Universidade de Lisboa

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Orientador: Professora Doutora Maria José de Oliveira Diógenes Nogueira

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Dissertação especialmente elaborada para obtenção do grau de Mestre em Neurociências

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I PUBLICATIONS

Papers where the author of this thesis participate during her master:

- Jerónimo-Santos A, Fonseca-Gomes J**,** Guimarães DA, **Tanqueiro SR**, Ramalho RM, Ribeiro JA, Sebastião AM, Diógenes MJ (2015). Brain-Derived Neurotrophic Factor mediates neuroprotection against Aβ-induced toxicity through a mechanism independent on adenosine 2A receptor activation. *Growth Factors*, 33: 298-308.

- Ribeiro FF, Xapelli S, Miranda-Lourenço C, **Tanqueiro SR**, Fonseca-Gomes J, Diógenes MJ, Ribeiro JA, Sebastião AM (2015). The role of purines in neuroregeneration and neuroprotection. *Neuropharmacology,* S0028-3908(15)30170-2.

I RESUMO

O fator neurotrófico derivado do cérebro (*Brain-derived neurotrophic factor* - BDNF) desempenha importantes funções no sistema nervoso central, nomeadamente diferenciação e sobrevivência neuronais e regulação da transmissão e plasticidade sinápticas. Em algumas doenças neurodegenerativas, como na doença de Alzheimer (*Alzheimer's disease* - AD) que se caracteriza por declínio cognitivo e perda de memória, sabe-se que a sinalização mediada pelo BDNF se encontra diminuída. De facto, tanto em doentes como em modelos animais de AD, existem evidências de que os níveis proteicos de BDNF e da isoforma completa do seu recetor, TrkB-FL (*full length* – FL), se encontram diminuídos. O BDNF tem também a capacidade de se ligar a recetores TrkB truncados (*truncated TrkB* – TrkB-TC), porém estes recetores são moduladores negativos de TrkB-FL, uma vez que são incapazes de iniciar as vias de sinalização mediadas pelo BDNF. Estudos recentes revelaram que o recetor TrkB-FL é clivado por um grupo de proteases, designadas por calpaínas, resultando na formação de um novo recetor TrkB truncado (TrkB-T') e de um fragmento intracelular (*intracellular domain* - ICD) que é translocado para o núcleo. Não se conhecem ainda em profundidade as ações destes novos fragmentos mas sabe-se que a função do BDNF fica severamente comprometida. As calpaínas são proteases dependentes de cálcio, sendo por isso ativadas por um aumento dos níveis intracelulares deste catião. Os recetores N-metil-D-aspartato (NMDARs), importantes mediadores da plasticidade sináptica, são permeáveis a cálcio e podem ser encontrados tanto na região sináptica como na extrassináptica. Enquanto a ativação dos recetores NMDAR sinápticos resulta em alterações que promovem a neuroprotecção, a ativação dos NMDARs extrassinápticos induz, preferencialmente, fenómenos de morte neuronal. Curiosamente, sabe-se que os eNMDARs se encontram sobreactivados em diversas condições patológicas, inclusivamente em modelos de AD, e que podem ter um importante papel na desregulação dos níveis de cálcio intracelular. Assim, o trabalho desenvolvido nesta tese teve como objetivo investigar se a ativação dos eNMDARs contribui para a ativação das calpaínas e consequente clivagem dos recetores TrkB-FL, assim como a perda da sinalização do BDNF.

Em primeiro lugar, investigou-se se a prevenção da ativação dos eNMDARs, em neurónios expostos ao péptido β amilóide (*amyloid* β – Aβ), inibia a clivagem dos recetores TrkB-FL pelas calpaínas. Para testar esta hipótese, culturas primárias de neurónios de rato Sprague-Dawley com 14 dias *in vitro* (DIV14), foram incubadas durante 24 h com $A\beta_{25-35}$ (25 μM), o mais pequeno fragmento tóxico do péptido Aβ, e memantina (1 μM), fármaco que bloqueia preferencialmente eNMDARs sobreactivados. Os resultados obtidos indicaram, como esperado, que $A\beta_{25-35}$ induz um aumento dos níveis dos produtos específicos da clivagem da αII-espectrina mediada pelas calpaínas (*specific spectrin breakdown products* – SBDP150) sugerindo que existe uma forte ativação destas proteases. Esta alteração traduziu-se na diminuição significativa dos níveis proteicos de TrkB-FL e num aumento nos níveis de TrkB-ICD. Por outro lado, os resultados mostraram, pela primeira vez, que os efeitos de $A\beta_{25-35}$ são prevenidos pela co-incubação com a memantina: i) a formação de SBDP150 diminuiu, ii) os níveis dos recetores TrkB-FL aumentaram e iii) os níveis de TrkB-ICD diminuíram. Assim, os resultados indicam que a ativação dos eNMDAR parece estar envolvida na ativação das calpaínas e, consequentemente, na clivagem dos recetores TrkB-FL.

Uma vez que a formação de novas sinapses são processos que estão na base da formação de memória, pensa-se que as alterações que decorrem no número de espinhas dendríticas num neurónio de um doente de AD tem um papel preponderante nos défices cognitivos que se desenvolvem possivelmente adjacentes à perda neuronal. Sabe-se que o BDNF aumenta o número de espinhas dendríticas, protusões sinápticas através das quais a maioria das sinapses excitatórias ocorre e que correspondem à força de atividade sináptica de um neurónio. Assim, propusemo-nos avaliar, através de imunocitoquímica, se a ativação dos eNMDARs está relacionada com a perda de espinhas dendríticas num neurónio. Os resultados foram obtidos a partir de neurónios provenientes da cultura primária de rato a DIV14. O número de protusões (espinhas dendríticas e filopodia, protusões mais finas) foi quantificado em frações de 10 μm da dentrite-mãe a uma distância de 25 μm do corpo celular do neurónio.

Os resultados indicam, como esperado, que $A\beta_{25-35}$ diminui significativamente o número de protusões e que o BDNF aumenta o número de protusões *per se*. Na presença de $A\beta_{25-35}$, os resultados sugerem que o BDNF perde a sua ação no aumento

do número de protusões. Essa função é recuperada aquando bloqueio dos eNMDARs com memantina, assim como bloqueio da atividade das calpaínas com MDL28170 (20 μM). Estes resultados propõem que os mecanismos através dos quais Aβ interfere com as espinhas dendríticas envolvem não só a ativação das calpaínas, como visto anteriormente, como também a ativação dos eNMDARs.

Por outro lado, foi também nosso propósito avaliar se o bloqueio dos eNMDARs restaurava o efeito do BDNF na potenciação de longa duração (long-term potentiation – LTP) na área CA1 do hipocampo, o mecanismo fisiológico da aprendizagem e memória, cuja magnitude se encontra diminuída na presença de Aβ.

Para tal, foram preparadas fatias de hipocampo de rato Wistar com 8-12 semanas de vida e após incubação durante 3h com $A\beta_{25-35}$ (25 μM) e/ou memantina (1 μM) procedemos ao registo extracelular dos potenciais pós-sinápticos excitatórios de campo (field excitatory *postsynaptic potentials* – fEPSP) e à indução de LTP na ausência ou presença de BDNF (20 ng/mL). Os dados sugerem, como esperado que, em fatias incubadas exclusivamente com líquido cefalorraquidiano artificial (*artificial cerebrospinal fluid* - aCSF), o BDNF induz um aumento significativamente da magnitude da LTP e que, na presença de Aβ, o BDNF perde a sua ação na LTP. Curiosamente, os nossos resultados indicam, pela primeira vez, que a co-incubação de memantina e $A\beta_{25–35}$ restaura a capacidade do BDNF em facilitar a LTP. Estes resultados indicam que a sinalização mediada pelo BDNF na LTP se encontra diminuída na presença de Aβ e que esta diminuição pode ser mediada pela ativação dos eNMDARs.

Em conclusão, os resultados sugerem que, na presença de Aβ, a sinalização mediada pelo BDNF se encontra severamente diminuída, afetando as suas ações sinápticas, através de um mecanismo que possivelmente é mediado pela ativação dos eNMDARs. Estas evidências realçam a consequência funcional da clivagem dos recetores TrkB-FL induzida pelo Aβ e propõem a modulação dos eNMDARs de modo a prevenir a desregulação dos níveis intracelulares de cálcio e, consequentemente, a perda dos mecanismos neuroprotetores mediados pelo BDNF.

Palavras-Chave: BDNF, péptido β-amilóide, recetor TrkB, calpaínas, recetores NMDA extrassinápticos

| ABSTRACT

The brain-derived neurotrophic factor (BDNF) plays important functions in the central nervous system, such as cell survival, neuronal outgrowth, differentiation and plasticity**.** In contrast, BDNF signaling is known to be impaired in some neurodegenerative diseases, including Alzheimer's disease (AD), which is characterized by cognitive decline and loss of memory. In fact, in AD patients and in several AD models a decrease in BDNF and its main receptor, TrkB-full length (TrkB-FL), has been reported.

BDNF can also bind to truncated TrkB (TrkB-TC), however these receptors act as dominant negative inhibitor of TrkB-FL since they cannot initiate BDNF signaling. Recent evidences revealed that TrkB-FL is processed by calpains, which results in the formation of a new truncated TrkB (TrkB-T') and in the formation of an intracellular domain (ICD) fragment. Thus, this cleavage culminates in the receptor loss of function. Calpains are Ca^{2+}-dependent proteases that are activated by increased intracellular levels of this cation. N-methyl-d-aspartate receptors (NMDARs), which are known to be permeable to Ca^{2+}, are essential mediators of brain synaptic plasticity and can be found at synaptic and extrasynaptic sites. Synaptic NMDARs are neuroprotective, whereas extrasynaptic NMDARs (eNMDARs) preferentially initiate cell death pathways. Importantly, eNMDARs are known to be over activated in AD. Furthermore, NMDARs have been proposed as one of the molecules that might be involved in intracellular Ca^{2+} deregulation. Thus, we purposed to investigate if, by preventing eNMDAR activation in primary rat neurons or hippocampal slices exposed to the active fragment of amyloid β ($A\beta_{25-35}$) (25 μM), one of the main neurotoxic species that contribute to AD progression, we could inhibit the truncation of TrkB-FL by calpains, restoring the functions of BDNF. Our results have shown that the inhibition of eNMDAR by memantine (1 μM), which preferentially blocks extrasynaptic receptors over synaptic receptors, reduces significantly the activation of calpains. These findings are related with an increase in TrkB-FL levels and a decrease in ICD levels. Moreover, it is known that BDNF increases the number of spines in one neuron, which are synaptic protrusions where the majority of excitatory post-synaptic domains are localized and that can highly predict the strength of synaptic activity. Our results indicate that, in the

presence of Aβ, BDNF loses its function upon spine density, which is prevented when calpains activity is inhibited with MDL28170 (20 μM) or while eNMDAR blockade. Finally, data suggest that the inhibition of eNMDAR restores the capacity of BDNF to enhance long-term potentiation in hippocampal slices, the physiological basis for learning and memory that is known to be impaired in the presence of Aβ. Finally, the focus of our work was to clarify the mechanism by which BDNF loss its function upon synapses. In conclusion, data suggest that, in primary neuronal cultures and hippocampal slices, Aβ severely impairs BDNF/TrkB-FL signaling affecting the synaptic actions of BDNF by a mechanism that is, at least in part, mediated by eNMDARs activation. These findings highlight the functional consequence of the Aβ-induced cleavage of TrkB receptors and propose eNMDAR modulation to prevent the disruption of Ca^{2+} homeostasis and, consequently, the loss of physiological mechanisms that depend on BDNF.

Keywords: BDNF, β-amyloid peptide, TrkB receptor, calpains, extrasynaptic NMDA receptors

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IABBREVIATIONS LIST

Aβ Amyloid-β
aCSF Artificial cerebrospinal fluid
AD Alzheimer's disease
AMPA Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR AMPA receptor
APOE Apolipoprotein E
APP Amyloid precursor protein
BAD Bcl-2-associated death promoter
BDNF Brain-derived neurotrophic factor
BSA Bovine serum albumin
CA1 Cornu ammonis area 1
CA3 Cornu ammonis area 3
CaMK Ca^{2+}-calmodulin-regulated Kinase
CDK5 Cyclin-dependent kinase 5
CNS Central nervous system
CREB CRE binding protein
CTR Control
DAG Diacylglycerol
DIV Days *in vitro*
DTT 1,4-dithiothreitol
EDTA Ethylenediaminetetraacetic acid
E-LTP Early LTP
ER Endoplasmic reticulum
ERK Extracellular signal-regulated kinase
eNMDAR Extrasynaptic NMDA receptor
fEPSP Field excitatory postsynaptic potential
FL Full-length
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
HBSS Hanks' balanced salt solution
HFS High frequency stimulation
ICD Intracellular domain
IkB Inhibitor of kappa B
IP3 Inositol 1,4,5-triphosphate
IP3R IP3 receptor
L-LTP Late phase of LTP
LTP Long-term potentiation
MAP Microtubule-associated protein
MAPK Mitogen-activated protein kinase
MDL28170 N-[N-[(phenylmethoxy)carbonyl]-L-valyl]-phenylalaninal
MEK Mitogen-activated protein kinase kinase
NFkB Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NFT Neurofibrillary tangles
NGF Nerve growth factor
NMDAR N-Methyl-D-aspartate receptor
NT Neurotrophin
p75NTR p75 neurotrophin receptor
PBS Phosphate buffered saline
PI3K Phosphatidylinositol 3-kinase
PIP_3 Phosphatidylinositol-3,4,5-trisphosphate
PKC Protein kinase C
PLC Phospholipase C
PSD Postsynaptic density
PVDF Polyvinylidene difluoride
RIPA Radioimmunoprecipitation assay buffer
ROS Reactive oxygen species
RT Room temperature
SBDP Spectrin breakdown product
SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM Standard error of the mean
sNMDAR Synaptic NMDA receptor
TC Truncated
TBS-T Tris-buffered saline-Tween 20
Trk Tropomyosin-related kinase
TrkB-FL TrkB full-length
TrkB-T' TrkB truncated (calpain-generated)
TrkB-T1 TrkB truncated isoform 1
TrkB-T2 TrkB truncated isoform 2
TrkB-TC TrkB truncated (total pool)
TrkB-ICD TrkB intracellular domain
TRPC Canonical transient receptor potential

1 | INTRODUCTION

1.1| Alzheimer's Disease

1.1.1| Overview

Alzheimer's disease (AD) is the most common form of dementia and the most prevalent neurodegenerative disease in the elderly population, affecting almost 40 million people worldwide (Brookmeyer et al., 2007, Alzheimer's, 2015). AD progression has been associated with a gradual damage in function and structure in the hippocampus and neocortex, brain areas involved in memory and cognition (Braak et al., 1993). As a consequence, the most common symptom is the gradually worsening ability to remember new information. AD is ultimately fatal due to brain changes that severely impair basic physiological functions (Alzheimer's, 2014).

The precise pathophysiological changes that trigger the development of AD remain largely unknown. Only approximatly 1% of AD cases are caused by three known genetic mutations, involving the gene for the amyloid precursor protein (APP) and the genes for the presenilin 1 and presenilin 2 proteins (Rogaeva, 2002). The majority of cases, however, are sporadic, with the disease onset starting usually after 65 years old. Thus, as a multifactorial disease, increased age is considered the main risk factor for developing AD (Guerreiro et al., 2012). In addition, individuals with the ε4 form of the gene apolipoprotein E (APOE ε4) for brain cholesterol transport are also at increased risk (Kim et al., 2009). Moreover, a diagnosis of mild cognitive impairment, cardiovascular disease, high blood cholesterol, diabetes, obesity and traumatic brain injury are also associated with a higher risk of developing AD (Boyle et al., 2006, Stampfer, 2006, Barbagallo and Dominguez, 2014, Naderali et al., 2009).

Although first described in 1906 by the neuropathologist Alois Alzheimer, the research into AD symptoms, causes and risk factors has only gained impetus in the last 25 years, while efficient treatment is still lacking (Goedert and Spillantini, 2006).

1.1.2| Pathophysiology

More than 100 years ago, Dr. Alois Alzheimer identified neurofibrillary tangles (NFT) and neuritic plaques in a 51-years-old brain's patient that presented a severe cognitive decline by the time of death (Hippius and Neundorfer, 2003). Later, the identification of tau protein as the major NFT component and amyloid β (Aβ) peptide as the major plaque component (Figure 1) led to a new era of research on AD (Kidd, 1963, Terry et al., 1964).

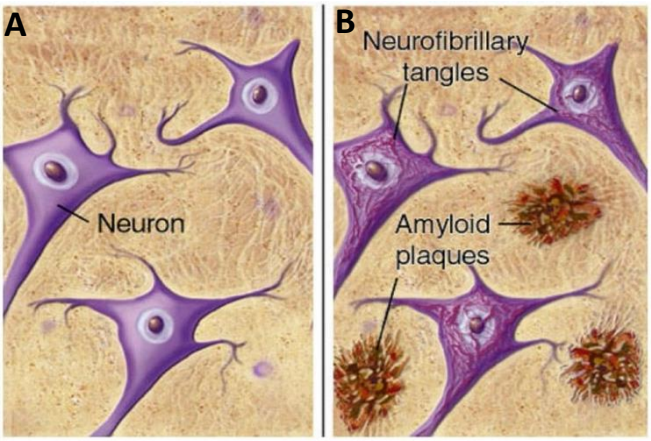


Figure 1 - **Schematic representation of (A) healthy neurons and (B) the abnormal deposits described by Dr. Alzheimer.** Amyloid plaques are localized extracellularly whereas NFT are localized inside neurons (Mitra and Dey, 2013).

