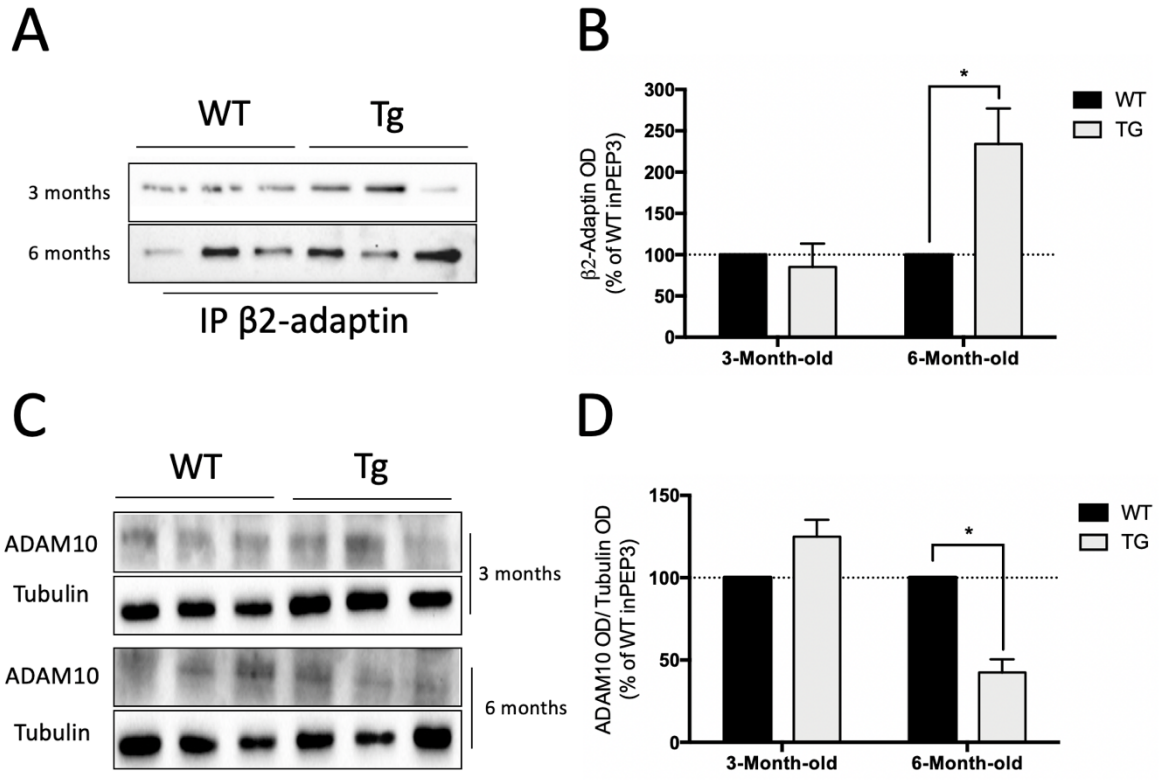
*(Figure 5) Behavioral read-out of the effect of PEP3 was performed via NORT with APP/PS1 and wild-type mice treated 14 days with either the PEP3 or inPEP3. (A) Representation of preference index for familiar and novel object in wild-type mice treated with inPEP3. A significant preference for the novel object was observed in this group after 24 hours (WT preference index FAMILIAR: 0.4575 ±0.02540, N=10; WT preference index NOVEL: 0.5425 ±0.02540, N=10; t-test: *p=0.0294 Novel vs Familiar). (B) Representation of preference index for familiar and novel object in APP/PS1 mice treated with inPEP3. No significant differences of preference index were observed in this group after 24 hours (Tg inPEP3 preference index FAMILIAR: 0.5370 ±0.05548, N=10; Tg inPEP3 preference index NOVEL: 0.4630 ±0.05548, N=10). (C) Representation of preference index for familiar and novel object in APP/PS1 mice treated with PEP3. No significant differences of preference index were observed in this group after 24 hours (Tg PEP3 preference index FAMILIAR: 0.5346 ±0.04969, N=10; Tg PEP3 preference index NOVEL: 0.4654 ±0.04969, N=10). (D) Representation of preference index for novel object for the three different experimental conditions.* Data analysis shows significant single t-test of Novel object preference index for wild-type treated with inPEP3 (Novel object Actual Mean 0.614> *chance level 0.50, N=10; single t-test* **p=0.0477).

1.6. Screening for pathology onset in APP/PS1 mice

These interesting data obtained treating APP/PS1 mice at full-blown pathology with PEP3 showed us the potential of such therapeutic action targeting ADAM10. Moreover, we also observed a similar defect of ADAM10 local trafficking mechanisms, as observed in AD patients, such as an increase in ADAM10/AP2 interaction and a decrease of ADAM10 synaptic levels compared to physiological levels. These biochemical changes are most likely to impact mice cognition at full-blown pathology, however at this stage the biochemical changes must be combined and diluted with different pathological mechanisms far too advanced such as accumulation of amyloid plaques, neuronal loss and a strong inflammatory response that could affect the behaviour. In order to assess the efficiency of PEP3 on the behaviour *via* its action on ADAM10 synaptic localization we decided to screen for the onset window during which these biochemical changes appear.

We assessed ADAM10 interaction with AP2 and its synaptic levels at different time points to be able to treat during the onset of the pathological changes in ADAM10 trafficking. The analysis revealed that changes appear before 9-month-old, as already at 6-months-old, since an increase of ADAM10/AP2 association is observed together with a decrease of ADAM10 synaptic levels in APP/PS1 mice. Indeed, co-immunoprecipitation of ADAM10 and the AP2 subunit β2-adaptin revealed significantly higher levels of association with β2-adaptin in the 6-month-old APP/PS1 transgenic mice (Tg β2-adaptin 6mo: +134% ±43.1%, N=3) compared to the wild-type mice at the same age (Figure-6 A and B). Western blotting revealed significantly lower synaptic levels of ADAM10 in the 6-month-old APP/PS1 transgenic mice (Tg ADAM10 6mo: -57.5% ±7.9%, N=3) compared to the wild-type mice at the same age (Figure-6 C and D). These differences observed at 6-month-old were not observed in APP/PS1 mice at the age of 3 months. Therefore, in light of these results we decided to treat APP/PS1 mice at the age of 6 months, at the onset of the ADAM10 trafficking alteration.

Fig.6



(Figure 6) Assessment of target parameters of PEP3 in APP/PS1 mice and wildtype mice at 3- and 6-month *of age. (A) Brain homogenates were immunoprecipitated using anti-ADAM10 antibody and β2-adaptin co-precipitation was evaluated considering Optical Density. (B) A significant increase of the interaction was observed in the 6-month-old transgenic mice (Tg β2-adaptin 6mo: 234.0 ±43.1, N=3; t-test: *p=0.0358; Two-way ANOVA: *p=0.0202) compared to wildtype mice at the same age. No significant changes in ADAM10 interaction with AP2 were observed at 3-month-old. (C) Representative immunoblot of ADAM10 WB analysis in TIF fraction.* (D) *OD quantification of WB experiments in TIF of transgenic* mice and wildtype mice at 3- and 6-month of age*. In the TIF fraction, OD ratio between ADAM10 and tubulin revealed a significant decrease in ADAM10 synaptic levels in 6-month-old transgenic mice (ADAM10 Tg 6mo: 42.5 ±7.9, N=3; t-test: *p=0.0184) compared to the wild-type mice at the same age. No significant change in ADAM10 synaptic levels were observed at 3-months-old.*

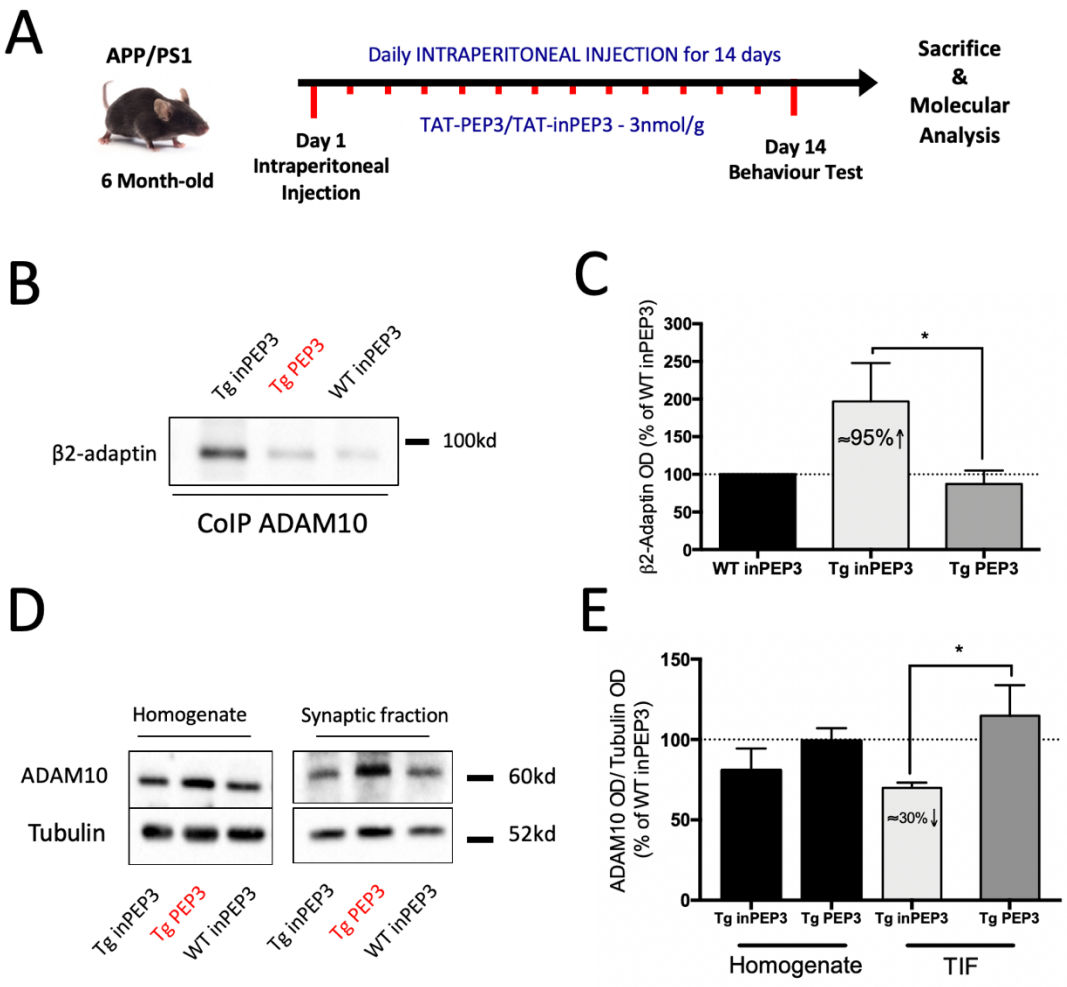
2. Testing PEP3 treatment at early stage of Alzheimer's disease in APP/PS1 mice.

2.1. Treatment with PEP3 efficiently increases ADAM10 synaptic localization at early stage of the disease

We have performed a 14-days treatment of daily intraperitoneal injections of 3nmol/g of either PEP3 or inPEP3 in 6-month-old APP/PS1 and wild-type mice (Figure-7 A). Following the treatment, the forebrain of the animals was lysated and the homogenate was used to assess ADAM10/AP2 interaction. As previously shown, a co-immunoprecipitation was performed in order to confirm the efficacy of the peptide in disrupting the interaction between ADAM10

and AP2. We have precipitated the complex using anti-ADAM10 antibody which confirmed that the APP/PS1 mice treated with inPEP3 present an increased association between ADAM10 and the β2-adaptin subunit of AP2 by around 95% compared to wild-type animals. The PEP3 treatment significantly reduces this aberrant increased association and restores the levels of interaction to the ones detected in wild-type mice.

Fig.7



*(Figure 7) Assessment of PEP3 effect on 6-month-old APP/PS1 and wild-type mice. (A) Experimental paradigm scheme of the 14 days treatment with daily intraperitoneal injections with either PEP3 or inPEP3 at a concentration of 3nM/g of body weight. (B) Brain homogenates were immunoprecipitated using anti-ADAM10 antibody and β-adaptin co-precipitation was evaluated considering Optical Density. (C) OD quantification of co-immunoprecipitation experiment in B. A significant increase of the interaction was observed in the transgenic mice treated with inPEP3 (β2-adaptin Tg inPEP3: 196.8 ±51.18, +96.8% ±51.1% N=9) while the treatment with PEP3 restored the AP2/ADAM10 interaction levels to levels similar to the ones of inPEP3-treated wild-type mice (β2-adaptin Tg PEP3: 87.22 ±17.94, -17.78% ±17.94%, N=9, One-way ANOVA: *p=0.0314). (D) Representative immunoblot of ADAM10 WB analysis in Homogenate and TIF fraction. (E) OD quantification of WB experiment in D. No significant differences were observed in the homogenate. In the TIF fraction, optical density ratio between ADAM10 and tubulin revealed a significant (t-test: *p=0.0382) decrease in ADAM10 synaptic levels in transgenic animals treated with the inPEP3 (ADAM10 Tg inPEP3: 69.94 ±3.3, -30.1% ±3.3%, N=7) compared to the animals treated with the PEP3 (ADAM10 Tg PEP3: 114.8 ±18.97, +14.8% ±18.9%, N=7) which have levels comparable to wild-type mice.*

Indeed, the quantitative analysis showed that the transgenic animals treated with PEP3 have significantly lower ADAM10/AP2 interaction levels (Tg PEP3: 82.22% ±17.94% N=9) compared to the transgenic mice treated with inPEP3 (Tg inPEP3: 196.8% ±51.1% N=9) (Figure-7 B and C). To assess the functional efficacy of the peptide in increasing ADAM10 synaptic levels at early stage of the disease we performed a TIF purification of the forebrain from the same pool of 6-month-old mice.