Under physiological conditions, phosphorylation of tau is important to maintain the structure of cytoskeleton. The balance of phosphorylated and unphosphorylated tau regulates the stability of microtubules, which defines the normal morphology of neurons and maintain the axoplasmic flow (Kosik, 1993). In AD, however, hyperphosphorylated tau protein accumulates inside the cell, dimerizing to paired helical filaments, which aggregate to form the typical NFTs within neurons (Goedert et al., 2006). An imbalance between the activation of phosphorylating protein kinases and dephosphorylating protein phosphatases is thought to occur in AD, leading to excessive tau phosphorylation, microtubule instability, axonal transport impairment and, consequently, cell death (Mandelkow et al., 1995).

On the other hand, extracellular amyloid plaques, also called senile or neuritic plaques, are mainly composed by Aβ peptide. These amyloid plaques are preferential

localized in the cortex and hippocampus and correlated with the primary cognitive and memory disturbances (Killiany et al., 2002).

Due to its neurotoxic effects and accumulation in AD, Aβ is believed to play a central role in the pathogenesis of the disease (Hardy and Higgins, 1992). Moreover, since it is known that Aβ aggregates into toxic plaques up to several years before the first clinical symptoms appear, their detection and monitoring became of primary interest, both for diagnostic purposes and for fundamental research (Goedert and Spillantini, 2006).

1.1.3| Aβ peptide

In 1991, Hardy and Allsop proposed, for the first time, the amyloid hypothesis for AD pathogenesis and this postulate still continues to be the hypothesis best scientifically supported nowadays (Carrillo-Mora et al., 2014). It assumes that Aβ, in multiple forms, triggers several cascades that can led to synapse loss and neurodegeneration (Morris et al., 2014). In fact, it was demonstrated that Aβ production are increased in familial forms of AD (Vetrivel and Thinakaran, 2006), and several toxic effects of this peptide have been described both *in vitro* and *in vivo* studies (Atwood et al., 2003). A study using two mouse AD lines (AβPPPS1-21 and Tau22 mice) described that AβPPPS1-21 transgenic mice were impaired in spatial, fear, aversion and extinction learning deficits, whereas Tau22 animals were impaired in appetitive responding. The deficits in AβPPPS1-21 mice suggested that amyloid-related pathology might be more pervasive and/or widespread than tau pathology (Lo et al. 2013). Furthermore, research on the pathological changes in AD indicates that accumulated Aβ *in vivo* may initiate the hyperphosphorylation of tau (Huang and Jiang 2009). Thus, an excessive production of Aβ or an impairment in its adequate clearance have been suggested as key events in the origin and progression of the neuronal damage (Mawuenyega et al., 2010).

Aβ is a peptide of 39 to 42 aminoacids produced in all neurons through proteolitic processing of the transmembrane amyloid precursor protein (APP) by β- and γ-secretases. The cleavage site used by γ-secretase in the amyloidogenic pathway determines whether the predominant $A\beta_{1-40}$ or the more aggregation-prone and neurotoxic $A\beta_{1-42}$ species of the peptide is generated. Within the nonamyloidogenic

pathway, APP is cleaved by α-secretase, preventing Aβ formation (Figure 2) (Hardy and Selkoe, 2002).

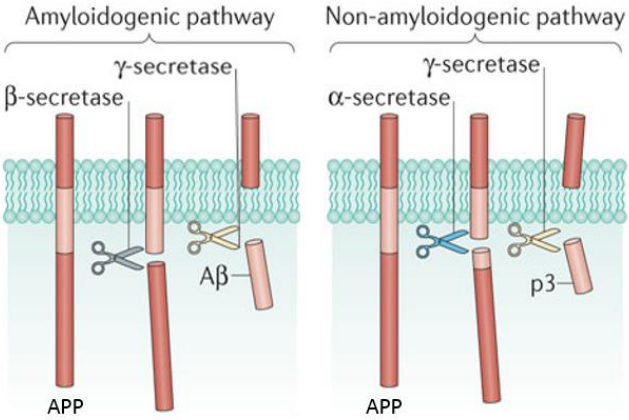


Figure 2 - **Generation of Aβ through proteolytic processing of APP.** Cleavage by β-secretase generates the N-terminal and intramembranous cleavage by γ-secretase gives rise to the C-terminal of Aβ. Cleavage by α-secretase precludes Aβ formation, producing another fragment (figure adapted from Heppner et al., 2015).

Interestingly, Aβ has been shown to have physiological functions within the brain at pM concentrations (Puzzo et al., 2008). In fact, Aβ can enhance synaptic plasticity, increase synaptic vesicles release probability (Abramov et al., 2009) and even protect against excitotoxic insults (Giuffrida et al., 2009). In contrast, at supraphysiological concentrations, Aβ self-aggregates into higher order structures. Initially, soluble Aβ acquires higher β-sheet content, leading to self-dimerization. This is followed by oligomerization into soluble high molecular aggregates, protofibril formation and finally aggregation into insoluble fibrillary structures characteristic of amyloid plaques (Figure 3) (Serpell, 2000).

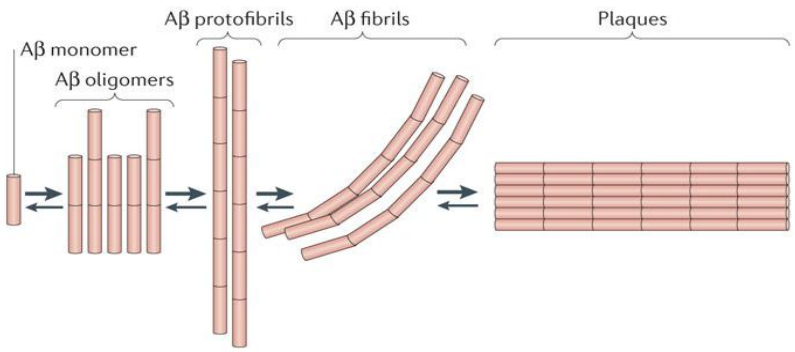


Figure 3 – **Formation of amyloid plaques.** Aβ monomers aggregate to form oligomers, fibrils and/or plaques, depending on mutations in the Aβ coding region of APP and/or post-translational modifications (figure adapted from Heppner et al., 2015).

In AD brain, insoluble extracellular amyloid plaques aggregate around neurons and glia and their main component is $A\beta_{1-42}$ (Hardy and Higgins, 1992). $A\beta_{1–42}$, with its two additional hydrophobic amino acids, has a higher tendency to aggregate than $A\beta_{1–40}$ and has been ascribed to be the main pathogenic form (Citron, 2010). Furthermore, since evidences suggest that Aβ toxicity implicate oligomers as the primary toxic species (Haass and Selkoe, 2007, Kayed and Lasagna-Reeves, 2013, Ferreira et al., 2007), a shift had occurred in amyloid hypothesis focus from plaque to soluble forms of Aβ.

1.1.4|Neurotoxicity and synaptic failure mediated by Aβ peptide

The accumulation of intracellular Aβ occurs early in the neuropathological phenotype of AD, even before the formation of NTFs and plaque deposition (Gouras et al., 2000). Although extracellular Aβ can bind to several receptors, producing Aβ-receptor complexes that can also be internalized into early endosomes, the amyloidogenic cleavage of APP also occurs in Golgi and endoplasmic reticulum membranes (LaFerla et al., 2007). Therefore, Aβ can accumulate in mitochondria, culminating in reactive oxygen species (ROS) production, leading to oxidative stress (Manczak et al., 2006, Caspersen et al., 2005). Furthermore, mitochondrial Aβ also can initiate a cascade of events that activates caspase-3, triggering the intrinsic apoptotic pathway (D'Amelio et al., 2011).

On the other hand, extracellular Aβ oligomers can also lead to the dysregulation of different signaling pathways that culminate in multiple mechanisms of synaptic failure (Kayed and Lasagna-Reeves, 2013). Most of the mechanisms whereby both intracellular and extracellular Aβ leads to synaptic dysfunction and neurodegeneration will be schematized later when all of them were described.

Interestingly, the notion that dementia is a consequence of synaptic degeneration was raised when Santiago Ramon y Cajal suggested that "dementia could result when synapses between neurons are weakened as a result of a more or less pathological condition, that is, when processes atrophy and no longer form contacts, when cortical mnemonic or association areas suffer partial disorganization" (Shankar and Walsh, 2009). Accordingly, several studies have shown that neuronal death in AD patients is closely associated with extensive synapse loss in the neocortex (Terry et al., 1991) and decreased synapse density is the strongest neuropathological correlate of the

degree of dementia in AD (Arendt, 2009, Masliah et al., 2006). Importantly, Aβ exerts neurotoxic effects by disrupting the integrity of both plasma and intracellular membranes (Demuro et al., 2005) and by accumulating at excitatory synapses, impairing synapse function (De Felice et al., 2007, Deshpande et al., 2009). Furthermore, it has been reported that Aβ can disrupt the postsynaptic density, which organizes synaptic proteins to mediate the functional and structural plasticity of the excitatory synapse and to maintain synaptic homeostasis (Gong and Lippa, 2010, Lacor et al., 2004, Reddy et al., 2005, Snyder et al., 2005, Hsieh et al., 2006).

1.2| The NMDA receptor

Glutamate is the major fast excitatory neurotransmitter and it is involved in almost all central nervous system (CNS) functions, particularly in cortical and hippocampal regions (Parsons et al., 1998), including synaptic transmission, neuronal growth, cell differentiation, synaptic plasticity, learning and memory (Butterfield and Pocernich, 2003, Francis, 2003).

The ionotropic glutamate receptors subtype N-methyl D-aspartate (NMDARs), which are known to be concentrated on postsynaptic spines of neuronal dendrites (Mattson et al., 1998), are cationic channels gated by glutamate, permeable to Na^+, K^+ and Ca^{2+} (Danysz and Parsons, 2012). At the resting membrane potential, the Ca^{2+} channel of the NMDA receptor is blocked by Mg^{2+} ions, which is associated with a low background level of postsynaptic intracellular Ca^{2+}. Mg^{2+} ion removal from the pore, which will allow the flow of ions, requires a membrane depolarization of sufficient amplitude and duration (Duguid IC, 2009). Importantly, NMDARs can differ in their subunit composition, a characteristic that also varies across CNS regions during development and in disease states, subcellular localization, pharmacological properties and their interacting proteins.

1.2.1| NMDAR composition

NMDAR subunits are encoded by three families of genes coding for GluN1, GluN2 and GluN3 subunits (Cull-Candy et al., 2001). Functional NMDARs are heterotetramers composed by two glycine or D-serine-binding GluN1 subunits and two glutamate binding GluN2 (GluN2A-D) subunits or, in some cases, glycine binding GluN3 (GluN3A/B)

subunits. The most widely expressed NMDARs contain the obligatory subunit GluN1 plus either GluN2B or GluN2A or a mixture of the two (Kohr, 2006). GluN3A and GluN3B are mostly expressed in oligodendrocytes and astrocytes (Cull-Candy et al. 2001).

Interestingly, during development and even in some adult synapses, the composition of NMDARs changes in response to neuronal activity. In early postnatal development, NMDARs switch their subunit composition from primarily containing GluN2B subunits to predominantly containing GluN2A subunits. Nevertheless, GluN2B subunits still populate many regions of the adult forebrain (Figure 4) (Paoletti et al., 2013).

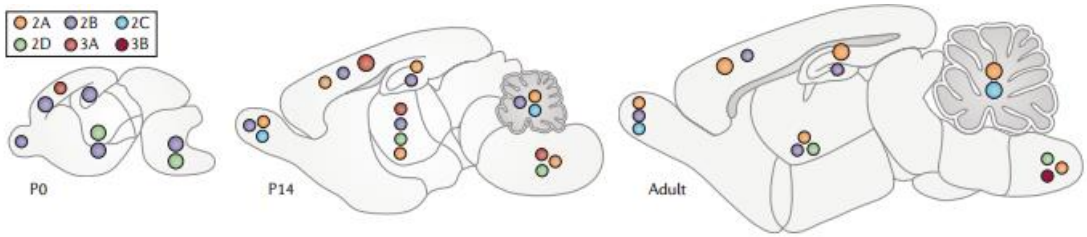


Figure 4 - **Expression of NMDARs subunits in the mouse brain at postnatal day 0 (P0), which is the day of birth, 2 weeks following birth (P14) and at the adult stage**. In adults, GluN2A is ubiquitously expressed in the brain, GluN2B is mostly restricted to the forebrain, GluN2C is limited to the cerebellum, and GluN2D is expressed in small numbers of cells in selected brain regions (Paoletti et al., 2013).

1.2.2| NMDAR and synaptic plasticity

Long term potentiation (LTP), which is a long-lasting increase in synaptic strength, is widely accepted as the neurophysiological basis for learning and memory (Bliss and Collingridge, 1993). The best-characterized form of LTP occurs between pyramidal neurons of Cornu Ammonis 3 (CA3) and Cornu Ammonis 1 (CA1) in the hippocampus (Figure 5) (Malenka, 1994). “CA” refers to cornu Ammon, the latin for Ammon’s horn, since the ram’s horn resembles the shape of the hippocampus (Purves D, 2001).

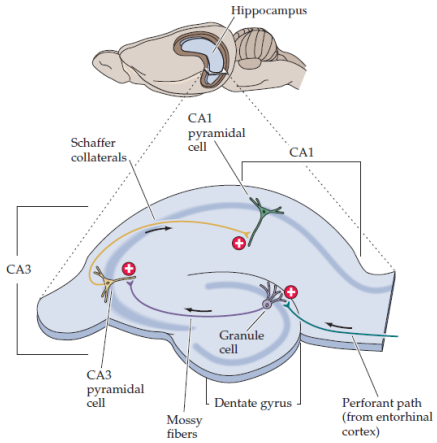


Figure 5 – **Schematic representation of a hippocampus slice**. The arrangement of neurons allows the hippocampus to be sectioned in slices, maintaining the relevant circuitry intact. The cell bodies of the pyramidal neurons lie in a single densely packed layer. This layer is divided into CA1 and CA3, where the dendrites of pyramidal cells in CA1 (stratum radiatum) receive synapses from the Schaffer collaterals, which are axons of pyramidal cells in the CA3 region (Purves D, 2001).

Electrical stimulation of Schaffer collaterals generates excitatory postsynaptic potentials (EPSPs) in the postsynaptic CA1 cells. A brief, high-frequency stimulation (HFS) to the same axons causes LTP, which is evident as a long-lasting increase in EPSP amplitude (Purves D, 2001). In addition, the mechanisms underlying electrically induced LTP, which can performed in laboratory with appropriate protocols, remains as a model of synaptic and cellular events that may underlie synaptic changes in brain during learning and memory formation. Supporting the emerging interest in LTP as a potential mechanism of memory, LTP exhibits numerous properties expected of a synaptic associative memory mechanism, such as rapid induction, synapse specificity, associative interactions, persistence, and dependence on correlated synaptic activity (Escobar and Derrick, 2007). Although LTP has become one of the most extensively studied topics in neuroscience, the molecular mechanisms underlying LTP are still not fully understood (Lømo, 2003). It is known that LTP is divided into early LTP (E-LTP) and late LTP (L-LTP). E-LTP requires modifications in existing proteins, whereas L-LTP is only induced by strong stimulation and requires synaptic growth, gene transcription and new protein synthesis (Pang and Lu, 2004).

It is clear that some types of LTP do not involve NMDAR (Johnston et al., 1992), however NMDAR-dependent LTP, which has received more attention, is known to occur in the CA1 area of the hippocampus (Lüscher et al., 2012). In NMDAR-dependent LTP,

during HFS, glutamate binds to Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid receptors (AMPARs), also ionotropic glutamate receptors, leading to cell depolarization, which allows the removal of Mg^{2+} from NMDAR channel. Thus, NMDARs are activated and, due to the Ca^{2+} influx through these channels, a rapid rise of intracellular Ca^{2+} occurs within the dendritic spines of the postsynaptic cell (Purves D, 2001). Dendritic spines are postsynaptic protrusions along dendrites, which can assume different morphologies (Figure 6B), where excitatory glutamatergic synapses occur (Figure 6A). They are highly motile, can undergo remodeling even in the adult nervous system and their number highly predict the strength of synaptic activity in one neuron (Knobloch and Mansuy, 2008).

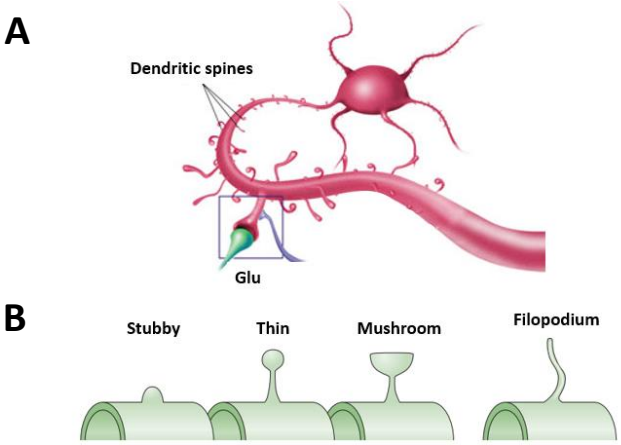


Figure 6 – **Schematic representation of dendritic spines**. (A) Glutamatergic synapse occurring through spines of two different neurons. (B) Examples of different spine morphologies (adapted from Yuste and Bonhoeffer, 2004).

Upon Ca^{2+} influx within the dendritic spine through activated NMDAR, Ca^{2+}-dependent proteins, such as calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC), are activated, inducing different intracellular cascades necessary for LTP induction (Figure 7) (Miyamoto, 2006). AMPARs, which can be phosphorylated in this process (Derkach et al., 1999), are further recruited to post-synaptic density (PSD), resulting in a greater post-synaptic response to glutamate (Malenka and Bear, 2004).

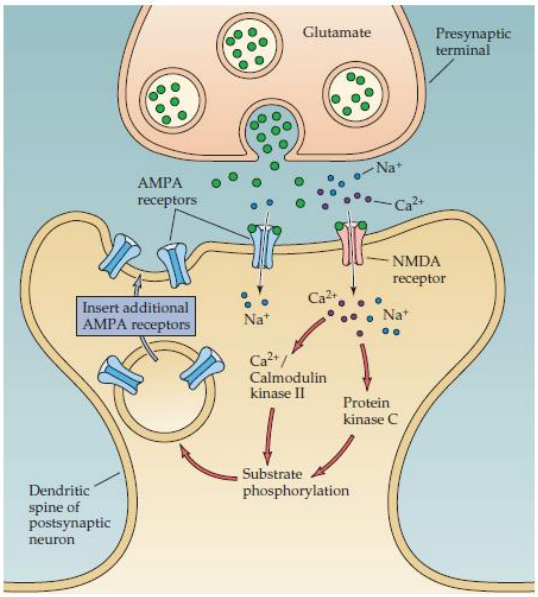


Figure 7 – **Molecular mechanisms underlying LTP.** During HFS, glutamate binds to AMPARs and, if the postsynaptic cell is sufficiently depolarized, NMDARs are activated. The Ca^{2+} ions that enter the cell through these channels activate postsynaptic protein kinases. These kinases may act postsynaptically to insert new AMPARs into the postsynaptic spine, thereby increasing the sensitivity to glutamate (Purves D, 2001).

AMPAR are tightly anchored in the PSD by numerous scaffolding proteins linking them to cytoskeletal elements, including actin. The insertion of additional receptors therefore is likely to affect synapse structure, and in fact, spines associated with synapses that underwent LTP become enlarged (Matsuzaki et al., 2004, Harvey and Svoboda, 2007, Holtmaat and Svoboda, 2009, Kasai et al., 2010).

L-LTP involves interactions with transcription factors, both local dendritic and nuclear transcription, and somatic translation, where the synthesis of required proteins for the maintenance of functional and structural plasticity after LTP triggering occurs (Nguyen et al., 1994). Moreover, LTP is correlated with the formation of new spines within minutes of induction, which leads to an increase in spine density (Figure 8) (Toni et al., 1999).

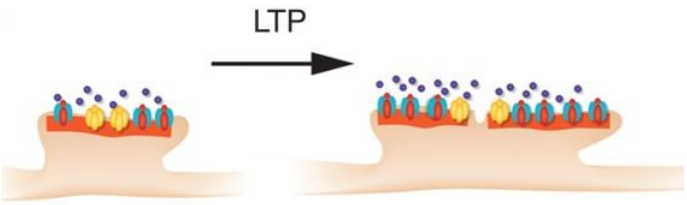


Figure 8 - **Structural changes associated with LTP.** Synaptic strength correlates with spine volume and the area of PSD. LTP can also lead to the formation of new spines (Luscher and Malenka, 2012).

Importantly, there are evidences that Aβ peptides could impair LTP (Lambert et al., 1998). In fact, perfusion of rat hippocampal slices with low concentrations (200 nM or 1 mM) of $A\beta_{1-42}$, $A\beta_{1-40}$ or their active fragment $A\beta_{25-35}$ has known to significantly impair LTP (Chen et al., 2000, Lee et al., 2000). On the contrary, in previous studies performed in our laboratory any significant change in LTP magnitude induced by a very-weak θ-burst in hippocampal slices exposed to Aβ was detected (Jeronimo-Santos et al., 2015). The stimulation protocol, the Aβ preparation, the developmental age or genetic background of the animals used (Smith et al., 2009) could explain this absence of Aβ effect upon LTP. Soluble oligomeric $A\beta_{1–42}$ significantly blocked hippocampal LTP when induced by HFS but not by θ-burst (Smith et al., 2009), the type of stimulation used.

Remarkably, synaptic dysfunction and loss caused by age-dependent accumulation of $A\beta_{1-42}$ in AD brains has been proposed to underlie cognitive decline (McLean et al., 1999, Lue et al., 1999, Wang et al., 1999, Izzo et al., 2014). Curiously, the loss of dendritic spines has also been described in AD (Spires et al., 2005, Spires-Jones et al., 2007). Since spine remodeling and the formation of new synapses are activity-dependent processes that provide a basis for memory formation, alterations in spine density are thought to be responsible for cognitive deficits long before or even in the absence of neuronal loss (Knobloch and Mansuy, 2008). Furthermore, studies demonstrated that the blockade of NMDARs prevent the decrease in synaptic density observed with AD animal models. Thus, it was suggested that NMDAR activation is required for Aβ to exert its effects on spines (Shankar et al., 2007, Shankar et al., 2008, Wei et al., 2010). Another reasonable causal hypothesis for the loss of synapses that leads to functionally disconnection between regions of the neocortex involved in cognition might involve deficiency or failure of delivery of trophic factors (Selkoe, 2002) which will also be discussed later on.

1.2.3| Synaptic and extrasynaptic NMDARs

NMDARs play an important role in several cell processes, including LTP, as described above. However, they can also lead to neurodegeneration. An important work has revealed that the localization of NMDARs influences whether they are coupled to pro-death or pro-survival signals (Hardingham and Bading, 2010).

NMDARs can be synaptic (sNMDAR), localized in synapses and gated by the co-agonist D-serine released by astrocytes (Kang et al., 2013), or extrasynaptic (eNMDAR), localized outside synapses and gated by the co-agonist glycine released by both astrocytes and neurons (Holopainen and Kontro, 1989). sNMDARs are neuroprotective, whereas eNMDARs preferentially initiate cell death pathways (Hardingham and Bading, 2010). In fact, low levels of eNMDARs activation have no effects on neuronal survival but high levels of eNMDAR activity enhances cell death pathways and exacerbates certain neurodegenerative processes, reducing, consequently, neuronal survival (Figure 9) (Hardingham and Bading, 2010).

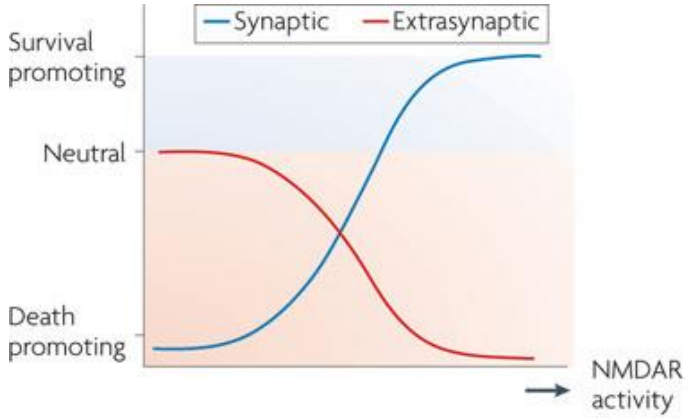


Figure 9 - **The NMDA paradox.** The ascending curve represents increased neuroprotection due to increased synaptic NMDAR activity, which is superimposed on a descending curve that illustrates the progressive decrease in neuroprotection due to increasing extrasynaptic NMDAR activity (Hardingham and Bading, 2010).

The activation of sNMDAR promotes neuroprotection, resulting from changes in gene expression that have multiple effects within the cell. Activation of sNMDARs initiates a chain of reactions that enhances antioxidant defenses, which contributes to neuroprotection against oxidative insults (Papadia et al., 2008). sNMDARs activity can also have anti-apoptotic effects, including the inactivation of pro-death transcription factors, such as forkhead box protein O (FOXO) and p53, and the transcription of pro-

survival factors, including CREB (cyclic-AMP response element binding protein) (Hardingham and Bading, 2010), that results in Brain-Derived Neurotrophic factor (BDNF) transcription (Tao et al., 1998).

In contrast, increased eNMDAR activity preferentially induces pro-death effects, such as the activation of CREB shut-off pathway, which block BDNF expression (Hardingham et al., 2002), ERK1/2 inactivation, which is necessary for BDNF function on spines (Hardingham et al., 2002), FOXO activation, the Ca^{2+}-dependent cysteine proteases calpains activation and subsequent striatal enriched tyrosine phosphatase (STEP) cleavage that prevents STEP from inhibiting p38 MAP kinase, further contributing to neuronal death (Figure 10).

Concerning the calpains, they are involved in multiple cell functions, including proliferation, differentiation, growth cone motility and guidance, and apoptosis. They are also known to play an important role in learning and memory in physiological conditions (Goll et al., 2003, Ono and Sorimachi, 2012). However, when overactivated, calpains cleave and change the function of several proteins, such as synaptic, structural or signaling proteins (Lee et al., 2000). For example, calpains activate cyclin-dependent kinase 5 (CDK5) by cleaving the CDK5-modulator p35 into p25. Increased CDK5 activity leads to tau hyperphosphorylation and to AD progression (Lee et al., 2000). Furthermore, there are two types of calpains: μ-calpain, which is activated in the presence of μM concentrations of Ca^{2+} and m-calpain that requires mM concentrations for its activation. These local Ca^{2+} concentrations can trigger calpain activation in distinct subcellular domains and calpains will regulate different substrates to produce opposite effects on neuronal fate (Wang et al., 2013). Synaptic NMDAR activation stimulates μ-calpain, resulting in the activation of pro-survival pathways, and extrasynaptic NMDAR activation induces m-calpain activation, resulting in cell death (Figure 10) (Xu et al., 2009, Ferreira, 2012).

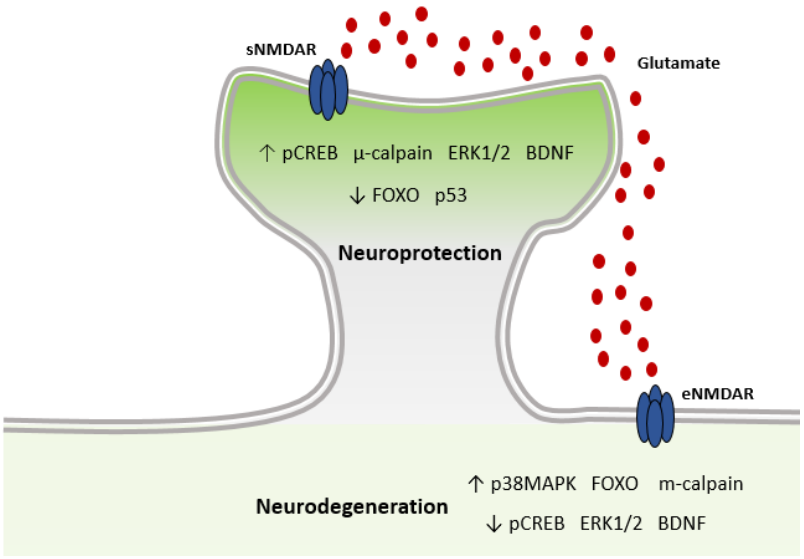


Figure 10 - **Mediators of synaptic and extrasynaptic NMDAR activity effects**. The activation of sNMDARs and eNMDARs triggers different pathways, such as the increase or decrease in the activation, expression or function of a particular intracellular signal that culminates in neuroprotection or neurodegeneration, respectively.

Although the proportion of sNMDARs increases with development, a significant population of NMDARs remains extrasynaptic in adulthood (Petralia et al., 2010). Physiological studies indicate that about 75% of NMDARs are extrasynaptic at 7 days *in vitro* (DIV), decreasing the levels to 20–50% by DIV14 (Gladding and Raymond, 2011). Additionally, a hippocampal slice study indicates that about 36% of NMDARs are extrasynaptic at DIV14-21 (Petralia et al., 2012). However, it is known that pathological conditions can increase the expression or activation of eNMDARs and thus favor these pro-death pathways (Danysz and Parsons, 2012).

1.2.4| Extrasynaptic NMDARs in AD

Studies have been indicating that activation of NMDARs by Aβ accumulation may occur at early stages of AD (Parameshwaran et al., 2008). Accordingly, it was shown that Aβ induces a sustained Ca^{2+} influx by interacting directly with NMDAR (Alberdi et al., 2010, Texido et al., 2011, Ferreira et al., 2012), modulating its properties (Hu et al., 2009). Interestingly, it was proposed that Ca^{2+} entry through NMDARs is particularly effective at killing neurons compared to entry through other channels (Tymianski et al., 1993). Early neuronal dysfunction induced by Aβ is known to be mediated by an activation of GluN2B subunits, the most abundant subunit of eNMDAR, in primary

neuronal cultures and hippocampal slices from rat and mouse (Rönicke et al., 2011). In fact, other reports also showed that Aβ oligomeric species evoke an immediately rise in intracellular Ca^{2+} in cultured cortical neurons through activation of GluN2B-containing NMDARs, suggesting that the activation of this subunit is involved in Aβ-induced Ca^{2+} homeostasis deregulation (Ferreira et al., 2012a). Importantly, by increasing the Ca^{2+} influx, Aβ, among other things, leads to an overactivation of calpains (Figure 11).

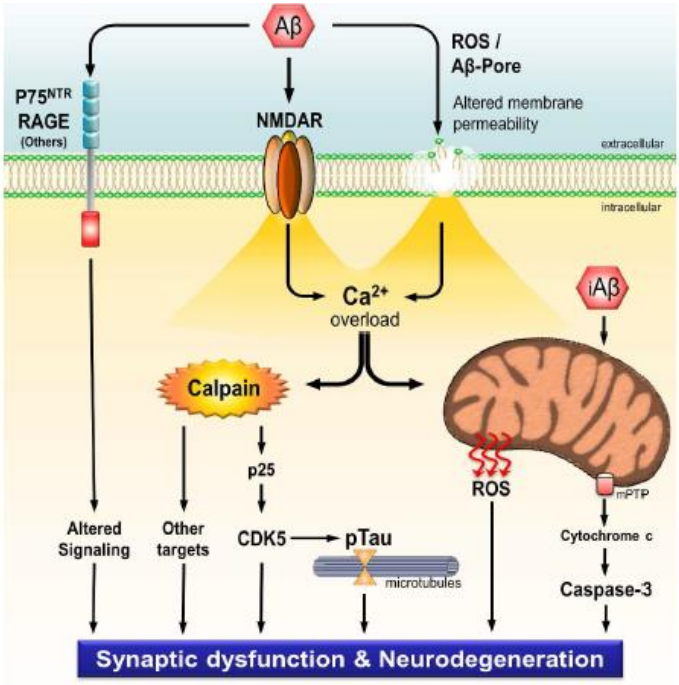


Figure 11 – **Schematic representation of the mechanisms whereby Aβ leads to synaptic dysfunction and neurodegeneration**. Intracellular Aβ accumulates in mitochondria, leading to ROS production and the activation of pro-apoptotic pathways. Extracellular Aβ oligomers interact with several receptors, such as p75NTR and RAGE, leading to the dysregulation of different signaling pathways that culminate in synaptic dysfunction and neurodegeneration. By interacting directly with NMDARs or by the formation of cation conducting pores, Aβ causes a sustained Ca^{2+} influx that culminates in calpains activation. Overactivated calpains change the function of several important proteins, leading to tau hyperphosphorylation and to AD progression (Jerónimo-Santos, 2014).

Interestingly, inhibition of calpain activity prevents excitotoxic neuronal death *in vitro* (Caba et al., 2002) and restores normal synaptic function and spatial memory in AD animal models (Trinchese et al., 2008, Granic et al., 2010, Medeiros et al., 2012). Since calpastatin, the endogenous inhibitor of calpains, is known to be depleted in AD brains (Rao et al., 2008), it would be important to find an alternative way that could modulate calpain activation. General pharmacological calpain inhibitors would not be the best solution, given the important physiological functions of these proteases. Thereby,

factors that selectively affect NMDA receptor-mediated Ca^{2+} influx could be candidates to efficiently modulate calpain activation and its deleterious effects in neurons (Bullock, 2006).

1.3| Neurotrophins

The discovery of neurotrophins family began with Levi-Montalcini, Cohen and Hamburger, who discovered the nerve growth factor (NGF), a factor required for axonal growth (Levi-Montalcini, 1987). Only about 30 years later, BDNF, neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), NT-6 and NT-7 were identified (Nilsson et al., 1998, Gotz et al., 1994).