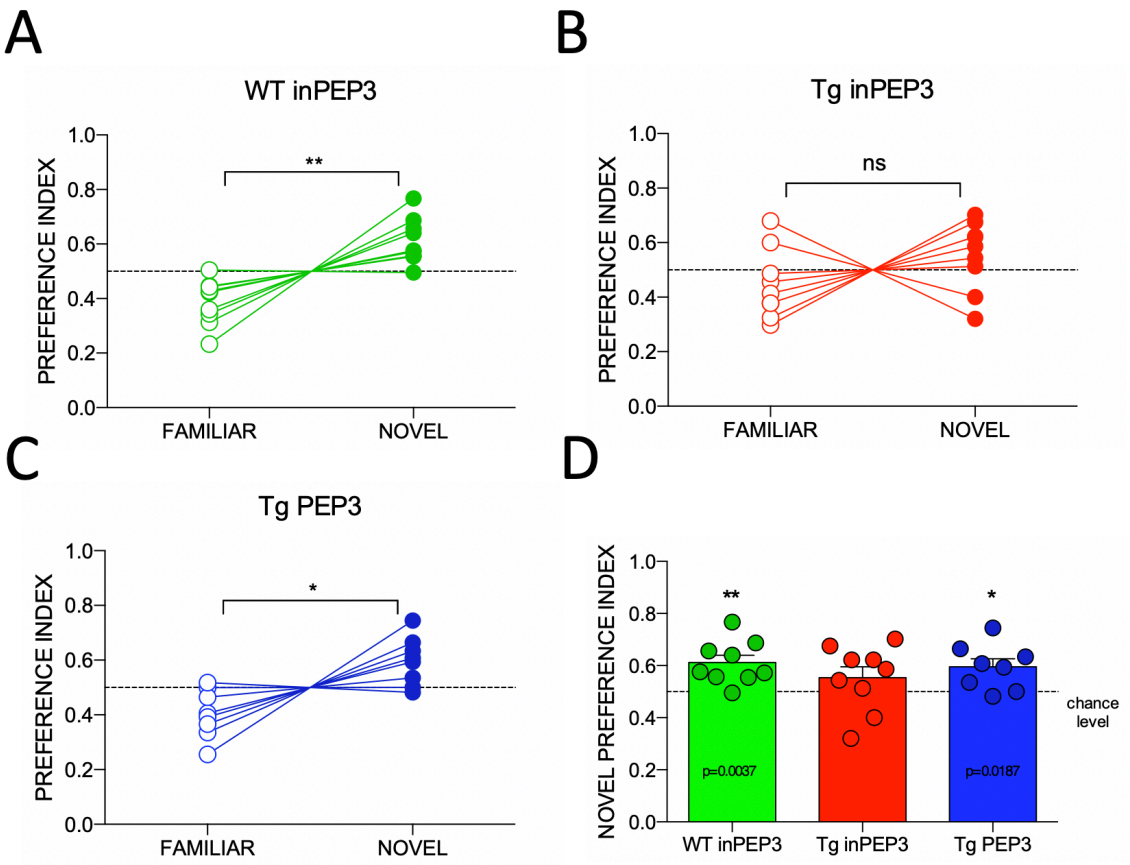
The statistical analysis revealed that APP/PS1 mice treated with inPEP3 presented significantly lower ADAM10 synaptic levels by around 30 % (ADAM10 Tg inPEP3: 69.94% ±3.3%, N=7) compared to wild-type animals treated with inPEP3. The treatment with PEP3 reverses the ADAM10 synaptic levels to wild-type levels, restoring its synaptic availability (ADAM10 Tg PEP3: 114.8% ±18.97%, N=7). No significant differences in ADAM10 total levels were observed in the homogenates (Figure-7 D and E). These encouraging data confirmed the efficacy of the peptide PEP3 in acting on ADAM10 synaptic levels at early stage of the pathology.

2.2. Treatment with PEP3 improves cognition in APP/PS1 mice at early stages of the disease

In order to test the effect of PEP3 on the behaviour of the APP/PS1 mice line previously used for molecular analysis, a behaviour test was performed to assess cognitive function of these mice. Animals treated during this experiment were 6-month-old APP/PS1 mice as considered at early stage of the disease. Like previously, a NORT (Novel Object Recognition Test) was performed at the end of a 14-days treatment with either inPEP3 or active PEP3. As observed at full-blown pathology, the analysis of the videos captured during the test, showed that the wild-type mice prefer to explore the novel object (WT preference index NOVEL: 0.6119 ±0.0276, N=9) compared to the familiar object (WT preference index FAMILIAR: 0.3881 ±0.0276, N=9). The results of this test have confirmed the conserved cognitive function of wild-type animals (Figure-8 A). No differences of preference index were observed in transgenic mice treated with the inPEP3 (Figure-8 B). These mice did not show a clear preference for the novel or familiar object, indicating that the mice were unable to discriminate between the two objects, a feature underlying a cognitive impairment. Interestingly, in mice treated with PEP3 a clear preference was observed for the novel object

(Tg PEP3 preference index NOVEL: 0.5953 ±0.0311, N=8) compared to the familiar object (Tg PEP3 preference index FAMILIAR: 0.4047 ±0.0311, N=8) (Figure-8 C). By comparing the preference index against the chance level, it is clear that PEP3 treatment completely reverts the cognitive impairment of the APP/PS1 mice (Figure-8 D).

Fig.8

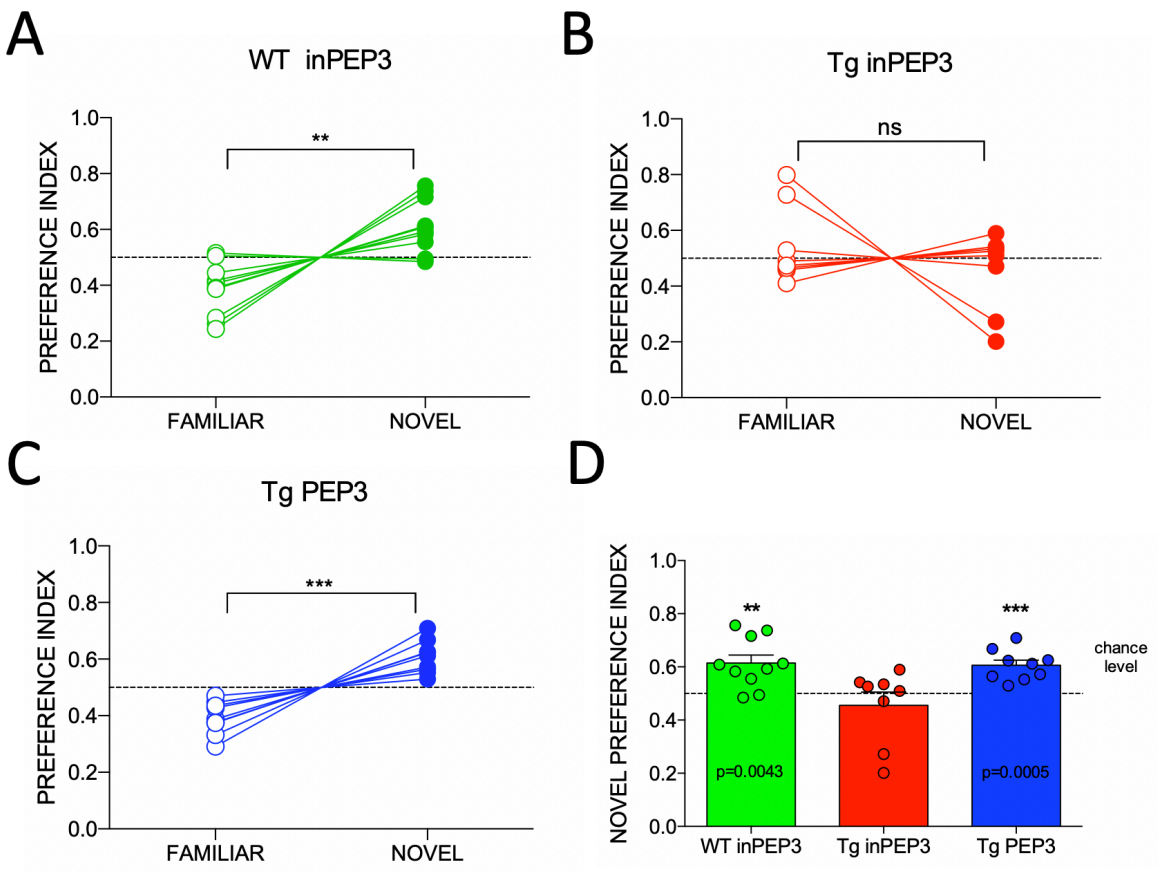


(Figure 8) Novel object recognition test performed on 6-month-old transgenic and wild-type mice treated 14 days with either inPEP3 or PEP3. Statistical analysis was performed by calculating the preference index for the Familiar object (time exploring familiar/(time exploring familiar + time exploring novel)) and Novel object (time novel/(time familiar + time novel)). (A) Data from NORT. Representation of data showing a significantly higher preference index of the wild-type mice for the Novel object (WT inPEP3 preference index NOVEL: 0.6119 ±0.0276 N=9) compared to the familiar object (WT inPEP3 preference index FAMILIAR: 0.3881 ±0.0276 N=9; t-test **p=0.0037). (B) Data from NORT. Representation of data showing no significant difference of preference index between Familiar and Novel object in transgenic mice treated with inPEP3. (C) Data from NORT. Representation of data showing a *significantly higher preference index of the transgenic mice treated with PEP3 for the Novel object (Tg PEP3 preference index NOVEL: 0.5953 ±0.0311, N=9) compared to the familiar object (Tg PEP3 preference index FAMILIAR: 0.4047 ±0.0311 N=8; t-test *p=0.0183 Familiar vs Novel). (D) Representation of preference index for novel object for the three different experimental conditions.* Data analysis shows significant single t-test for Novel object preference index for wild-type mice treated with inPEP3 (Novel object Actual Mean: 0.611> *chance level: 0.50, N=9; single t-test* **p=0.0037) and transgenic mice treated with PEP3 (Novel object Actual Mean: 0.595> *chance level: 0.50, N=8; single t-test* *p=0.0187).

These results suggest that PEP3 treatment induces an improvement of the cognitive functions. In order to confirm these interesting results, and to obtain a second behavioral read-out of the

effect of PEP3 on the cognitive function, we performed a Y-maze test. This test is based on the free exploration of an apparatus with three arms; in the first 5 minutes of exploration, one arm is closed while in the second phase of 5 minutes of exploration all the arms are accessible. The Y-maze test involves the hippocampus through spatial recognition and memory processes.

Fig.9



(Figure 9) Y-Maze test performed on 6-month-old transgenic and wild-type mice treated 14 days with either inPEP3 or PEP3. Statistical analysis was performed by calculating the preference index for the Familiar arm (time exploring familiar/(time exploring familiar + time exploring novel)) and Novel arm (time exploring novel/(time exploring familiar + time exploring novel)). (A) Data from Y-maze test. Representation of data showing a significantly higher preference index of the wild-type mice for the Novel arm (WT inPEP3 preference index NOVEL: 0.6142 ±0.0301, *N=10)* compared to the familiar arm (*WT* inPEP3 preference index FAMILIAR: 0.3858 ±0.0301, *N=10; t-test* **p=0.0043 Familiar vs Novel). (B) Data from Y-maze test. Representation of data showing no significant difference of preference index between Familiar and Novel arm in transgenic mice treated with inPEP3. (C) Data from Y-maze test. Representation of data showing a significantly higher preference index of the transgenic mice treated with PEP3 for the Novel arm (Tg PEP3 preference index *NOVEL: 0.6065 ±0.191, N=9)* compared to the familiar arm (*Tg PEP3 preference index* FAMILIAR: 0.3935 ±0.191, *N=9; t-test* ***p=0.0005 Familiar vs Novel). (D) *Representation of preference index for novel object for the three different experimental condition.* Data analysis shows significant single t-test of Novel arm preference index for wild-type mice treated with inPEP3 (Novel object Actual Mean: 0.614> *chance level 0.50, N=10; single t-test* **p=0.0043) and transgenic mice treated with PEP3 (Novel arm Actual Mean: 0.606> *chance level, 0.50, N=9; single t-test* ***p=0.0005).