Neurotrophins are dimers, which are secreted in an unprocessed form called pro-neurotrophins, synthesized in the hippocampus and cortex and retrogradely transported to the basal forebrain (Holsinger et al., 2000). They mediate their effects through binding to two different receptors – tropomyosin-related kinase (Trk) receptors and the p75 neurotrophin receptor (p75NTR) (Arevalo and Wu, 2006). p75NTR is known to bind the unprocessed pro-neurotrophins (Roux and Barker, 2002) and several reports support a dual role of p75NTR in cell death, as well as in survival, depending on the physiological or developmental stage of the cells (Mamidipudi and Wooten, 2002). Moreover, when expressed within the same cell, p75NTR can interact with Trk receptor activating a set of signaling pathways that are synergistic to those activated by Trk receptors (Huang and Reihardt, 2003).

Focusing on Trk receptors, they are subdivided in three types of receptors, each of which is a single transmembrane protein with a cytoplasmic tyrosine kinase domain that dimerizes and become active upon neurotrophin ligand binding. TrkA is primarily a receptor for NGF, TrkB a receptor for BDNF and NT-4/5, and TrkC a receptor for NT-3 (Figure 12). Neurotrophins bind with equal affinity to p75NTR and preferentially to specific Trk receptors (McAllister et al., 1999).

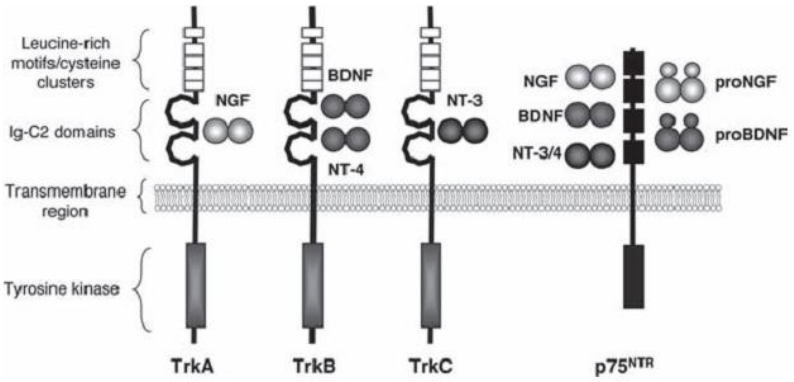


Figure 12 - **Neurotrophin receptors and their specificity for the neurotrophins.** Neurotrophins are important regulators of neuronal survival, development, function, and plasticity. NGF binds to TrkA, BDNF and NT-4 bind to TrkB and NT-3 binds to TrkC. All of these neurotrophins binds to p75NTR, however pro-neurotrophins bind to this receptor with more affinity (McAllister et al., 1999).

Regarding to TrkB, it is important to clarify that, in the human brain, multiple isoforms of this receptor are expressed. There are three major isoforms of the TrkB receptor characterized: the full-length (TrkB-FL) and two C-terminal truncated TrkB receptors (TrkB-TC). Both C-terminal truncated isoforms are generated by alternative splicing of the TrkB pre-mRNA but differ in that each contain unique amino acid sequences at their C-terminal. TrkB-Shc isoform was also indentified and includes the sarc homology containing (Shc) binding domain that is absent in TrkB-TC (Figure 13) (Stoilov et al., 2002).

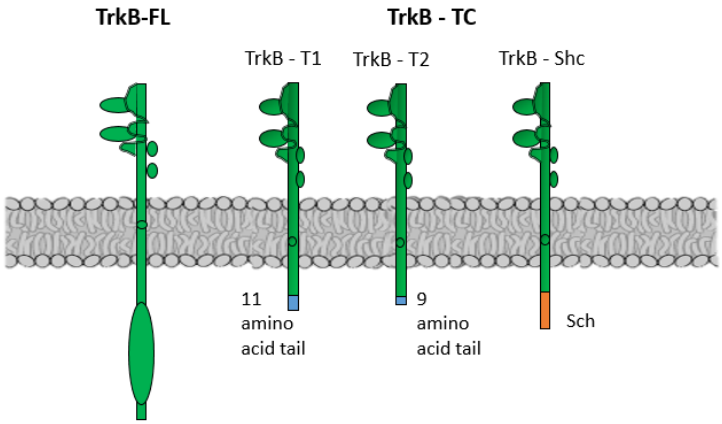


Figure 13 – **TrkB isoforms.** The full-length (TrkB-FL) isoform and three C-terminal truncated TrkB receptors (TrkB-TC): TrkB-T1 with an 11 amino acid tail, TrkB-T2 with a unique 9 amino acid tail and TrkB-Shc, the sarc homology containing TrkB.

1.3.1| BDNF signaling and its function

BDNF is widely expressed in the CNS (Durany et al., 2000) and it has been demonstrated, in several cell culture and animal models, that its signaling is critical for neuronal differentiation, survival, plasticity and cognition (Bartkowska et al., 2010, Lowenstein and Arsenault, 1996).

Upon BDNF binding, monomeric TrkB-FL dimerizes and tyrosine kinase domains activate, leading to a cross-phosphorylation of the tyrosine residues on each domain. Phosphorilated tyrosine residues can act as a "docking" site for signaling molecules, initiating different signaling pathways that promote neuronal survival (via phosphatidylinositol-3-kinase (PI3K)/Akt pathway), growth and differentiation (via Ras/MAPK pathway) and activity-dependent plasticity (via PLCγ) (Segal and Greenberg, 1996, Blum and Konnerth, 2005, Arevalo and Wu, 2006).

There are two tyrosine phosphorylation residues outside the kinase activation domain of TrkB. TrkB phosphorylation site on Tyr^{515} recruits Shc to TrkB and phosphorylates it, thus allowing the activation of PI3K pathway (Reichardt, 2006). Activation of PI3K changes the composition of inositol phospholipids in the inner leaflet of the plasma membrane. This results in the translocation of Akt/protein kinase B to the plasma membrane. Akt then phosphorylates and controls the biological functions of several proteins important cell survival modulation (Yuan and Yankner, 2000), such as BAD, a Bcl-2 family member that promotes apoptosis, or IκB (Datta et al., 1999), whose phosphorylation leads to degradation and activation of NFκB, resulting in transcription to promote neuronal survival (Middleton et al., 2000).

Recruitment of Shc to the Trk receptors also allows the activation of Ras that activates the downstream kinase B-raf, MEK and ERK family of MAP kinase (Grewal et al., 1999, Huang and Reichardt, 2003). MEK-MAPK/ERK signaling influences transcription events (Xing et al., 1998), such as the activation of the CREB transcription factor (Shaywitz and Greenberg, 1999). CREB regulates genes whose products are essential for prolonged neurotrophin-dependent survival of neurons (Riccio et al., 1999). MAPK signaling pathway is involved in the structural remodeling of excitatory spine synapses triggered by neurotrophins (Alonso et al., 2004), as well as in dendritic growth (Kumar et al., 2005).

TrkB phosphorylation site on Tyr^{816} recruits and activates PLCγ, which hydrolyses phosphatidyl inositides to generate inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Vetter et al., 1991). IP_3 induces the release of Ca^{2+} from intracellular stores, increasing levels of intracellular Ca^{2+}. This results in activation of various enzymes regulated by Ca^{2+}, including protein kinases and phosphatases regulated by Ca^{2+}-calmodulin. The formation of DAG stimulates the activity of protein kinase C (PKC) isoforms (Arevalo and Wu, 2006), that have an important role in LTP, as described above (Figure 14).

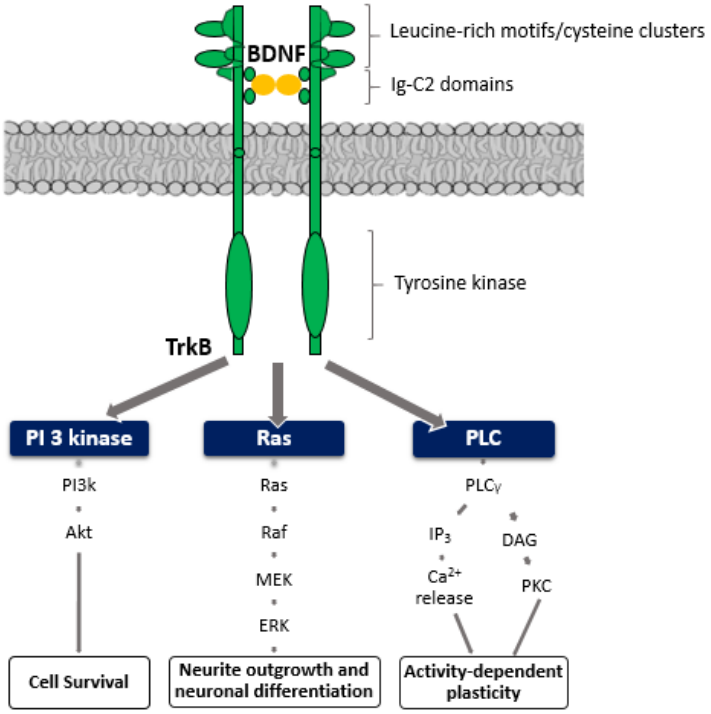


Figure 14 - **BDNF/TrkB-FL signaling pathway.** BDNF binds to TrkB-FL inducing receptors dimerization and after tyrosine kinase domains activation, different signaling pathways are initiated. Activation of PI3 kinase through Ras promotes survival and growth of neurons and other cells. Activation of Ras results in activation of the MAP kinase-signaling cascade, which promotes neuronal differentiation, including neurite outgrowth. Activation of PLCγ results in activation of PKC-regulated pathways that promote synaptic plasticity.

In fact, several studies have reinforced that, in the adult brain, BDNF facilitate spine formation (Tartaglia et al., 2001). Indeed, chronic treatment of hippocampal slice cultures with BDNF increases synapse number and spine density in apical dendrites of CA1 pyramidal neurons. Consistently, BDNF can also enhance synaptic transmission and synaptic plasticity (Lu et al., 2008, Korte et al., 1998), which is particularly favored when weak θ-burst stimuli is applied at the synapses from CA3 pyramidal neurons onto CA1

pyramidal neurons (Diogenes et al., 2011). Accordingly, impaired LTP in BDNF knockout mice can be recovered by direct application of BDNF (Pozzo-Miller et al., 1999, Patterson et al., 1996). Furthermore, it has been provided evidences about how BDNF facilitates LTP at glutamatergic hippocampal synapses. It is know that endogenous BDNF is released from glutamatergic synapses, in a Ca^{2+}-dependent way, in response to stimulus used to induce LTP (Balkowiec and Katz, 2002, Aicardi et al., 2004). Released BDNF can facilitate LTP at excitatory CA1 synapses, by increasing presynaptic release of glutamate, and by amplifying the postsynaptic response to this neurotransmitter (Carvalho et al., 2008). Through its postsynaptic TrkB-FL receptor, BDNF stimulates tyrosine kinase Fyn, which in turn phosphorylates the NMDAR and increases its activity (Levine et al., 1998, Mizuno et al., 2003). Moreover, BDNF/TrkB-FL signaling can induce cation influx through canonical transient receptor potential channels (TRPC). In this case, activation of TrkB-FL and PLCγ leads to IP_3-dependent Ca^{2+} store depletion, which activates the influx of Ca^{2+} and Na^{+} through TRPC3, altering membrane potentials that might, in turn, facilitate synaptic Ca^{2+} entry through voltage-gated channels or NMDARs (Li et al., 1999, Amaral and Pozzo-Miller, 2007). Ca^{2+} influx through NMDARs activates CaMKII, which, in turn, contributes to the induction and expression of LTP. Finally, BDNF/TrkB-FL signaling modulates AMPAR expression and trafficking to postsynaptic terminal, a mechanism also dependent on IP_3 receptor and TRPC calcium signaling (Figure 15) (Caldeira et al., 2007, Nakata and Nakamura, 2007).

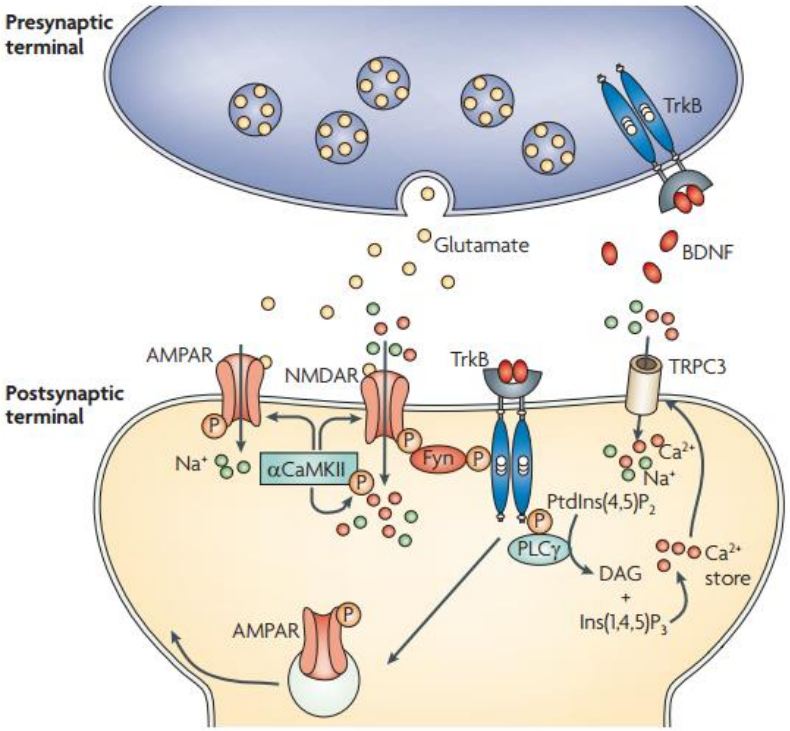


Figure 15 – **BDNF facilitation upon LTP in a glutamatergic synapse.** Presynaptic TrkB activation by BDNF increases glutamate release. Postsynaptic activation of TrkB: increases the open probability of NMDAR by activating the protein tyrosine kinase Fyn; promotes the influx of cations, through TRPC, depolarizing the postsynaptic terminal and facilitating the Ca^{2+} entry through voltage-gated channels or NMDARs; and modulates AMPAR expression and trafficking (Minichiello, 2009).

Ample studies indicate that BDNF is also involved in L-LTP. Acute application of BDNF to hippocampal slices induces synaptic potentiation in the hippocampal CA1 region (Kang and Schuman, 1996). Furthermore, evidences suggest that pairing BDNF perfusion and weak θ-burst stimulation produces a reliable L-LTP in CA1 area of hippocampus by regulating local dendritic protein translation and concomitantly increasing synthesis of LTP-associated proteins, such as CaMKII. BDNF can also regulate actin cytoskeletal dynamics, which are required for structural changes of synapses and L-LTP formation (Pang et al., 2004). Briefly, BDNF signaling, in the presence of synaptic activity, can facilitate spine morphogenesis, particularly because similar changes in spine morphology have been associated with long-term synaptic potentiation, where considerable evidence for the requirement of TrkB-FL signaling exists. Importantly, increase in glutamatergic synapse activity stimulates the release of BDNF (Hartmann et al., 2001). BDNF, in turn, facilitates the growth of immature spines into mature spines (Yoshii and Constantine-Paton, 2010).

The regulation of BDNF upon synaptic plasticity and synaptic growth suggests that it has a crucial role in cognitive functions. Accordingly, a genetical or pharmacological reduction of hippocampal BDNF levels leads not only to impaired LTP and reduced number of synapses, but also causes deficits in the formation and consolidation of hippocampus-dependent memory (Mu et al., 1999, Bekinschtein et al., 2008, Bekinschtein et al., 2007).

1.3.2| TrkB-FL cleavage and loss of BDNF signaling in AD

Importantly, BDNF/TrkB-FL signaling is modulated by alterations in TrkB-FL/TrkB-TC ratio (Eide et al., 1996). Since they lack the tyrosine kinase domain, truncated receptors cannot initiate BDNF signaling and act as a dominant-negative inhibitor of TrkB-FL, by sequestering BDNF so that it cannot bind to TrkB-FL, or by the formation of non-funtional heterodimers with TrkB-FL (Figure 16) (Wong, 2013). Thus, TrkB-FL isoform is the principal mediator of the neurotrophic effects of BDNF (Biffo et al., 1995, Eide et al., 1996).

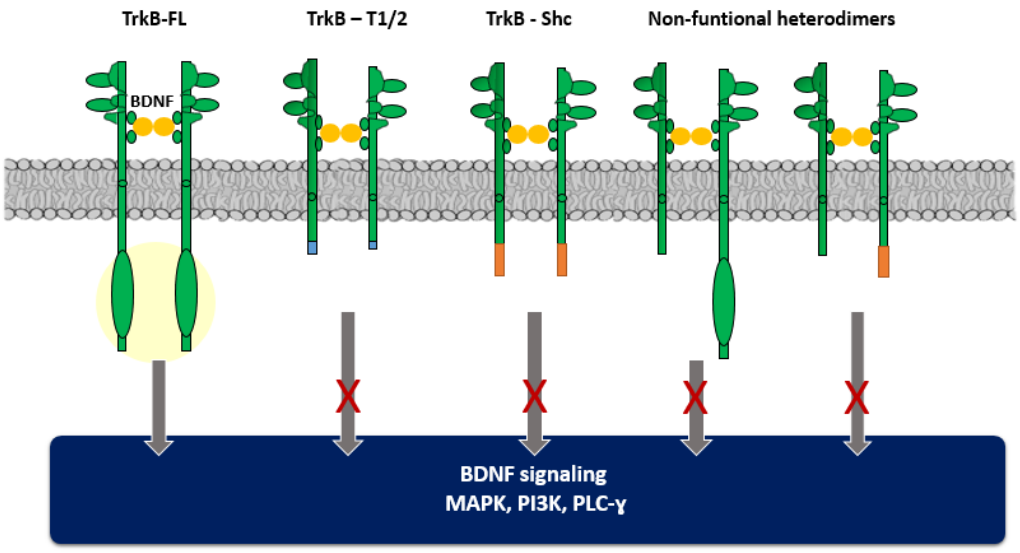


Figure 16 - **TrkB receptor dimer combinations.** All dimer combinations of TrkB receptors can bind to BDNF. However, only a homodimer of TrkB-FL can initiate TrkB signaling pathways.

Particularly, on primary neuronal cultures, Aβ is known to induce a decrease in TrkB-FL receptors and an increase in truncated TrkB receptors, which is independent of

the presence of glial cells (Kemppainen et al., 2012). These effects of Aβ exposure on TrkB receptors are time and concentration-dependent, thus longer incubation times with Aβ or higher concentrations of Aβ produce a more robust change on TrkB isoforms levels (Rodrigues et al., 2000). Importantly, corroborating this data, TrkB-FL isoform was also found to be decreased (Connor and Dragunow, 1998), whereas TrkB-TC isoforms were found to be increased in *postmortem* brain samples of AD patients (Connor et al., 1996, Ferrer et al., 1999).

It is well-known from previous studies that the influence of Aβ upon TrkB-FL occurs at the post-translational level, since Aβ strongly reduces TrkB-FL protein levels. In fact, Aβ selectively increases mRNA levels for the truncated TrkB isoforms without affecting TrkB-FL mRNA levels (Jeronimo-Santos et al., 2015). Regarding the unbalance in the ratio of TrkB isoforms, it was proposed a calpain processing of TrkB-FL as a possible mechanism of TrkB regulation (Vidaurre et al., 2012). Indeed, Aβ induces a calpain-mediated cleavage of TrkB-FL receptors, originating a new truncated TrkB receptor (TrkB-T') and an intracellular fragment (TrkB-ICD) (Figure 17). TrkB-T' is heavier than the natural truncated TrkB isoforms T1 and TrkB-ICD is a fragment of approximately 32 kDa. Notably, this intracellular fragment is also detected in *postmortem* human brain samples, showing that human endogenous calpains can also cleave human TrkB-FL receptor (Jeronimo-Santos et al., 2015). Interestingly, it is suggested that ICD fragments, which result from the proteolytic cleavage of some members of the receptor tyrosine kinase family, can have a biological function, since they can bind to transcription factors in the nucleus (Ancot et al., 2009). Recent unpublished data of our lab has been indicating that TrkB-ICD is translocated to the nucleus, but its function is still not clear.

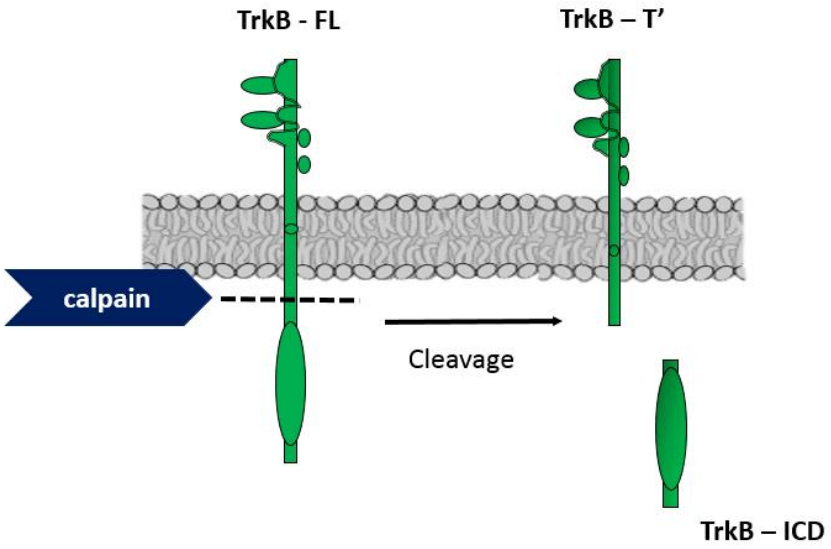


Figure 17 – **TrkB-FL cleavage.** The cleavage of TrkB-FL is mediated by calpains, resulting in the formation of a new truncated TrkB receptor (TrkB-T'), which is incapable of initiating TrkB signaling, thus impairing BDNF functions, and an intracellular fragment (TrkB-ICD) whose function is not known yet.

Moreover, it was also demonstrated that, upon calpain-dependent TrkB truncation, BDNF becomes unable to modulate neurotransmitter release from hippocampal nerve terminals, as well as LTP in hippocampal slices (Jeronimo-Santos et al., 2015). This suggests that calpains overactivation induced by Aβ also affects BDNF synaptic actions.

Taken together, the observations that BDNF and TrkB-FL receptor are required for synaptic plasticity and neuronal survival on CNS (Alcantara et al., 1997) and that increased TrkB-FL and BDNF signaling ameliorate the neurodegeneration and cognitive impairment in multiple AD models (Lu et al., 2013) lead to the hypothesis that the loss of BDNF signaling might be involved in AD pathology (Arancio and Chao, 2007, Schindowski et al., 2008).

2 I AIM

Aβ peptide induces an increase in intracellular Ca^{2+} levels, which results in calpains activation. These proteases lead to TrkB-FL truncation and, consequently, to an impairment of BDNF signaling. However, the causes for this intracellular Ca^{2+} deregulation remains to be clarified.

Extrasynaptic NMDARs, which are known to be overactivated in AD and associated to harmful effects in neurons, are permeable to Ca^{2+}, allowing the influx of this ion to the cell. Therefore, in the present work, the hypothesis that these receptors could have a central role, contributing to the calpains activation, was considered. Accordingly, the aim of this thesis was to clarify whether the role of BDNF could be recovered by inhibiting the activation of eNMDARs, in neuronal cultures or hippocampal slices exposed to Aβ.

Taking into account the main aim of this work, two specific objectives were proposed: i) to explore if the inhibition of eNMDAR could limit the activation of calpains and, ii) to study whether eNMDAR inhibition could prevent the truncation of TrkB-FL induced by Aβ and therefore facilitate the BDNF mediated actions.

3 | MATERIALS AND METHODS

3.1| Primary Neuronal Cultures and drug treatments

Primary neuronal cultures were obtained from fetuses of 18/19-day pregnant Sprague-Dawley females. Animals were purchased from Charles River (Barcelona, Spain) and were handled according to European Community guidelines and Portuguese law concerning animal care (86/609/EEC). Unless stated otherwise, culture reagents and supplements were from Gibco (Paisley, UK). The fetuses were collected in Hanks' balanced salt solution (HBSS). After brain dissection, the cerebral cortex was isolated and meninges were removed. The tissue was mechanically fragmented and its digestion was performed with 0,025% of trypsin solution in HBSS for 15 min at 37°C. After tissue digestion, cells were precipitated by centrifugation at 1200 rpm. The supernatant was removed and 20% of Fetal Bovine Serum (FBS) was added to HBSS. Cells were again precipitated by centrifugation, the supernatant removed and 2 mL of HBSS were added to the solution. Cells resuspension by pipete aspiration was required between centrifugations in order to dissociate cells. This washing process was repeated four more times to neutralize trypsin. After washed, cells were resuspended in Neurobasal medium supplemented with 0.5 mM L-glutamine, 25 mM glutamic acid, 2% B-27, and 25 U/mL penicillin/streptomycin. To obtain single cells and avoid cellular clusters or tissue fragments, the suspension was filtrated with a nylon filter (BD Falcon™ Cell Strainer 70 µM, Thermo Fisher Scientific, Waltham, MA, USA). Cells were plated at 6 x 10^4 cells/cm^2 and 5 x 10^4 cells/cm^2 on coverslips to perform western blotting and immunocytochemistry, respectively, and maintained at 37°C in a humidified atmosphere of 5% CO_2. This coverslips were previously sterilized under UV light and coated with 10 µg/mL of poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA), which is a synthetic amino acid that enhance cell attachment and adhesion to both plasticware and glass surfaces, overnight and washed with sterile H_2O.

Primary neuronal cultures were incubated with 25 µM of $A\beta_{25-35}$ (Bachem (Bubendorf, Switzerland) at DIV13 for 24 hours at 37°C, as previously described (Kemppainen et al., 2012). In these experiments, cells were also co-incubated with Aβ peptide and 1 µM of Memantine, a NMDAR antagonist, which was a gift by Merz (Frankfurt, Germany). Finally, for immunocytochemistry, cells were co-incubated with

the same drugs and 20 µM of N-[N-(Phenylmethoxy)carbonyl]-L-valyl]-phenylalaninal (MDL28170) (Tocris Bioscience, Bristol, UK), in the presence or absence of 20 ng/mL of BDNF, a gift from Regeneron Pharm (Tarrytown, NY, USA).

3.2| Western Blotting

After treatments, primary neuronal cultures at DIV14 were washed with ice-cold phosphate-buffered saline (PBS) and lysed with Radio Immuno Precipitation Assay (RIPA) buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5mM ethylenediamine tetra-acetic acid (EDTA), 0.1% SDS and 1% Triton X-100) containing protease (Roche, Penzberg, Germany). RIPA contains ionic detergents that bring the proteins into solution, allowing them to migrate individually through a separating gel. Adherent cells were scraped off the dish using a plastic cell scraper and the cell suspension were centrifugated at 13 000 g, 4°C during 10 min. The supernatant were aspired, discarding the pellet, and placed in fresh tubes.

The amount of protein was determined by Bio-Rad DC reagent (Bio-Rad Laboratories, Berkeley, CA, USA) and all samples were prepared with same amount of total protein (30 µg). In order to enable the access of the antibody to the protein epitope, a loading buffer (350 mM Tris pH=6.8, 10% SDS, 30% glycerol, 600 mM DTT, 0.06% bromophenol blue) was added and the mixture was boiled at 95-100°C for 5 min to denature proteins.

Next, all samples as well as the molecular weight marker (Thermo Fisher Scientific) were loaded and separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) within a standard migration buffer (25 mM Tris pH= 8.3, 192 mM Glycine, 10% SDS), at a constant voltage between 80-120 mV. Then, proteins were transferred onto PVDF membranes (GE Healthcare, Buckinghamshire, UK), previously soaked in methanol for 5 min, within the standard buffer (25 mM Tris pH= 8.3, 192 mM Glycine, 15% methanol) for wet transfer conditions. After 1h30 of transfer, membranes were soaked again in methanol for 5 min and then stained with Ponceau S solution to evaluate protein transference efficacy.

Before incubations with the primary (overnight at 4°C) and secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA; Invitrogen, Waltham, MA, USA; and Sigma-Aldrich) for 1 h at room temperature (RT) as identified below in 3.7 section (Table 3),

membranes were blocked with a 5% (w/v) nonfat dry milk solution in TBS with the detergent Tween-20 (TBST) (20 mM Tris base, 137 mM NaCl and 0.1% Tween-20) to avoid non-specific binding.

Finally, immunoreactivity was visualized using ECL chemiluminescence detection system (GE Healthcare), band intensity was measured using ChemiDoc (Bio-Rad Laboratories) and quantified by the digital densitometry ImageJ 1.45 software (MD, USA). The intensity of GAPDH was used as loading control.

3.3| Immunocytochemistry

Primary neuronal cultures at DIV14 were washed with PBS and then fixed in 4% paraformaldehyde in PBS (pH=7.4) for 15 min at RT. Cells were incubated with the blocking solution (3% (w/v) bovine serum albumin (BSA)) (Sigma-Aldrich) in PBS with 0.1% (v/v) Triton X-100) for 1 h to block unspecific binding of the antibodies. After new washes with PBS, cells were incubated with mouse microtubule-associated protein 2 (MAP2) primary antibody (Millipore, Billerica, MA, USA), to specifically detect neurons, overnight at 4°C. After this, cells were washed with PBS and then incubated with Anti-IgG – Alexa Fluor® 568 secondary antibody (1:200 in blocking solution) (Invitrogen), for 1h at RT, in the dark. The secondary antibody solution was decanted and rinsed with PBS. Then, cells were incubated with Alexa Fluor® 488 Phalloidin (1:40 in PBS) (Invitrogen), which recognizes filamentous actin (F-actin), for 30 min. F-actin has an important role in the constitution of the cytoskeleton of dendritic spines.

After washed, coverslips were mounted in Mowiol mounting solution. Fixed cells were observed by the inverted fluorescent microscope Axiovert 135 TV (Carl Zeiss Microscopy, Thornwood, NY, USA) with a zoom of 630x. Accordingly with the emission and absorption peak of fluorescent dyes conjugated with antibodies and the microscope filters used, neurons were labeled in red and protrusions (filopodia and spines) in green.

We consider spine density as the number of protrusions per 10 µm of the parent dendrite with a distance of 25 µm from the cell body. We analyze 6 neurons per immunocytochemistry condition and in each neuron we count protrusions, according to the rules we stablished, in 3 different dendrites.

3.4| Freshly Prepared Hippocampal Slices

Male Wistar rats (8–12 weeks old) were purchased from Charles River and were handled according to European Community guidelines and Portuguese law concerning animal care (86/609/EEC). Animals were deeply anesthetized with isoflurane (Esteve, Barcelona, Spain) before decapitation. The brain was quickly removed into ice-cold continuously oxygenated (O_2/CO_2: 95%/5%) artificial cerebrospinalfluid (aCSF) (124 mM NaCl, 3 mM KCl, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM CaCl2, 1 mM $MgSO_4$ and 10 mM glucose, pH 7,4) and the hippocampi were dissected out. The hippocampal slices were cut perpendicularly to the long axis of the hippocampus with a McIlwain tissue chopper (400 µM thick) (Figure 18).

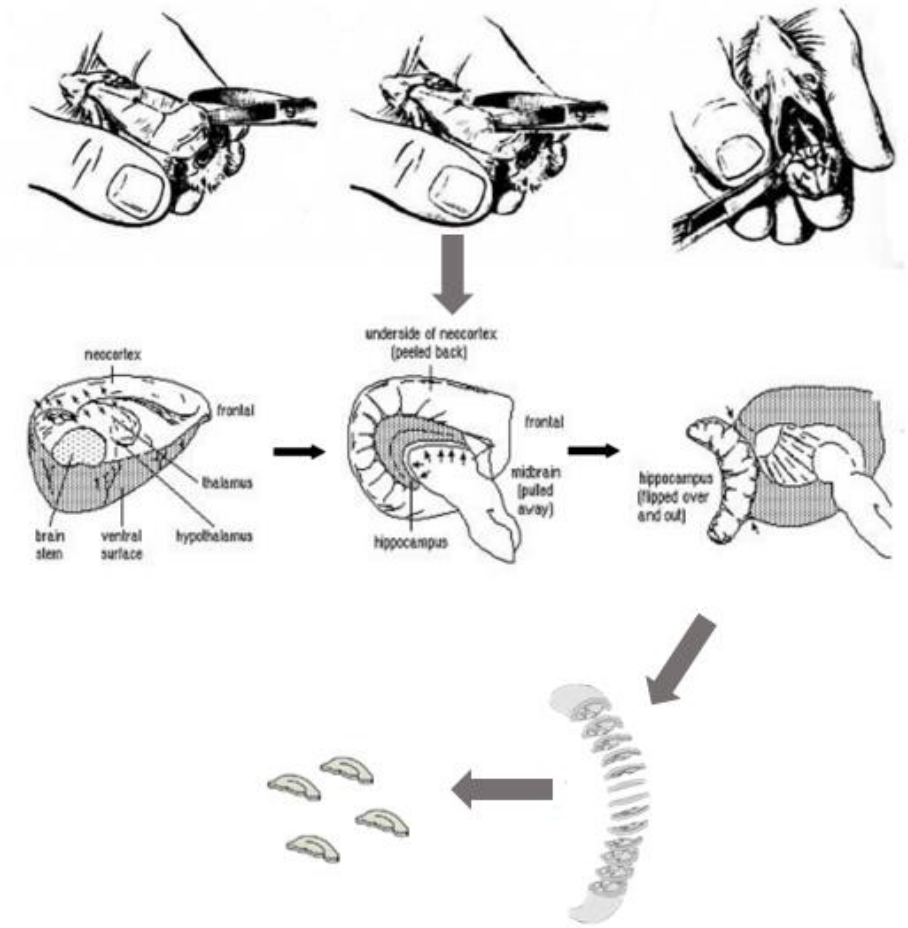


Figure 18 – **Rat hippocampal slices preparation.** The brain is removed and after that hemispheres are separated. The hippocampus is isolated and cut (400 µM thick) to obtain hippocampal slices used to perform electrophysiological (adapted from Palkovits e Brownstein, 1983).

Slices recovered functionally and energetically for at least 1 h in a resting chamber filled with continuously oxygenated aCSF at RT. After recovering, slices were

incubated for 3 h, the minimum time required to observe changes in TrkB-FL receptor cleavage (Jeronimo-Santos et al., 2015). Slices were incubated with oxygenated aCSF (control), with aCSF containing 25 µM of $A\beta_{25–35}$ peptide, containing 1 µM of memantine or containing $A\beta_{25–35}$ plus memantine. After this incubation period, the slices were used for electrophysiology recordings.