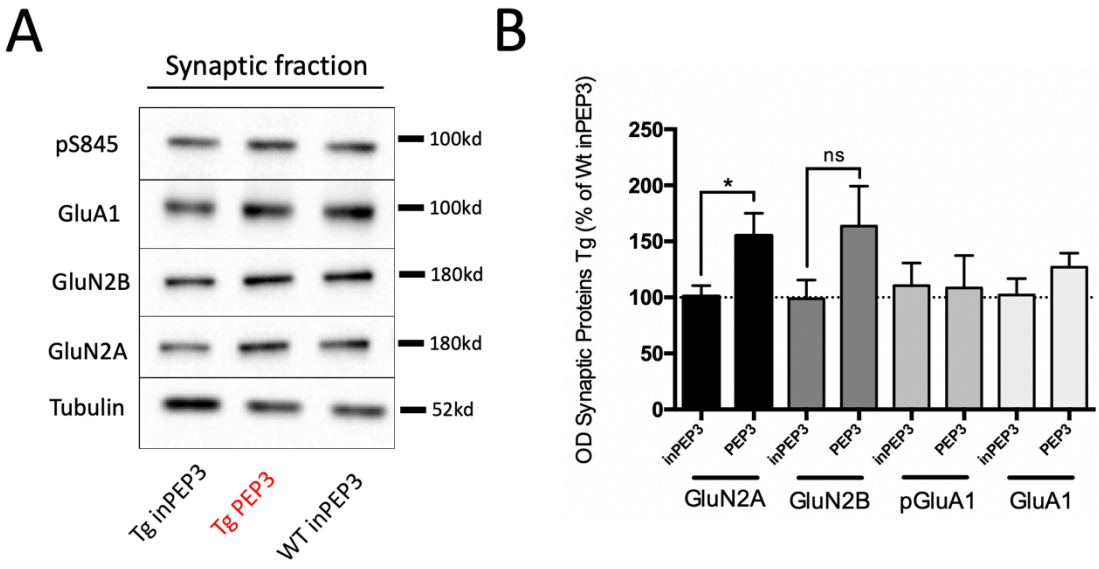
Like previously the behaviour test was performed at the end of a 14-days treatment with either 3nmol/g of inPEP3 or PEP3 using intraperitoneal administration route. Following the treatment, the test was performed in accordance to standard protocol in use for this test. As observed in the NORT, the analysis of the videos captured during the test, showed that the wild-type mice treated with inPEP3 prefer to explore the Novel arm (WT inPEP3 preference index NOVEL: 0.6142 ±0.0301, N=10) compared to Familiar arm (WT inPEP3 preference index FAMILIAR: 0.3858 ±0.0301, N=10). These results confirmed the conserved cognitive function of the wild-type animals (Figure-9 A). No differences of preference index were observed in transgenic mice treated with inPEP3 (Figure-9 B). These mice did not show a preference for the Novel or Familiar arm, indicating that they were unable to discriminate between the two objects, which indicate a cognitive impairment. Confirming data obtained with NORT, the transgenic mice treated with PEP3 showed a preference for novel arm (Tg PEP3 preference index NOVEL: 0.6065 ±0.191, N=9) compared to the Familiar arm (Tg PEP3 preference index FAMILIAR: 0.3935 ±0.191, N=9) (Figure-9 C). By comparing the preference index against the chance level, it is clear that PEP3 treatment completely reverts the cognitive impairment of the APP/PS1 mice as previously observed in the NORT (Figure-9 D).

2.3. Treatment with PEP3 affects synaptic levels of NMDAR subunits

In light of these results, we decided to assess different synaptic proteins to understand the impact of PEP3 on major actors implicated in the synaptic transmission. Prior results at full-blown pathology showed a decrease of GluN2A synaptic levels in the APP/PS1 mice but no differences in the homogenates. We decided to focus on the analysis of the TIF fraction from the hippocampus as its involvement in learning and memorisation process is crucial. We screened major subunits of NMDAR and AMPAR that are underlying synaptic transmission and plasticity mechanisms. No significant differences were observed for GluA1 subunit of the AMPAR and its level of phosphorylation at Ser845. Interestingly, no difference in GluN2A subunit synaptic level were observed in the transgenic mice treated with inPEP3 (GluN2A Tg inPEP3: 107% ±9%, N=9) compared to wild-type mice. However, the transgenic mice treated with PEP3 presented significantly higher GluN2A synaptic levels (GluN2A Tg PEP3: 154.6% ±20%, N=9) compared to transgenic mice treated with inPEP3 (Figure-10 A and B). This observation regarding GluN2A confirmed the effect of PEP3 as

enhancer of the synaptic levels of GluN2A subunits like previously shown in mice at full-blown pathology. At early stages of the disease, GluN2B subunits are also tending to increase in the transgenic mice treated with PEP3 (GluN2B Tg PEP3: 163.5% ±35%, N=9), however this change is not observed to be significant as compared with inPEP3 condition. NMDAR are essential in synaptic plasticity and synapse formation underlying learning and memory processes. This effect of PEP3 on the synaptic levels of the subunit of NMDAR clearly represents an important mechanism by which PEP3 could improve synaptic transmission in transgenic mice. Therefore, investigation of the effect of the peptide on synaptic transmission was investigated during a secondment in Janssen Pharmaceuticals.

Fig.10



(Figure 10) Synaptic proteins assessment after treatment of *9-month-old APP/PS1 and wild-type mice* with either inPEP3 or PEP3. *(A) Representative immunoblot of synaptic proteins WB analysis from hippocampus TIF of the treated transgenic mice. (B) OD quantification of WB experiment in A. Significantly higher GluN2A levels were observed in the transgenic mice treated with PEP3 (GluN2A Tg PEP3: 154.6 ±20.26, N=9; t-test: **p<0.0290) compared to transgenic mice treated with inPEP3 (GluN2A Tg inPEP3: 100.7 ±9.73, N=9) in which GluN2A levels is comparable to wild-type levels. No significant difference was observed in Ser-845 GluA1 phosphorylation levels nor in total GluA1 levels. A trend regarding GluN2B was observed as PEP3 tend to increase GluN2B synaptic levels (GluN2B Tg PEP3: 163.5 ±35.67, N=9) however, no significant difference was observed as compared to inPEP3 condition (GluN2B Tg inPEP3: 98.86 ±16.76, N=7).*

2.4. Electrophysiological effect of the treatment with PEP3

During my secondment in Janssen Pharmaceuticals in Belgium, we tested the effect of PEP3 on the neuronal activity in the hippocampus. Using Multi Electrode Array setup, we recorded field Excitatory Postsynaptic Potential (fEPSP) of AMPA/NMDA and NMDA-isolated components of the response from acute hippocampal slices of treated mice. The experimental

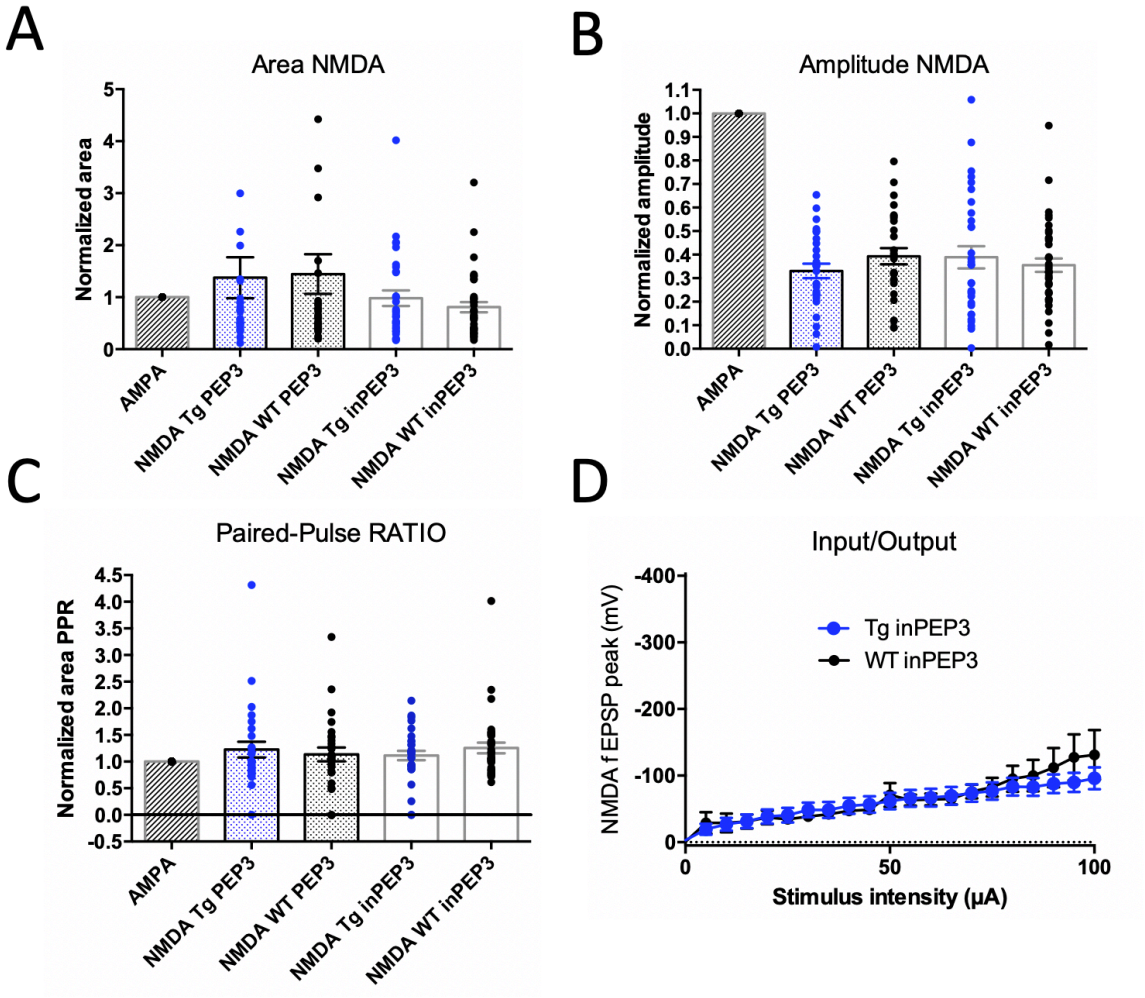
plan was composed of four groups: wild-type mice and transgenic APP/PS1 mice treated with inPEP3 and PEP3 (APP/PS1 line:"B6.Cg-Tg(Thy1-APPSw,Thy1PSEN1*L166P)21Jckr").[141]

No significant differences between the experimental groups were observed in the area and amplitude of the NMDA components (Figure-11 A and B). A paired-pulse ratio protocol was also performed while recording the NMDA component, however no differences and potential implication of pre-synaptic mechanisms were observed (Figure-11 C). Finally, the results obtained via input/output protocols confirmed the absence of impairment of the synaptic transmission in the transgenic animals as the responses to the protocol were similar to the wild-type responses (Figure-11 D).

Overall, these results do not show any difference in NMDAR and AMPA currents. However, the transgenic line used in Janssen is slightly different from the APP/PS1 line used for the biochemical and behavioural experiments in Milan, since these mice overexpress a human APP transgene (HuAPP-KM670/671NL), while the APP/P1 mice used in biochemical and behavioural experiments based in Milan overexpress a chimeric mouse/human APP (Mo/HuAPP695swe).[141]

The APP/PS1 mice used for the electrophysiological experiments did not display synaptic deficit, neither cognitive deficits and nor decrease in ADAM10 synaptic levels revealed by further biochemical analysis (Data not shown). Therefore, we decided to focus our research on APP/PS1 mice showing impairment of ADAM10 synaptic availability.

Fig. 11



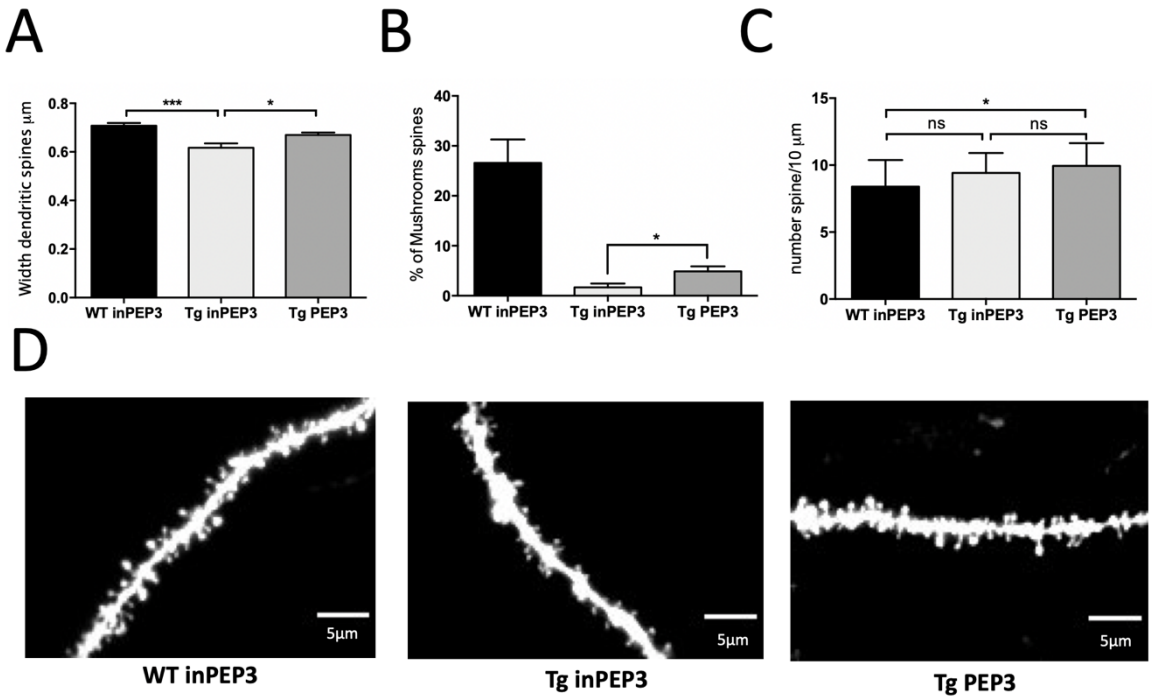
(Figure 11) Electrophysiological Multi-Electrode Array (MEA) experiment performed on acute hippocampal slices from treated transgenic or wild-type mice for 14 days with either inPEP3 or PEP3. (A) Analysis of AMPA-normalized area of NMDA f-EPSP component from APP/PS1 and wild-type animals treated with PEP3 or inPEP3. No significant difference was observed among the different experimental groups (NMDA Tg PEP3: 1.375 ±0.3953, N=27; NMDA WT PEP3: 1.444 ±0.3809, N=29; NMDA Tg inPEP3: 0.9805 ±0.1491, N=31; NMDA WT inPEP3: 0.8096 ±0.0985, N=39). (B) Analysis of AMPA-normalized amplitude of NMDA f-EPSP component from transgenic and wild-type animals treated with PEP3 or inPEP3. No significant difference was observed among different experimental groups (NMDA Tg PEP3: 0.3307 ±0.03059, N=28; NMDA WT PEP3: 0.3930 ±0.3428, N=29; NMDA Tg inPEP3: 0.3889 ±0.4704, N=31; NMDA WT inPEP3: 0.3554 ±0.02845, N=39). (C) Analysis of Paired-pulse ratio protocol using AMPA-normalized area of NMDA f-EPSP component from transgenic and wild-type animals treated with PEP3 or inPEP3. No significant difference was observed among the different experimental groups (NMDA Tg PEP3: 1.225 ±0.1480, N=28; NMDA WT PEP3: 1.135 ±0.1276, N=29; NMDA Tg inPEP3: 1.114 ±0.08735, N=31; NMDA WT inPEP3: 1.256 ±0.09887, N=37). (D) Analysis of Input/Output protocol using NMDA f-EPSP peak component from transgenic and wild-type animals treated with PEP3 or inPEP3. No significant difference was observed among different experimental groups (NMDA fEPSP Tg inPEP3: -58.24 ±5.464, N=21; NMDA fEPSP WT inPEP3: -63.12 ±7.622, N=21).

2.5. Treatment with PEP3 increases spines width and density

To assess whether the PEP3 treatment could affect spine shape, using Dil membrane staining the morphology and density of spines in the hippocampus was analysed. Transgenic mice treated with inPEP3 showed a significant shrinkage of the spines compared to wild-type

mice. Interestingly, transgenic mice treated with PEP3 showed significantly increased dendritic spines width (Tg PEP3: 0.669 ±0.009, N=17) compared to APP/PS1 mice treated with inPEP3 (Tg inPEP3: 0.617 ±0.017, N=12) (Figure-12 A). Moreover, analysis of the different type of spines revealed that the percentage of mushroom spines drastically decreased in the transgenic mice treated with inPEP3 compared to wild-type mice. Interestingly, transgenic mice treated with PEP3 showed a significantly increased percentage of mushroom spines (Tg PEP3: 4.868% ±1%, N=15) compared to transgenic mice treated with inPEP3 (Tg inPEP3: 1.697% ±0.737%, N=10) (Figure-12 B).

Fig.12



(Figure 12) Spine morphology investigation after treatment of APP/PS1 or wild-type mice with either PEP3 or inPEP3 (A) Representation of statistical analysis of dendritic spines width (µm). Transgenic mice treated with inPEP3 showed a significant decrease of spines width compared to wild-type mice (Tg inPEP3: 0.617 *±0.017, N=12; WT inPEP3: 0.707 ±0.011, N=23; One-way ANOVA ****p<0.0001). Transgenic mice treated with PEP3 showed significantly higher spine width compared to transgenic mice treated with inPEP3 (*Tg inPEP3: 0.617 *±0.017, N=12; Tg PEP3: 0.669 ±0.009, N=17; One-way ANOVA *p=0.0264). (B) Representation of statistical analysis of mushroom spines percentage. Transgenic mice treated with inPEP3 showed a significant decrease of mushroom spines percentage compared to wild-type mice (*Tg inPEP3: 1.697% *±0.737%, N=10; WT inPEP3: 26.60% ±4.682%, N=23; One-way ANOVA ***p=0.0004). Tg mice treated with PEP3 showed a significant increase in percentage of mushroom spines compared to transgenic mice treated with inPEP3 (*Tg inPEP3: 1.697% *±0.737%, N=10; Tg PEP3: 4.868% ±1%, N=15; One-way ANOVA ****p<0.0001). (C) Representation of statistical analysis of spines density (number of spines per 10µm long dendrite area). No significant difference of spine density was observed between transgenic mice treated with inPEP3 and wild-type mice. Significant increase of spine density was observed in transgenic mice treated with PEP3 compared to wild-type mice (*WT inPEP3: 8.390 *±0.4157, N=23; Tg PEP3 Mean: 9.946 ±0.4122, N=17; One-way ANOVA *p=0.0246). (D) Panels showing potential changes in dendritic spines shape and count in regard of experimental groups.

Regarding the overall spine density, the transgenic mice treated with inPEP3 have similar spine density to wildtype mice as no significant differences were observed between the two

groups. However, the transgenic mice treated with PEP3 showed a significant increase of spines density (Tg PEP3 Mean: 9.946 ±0.4122, N=17) compared to wild-type mice treated with inPEP3 (WT inPEP3: 8.390 ±0.4157, N=23) (Figure-12 C and D).These morphological changes in spines observed in the hippocampus of the mice treated with PEP3 could represent a functional mechanism by which the treatment improved APP/PS1 mice cognition since spine morphology is known to strongly correlates with cognitive performance.[142] ADAM10 has many substrates known to act as cell-adhesion molecules and therefore having important roles in spine and synapse morphological modulation.[114] Among these substrates we investigated the effect of increased ADAM10 synaptic localization on the processing of APP and N-cadherin. Investigation of Notch processing was also performed as it is an important player in neurogenesis.

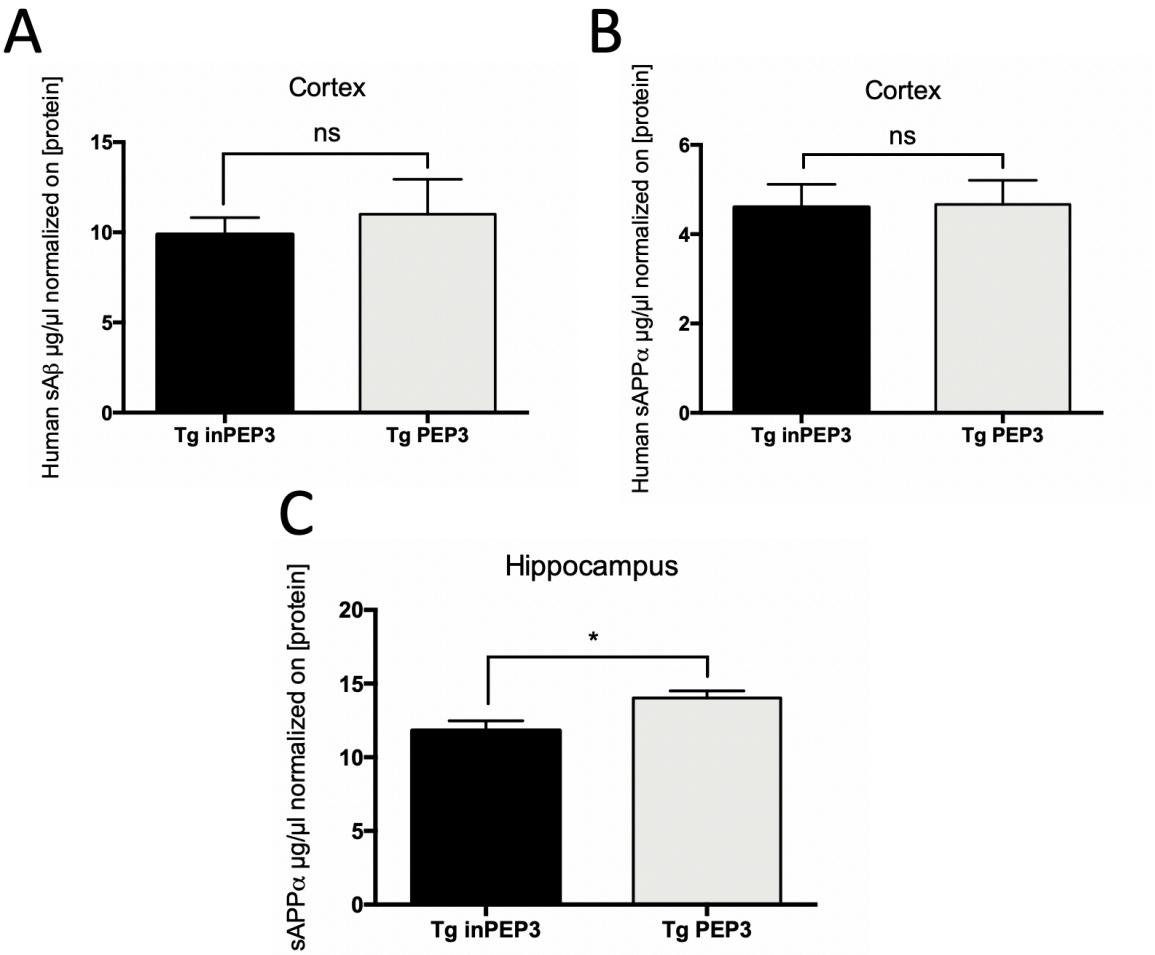
2.6. PEP3 treatment increases endogenous sAPPα levels in APP/PS1 mice without changing N-Cadherin and Notch shedding

To investigate the effect of PEP3 on the activity of the α-secretase towards APP, we measure the levels of $A\beta_{42}$ and sAPPα by ELISA. Interestingly no changes were observed in human $A\beta_{42}$ and human sAPPα levels in the cortex of transgenic mice treated with PEP3 (Figure-13 A and B). This is likely to be due to the strong 3-fold overproduction of humanized APP in this mouse model together with the increase cleavage of mutated PS1 leading to overproduction of $A\beta_{42}$ peptide. Therefore, we hypothesized that PEP3 could not counteract the effect of the Swedish mutation on APP metabolism. However, to assess if ADAM10 activity was increased by the treatment we measured mouse sAPPα levels in the hippocampus.

The results obtained using a mouse/rat sAPPα ELISA kit showed a significant increase of sAPPα levels in APP/PS1 mice treated with PEP3 (PEP3: 14,02 ± 0,491, N=8) compared to transgenic mice treated with inPEP3 (inPEP3: 11,81 ± 0,658, N=7) (Figure-13 C). This interesting result suggests an increase of ADAM10 activity on the endogenous APP protein. In consideration of previous data observing no changes in humanized metabolites, this observation supports the action of PEP3 in increasing ADAM10 relative activity towards

APP, however its action is not enough to significantly change overexpressed humanized APP and overproduction of $A\beta_{1-42}$ caused by mutations in this model.

Fig.13

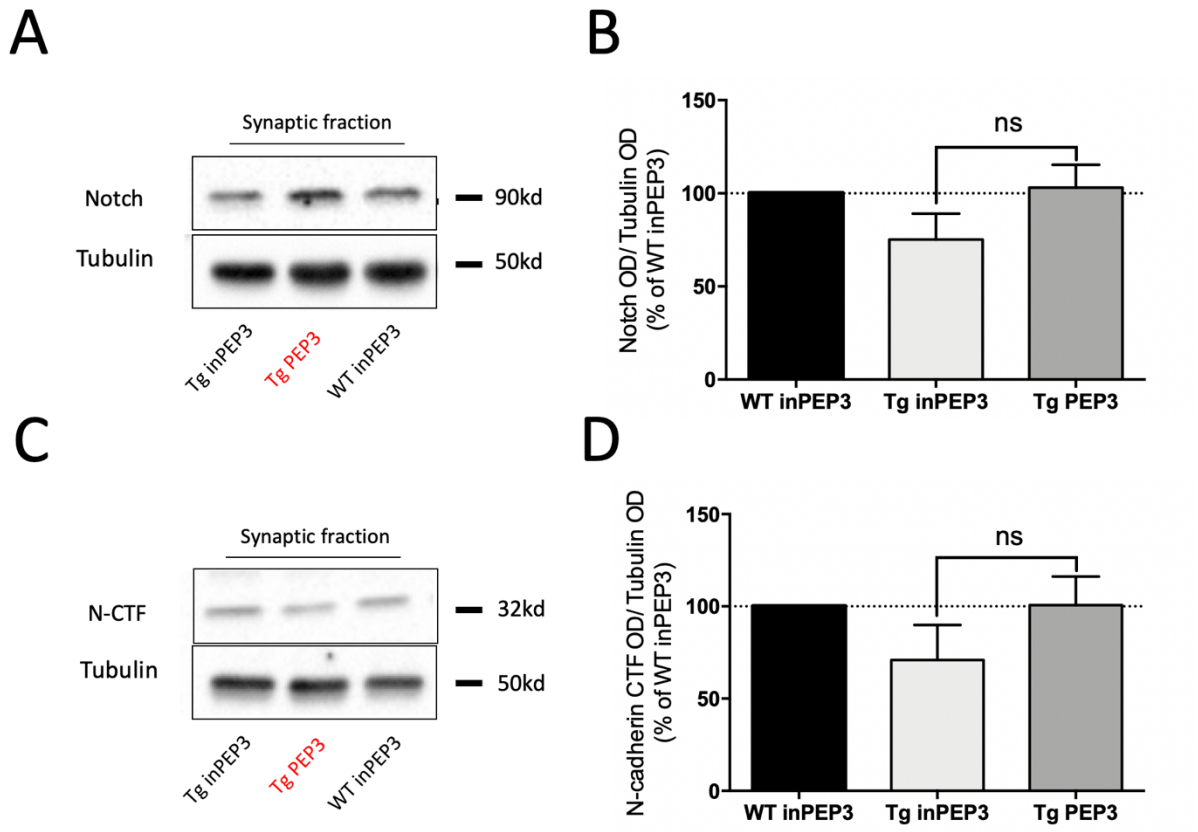


(Figure 13) ELISA assays assessing metabolites levels in the cortex and hippocampus of the treated *6-month-old APP/PS1 mice* with either inPEP3 or PEP3. *(A) ELISA assays performed to assess Human $A\beta_{42}$ levels in the cortex of transgenic mice after treatment. No significant difference in $A\beta_{42}$ levels was observed in the transgenic mice treated with either active PEP3 or inactive PEP3 ($A\beta_{42}$ PEP3 Mean 11,01 ± 1,932, N=9; inPEP3 Mean: 9,88 ± 0,927, N=9). (B) ELISA assays performed to assess Human sAPPα levels in the cortex of the transgenic mice after treatment. No significant difference was observed in the transgenic mice treated with either active PEP3 or inactive PEP3 (sAPPα PEP3 Mean: 4,670 ± 0,5401, N=9; inPEP3 Mean: 4,602 ± 0,5172, N=9) (C) ELISA assays performed to assess sAPPα levels in the hippocampus of the transgenic mice after treatment. Significant increase of sAPPα levels was observed in the transgenic mice treated with PEP3 compared to transgenic mice treated with inactive PEP3 (PEP3: 14,02 ± 0,491, N=8; inPEP3: 11,81 ± 0,658, N=7; t-test *p=0,0017).*

To assess ADAM10 activity, we measured also Notch and N-Cadherin cleavage. Analysis of Notch processing in 6-month-old APP/PS1 mice showed no difference between transgenic mice treated with the inPEP3 or PEP3. However, a trending decrease of the processing of Notch was observed in transgenic mice treated with inPEP3 compared to wild-type mice

treated with inPEP3 (Figure-14 A and B). The processing of N-cadherin also showed no significant difference between transgenic mice treated with inPEP3 or PEP3. Nevertheless, a trend to a decrease of the processing of these proteins is observable when comparing the transgenic mice treated with the inPEP3 to the inPEP3-treated wild-type mice (Figure-14 C and D).