3.5| *Ex-Vivo* Electrophysiology Recordings - LTP induction

Hippocampal slices from 8 to 12 weeks old Wistar rats were transferred to a recording chamber continuously superfused with oxygenated aCSF at 32°C (flow rate of 3 mL/min in open system). Recordings were obtained with an Axoclamp 2B amplifier and digitized from Axon Instruments (Foster City, CA, USA). LTP induction and quantification were performed as described previously (Diógenes et al., 2011). Individual responses were monitored, and averages of six consecutive responses continuously stored on a personal computer with the LTP software (Anderson and Collingridge, 2001). The recording configuration used to obtain fEPSPs in the stratum radiatum of CA1 area of a hippocampal slice is represented in figure 19. These responses are evoked by stimulation of two separate sets of Shaffer collaterals (S1 and S2). LTP was induced by weak θ-burst protocol consisting of 3 trains of 100 Hz, 3 stimuli, separated by 200 ms (Figure 19C). This pattern of stimulation was used because it is considered to be closer to what occurs physiologically in the hippocampus during episodes of learning and memory in living animals (Albensi et al., 2007).

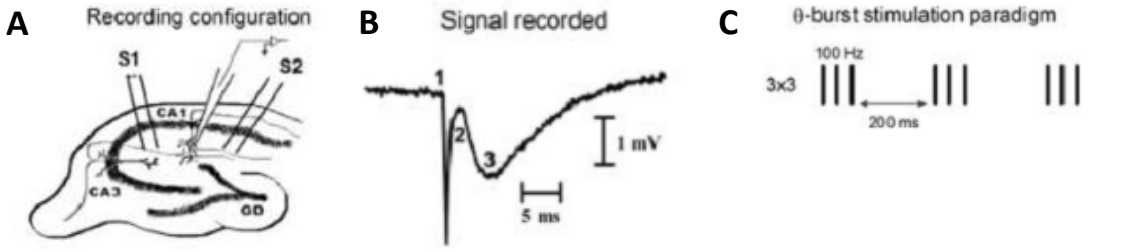


Figure 19 - **Schematic representation of extracellular recordings in hippocampal slices.** (A) Hippocampal transverse slice preparation showing the recording configuration used to obtain extracellular responses in the CA1 dendritic layer evoked by stimulation of two separate sets of the Schaffer pathway (S1 and S2). (B) Representative trace obtained after stimulation composed by (1) the stimulus artifact, (2) the pre-synaptic volley and (3) the field excitatory post-synaptic potentials (fEPSPs). (C) Schematic representation of the stimulation protocol used in LTP induction (Diógenes et al., 2011).

The facilitatory action of BDNF upon LTP is mostly seen under weak θ-burst stimulation (Fontinha et al., 2008). Therefore, we selected the optimal stimulation paradigm to observe an effect of BDNF upon LTP, so that we could evaluate the influence of Aβ and memantine upon the effect of BDNF.

When fEPSP slope was stable, LTP was inducted in the first pathway. At 1 h after LTP induction in the first pathway, BDNF (20 ng/mL) was added to the superfusion solution. The intensity of stimulation was adjusted for similar values recorded before BDNF application. After guarantying at less 20 min of BDNF perfusion, LTP was induced in the second pathway.

LTP was quantified as % change in the average slope of the fEPSP taken from 46 to 60 min after LTP induction in relation to the average slope of the fEPSP measured during the 10 min before the induction of LTP. The effect of BDNF upon LTP was evaluated by comparing the magnitude of LTP in the first pathway (control pathway) with the magnitude of LTP in the second pathway (test pathway) in the presence of BDNF.

3.6| Data analysis

Data are expressed as mean ± standard error of the mean (SEM) of the n number of independent experiments. Independent experiments are considered the results observed in different primary neuronal cultures obtained from fetuses of different pregnant-Sprague Dawley females and the results acquired from hippocampal slices of different Wistar rats.

One-way ANOVA followed by a Bonferroni *post-test* was used to compare means of > 2 conditions, which is the case of western blotting and immunocytochemistry, and student's t-test was used to evaluate the significance of differences between the means of 2 conditions in LTP analyses.

Values of $P< 0.05$ were considered to represent statistically significant differences. Prism GraphPad software (CA, USA) was used for statistical analysis.

3.7| Materials

Table 1 - Reagents used and respective function

Reagents	Function/Description
Hanks' balanced salt solution (HBSS)	Maintains proper pH and the osmotic balance
Trypsin	Proteolitic enzyme used to dissociate cells from tissue
Fetal bovine serum (FBS)	Contain α1-antitrypsin protein used to neutralize trypsin
Neurobasal medium™ (NB)	Meets the special cell culture requirements of pre-natal and embryonic neuronal cells
B-27	Supplement for growth and maintenance which was developed for the long-term culture of cortical neurons
L-Glutamic acid	Commonly referred as "glutamate", the main excitatory amino acid neurotransmitter in brain that, at low concentrations, promote neuronal viability
Glutamine	Amino acid essential for cell growth and function, since participates in the formation of L-glutamate, and other amino acids, as well as in protein synthesis and glucose production.
Penicillin/streptomycin	Prevent bacterial contamination of cell cultures due to their effective combined action against gram-positive and gram-negative bacteria.

Table 2 – Drug treatments

Treatments	Function	Concentration	Company
$A\beta_{25-35}$	Amyloid β peptide	25 µM	Bachem
BDNF	Neurotrophic factor	20 ng/mL	Regeneron Pharm
Memantine	Antagonist of eNMDAR	1 µM	Sigma-Aldrich
MDL28170	Calpain inhibitor	20 µM	Tocris Bioscience

Table 3 – Antibodies and probes

Primary antibodies	Technique	Dilution	Company
Rabbit Trk-FL polyclonal antibody (raised against the intracellular domain)	WB	1:2000	Santa Cruz Biotechnology
Mouse αII-Spectrin monoclonal antibody	WB	1:2500	Santa Cruz Biotechnology
Mouse GAPDH monoclonal antibody	WB	1:5000	Invitrogen
Rabbit anti-GluN2B polyclonal antibody	WB	1:1000	Sigma-Aldrich
Mouse anti-GluN1 polyclonal antibody	WB	1:1000	Sigma-Aldrich

Mouse anti-MAP2 monoclonal antibody	ICC	1:200	Millipore

Secondary antibodies	Technique	Dilution	Company
IgG-horseradish peroxidase-conjugated goat anti-mouse	WB	1:10000	Santa Cruz Biotechnology
IgG-horseradish peroxidase-conjugated goat anti-rabbit	WB	1:10000	Santa Cruz Biotechnology
Rabbit Anti-IgG – AlexaF568	ICC	1:200	Invitrogen

Probes	Technique	Dilution	Company
Alexa Fluor 488 Phalloidin	ICC	1:40	Invitrogen

4 | RESULTS

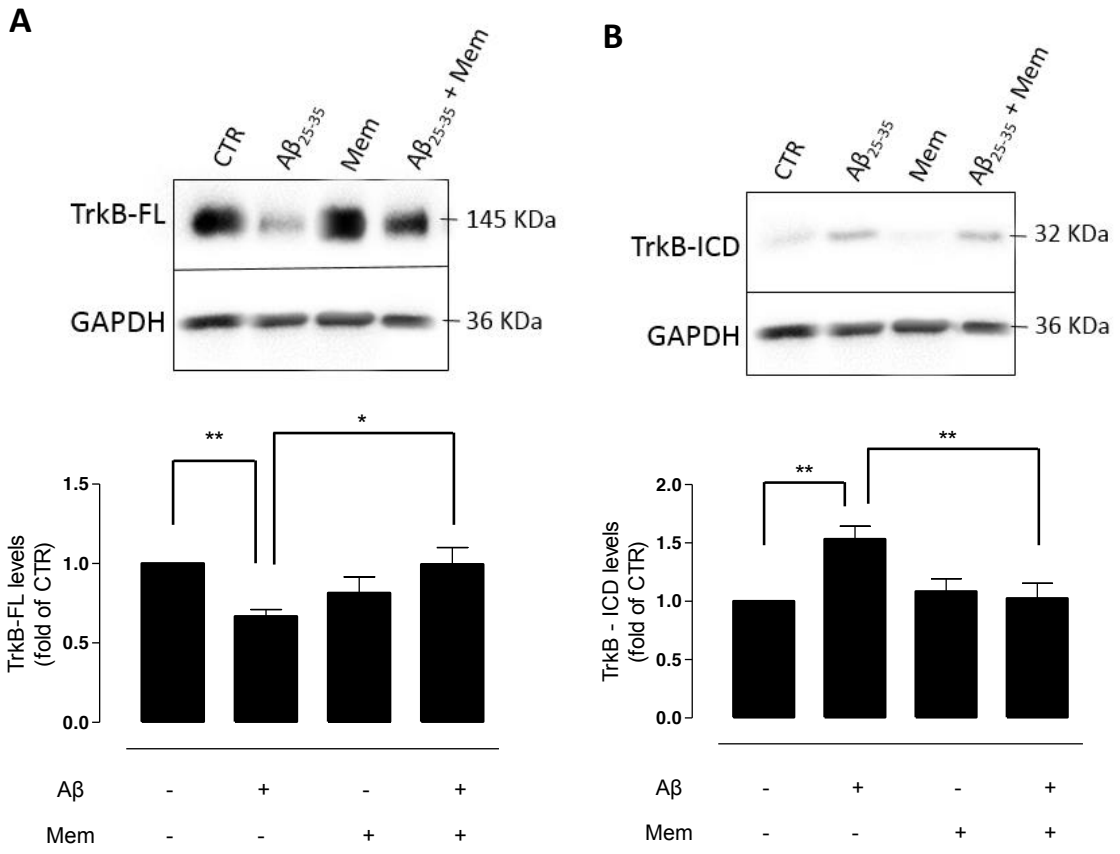
4.1| The blockade of eNMDAR can limit TrkB-FL truncation induced by Aβ and restore the ability of BDNF to increase spine density on primary neuronal cultures

It has been reported that, in primary neuronal cultures, Aβ induces a decrease in TrkB-FL, and the formation of TrkB-T' and TrkB-ICD fragments in a mechanism dependent on calpain processing of TrkB-FL (Jeronimo-Santos et al., 2015). This is correlated with a loss of BDNF signaling (Jeronimo-Santos et al., 2015), which appears to be involved in AD pathology (Arancio and Chao, 2007, Schindowski et al., 2008). In addition, neuronal dysfunction induced by Aβ is known to be mediated by excessive eNMDAR activity (Rönicke et al., 2011) which evokes an immediate rise in intracellular Ca^{2+} (Ferreira et al., 2012a). Thus, we hypothesized that eNMDAR activation by Aβ contributes to the increase of intracellular Ca^{2+} levels, activating calpains that ultimately will cleave TrkB-FL.

To test this hypothesis, primary cortical neurons were cultured for DIV14 and treated with 25 μM $A\beta_{25-35}$ peptide for 24 h, in the presence or absence of 1 μM memantine, a NMDAR non-competitive antagonist with higher specificity to activated eNMDAR. Then, total proteins were isolated and TrkB-FL and TrkB-ICD levels were evaluated by western-blotting, using an antibody recognizing the intracellular C-terminal domain of TrkB-FL.

Consistently with previous results (Jeronimo-Santos et al., 2015), incubation of primary cortical neurons with $A\beta_{25-35}$ induced a dramatic decrease in TrkB-FL levels of approximately 33 % when compared to the control ($TrkB\text{-}FL_{A\beta25-35}$: 0.67 ± 0.043; $TrkB\text{-}FL_{CTR}$: 1, n = 10, $p<0.01$, ANOVA, Figure 20A), whereas TrkB-ICD levels increased up to 53 % ($TrkB\text{-}ICD_{A\beta25-35}$: 1.53 ± 0.11; $TrkB\text{-}ICD_{CTR}$: 1, n=10, $p<0.01$, ANOVA, Figure 20B). Additionally, our data showed that these $A\beta_{25-35}$ effects were significantly prevented by the co-incubation of memantine. In fact, TrkB-FL levels increased up to 0.99 ± 0.11 (n=9, $p<0.05$, ANOVA, Figure 20A) and TrkB-ICD levels decreased to 1.02 ± 0.13 (n=11, $p<0.01$, ANOVA, Figure 20B) when compared to cells that were only incubated with $A\beta_{25-35}$. Memantine alone had no significant effect on TrkB-FL (n=9, Figure 20A) or TrkB-ICD levels (n=12, Figure 20B).

To ensure that these changes in TrkB-FL and TrkB-ICD were due to calpain activation, we also measured αII-spectrin levels and the formation of the calpain-specific spectrin breakdown products (SBDPs), a standard assay for monitoring calpain activity. αII-spectrin (250 kDa) is a major substrate for calpain and caspase-3 proteases, and can produce breakdown products with distinct molecular sizes. Particularly, calpain mediates the degradation of αII-spectrin in highly stable 150 kDa SBDPs (SBDP150). The presence of the calpain-cleaved fragments occurs early in neural cell pathology and may be indicative of necrotic and excitotoxic neuronal injury and death (Yan et al., 2012). Our results showed that the exposure of neuronal cultures to $A\beta_{25-35}$ resulted in high levels of SBDP150, up to 3 fold ($SBP150_{A\beta25-35}$: 3.22 ± 0.97) compared to the control ($SBP150_{CTR}$: 1), suggesting a strong activation of calpains (n=7, $p<0.01$, ANOVA, Figure 20C), as previously confirmed (Jeronimo-Santos et al., 2015). Importantly, memantine was able to revert this effect, by reducing the levels of SBDP150 to 1.14 ± 0.25 (n=7-8, $p<0.01$, ANOVA as compared to the Aβ). Memantine alone had no significant effect (n=12, Figure 20C).



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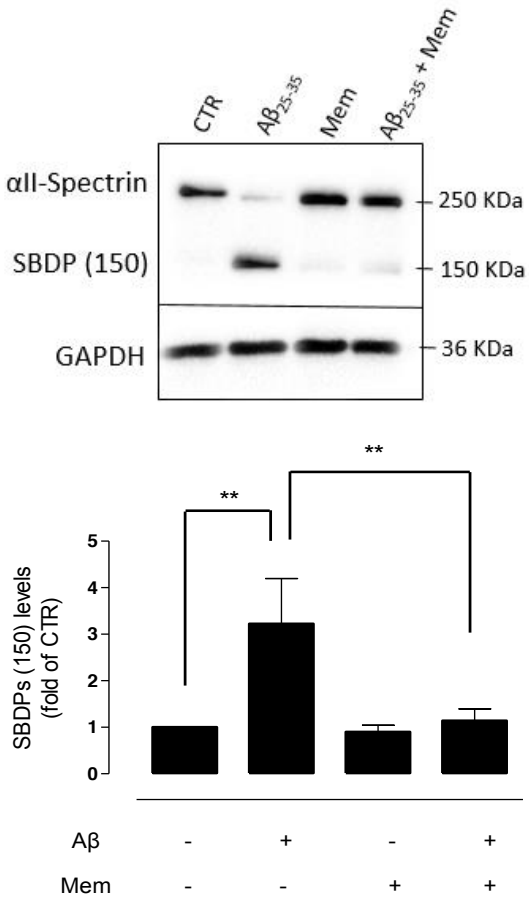


Figure 20 – **The inhibition of eNMDAR reduces the cleavage of TrkB-FL by modulating calpains activation. (A)** TrkB-FL protein levels. Representative western-blots of DIV14 neuronal cultures showing the effect of 24 hours of 25 µM $A\beta_{25-35}$ and 1 µM memantine on TrkB-FL (~145 KDa). The panel shows the average band intensity of TrkB-FL (*p<0.05, **p<0.01, n=9-10, one-way ANOVA with Bonferroni's multiple comparison test). **(B)** TrkB-ICD protein levels. Representative western-blots of DIV14 neuronal cultures showing the effect of 24 hours of 25 µM $A\beta_{25-35}$ and 1 µM memantine on TrkB-ICD (~32 KDa). The panel shows the average band intensity of TrkB-ICD (**p<0.01, n=10-12, one-way ANOVA with Bonferroni's multiple comparison test) and **(C)** Calpains activation. Representative western-blots of DIV14 neuronal cultures showing the effect of 24 hours of 25 µM $A\beta_{25-35}$ and 1 µM memantine on SBPD (150 KDa). The panel shows the average of the ratio of the SBDP (150) to intact spectrin (**p<0.01, n=7-12, one-way ANOVA with Bonferroni's multiple comparison test). GAPDH was used as loading control. Values are mean ± SEM.

Our data indicates that Aβ-induced TrkB-FL cleavage by calpains involves the activation of eNMDAR, which can be prevented when these receptors are inhibited.