Fig.14



(Figure 14) Substrates assessment after treatment of *6-month-old APP/PS1 and wild-type mice* with either inPEP3 or PEP3. *(A) Levels of the synaptic Notch protein were assessed performing western blotting from TIF of forebrain of the treated transgenic mice. (B) OD quantification of WB experiment in A. No significant difference in Notch levels was observed between transgenic mice treated with PEP3 or inPEP3 (*Tg inPEP3: 75.14% *±13.94%, N=7; Tg PEP3: 103% ±12.25%, N=9). (C) Levels of the synaptic N-cadherin protein were assessed performing WB from TIF of hippocampus of the treated transgenic mice. (D) OD quantification of WB experiment in C. No significant difference in N-cadherin CTF synaptic levels was observed between transgenic mice treated with PEP3 or inPEP3 (*Tg inPEP3: 70.93% *±18.95%, N=6; Tg PEP3: 100.6% ±15.40%, N=7).*

In light of these results, the increase in ADAM10 synaptic levels through the action of PEP3 can be considered determinant to increase endogenous sAPPα and trigger morphological changes in spines, independently to its action towards other substrates as N-cadherin. The PEP3 agent showed interesting potential as therapeutic agent in the frame of AD. Therefore, improvement of its drug potential was decided.

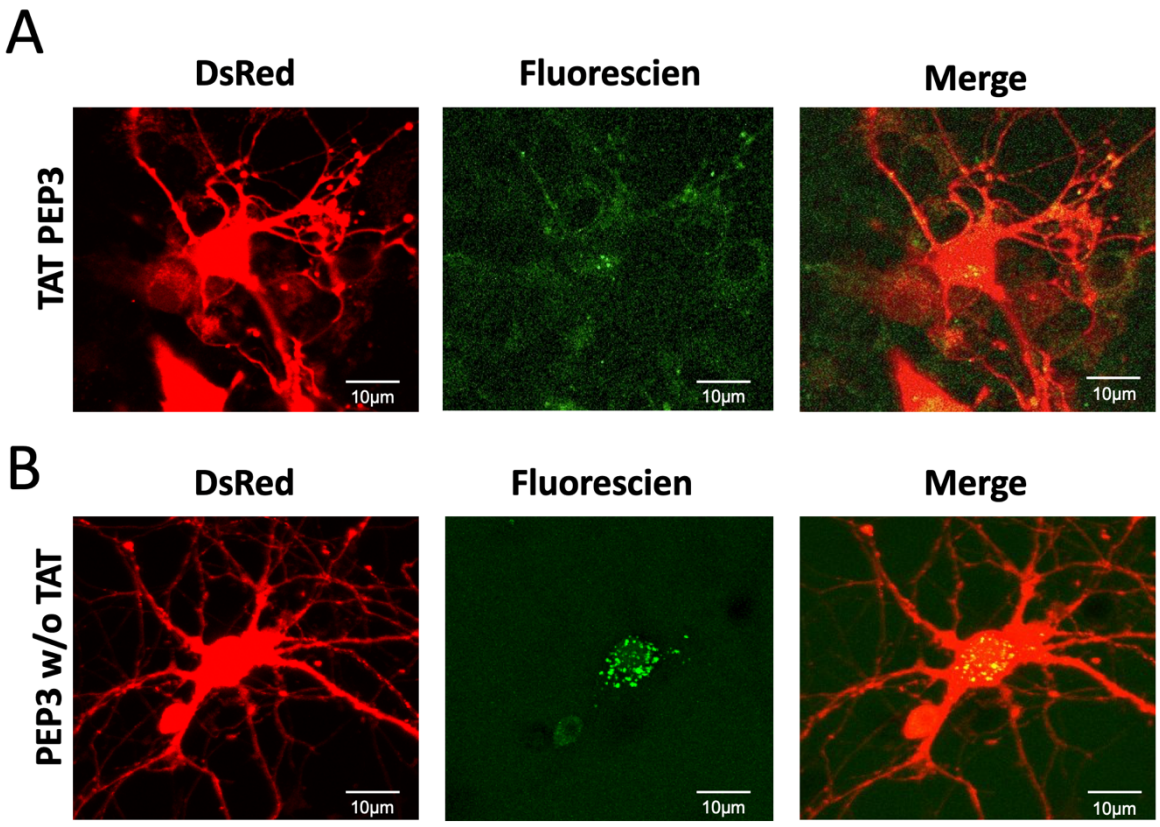
3. PEP3 lacking TAT sequence is able to cross cells' membrane and increase ADAM10 synaptic levels by interfering with the formation of ADAM10/AP2 complex

In order to improve the druggability of the cell-permeable peptide PEP3, we have tested the capacity of the PEP3 without the TAT sequence (named 3R) to penetrate the cells. As control we used the inPEP3 sequence without TAT and we named it 3E.

Indeed, modelling studies indicate that such sequences have an alpha-helical structure that can confer cell penetrance. Firstly, 3R peptide tagged with fluorescein was tested for its capacity to cross cell membranes. Cells were transfected with dsRed to detect cell morphology. Live imaging was performed to analyse the localization of the peptides.

We used TAT peptide as positive control. Hippocampal neuronal culture from rat embryos were treated with the peptides at 1μM. Images show a clear penetration of TAT peptide into the cells, while 3R accumulate at the inner side of the plasma membrane site
(Figure-15 A and B).

Fig.15



(Figure 15) Live imaging of cell penetration capacity of PEP3 lacking TAT sequence. (A) cells expressing DsRed were imaged after application of TAT PEP3 showing endogenous signal from the peptide fluorescein to colocalize inside the cells. (B) cells expressing DsRed were imaged after application of PEP3 lacking TAT sequence showing endogenous signal from the peptide fluorescein inside the cells.

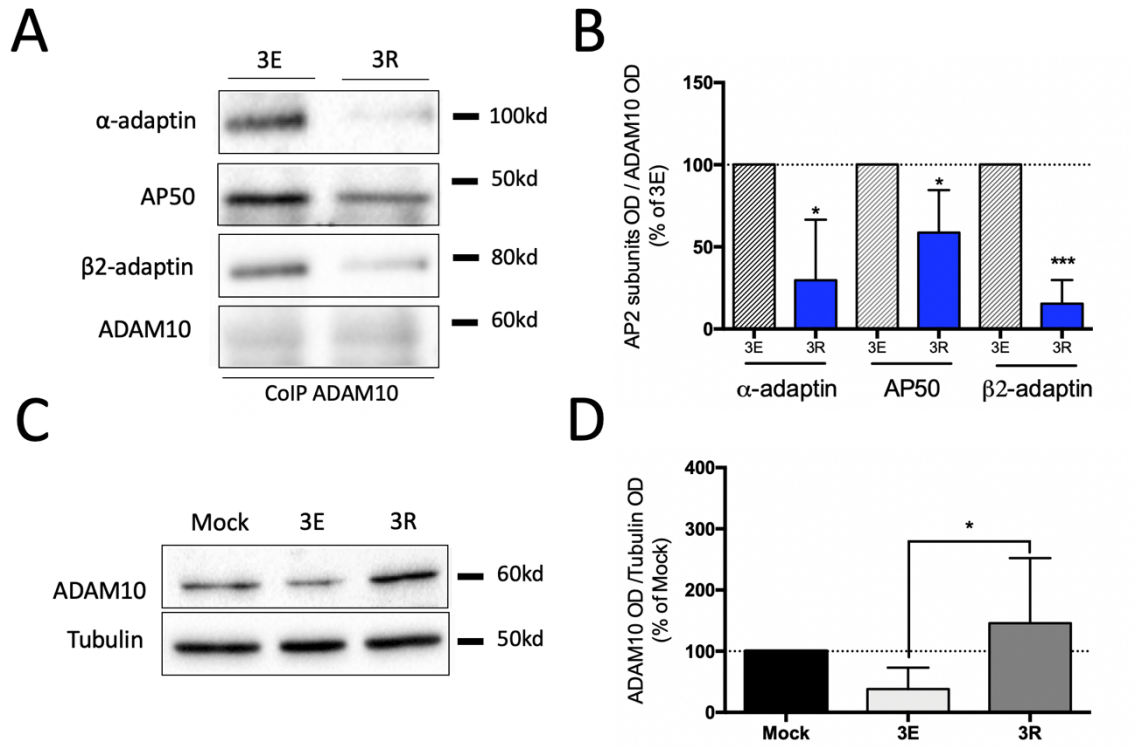
Secondly, the parameters of the efficacy of 3R peptide in targeting ADAM10 endocytosis were also assessed *in-vitro* on hippocampal neuronal culture from rat embryos. Treatment with these peptides at 1µM were applied on cells for 30 minutes. Co-immunoprecipitation was performed from the neuronal lysates to analyze the effect of the peptide on ADAM10/AP2 interaction, followed by western blotting to assess ADAM10 synaptic levels.

Immunoprecipitation of ADAM10 revealed significantly lower levels of β2-adaptin, α-adaptin and AP50 subunits of AP2 in the immunocomplex from cells treated with 3R when compared to cells treated with 3E (Figure-16 A and B). Data obtained with 3R were similar to previous *in vitro* laboratory work showing reduction of the ADAM10/AP2 complex interaction by 89% for α-adaptin subunit and by 78% for µ2-subunit when treating cells with TAT-containing PEP3 (Data not shown). Therefore, 3R has similar disruptive effect on ADAM10/AP2 complex as its TAT-containing equivalent PEP3.

Finally, these effects of 3R on the interaction led to an increase of ADAM10 synaptic levels, similar to what has been observed with the peptide PEP3 during *in-vivo* testing described above.

Significantly higher levels of synaptic ADAM10 were observed in cells treated with 3R compared to cells treated with 3E (Figure-16 C and D). Overall, these outcomes demonstrate that the 3R peptide represents the starting point for the design of a new peptidomimetic compound able to improve the pharmacokinetics properties.

Fig.16



(Figure 16) Efficacy assessment of PEP3 lacking TAT sequence, named 3R. (A) After treatment *cells were lysated and immunoprecipitated using anti-ADAM10 antibody and AP2-subunits co-precipitation was evaluated. (B).* Quantification of experiments in A. OD analysis showing treating cells with 3R significantly reduce co-immunoprecipitation of ADAM10 with α-Adaptin, AP50 and β2-Adaptin (α-Adaptin 3R: 29.67 ±21.33, N=3, t-test *p=0.003; AP50 3R: 58.67 ±14.95, N=3, t-test *p=0.05; β2-Adaptin 3R: 15.39 ±8.358, N=3, t-test ***p=0.0005) compared to cells treated with 3E. (C) *Representative immunoblot of ADAM10 WB analysis from cells TIF fraction. (D)* Quantification of experiments in C. OD analysis showing significant increase of ADAM10 synaptic levels in cells treated with 3R compared to cells treated with 3E (ADAM10 3R: 145.9 ±47.60, N=5; ADAM10 3E: 38.21 ±13.19, N=7;t-test*p=0.0296.

DISCUSSION

AD is a very complex disease that represents a real challenge for drug development. Many drugs targeting the β-secretase have failed to improve cognition or have showed toxic side effects. Lately immunotherapies targeting Aβ peptide have also been failing as Roche Holding and partners AC Immune SA have recently stopped late stage clinical trial for their antibody Crenezumab. These unsuccessful clinical trials for disease modifying agents traduce the deep complexity of understanding AD mechanisms. However, new advances have been made in developing agents that could pharmacologically up-regulate the activity of the α-secretase ADAM10. Such promising compounds are currently in clinical trials and could represent potential new therapeutics in a close future.

In light of these considerations, the results described in this thesis support the use of α-secretase potentiator in AD. After observing promising *in-vitro* results with the CPP PEP3. This PhD project had for aim to test *in-vivo* the innovative peptide with the final objective of rescuing AD phenotype in a transgenic mouse model of the pathology. The results obtained during this project demonstrated that PEP3 is able to interfere with the association between ADAM10 and AP2 complex, and thereby to modify ADAM10 local trafficking and synaptic availability. Indeed, after a 14 days treatment of transgenic mice with the CPP, ADAM10 synaptic availability was observed to be higher in these animals. These promising results led us to set up a series of experiments to assess the effect of this increase on AD mice at full-blown pathology. At 9 month of age the transgenic mice treated with PEP3 showed a decrease in ADAM10/AP2 association and an increase in ADAM10 synaptic levels. Unfortunately, those changes induced by the peptide did not lead to an improvement of the strong phenotype of the APP/PS1 mice at full-blown pathology. This absence of effect on the phenotype might be due to the far too advanced stage of the disease in these mice. At this age the disease is to be considered severe as these mice present irreversible neuronal loss together with strong cognitive impairments. The treatment with PEP3, even though showing to trigger an increase of ADAM10 synaptic localization and modification in GluN2A subunit synaptic levels, is likely to have had too mild synaptic compensatory mechanisms to reverse the tremendous disturbance occurring at this stage of the disease, such as neuronal loss, gliosis and inflammatory response. Analysis of the metabolites resulting from APP processing revealed no changes in human soluble APPα levels suggesting no increase of α-secretase activity towards humanized APP. But paradoxically a decrease of mouse soluble Aβ was

observed and could suggest either an increase of endogenous APP cleavage or an increase of Aβ peptide clearance.

In light of these promising results, decision was made to treat APP/PS1 mice with PEP3 at early stage of the disease to modify its progression and act on pathological mechanisms observed in previous studies such as synaptic loss and changes in synaptic transmission.[143] A positive effect of PEP3 on synaptic loss could strongly improve the behavior of the mice, as spine density have been observed to correlate strongly with cognitive performance in AD patients.[67] In order to treat the mice during the onset window of the disease we have screened APP/PS1 mice of different age. Screening of 3- and 6-month-old APP/PS1 revealed that ADAM10/AP2 association was already increased at 6-month-old but not yet changed at 3-month-old. In the same manner, the decrease of ADAM10 synaptic levels was observed at 6-month-old but no change was yet to be observed at 3-month-old. In light of these results, the age of 6-month-old represents an early stage of the disease correlating with the onset of ADAM10 local trafficking impairment in these mice. Therefore, we treated a new pool of APP/PS1 mice of 6-month-old to act on the disease progression as from its onset. Experiments at early stage, showed the same rescue of ADAM10 association and synaptic levels with PEP3 treatment, confirming the results previously obtained at full-blown pathology on the efficacy of the peptide to act on the α-secretase synaptic availability. In contrast to what was observed at full-blown pathology the treatment at early stage of the pathology leaded to a significant improvement of the phenotype of the transgenic mice. This behavioral readout obtained in a Novel Object Recognition Test was confirmed later in a Y-maze test. Interestingly, the mice treated with the PEP3 displayed higher preference index for the novel object and arm translating an efficient recognition, learning and memory retention in these mice. The treatment clearly improved hippocampal function as this structure is supporting spatial memory, object recognition and learning processes involved in these behavior tasks. Interestingly a increase of the synaptic levels of GluN2A subunits together with a trending increase of GluN2B subunit of the NMDA receptors were observed in the hippocampus of the mice treated with PEP3. This represents a strong evidence of a therapeutic mechanism by which the peptide could increase synapses and restore deficits in synaptic transmission occurring in the transgenic mice.

In order to further investigate the effect of PEP3, we decided to repeat a new treatment on a different line of 6-month-old APP/PS1 mice during a secondment in JANSSEN pharmaceuticals and to assess via electrophysiology the effect of the peptide on the hippocampal synaptic activity. Field excitatory postsynaptic potential were recorded using MEA setups. Unfortunately, no synaptic deficits were observed in the transgenic mice as neither a cognitive deficit was observed in a V-maze test and nor a change in ADAM10 synaptic levels after biochemical investigation. This showing the absence of the "synaptic traits" of the disease in this line of mice at this age as in this specific setup environment. Those data do confirm that the peptide is not toxic and acts specifically on the synaptic traits that are characteristic of the pathology. As no impairment of ADAM10 was present in those mice, the peptide did not show is therapeutic potential. However, in patients presenting those AD-specific synaptic deficits, the treatment could have a deep disease modifying impact.

Decision was taken to further analysis mice previously treated in Milan to screen functional changes in the hippocampus. Morphological analysis revealed a shrinkage of the spines and a drop of the number of mushroom spines in the hippocampus of the transgenic mice. Treatment with PEP3 rescued the deficit in synaptic size and density observed in the transgenic mice which is in accordance with the previously observed, increase of synaptic GluN2A and GluN2B subunit levels in the hippocampus. The metabolites were also changed as an increase of mouse sAPPα was observed in mice treated with PEP3 which could trigger an increase in hippocampal synaptic density and an improvement of memory as observed in previous studies.[144] No changes in human sAPPα levels were observed. Similar to treatment at full-blown pathology, the effect of the PEP3 at early stage of the disease could shift endogenous APP processing, but the strong mutation bearing in the transgenic mice and their effects such as overexpression of humanized APP and upregulation of β-secretase activity, could not be counteracted. Supporting this hypothesis, absence of changes in humanized metabolites such as human soluble APPα and human soluble Aβ was observed after treatment at early stage.

In conclusion, we demonstrated that the administration of PEP3 in a mouse model of AD at early stage interferes with ADAM10/AP2 complex formation and increase the synaptic availability of ADAM10. This led to an increase of the sheddase's activity towards APP as increased of endogenous mouse sAPPα was observed. In AD patients, this causality between ADAM10 activity and sAPPα levels could also hypothetically lead to the use of sAPPα

levels in CSF as biomarker of ADAM10 synaptic activity. In our experiment, the increase in mouse sAPPα level suggests a shift of APP metabolism towards the non-amyloidogenic pathway. Moreover, this increase of soluble APPα triggered compensatory mechanisms leading to improvement of synaptic organization in the hippocampus. Results observed suggest an increase of spine width, mushroom spines and overall spine density together with increase of synaptic GluN2A and GluN2B containing NMDAR in the hippocampus. Therefore, the use of PEP3 can be considered as a potential disease-modifying tool since it can affect the primary mechanism of AD pathogenesis at the synapse, and could *via* its rescue of spine morphology and density improve synaptic transmission and *in fine* alleviate cognitive impairments related to the disease progression.

The second aim of the thesis was to enhance the drugability of the agent. With this objective, a specific PEP3 lacking the TAT sequence was created and tested *in-vitro*. This peptide was able to penetrate the cells and supposedly interact with the target as observed by live imaging. As its equivalent containing the TAT sequence, the peptide lacking the TAT sequence is able to interfere with the ADAM10/AP2 protein complex. And this effect is leading to a significant increase in ADAM10 synaptic availability. These effects are expected to be followed by mechanisms observed during *in-vivo* testing. The future objective is to test this peptide *in-vivo* and assess its potential curable effect on AD mouse models.

To conclude enhancing the action of ADAM10 on the amyloid cascade represent a serious therapeutic strategy. The action of PEP3 on the endocytosis of the sheddase in order to increase its synaptic availability without affecting its systemic activity represents a clever and promising strategy to enhance the activity of the secretase in the frame of AD. In order to fully validate this strategy to be used in patients, further investigation on ADAM10 function and partners is needed. The sheddase ADAM10 has many substrates that should be further studied as they could also impact the course of the disease as a downstream effect of the PEP3-mediated upregulation of the sheddase activity. Important substrates like N-cahderin that could impact cell adhesion and Notch that could impact neurogenesis need to be further studied for their interaction with ADAM10. Finally, the most important point to clarify is the implication of the sheddase in the cross-talk between Aβ and the synaptic function. Understanding the mechanisms of this interaction is essential to counteract the synaptic loss induced by Aβ.

The synaptic dysfunction observed in AD drives the cognitive decline and are known to precede neuronal death. Therefore, the deficit in synaptic transmission is not only a consequence of cell death but a real drive for the pathology. Impairments of ADAM10 local trafficking is associated with synaptic failure related to Aβ as it has been observed in AD patients and confirmed in this study on APP/PS1 mice.

In conclusion, the impairment of ADAM10 synaptic trafficking in AD patients could have a strong negative impact on the synapse, in this sense we need to better understand the role of the sheddase as an important player of the synapse.

The comprehension of the molecular pathways underlying Aβ-induced synaptic dysfunction and their interconnection with ADAM10 activity is essential in order to develop an innovative agent targeting directly the pathogenic mechanisms of the disease.

REFERENCES

1. Amouyel, P. Maladie d'Alzheimer et autres démences. *Medecine Therapeutique* 6, 843–848 (2000).

2. Bottino, A. Space , time and remembering in the orchard of laertes : A cognitive approach. Harvard Classics@. 1–35 (2013).

3. Berchtold, N. C. & Cotman, C. W. Evolution in the conceptualization of dementia and Alzheimer's disease: Greco-Roman period to the 1960s. *Neurobiol. Aging* 19, 173–189 (1998).

4. Andres-Barquin, P. J. Ramón y Cajal: a century after the publication of his masterpiece. *Endeavour 25, 13-17* (2001).

5. López-Muñoz, F., Boya, J. & Alamo, C. Neuron theory, the cornerstone of neuroscience, on the centenary of the Nobel Prize award to Santiago Ramón y Cajal. *Brain Res. Bull.* 70, 391-405 (2006).

6. Alzheimer, A. Über eine eigenartige Erkrankung der Hirnrinde. *Allg. Zeitschrift für Psychiatr. und Psych. Medizin* (1907).

7. Stelzmann, R. A., Norman Schnitzlein, H. & Reed Murtagh, F. An english translation of alzheimer's 1907 paper, ber eine eigenartige erkankung der hirnrinde *Clin. Anat.* 8, 429–431 (1995).

8. Gaugler, J., James, B., Johnson, T., Scholz, K. & Weuve, J. 2016 Alzheimer's disease facts and figures. *The journal of the Alzheimer's association* 12, 459-509 (2016).

9. Givens, J. L., Jones, R. N., Shaffer, M. L., Kiely, D. K. & Mitchell, S. L. Survival and comfort after treatment of pneumonia in advanced dementia. *Arch. Intern. Med. 170, 1102-1107* (2010).

10. Carter, C. L., Resnick, E. M., Mallampalli, M. & Kalbarczyk, A. Sex and Gender Differences in Alzheimer's Disease: Recommendations for Future Research. *J. Women's Heal.* 21 (2012).

11. DiLuca, M. & Olesen, J. The cost of brain diseases: A burden or a challenge? *Neuron* 82, 1205–1208 (2014).

12. Prince, M. *et al.* World Alzheimer Report 2015: The Global Impact of Dementia - An analysis of prevalence, incidence, cost and trends. *Alzheimer's Dis. Int.* 84 (2015).

13. Gustavsson, A. *et al.* Cost of disorders of the brain in Europe 2010. *Eur. Neuropsychopharmacol.21, 18-79* (2011).

14. Wu, L. *et al.* Early-onset familial alzheimer's disease (EOFAD). *Canadian Journal of Neurological Sciences* 39, 436-445 (2012).

15. Bertram, L. & Tanzi, R. E. The genetics of Alzheimer's disease. *Prog. Mol. Biol. Transl. Sci.* 107, 79-100 (2012).

16. Blennow, K., Deleon, M., Zetterberg, H. Alzheimer 's Disease. The lancet neurology. 368, 387-403 (2006).

17. Crous-Bou, M., Minguillón, C., Gramunt, N. & Molinuevo, J. L. Alzheimer's disease prevention: From risk factors to early intervention. *Alzheimer's Res. Ther.* 9, 1–9 (2017).

18. Hersi, M. *et al.* Risk factors associated with the onset and progression of Alzheimer's disease: A systematic review of the evidence. *Neurotoxicology* 61, 143-187 (2017).

19. Serrano-Pozo, A., Frosch, M. P., Masliah, E. & Hyman, B. T. Neuropathological alterations in Alzheimer disease. *Cold Spring Harb. Perspect. Med.* 1, 1–23 (2011).

20. Kosik, K. S., Joachim, C. L. & Selkoe, D. J. Microtubule-associated protein tau (T) is a major antigenic component of paired helical filaments in Alzheimer disease. *Proc Natl Acad Sci USA* 83, 4044–4048 (1986).

21. Caine, Wong; George, G. Alzheimer's disease and Down's syndrome: Sharing of a unique cerebrovascular amyloid fibril protein. *Biochem. Biophys. Res. Commun.* 122, 1131–1135 (1984).

22. Ye Ingrid, Y. *et al.* γ-Secretase Substrate Concentration Modulates the AB 42 / AB 40 Ratio. *J. Biol. Chem.* 282, 23639–23644 (2007).

23. Deng, Y. *et al.* Deletion of Presenilin 1 Hydrophilic Loop Sequence Leads to Impaired Gamma Secretase Activity and Exacerbated Amyloid Pathology. *J. Neurosci.* 26, 3845–3854 (2006).

24. Cammarata, S. *et al.* Amyloid-β42 Plasma Levels are Elevated in Amnestic Mild Cognitive Impairment. *J. Alzheimer's Dis.* 18, 267–271 (2009).

25. Haass, C. & Selkoe, D. J. Cellular processing of β-amyloid precursor protein and the genesis of amyloid β-peptide. *Cell* 75, 1039-1042 (1993).

26. Musardo, S., Marcello, E., Gardoni, F. & Di Luca, M. ADAM10 in synaptic physiology and pathology. *Neurodegener. Dis.* 13, 72–74 (2014).

27. Hardy, J. A. & Higgins, G. A. Alzheimer's disease: The amyloid cascade hypothesis. *Science* 256, 184-185 (1992).

28. Taylor, P. & Brown, J. H. in *Basic Neurochemistry : Molecular , Cellular and Medical Aspects. 6th edition.* (1943).

29. Chua, J. J. E., Kindler, S., Boyken, J. & Jahn, R. The architecture of an excitatory synapse. *J. Cell Sci.* 62, 405-496 (2010).