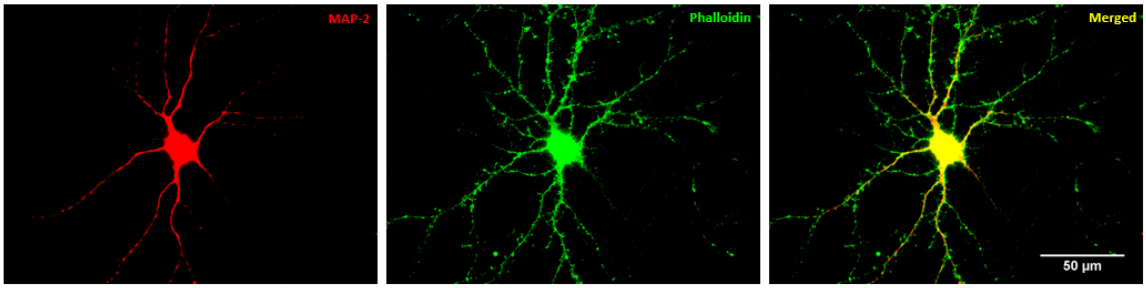
One of the BDNF abilities is to increase the number of dendritic spines in one neuron (Ji et al., 2005, Ji et al. 2010, Tyler and Pozzo-Miller, 2001, Kellner et al. 2014). In addiction, the loss of dendritic spines is a process that has been described in patients and several animal models of AD (Spires et al., 2005, Spires-Jones et al., 2007) and alterations in spine density are thought to be responsible for the cognitive deficits (Knobloch and Mansuy, 2008). Moreover, the inhibition of NMDARs prevents the decrease in synaptic density observed in animal models of AD, which suggests that NMDAR activation is required for Aβ to exert its effects on spines (Shankar et al., 2007, Shankar et al., 2008, Wei et al., 2010).

Thus, after biomolecular evaluation, we next aimed to assess, firstly, if spine density is affected when BDNF is in the presence of Aβ and, then, if eNMDAR blockade can restore BDNF function upon spine density in our experimental conditions.

Our results showed a significant reduction in the number of protrusions per 10 μm of the parent dendrite with a distance of 25 μm from the cell body in primary cortical neurons incubated with 25 μM $A\beta_{25-35}$ for 24 h, when compared to control ($A\beta_{25-35}$: 4.02 ± 0.30 vs CTR: 6.56 ± 0.35, n=6-8, $p<0.001$, ANOVA, Figure 21C). Interestingly, when neurons were treated with 20 ng/mL BDNF alone for 24 h, the number of protrusions significantly increased when compared to control (BDNF: 8.43 ± 0.38 vs CTR: 6.56 ± 0.35, n=4-8, $p<0.05$, ANOVA). In the presence of Aβ, however, BDNF loses its ability to increase the number of protrusions, that reach near to control condition numbers ($A\beta_{25-35}$+BDNF: 6.76 ± 0.41 vs BDNF: 8.43 ± 0.38, n=4-7, $p<0.05$, ANOVA). Curiously, in the presence of Aβ, BDNF partially loses its ability to increase spines, although it continues to exert some function upon spine density ($A\beta_{25-35}$+BDNF: 6.76 ± 0.41 vs $A\beta_{25-35}$: 4.02 ± 0.30, n=6-8, $p<0.001$, ANOVA). Then, we examine if the inhibition of calpains could revert Aβ-induced dendritic spine loss by exposing neurons to Aβ in the absence or presence of the calpain inhibitor 20 μM MDL28170 and 20 ng/mL BDNF for 24 h. The results showed that BDNF effect on spines number is rescued when cells are incubated with MDL28170 ($A\beta_{25-35}$+BDNF+MDL: 8.28 ± 0.20 vs $A\beta_{25-35+}$BDNF: 6.76 ± 0.41, n=4-7, $p<0.05$, ANOVA). MDL28170 alone had no significant effect. This data confirms that when calpains activation is inhibited, TrkB-FL cleavage induced by Aβ is reduced, restoring BDNF signaling, which, among other things, affects positively synaptic density.

Next, we evaluated if the results would be similar when eNMDAR were inhibited. Remarkably, when cells were co-incubated with 1 µM memantine for 24 h, BDNF recovered the capability of significantly induce the increase in the number of dendritic spines ($A\beta_{25-35}$+BDNF+Mem: 8.69 ± 0.19 vs BDNF: 8.43 ± 0.38, n=4-7, p>0.05, ANOVA).

A



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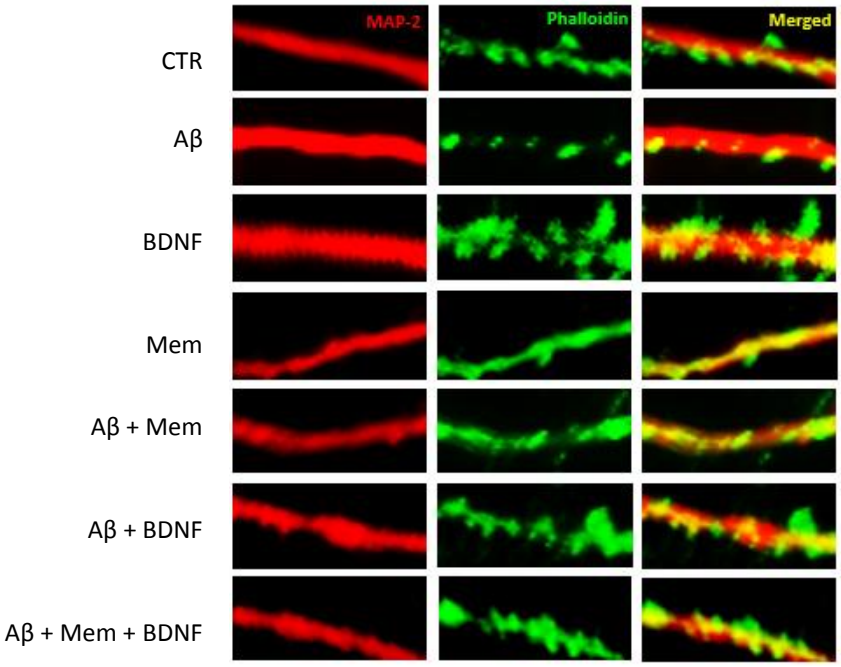


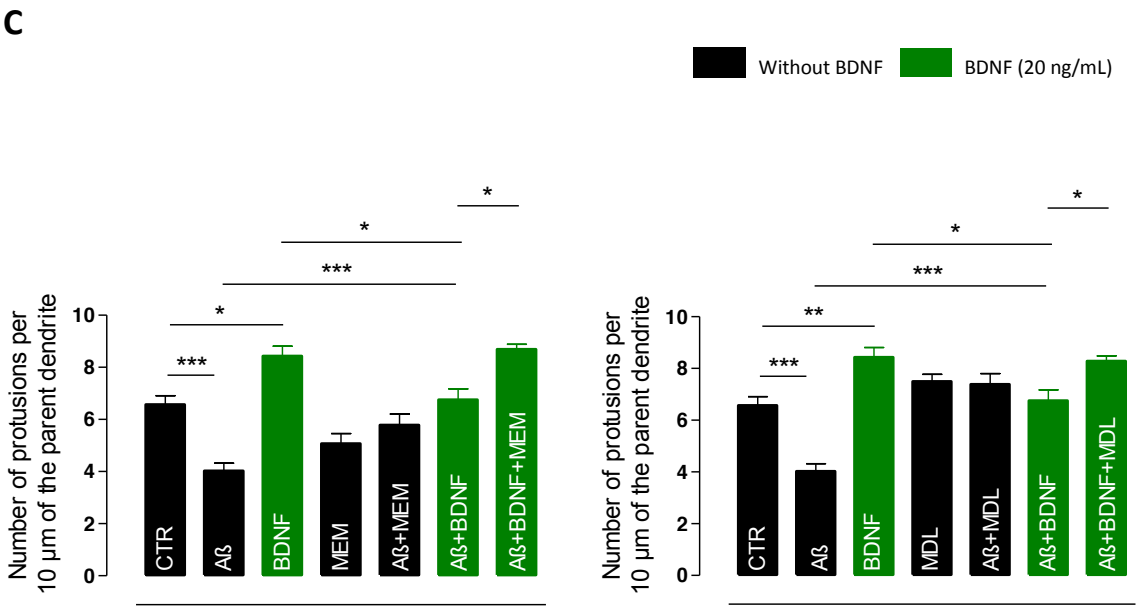


Figure 21 – **BDNF restores its capacity to increase dendritic spines number after inhibition of Aβ-induced eNMDAR activation. (A)** Representative image of a neuron obtained from primary cultures. DIV14 neurons were incubated with 20 ng/mL BDNF for 24h, in the presence or absence of 25 µM $A\beta_{25-35}$ and/or 20 µM MDL28170 and/or 1 µM memantine. MAP2, which specifically detect neurons, labeled in red and phalloidin, which recognizes F-actin, labeled protrusions (filopodia and spines) in green. The merge of both elements are represented in yellow. 6 neurons were analyzed per condition and spine density was considered, in each cell, as the number of protrusions per 10 µm of the parent dendrite with a distance of 25 µm from the cell body. Protrusions were counted in each neuron in 3 different dendrites. **(B)** Treatments effects on synaptic growth. Aβ significantly reduces the number of protrusions, whereas BDNF increases the number of protrusions when incubated alone. In the presence of Aβ, BDNF loses its ability to increase the number of protrusions, which is rescued when cells are incubated with memantine. **(C)** The panels show the average number of protrusions in different conditions when neurons were treated with memantine (left histogram) and MDL28170 (right histogram) (*p<0.05, **p<0.01, ***p<0.001, n=3-8, one-way ANOVA with Bonferroni's multiple comparison test). Values are mean ± SEM.

To evaluate if these alterations in the number of spines were correlated with Aβ-induced changes in NMDAR subunit levels and if memantine prevents those changes, we performed a western blot analysis using anti-GluN2B and GluN1 subunits antibodies. Interestingly, no significant changes were obtained in these NMDAR subunit levels (p>0.05, n=3-4, ANOVA, Figure 22).

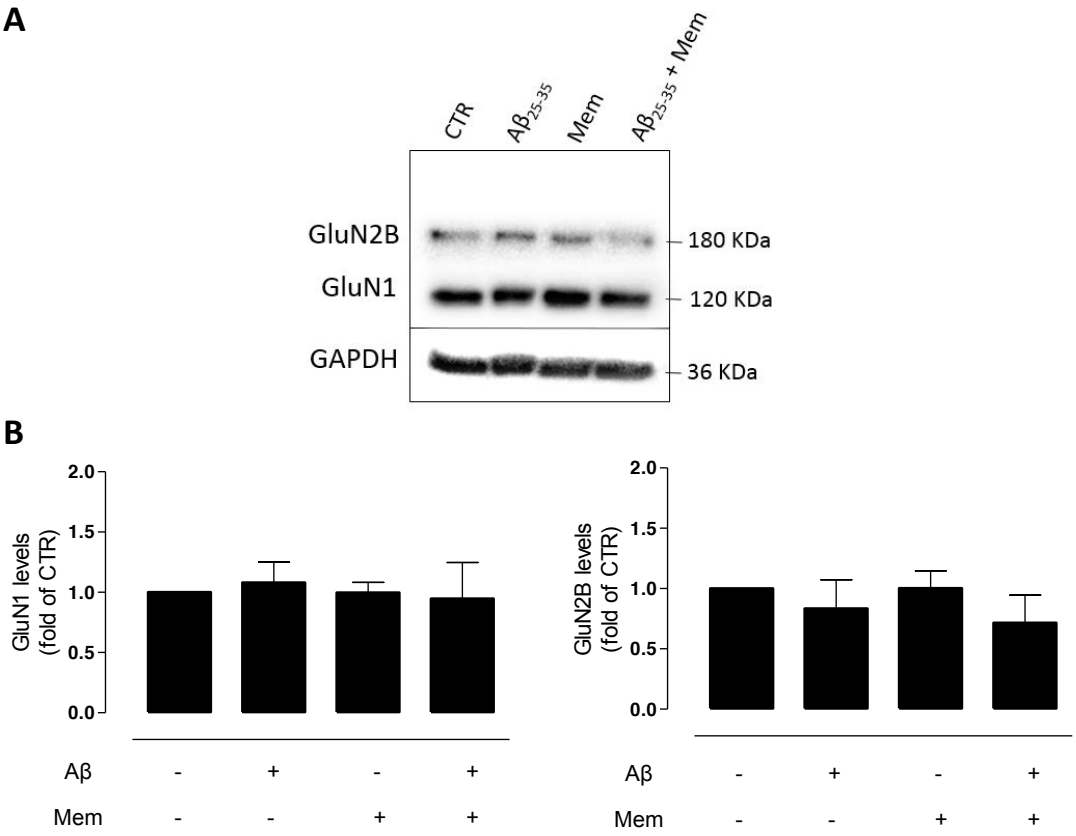


Figure 22 - **The alterations in the number of spines are not associated with changes in different NMDAR subunits expression. (A)** Representative western-blots of DIV14 neuronal cultures showing the effect of 24 hours of 25 µM $A\beta_{25-35}$ and 1 µM memantine on GluN1 and GluN2B. **(B)** The panels show the average band intensity of GluN1 (left histogram) and GluN2B (right histogram) ($p>0.05$, n=3-4, one-way ANOVA with Bonferroni's multiple comparison test). GAPDH was used as loading control. Values are mean ± SEM.

This data indicates that, in the presence of Aβ and/or memantine, the number of spines is probably not influenced by changes in NMDAR subunits expression, but rather by possibly interference with eNMDAR phosphorylation and its downstream effects.

Thus, our results propose that the inhibition of Aβ-induced eNMDAR activation is capable of restoring the ability of BDNF to increase the number of spines in each neuron, which may be related with a decrease in calpains activation.

4.2| The inhibition of eNMDAR can partially rescue the facilitatory effect of BDNF upon LTP on hippocampal slices

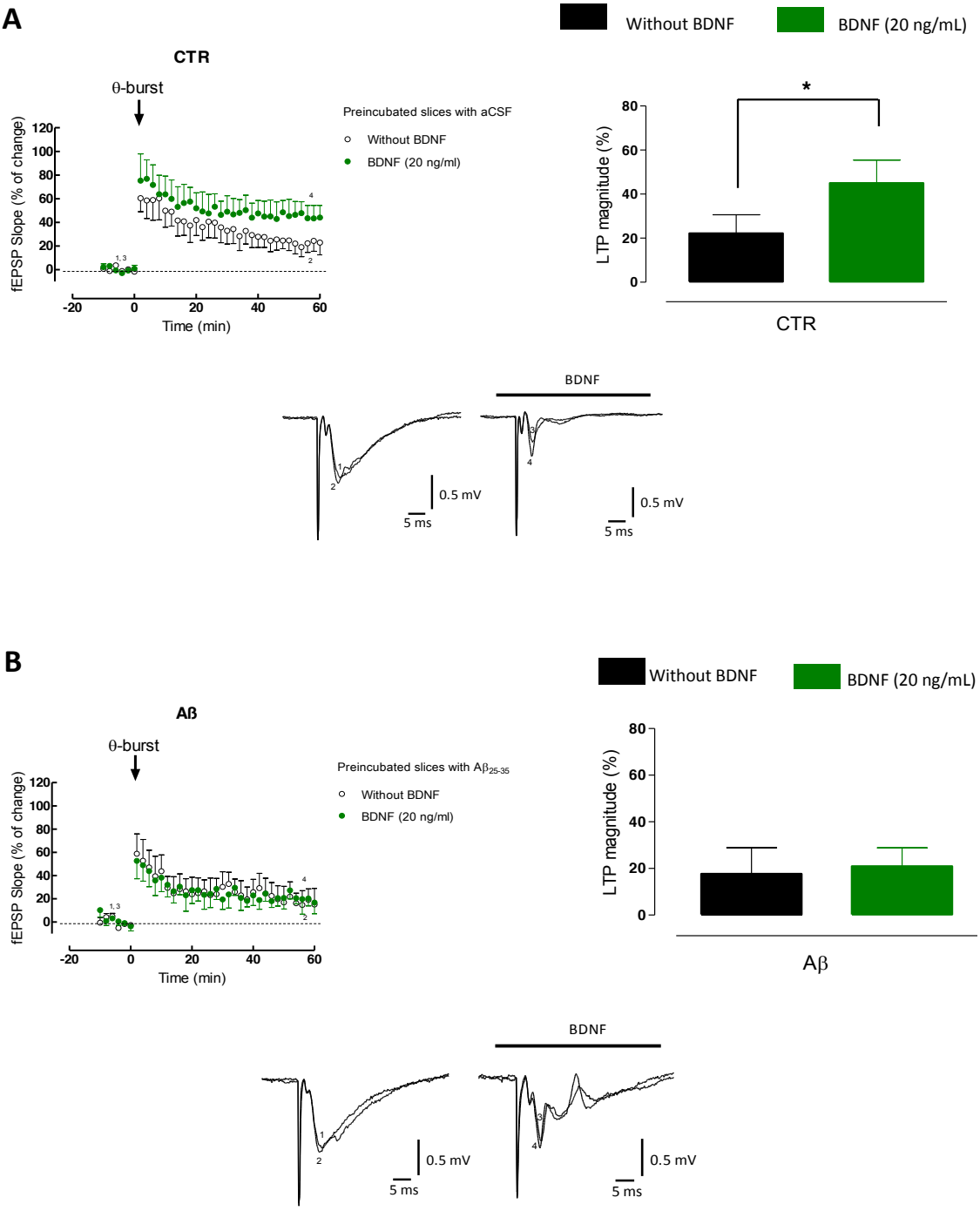
The ability of BDNF to increase LTP on hippocampal CA1 area through TrkB-FL activation is well described (Korte et al., 1995, Figurov et al. 1996). Recent studies demonstrated that BDNF loses its ability to facilitate LTP in the presence of Aβ

(Jeronimo-Santos et al., 2015). Therefore, and given the possibility raised in this thesis of eNMDAR activation being involved in TrkB-FL cleavage by calpains and consequently involved in the loss of BDNF function, we evaluated if eNMDAR inhibition could also rescue BDNF effect on LTP in the presence of Aβ.