30. Scheiffele, P. Cell-cell signaling during synapse formation in the CNS. Annu. Rev. Neurosci. 26, 485-508 (2003).

31. Buonarati, O. R., Hammes, E. A., Watson, J. F., Greger, I. H. & Hell, J. W. Mechanisms of postsynaptic localization of AMPA-type glutamate receptors and their regulation during long-term potentiation. *Sci. Signal* 12, 1-9 (2019).

32. Mayer, M. L. & Armstrong, N. Structure and Function of Glutamate Receptor Ion Channels. *Annu. Rev. Physiol.* 66, 161–181 (2004).

33. Özyener, F. Evaluation of intra-musclar oxygenation during exercise in humans. *J. Sport. Sci. Med.* 1, 15–19 (2002).

34. Bliss, T. V. P. & Collingridge, G. L. A synaptic model of memory: Long-term potentiation in the hippocampus. *Nature* 361, 31-39 (1993).

35. Malenka, R. C. & Bear, M. F. LTP and LTD- Review An Embarrassment of Riches. 44, 5–21 (2004).

36. Collingridge, G. L., Kehl, S. J. & McLennan, H. Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. *J. Physiol.* 334, 33-46 (1983).

37. Mulkey, R. M. & Malenka, R. C. Mechanisms underlying induction of homosynaptic long-term depression in area CA1 of the hippocampus. *Neuron* 9, 967-975 (1992).

38. Madden, R. The inner workings of the AMPA receptors. *Curr Opin Drug Discov devel* 5, 1-8 (2002).

39. Jurado, S. AMPA Receptor Trafficking in Natural and Pathological Aging. *Front. Mol. Neurosci.* 10, 1–14 (2018).

40. Zhu, J. J., Esteban, J. A., Hayashi, Y. & Malinow, R. Postnatal synaptic potentiation: Delivery of GluR4-containing AMPA receptors by spontaneous activity. *Nat. Neurosci.* 3, 1098-1106 (2000).

41. Burnashev, N., Monyer, H., Seeburg, P. H. & Sakmann, B. Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* 8, 189-198 (1992).

42. Kim, D. Y., Kim, S. H., Choi, H. B., Min, C. K. & Gwag, B. J. High abundance of GluR1 mRNA and reduced Q/R editing of GluR2 mRNA in individual NADPH-diaphorase neurons. *Mol. Cell. Neurosci.* 17, 1025-1033 (2001).

43. Nakazawa, K., Mikawa, S., Hashikawa, T. & Ito, M. Transient and persistent phosphorylation of AMPA-type glutamate receptor subunits in cerebellar Purkinje cells. *Neuron* 15, 697–709 (1995).

44. Lee, H. K. *et al.* Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell* 112, 631-643 (2003).

45. Lee, H.-K., Takamiya, K., He, K., Song, L. & Huganir, R. L. Specific Roles of AMPA Receptor Subunit GluR1 (GluA1) Phosphorylation Sites in Regulating Synaptic Plasticity in the CA1 Region of Hippocampus. *J. Neurophysiol.* 103 479-489 (2010).

46. Barria, A., Muller, D., Derkach, V., Griffith, L. C. & Soderling, T. R. Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. *Science 276, 2042-2045* (1997).

47. Esteban, J. A. *et al.* PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. *Nat. Neurosci.* 6, 136-143 (2003).

48. Derkach, V., Barria, A. & Soderling, T. R. Ca2+/calmodulin-kinase II enhances channel conductance of -amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. *Proc. Natl. Acad. Sci.* 96, 3269-3274 (1999).

49. Wright, A. & Vissel, B. The essential role of AMPA receptor GluR2 subunit RNA editing in the normal and diseased brain. *Front. Mol. Neurosci.* 5, 1-13 (2012).

50. Matsuda, S., Mikawa, S. & Hirai, H. Phosphorylation of serine-880 in GluR2 by protein kinase C prevents its C terminus from binding with glutamate receptor-interacting protein. *J. Neurochem.* 73, 1765-1768 (1999).

51. Petralia, R. S. & Wenthold, R. J. Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat brain. *J Comp Neurol* 318, 329-354 (1992).

52. Renner, M. C. *et al.* Synaptic plasticity through activation of GluA3-containing AMPA-receptors. *Elife* 6, 1-22 (2017).

53. Atanasova, T. *et al.* GluA4 Dependent Plasticity Mechanisms Contribute to Developmental Synchronization of the CA3–CA1 Circuitry in the Hippocampus. *Neurochemical Research* 44, 562-571 (2017).

54. Paoletti, P., Bellone, C. & Zhou, Q. NMDA receptor subunit diversity: Impact on receptor properties, synaptic plasticity and disease. *Nature Reviews Neuroscience* 14, 383–400 (2013).

55. Cull-Candy, S. G. & Leszkiewicz, D. N. Role of Distinct NMDA Receptor Subtypes at Central Synapses. *Sci. Signal.* 2004, 1-16 (2004).

56. Monyer, H., Burnashev, N., Laurie, D. J., Sakmann, B. & Seeburg, P. H. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12, 529-540 (1994).

57. Akazawa, C., Shigemoto, R., Bessho, Y., Nakanishi, S. & Mizuno, N. Differential expression of five N-methyl-D-aspartate receptor subunit mRNAs in the cerebellum of developing and adult rats. *J. Comp. Neurol.* 347, 150-160 (1994).

58. Sheng, M., Cummings, J., Roldan, L. A., Jan, Y. N. & Jan, L. Y. Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature* 368, 144-147 (1994).

59. Paoletti, P. Molecular basis of NMDA receptor functional diversity. *European Journal of Neuroscience* 33, 1351-1365 (2011).

60. Fellin, T. *et al.* Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. *Neuron* 43, 729-743 (2004).

61. Madara, J. C. & Levine, E. S. Presynaptic and Postsynaptic NMDA Receptors Mediate Distinct Effects of Brain-Derived Neurotrophic Factor on Synaptic Transmission. *J. Neurophysiol.* 100, 3175-3184 (2008).

62. Pin, J. P., Galvez, T. & Prézeau, L. Evolution, structure, and activation mechanism of family 3/C G-protein-coupled receptors. *Pharmacol. Ther.* 98, 325–354 (2003).

63. Mary, S. *et al.* The rat mGlu(1d) receptor splice variant shares functional properties with the other short isoforms of mGlu1 receptor. *Eur. J. Pharmacol.* 335, 65-72 (1997).

64. Lin, F. F. *et al.* Cloning and stable expression of the mGluR1b subtype of human metabotropic receptors and pharmacological comparison with the mGluR5a subtype. *Neuropharmacology* 36, 917-931 (1997).

65. Halina, K. W. (S)-3,5-DHPG: A Review. *CNS Drug Rev.* 2006, 557-588 (2001).

66. Selkoe, D. J. Alzheimer's disease is a synaptic failure. *Science* 298, 789-791 (2002).

67. DeKosky, S. T. & Scheff, S. W. Synapse loss in frontal cortex biopsies in Alzheimer's disease: Correlation with cognitive severity. *Ann. Neurol.* 27, 457-464 (1990).

68. Arendt, T. Synaptic degeneration in Alzheimer's disease. *Acta Neuropathologica* 118, 167-179 (2009).

69. Walsh, D. M. & Selkoe, D. J. Deciphering the molecular basis of memory failure in Alzheimer's disease. *Neuron 44, 181-193* (2004).

70. Glabe, C. G. Structural classification of toxic amyloid oligomers. *Journal of Biological Chemistry* 283, 29639-29643 (2008).

71. Hardy, J. & Selkoe, D. J. The amyloid hypothesis of Alzheimer's disease: Progress and problems on the road to therapeutics. *Science* 297, 353-356 (2002).

72. Wang, H. W. *et al.* Soluble oligomers of β amyloid (1-42) inhibit long-term potentiation but not long-term depression in rat dentate gyrus. *Brain Res.* 924, 133–140 (2002).

73. Hsiao, K. K. *et al.* Age-related CNS disorder and early death in transgenic FVB/N mice overexpressing Alzheimer amyloid precursor proteins. *Neuron* 15, 1203-1218 (1995).

74. Westerman, M. a *et al.* The relationship between Abeta and memory in the Tg2576 mouse model of Alzheimer's disease. *J. Neurosci.* 22, 1858-1867 (2002).

75. Terry, R. D. *et al.* Physical basis of cognitive alterations in alzheimer's disease: Synapse loss is the major correlate of cognitive impairment. *Ann. Neurol.* 30, 572-580 (1991).

76. Bertoni-Freddari, C., Fattoretti, P., Casoli, T., Caselli, U. & Meier-Ruge, W. Deterioration threshold of synaptic morphology in aging and senile dementia of Alzheimer's type. *Anal. Quant. Cytol. Histol.* 196, 1-13 (1996).

77. Moechars, D. *et al.* Early phenotypic changes in transgenic mice that overexpress different mutants of amyloid precursor protein in brain. *J. Biol. Chem.* 274, 6483-6492 (1999).

78. Lacor, P. N. Synaptic Targeting by Alzheimer's-Related Amyloid Oligomers. *J. Neurosci.* 24, 10191-10200 (2004).

79. Lacor PN *et al.* Abeta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. *J. Neurosci.* 27, 796-807 (2007).

80. Roselli, F. Soluble -Amyloid1-40 Induces NMDA-Dependent Degradation of Postsynaptic Density-95 at Glutamatergic Synapses. *J. Neurosci.* 25, 11061-11070 (2005).

81. Snyder, E. M. *et al.* Regulation of NMDA receptor trafficking by amyloid-β. *Nat. Neurosci.* 8, 1051-1058 (2005).

82. Shankar, G. M. *et al.* Natural Oligomers of the Alzheimer Amyloid- Protein Induce Reversible Synapse Loss by Modulating an NMDA-Type Glutamate Receptor-Dependent Signaling Pathway. *J. Neurosci. 27, 2866-2875* (2007).

83. Li, S. *et al.* Soluble A Oligomers Inhibit Long-Term Potentiation through a Mechanism Involving Excessive Activation of Extrasynaptic NR2B-Containing NMDA Receptors. *J. Neurosci.* 31, 6627-6638 (2011).

84. Talantova, M. *et al.* A induces astrocytic glutamate release, extrasynaptic NMDA receptor activation, and synaptic loss. *Proc. Natl. Acad. Sci.* 110, 13690-13691 (2013).

85. Chen, K. H., Reese, E. A., Kim, H. W., Rapoport, S. I. & Rao, J. S. Disturbed neurotransmitter transporter expression in Alzheimer's disease brain. *J. Alzheimer's Dis.* 26, 755-766 (2011).

86. Abramov, E. *et al.* Amyloid-B as a positive endogenous regulator of release probability at hippocampal synapses. *Nat. Neurosci.* 12, 1567-1576 (2009).

87. Scimemi, A. *et al.* Amyloid-β1-42slows clearance of synaptically released glutamate by mislocalizing astrocytic GLT-1. *Ann. Intern. Med. 33, 5312-5318* (2013).

88. Noda, M., Nakanishi, H. & Akaike, N. Glutamate release from microglia via glutamate transporter is enhanced by amyloid-beta peptide. *Neuroscience* 92, 1465-1474 (1999).

89. Cirrito, J. R. *et al.* Synaptic activity regulates interstitial fluid amyloid-β levels in vivo. *Neuron 48, 913-922* (2005).

90. Lesne, S. NMDA Receptor Activation Inhibits -Secretase and Promotes Neuronal Amyloid- Production. *J. Neurosci.* 25, 9367-9377(2005).

91. Hoey, S. E., Williams, R. J. & Perkinton, M. S. Synaptic NMDA Receptor Activation Stimulates -Secretase Amyloid Precursor Protein Processing and Inhibits Amyloid- Production. *J. Neurosci.* 29, 4442-4460 (2009).

92. Yamamoto-Sasaki, M., Ozawa, H., Saito, T., Rösler, M. & Riederer, P. Impaired phosphorylation of cyclic AMP response element binding protein in the hippocampus of dementia of the Alzheimer type. *Brain Res.* 924, 300-303 (1999).

93. Hardingham, G. E., Fukunaga, Y. & Bading, H. Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat. Neurosci.* 5, 405-414 (2002).

94. Smith. Reversal of long-term dendritic spine alterations in Alzheimer disease models. *Proc Natl Acad Sci USA* 29, 16877-16880 (2009).

95. Dieterich, D. C. *et al.* Caldendrin-Jacob: A protein liaison that couples NMDA receptor signalling to the nucleus. *PLoS Biol.* 6, 286-306 (2008).

96. Karpova, A. *et al.* Encoding and transducing the synaptic or extrasynaptic origin of NMDA receptor signals to the nucleus. *Cell* 152, 1119-1133 (2013).

97. Rönicke, R. *et al.* Early neuronal dysfunction by amyloid β oligomers depends on activation of NR2B-containing NMDA receptors. *Neurobiol. Aging* 32, 2219-2228 (2011).

98. Tao, X., Finkbeiner, S., Arnold, D. B., Shaywitz, A. J. & Greenberg, M. E. Ca2+influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. *Neuron* 20, 709-726 (1998).

99. Peng, S. *et al.* Decreased Brain-Derived Neurotrophic Factor Depends on Amyloid Aggregation State in Transgenic Mouse Models of Alzheimer's Disease. *J. Neurosci.* 29, 9321-9329 (2009).

100. Peng, S., Wuu, J., Mufson, E. J. & Fahnestock, M. Precursor form of brain-derived neurotrophic factor and mature brain-derived neurotrophic factor are decreased in the pre-clinical stages of Alzheimer's disease. *J. Neurochem.* 93, 1412-1421 (2005).

101. Blurton-Jones, M. *et al.* Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. *Proc. Natl. Acad. Sci.* 106, 13594-13599 (2009).

102. Caccamo, A., Maldonado, M. A., Bokov, A. F., Majumder, S. & Oddo, S. CBP gene transfer increases BDNF levels and ameliorates learning and memory deficits in a mouse model of Alzheimer's disease. *Proc. Natl. Acad. Sci.* 107, 22687-22692 (2010).

103. Danysz, W. & Parsons, C. G. Alzheimer's disease, β-amyloid, glutamate, NMDA receptors and memantine - Searching for the connections. *British Journal of Pharmacology* 167, 324-352 (2012).

104. Anders, A., Gilbert, S., Garten, W., Postina, R. & Fahrenholz, F. Regulation of the α-secretase ADAM10 by its prodomain and proprotein convertases. *FASEB J.* 15, 1837-1839 (2001).

105. Saftig, P. & Lichtenthaler, S. F. The alpha secretase ADAM10: A metalloprotease with multiple functions in the brain. *Progress in Neurobiology* 135, 1-20 (2015).

106. Schäfer, W. *et al.* Two independent targeting signals in the cytoplasmic domain determine trans-Golgi network localization and endosomal trafficking of the proprotein convertase furin. *EMBO J.* 14, 2424-2435 (1995).

107. Moss, M. L. *et al.* The ADAM10 prodomain is a specific inhibitor of ADAM10 proteolytic activity and inhibits cellular shedding events. *J. Biol. Chem.* 282, 35712-35721 (2007).

108. Postina, R. *et al.* A disintegrin-metalloproteinase prevents amyloid plaque formation and hippocampal defects in an Alheizmer disease mouse model. *J. Clin. Invest.* 113, 1456-1464 (2004).

109. Fahrenholz, F., Gilbert, S., Kojro, E., Lammich, S. & Postina, R. α-Secretase Activity of the Disintegrin Metalloprotease ADAM 10: Influences of Domain Structure. *Ann. N. Y. Acad. Sci.* 2297, 1-8 (2006).

110. Horiuchi, K. *et al.* Substrate selectivity of epidermal growth factor-receptor ligand sheddases and their regulation by phorbol esters and calcium influx. *Mol. Biol. Cell* 18, 176-188 (2007).

111. Lammich, S. *et al.* Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. *Proc. Natl. Acad. Sci.* 96, 3922-3927 (1999).

112. Matthews, A. L., Noy, P. J., Reyat, J. S. & Tomlinson, M. G. Regulation of A disintegrin and metalloproteinase (ADAM) family sheddases ADAM10 and ADAM17: The emerging role of tetraspanins and rhomboids. *Platelets* 28, 333-341 (2017).

113. Vincent, B. *et al.* The Disintegrins ADAM10 and TACE Contribute to the Constitutive and Phorbol Ester-regulated Normal Cleavage of the Cellular Prion Protein. *J. Biol. Chem.* 276, 37742-37743 (2001).

114. Reiss, K. *et al.* ADAM10 cleavage of N-cadherin and regulation of cell-cell adhesion and β-catenin nuclear signalling. *EMBO J.* 24, 742-752(2005).

115. Litterst, C. *et al.* Ligand binding and calcium influx induce distinct ectodomain/γ-secretase-processing pathways of EphB2 receptor. *J. Biol. Chem.* 282, 16155–16163 (2007).

116. Boller, F. & Forbes, M. M. History of dementia and dementia in history: An overview. *Journal of the Neurological Sciences* 158, 125–133 (1998).

117. Marcello, E., Borroni, B., Pelucchi, S., Gardoni, F. & Di Luca, M. ADAM10 as a therapeutic target for brain diseases: From developmental disorders to Alzheimer's disease. *Expert Opinion on Therapeutic Targets* 21, 1017-1026 (2017).

118. Marcello, E. *et al.* Endocytosis of synaptic ADAM10 in neuronal plasticity and Alzheimer's disease. *J. Clin. Invest.* 123, 2523–2538 (2013).

119. Marcello, E. *et al.* Synapse-Associated Protein-97 Mediates -Secretase ADAM10 Trafficking and Promotes Its Activity. *J. Neurosci.* 27, 1682–1691 (2007).

120. Wan, X.-Z. *et al.* Activation of NMDA Receptors Upregulates A Disintegrin and Metalloproteinase 10 via a Wnt/MAPK Signaling Pathway. *J. Neurosci.* 32, 3910–3916 (2012).

121. Marcello, E., Gardoni, F., Luca, M. Di & Pe, I. An Arginine Stretch Limits ADAM10 Exit from the. *J. Biol. Chem.* 285, 10376–10384 (2010).

122. Endres, K. & Deller, T. Regulation of Alpha-Secretase ADAM10 In vitro and In vivo: Genetic, Epigenetic, and Protein-Based Mechanisms. *Front. Mol. Neurosci.* 10, 1-18 (2017).

123. Marcello, E. *et al.* SAP97-mediated local trafficking is altered in Alzheimer disease patients' hippocampus. *Neurobiol. Aging* 33, 422-432 (2012).

124. Epis, R. *et al.* Blocking ADAM10 synaptic trafficking generates a model of sporadic Alzheimer's disease. *Brain* 133, 3323-3325 (2010).

125. Suh, J. *et al.* ADAM10 Missense Mutations Potentiate β-Amyloid Accumulation by Impairing Prodomain Chaperone Function. *Neuron 80, 385-401* (2013).

126. Francis, P. T., Palmer, A. M., Snape, M. & Wilcock, G. K. The cholinergic hypothesis of Alzheimer's disease: A review of progress. *Journal of Neurology Neurosurgery and Psychiatry* 66, 137-147 (1999).

127. Grutzendler, J. & Morris, J. C. Cholinesterase inhibitors for Alzheimer's disease. *Drugs* 61, 41-52 (2001).

128. Reisberg, B. *et al.* Memantine in Moderate-to-Severe Alzheimer's Disease. *N. Engl. J. Med.* 348, 1333-1341 (2003).

129. Yiannopoulou, K. G. & Papageorgiou, S. G. Current and future treatments for Alzheimer's disease. *Therapeutic Advances in Neurological Disorders* 6, 19-33 (2013).

130. Howard, R. *et al.* Donepezil and Memantine for Moderate-to-Severe Alzheimer's Disease. *N. Engl. J. Med.* 366, 893-903 (2012).

131. Kumar, A., Singh, A. & Ekavali. A review on Alzheimer's disease pathophysiology and its management: An update. *Pharmacological Reports* 67, 195-203 (2015).

132. Wong, G. T. *et al.* Chronic Treatment with the γ-Secretase Inhibitor LY-411,575 Inhibits γ-Amyloid Peptide Production and Alters Lymphopoiesis and Intestinal Cell Differentiation. *J. Biol. Chem.* 279, 12876-12882 (2004).

133. Huang, W., Yu, H., Sheng, R., Li, J. & Hu, Y. Identification of pharmacophore model, synthesis and biological evaluation of N-phenyl-1-arylamide and N-phenylbenzenesulfonamide derivatives as BACE 1 inhibitors. *Bioorganic Med. Chem.* 16, 10190-10197 (2008).

134. Choi, Y. H. *et al.* In vitro BACE-1 inhibitory phenolic components from the seeds of Psoralea corylifolia. *Planta Med.* 74, 1405-1408 (2008).

135. Coimbra, J. R. M. *et al.* Highlights in BACE1 Inhibitors for Alzheimer's Disease Treatment. *Front. Chem.* 6, 1-10 (2018).

136. Marcade, M. *et al.* Etazolate, a neuroprotective drug linking GABAA receptor pharmacology to amyloid precursor protein processing. *J. Neurochem.* 106, 392-404 (2008).

137. Vellas, B. *et al.* EHT0202 in Alzheimers Disease: A 3-Month, Randomized, Placebo-Controlled, Double-Blind Study. *Curr. Alzheimer Res.* 8, 203-212 (2011).

138. Jankowsky, J. L. *et al.* Mutant presenilins specifically elevate the levels of the 42 residue β-amyloid peptide in vivo : evidence for augmentation of a 42-specific γ-secretase. 13, 159–170 (2004).

139. Diering, G. H., Heo, S., Hussain, N. K., Liu, B. & Huganir, R. L. Extensive phosphorylation of AMPA receptors in neurons. 113, 4920-4927 (2016).

140. Jackson, R. J. *et al.* Human tau increases amyloid b plaque size but not amyloid b - mediated synapse loss in a novel mouse model of Alzheimer ' s disease. 44, 3056–3066 (2016).

141. Radde, R. *et al.* EMBO journal. Scientific report. 7, 940-946 (2006).

142. Corbett, N. J. & Hooper, N. M. Soluble Amyloid Precursor Protein a : Friend or Foe? Adv. Exp. Med. Biol. 1112, 177–183 (2018).

143. Hong, S. *et al.* Complement and microglia mediate early synapse loss in Alzheimer mouse models. *Science* 352, 712-716 (2016).

144. Roch, J. M. *et al.* Increase of synaptic density and memory retention by a peptide representing the trophic domain of the amyloid β/a4 protein precursor. *Proc Natl Acad Sci USA* 91, 7450–7454 (1994).

PhD Research activity

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COORDINATOR: Prof. Alberico L. Catapano
Academic years: 2017-2018

Poster presentations:

1 **European Synapse meeting (ESM), December 2017, MILAN, ITALY**
S. Therin, S. Musardo, F. Seibt, A. Ribeiro, D. Di Marino, C. Balducci, G. Forloni, V. Grieco, C. Giudice, F. Gardoni , J. Pita-Almenar, M. Di Luca, E. Marcello. ***An innovative tool to modulate ADAM10 synaptic localization and activity in a mouse model of Alzheimer's disease.***

2 **Federation of European Neuroscience societies (FENS), July 2018, BERLIN, GERMANY**
S. Therin, S. Musardo, F. Seibt, A. Ribeiro, D. Di Marino, C. Balducci, G. Forloni, V. Grieco, C. Giudice, F. Gardoni , J. Pita-Almenar, M. Di Luca, E. Marcello. ***An innovative tool to modulate ADAM10 synaptic localization and activity in a mouse model of Alzheimer's disease.***

3 **American Society for Neuroscience (SfN), November 2018, SAN DIEGO, USA**
S. Therin, S. Musardo, F. Seibt, A. Ribeiro, D. Di Marino, C. Balducci, G. Forloni, V. Grieco, C. Giudice, F. Gardoni , J. Pita-Almenar, M. Di Luca, E. Marcello. ***An innovative tool to modulate ADAM10 synaptic localization and activity in a mouse model of Alzheimer's disease***.

Data presentations:

University of Milan June 15th 2016	Data presentation during "Next step 7" conference. (200 people)
DZNE, Bonn, Germany May 9th-10th 2017	SyDAD Annual meeting Data presentation for EU committee. (European committee + SyDAD commitee)
University of Milan April 26th-28th 2017	Data presentation during PhD Spring Camp in Gargnano. (50 people)
University of Milan June 15th 2017	Data presentation during "Next Step 8" conference. (300 people)
University of Milan April 7th-8th 2018.	SyDAD annual Meeting Data presentation. (European committee + SyDAD commitee)
University of Milan April 14th 2018	Skype Data presentation during PhD Spring Camp. (50 people)
Bordeaux, France July 7th-11th 2018	SyDAD final annual Meeting Data presentation. (European committee + SyDAD commitee)

Attended courses/workshops:

2016

- Kick-off Meeting, Karolinska Institutet, April 21th-22th.
- Innovation Workshops, Serendipity Innovations and Axon Neuroscience, April 23th.
- Alzheimer Disease Course, Karolinska Institutet, April 25-29th. (ETCS 1,5)
- Transversal competence courses, Course A: Open access, University of Milan, September 26th.
- Transversal competence courses, Course B: Evaluation of research, University of Milan, October 3rd.
- Pharmacological properties of biotechnological drugs, University of Milan, June 14-17th. (ETCS 2)
- Advanced analytical approaches in clinical and experimental pharmacology, University of Milan, July 4th- 7th. (ETCS 2)

2017

- Synapse Methodology course Bordeaux, January 18-26th. (ETCS 1,5)
- Drug Discovery Course, Axon Neuroscience, March 26-28th. (ETCS 1,5)
- Molecular biology Course, University of Milan, 19th June; 4th July and 10th July. (ECTS 1)
- Genetic toxicological issues and methodological approaches in vitro and in vivo, University of Milan, July 13th. (ECTS 2)
- Neuropsychopharmacology Course, University of Milan, July 21th. (ECTS 2)

2018

- Workshop Project Management and career plan, University of Milano, May 9th. (ETCS 1)

Other activities & Outreach activities:

- Italian language course, University of Milan, September 2016 – February 2017.
- Introduction of pharmacology to the public at department booth during the European Researcher night, Milan September 2017
- Representing SyDAD program at European corner during the European Researcher night, Milan, September 2018.
- Teaching at Workshop on Advanced Methods for Preclinical Alzheimer Disease Research, Bordeaux School of Neuroscience, January 21th-February 2nd, 2019.

Patent and Publication in preparation:

Patent application:

Inventor in the Italian Patent Application No.102017000149130 filed on 22/12/17.
Under review for extension of the patent protection to US and Chinese regulatory agencies.

"Peptides able to activate ADAM10 enzyme, adequate for treatment of diseases characterized by an increased amyloid beta peptide production"

Publication combining PhD Data in preparation:

"Use of a Cell Permeable Peptide to modulate ADAM10 synaptic localization and activity in a mouse model of Alzheimer's disease"

PhD Secondment:

8 months PhD secondment in Janssen Pharmaceutica in Belgium: Electrophysiological assays following intraperitoneal administration of CPPs in APP/PS1 mice.