Hippocampal slices were prepared and two independent stimulation pathways were used as previously described in methods section. The LTP was firstly induced by θ-burst stimulation in the first pathway and its magnitude quantified 60 min after LTP induction. BDNF was then added to the perfusing aCSF and allowed to equilibrate for at least 20 min before inducing LTP in the second pathway.

As previously shown (Fontinha et al., 2008, Jeronimo-Santos et al., 2015), the θ-burst stimulus applied in the presence of BDNF (20 ng/mL) induced a robust LTP, which was significantly higher than that obtained in the absence of BDNF (LTP_{BDNF}: 45.03± 10.43 % vs LTP_{CTR}: 22.15± 8.49% increase in fEPSP slope, n = 5, $p< 0.05$, student's t-test, Figure 23A). However, when hippocampal slices were pretreated with 25 µM $Aβ_{25-35}$ for 3 h, BDNF (20 ng/mL) failed to enhance LTP magnitude ($LTP_{Aβ}$: 17.68 % ± 11.25 % vs $LTP_{Aβ+BDNF}$: 20.93 % ± 7.95 % increase in fEPSP slope, n = 3, $p>0.05$, student's t-test, Figure 23B). To explore whether eNMDAR activation played a role in the Aβ-induced loss of BDNF effect upon LTP, hippocampal slices were treated simultaneously with the 25 µM $Aβ_{25–35}$ and 1 µM memantine for 3 h. In fact, the co-incubation of memantine and $Aβ_{25–35}$ restored the enhancement of LTP magnitude when BDNF was added ($LTP_{Aβ+Mem+BDNF}$: 30.62 % ± 8.96 % vs $LTP_{Aβ+Mem}$: 15.12 % ± 5.78 % increase in fEPSP slope n=7, $p<0.05$, student's t-test, Figure 23D). The number of experiences performed when slices were incubated only with memantine, although not sufficient yet, showed a small effect of BDNF upon LTP ($LTP_{Mem+BDNF}$: 10.75 % ± 7.48 % vs LTP_{Mem}: 4.00 % ± 6.82 % increase in fEPSP slope, n=3, $p>0.05$, student's t-test, Figure 23C).

Altogether, our results suggest that BDNF lose its ability to increase LTP magnitude in hippocampal slices pretreated with Aβ. However, when hippocampal slices are pre-exposed simultaneously with Aβ and memantine there is a partially recovery of BDNF function upon LTP.


A
Without BDNF
BDNF (20 ng/mL)
CTR
θ-burst
fEPSP Slope (% of change)
Time (min)
Preincubated slices with aCSF
Without BDNF
BDNF (20 ng/ml)
LTP magnitude (%)
*
CTR
BDNF
0.5 mV
5 ms
0.5 mV
5 ms
B
Without BDNF
BDNF (20 ng/mL)
Aß
θ-burst
fEPSP Slope (% of change)
Time (min)
Preincubated slices with Aβ25-35
Without BDNF
BDNF (20 ng/ml)
LTP magnitude (%)
Aβ
BDNF
0.5 mV
5 ms
0.5 mV
5 ms

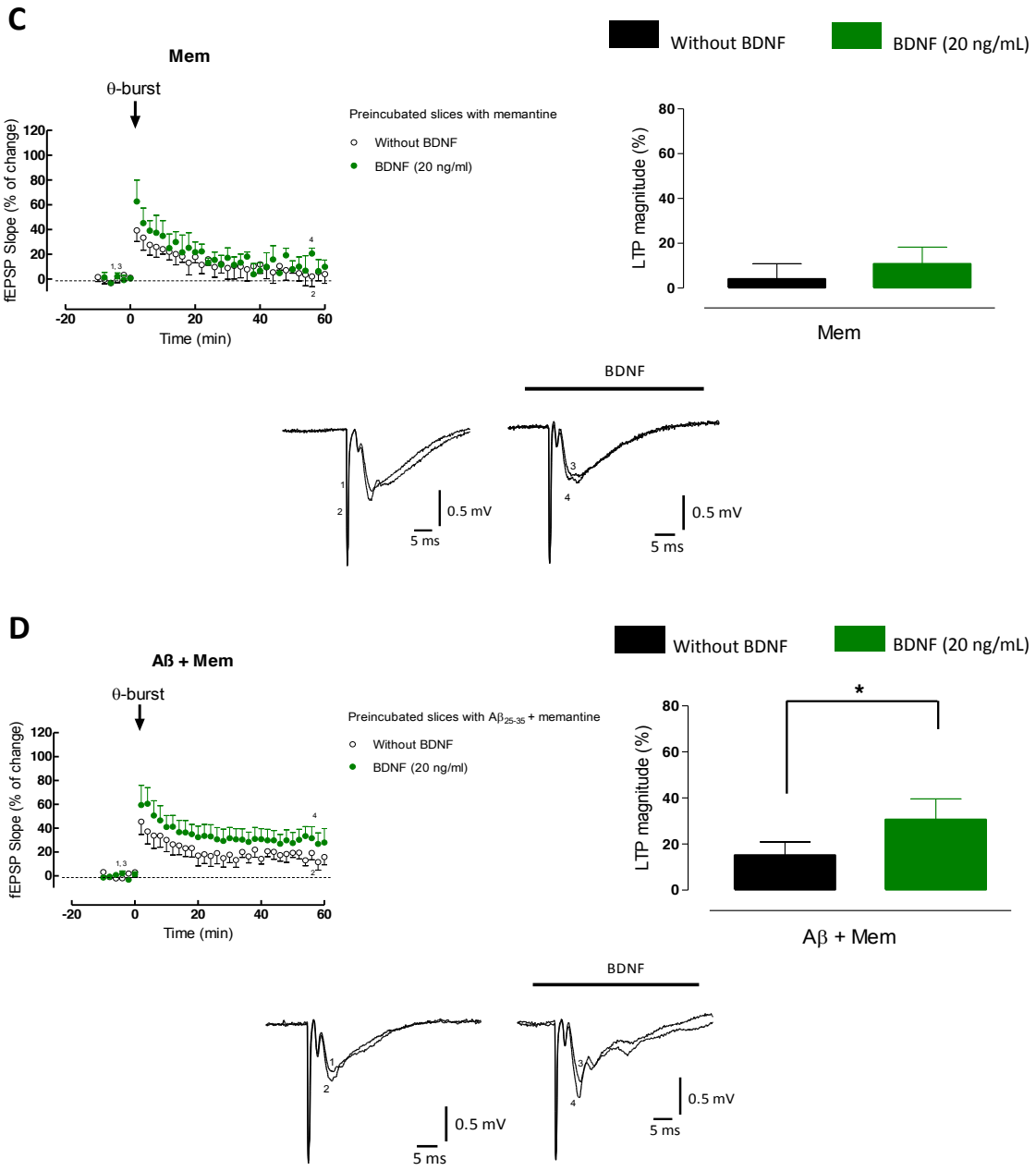


Figure 23 – **The inhibition of eNMDAR activation by Aβ restores the facilitatory effect of BDNF upon θ-burst-induced LTP.** Panels A–D show the averaged time courses changes in field excitatory post-synaptic potential (fEPSP) slope induced by a θ-burst stimulation in the absence or in the presence of BDNF (20 ng/mL) in the second stimulation pathway in rat hippocampal slices without (A, n=5) or with a pre-exposure for 3 h to aCSF solution containing 25 µM $A\beta_{25–35}$ (B, n=3) or 1 µM Mem (C, n=3) and 25 µM $A\beta_{25–35}$ in the presence of 1 µM Mem (D, n=7). The traces from representative experiments are shown below panels A–D; each trace is the average of 6 consecutive responses obtained before (1 and 3) and 46–60 min after (2 and 4) LTP induction. The traces are composed by the stimulus artifact, followed by the pre-synaptic volley and the fEPSP. The traces (1 and 2) and traces (3 and 4) were obtained in the absence and presence of BDNF, respectively (*$p<0.05$, n=3-7, student's t-test). Values are mean ± SEM.

Thus, these data suggest that Aβ severely impairs BDNF action on hippocampal LTP by a mechanism dependent on eNMDAR activation.

Briefly, our results suggest that TrkB-FL cleavage occurs by calpains that are activated, at least partially, by a Ca^{2+} influx through Aβ-induced eNMDAR activation. Importantly, when eNMDAR are inhibited, BDNF signaling is rescued, which is confirmed by its capacity to increase the number of protusion and to facilitate LTP.

5 | DISCUSSION

5.1| The inhibition of eNMDAR limits Aβ-induced TrkB-FL truncation and restores the ability of BDNF to increase spine density on primary neuronal cultures

Taken together the facts that: i) the loss of BDNF function induced by Aβ is due to TrkB-FL cleavage by calpains, which are known to be overactivated under increased levels of intracellular Ca^{2+} (Jeronimo-Santos, et al., 2015); ii) GluN2B activation has a crucial role on Ca^{2+} homeostasis dysregulation (Ferreira et al., 2012b); iii) eNMDARs, which are excessively activated under pathological conditions, including AD, are mainly composed by GluN2B subunits (Stocca and Vicini, 1998, Tovar and Westbrook, 1999, Cull-Candy et al., 2001, Hanson et al., 2013, Papouin et al., 2012); we were prompted to, by the use of the pharmacological tool available, memantine, investigate if the activation of eNMDA receptors was involved in the loss of BDNF function in cells exposed to Aβ.

Memantine was used at the concentration of 1 μM, which corresponds to a concentration reported to be in the NMDAR-blocking therapeutic range (Lipton, 2007, Parsons et al., 2007). Furthermore, it is well described that at this low concentrations it acts as an effective antagonist of excessive eNMDAR stimulation (Xia et al., 2010).

In the present work, we firstly replicated the results previously obtained (Jeronimo-Santos et al., 2015), to confirm, in our experimental conditions, that Aβ indeed promotes calpain-mediated cleavage of TrkB-FL receptor, consequently impairing BDNF functions. In fact, Aβ induced i) TrkB-FL cleavage due to calpains activation and, consequently ii) increased the levels of TrkB-ICD. Afterwards, we investigated if eNMDARs inhibition could prevent the cleavage of TrkB-FL in the presence of Aβ. Remarkably, the results show that indeed, memantine can restore the expression levels of TrkB-FL and TrkB-ICD near to control. This suggests that eNMDAR activation has a significant contribution for the increased intracellular Ca^{2+} levels responsible for calpain activation and consequent to the TrkB-FL truncation. In line with these results are the previous works showing that antagonists of NMDARs prevent the activation of calpains (del Cerro et al., 1994) suggesting that calpains activation in hippocampal neurons depends on NMDAR activation (Adamec et al., 1998). Curiously, it

was also described that GluN2B antagonists can prevent neuronal disruption of intracellular Ca^{2+} homeostasis induced by Aβ (Ferreira et al., 2012).

The role of BDNF in spine density has been widely described. Previously published data showed a highly significant increase in dendritic spine density and alterations in spine morphology upon the application of exogenous BDNF to mature hippocampal primary neurons (Ji et al., 2005, Ji et al. 2010) or to hippocampal slice cultures (Tyler and Pozzo-Miller, 2001). Accordingly, recent work has shown that the removal of BDNF results in a significant reduction in dendritic spine density (Kellner et al. 2014). Moreover, Aβ, which is known to impair BDNF signaling (Jeronimo-Santos et al., 2015) also induces a decrease on spine density in hippocampal slices (Shrestha et al., 2006) and in APP/PS1 transgenic mice (Smith et al., 2009).

Our data indicate that, in the presence of Aβ, BDNF partially loses its ability to increase spine density. However, this effect was rescued when calpain activation was inhibited by MDL28170. The same was observed when eNMDARs were blocked by memantine, indicating that eNMDAR activation, which probably mediates calpain activity, contributes to Aβ-mediated BDNF loss of function upon synapses. Accordingly, it has been reported that NMDAR signaling plays a critical role in regulating spine size and density in the developing cortex (Ultanir et al., 2007). Consistently, studies have shown that GluN2B-NMDAR antagonists prevent the synapse loss induced by incubation with exogenous Aβ in primary neuronal cell culture and hippocampal slices from rat and mouse (Ronicke et al., 2011).

To assess if the detrimental Aβ effects mediated by GluN2B-containing NMDARs were related with changes in NMDAR subunits expression and if memantine was capable of preventing this alteration, we also evaluated the levels of GluN1 and GluN2B subunits. However, no significant changes were found. Although other studies evidenced that the expression levels of mRNA of GluN1 (Bi and Sze, 2002) are unchanged in AD patient's brains, the results now obtained concerning GluN2B protein levels are not in total agreement with previous studies. In fact, it has been reported that expression levels of both GluN2B mRNA and protein are decreased in susceptible regions of *postmortem* human AD brain, such as hippocampus and cortex (Mishizen-Eberz et al., 2004, Bi and Sze, 2002, Hynd et al., 2004). It is possible that certain subunit combinations may be lost in AD because of selective neuronal loss. If so, the retention of

GluN1 trancripts suggests that neurons that express GluN2B NMDAR are comparatively more susceptible to neurotoxicity (Hynd et al., 2004). However, the discrepancies between our results and data obtained in these studies might be explained by the difference in samples origin. Furthermore, it is worthwhile to note that although our primary neuronal cultures were treated with relatively high concentrations of Aβ (25 μm), the exposure period to the peptide was much smaller (24 h) than what happens in human AD patients (several years), which could not be sufficient to induce alterations in NMDAR subunits expression. Even though this work shows that, in primary neuronal cultures, Aβ affects spine density in a mechanism probably mediated by eNMDARs, this occurs without changes in the expression levels of the studied NMDAR subunits. Instead, Aβ possibly interferes with the NMDARs subunits, modulating the activity of these receptors. In fact, it is described that Aβ enhances GluN2B-mediated NMDA currents and extrasynaptic responses (Li et al., 2011).

Curiously, in cultured rat hippocampal neurons, the stress-released neuropeptide corticotropin-releasing hormone (CRH), which is one of the molecular mediators of the structural effects of stress in hippocampus, also induces dendritic spine loss by a mechanism that requires the activation of NMDAR and the subsequent influx of Ca^{2+} and calpains activation (Andres et al., 2013). As stated before, calpain substrates include spectrin, which cross-link and stabilize actin filaments. Spectrin cleavage disrupts the spine cytoskeleton, as well as the organization of the postsynaptic density leading to spine loss (Andres et al., 2013).

The presented results show that the blockade of eNMDARs inhibits the activation of calpains and the consequent cleavage of TrkB-FL, restoring the BDNF/TrkB-FL signaling upon spine density in primary neuronal cultures.

5.2| The inhibition of eNMDAR can partially restore BDNF synaptic function in the presence of Aβ

Although Aβ impaired BDNF-mediated facilitation of LTP in hippocampal slices, as expected (Jeronimo-Santos et al., 2015), it was possible to partially rescue the effect of BDNF by memantine. This indicates that the Aβ-induced BDNF loss of function upon LTP is, at least in part, mediated by eNMDAR activation. Consistently, others have shown that the impairment of LTP by acute application of exogenous Aβ is mitigated or

prevented by antagonists that are selective for GluN2B-NMDARs (Olsen and Sheng, 2012, Rammes et al., 2011, Ronicke et al. 2011). In fact, several findings suggest that the effects of Aβ on NMDAR impair synaptic plasticity, but the detailed mechanism remains unknown. Here, we purpose eNMDAR activation as one of the triggers by which levels of intracellular Ca^{2+} are increased, which, consequently, leads to calpain activation and TrkB-FL truncation. Interestingly, recent studies showed that the impairment of BDNF actions upon LTP induced by Aβ is also prevented by inhibitors of calpains (Jeronimo-Santos et al., 2015).

Given our previous results, in primary neuronal cultures, indicating that memantine, in the presence of Aβ, could decrease calpains activation and that inhibitors of calpain activity could rescue the BDNF effect upon LTP (Jeronimo-Santos et al., 2015), we proposed to evaluate if memantine could also rescue the BDNF effect upon LTP in slices pre-incubated with Aβ.

There are evidences that, in rat hippocampal slices, $Aβ_{1-42}$, $Aβ_{1-40}$ or their active fragment $Aβ_{25-35}$ significantly impair LTP (Chen et al., 2000, Lee et al., 2000). However, according to our results and to the previous studies, also performed in our laboratory, no significant change in LTP magnitude induced by a very-weak θ-burst in hippocampal slices exposed to Aβ was detected (Jeronimo-Santos et al., 2015). The stimulation protocol, the Aβ preparation, the developmental age or genetic background of the animals used (Smith et al., 2009) could explain this absence of Aβ effect upon LTP.

Curiously, we also noticed that when hippocampal slices or primary cultures were treated only with memantine, in the absence of Aβ insult, both LTP magnitude and spine density presented a slight decrease when compared to control conditions. In fact, it makes sense that in physiological conditions, when NMDAR are not overactivated and intracellular Ca^{2+} levels are regulated, the blockade of NMDAR will probably dysregulate some processes required for neuronal maintaining. However, studies from our laboratory with a representative number of experiences revealed that 1 μM of memantine alone had no effect on LTP magnitude induced by θ-burst stimulation in rats with ages between 8-12 weeks, the same conditions used in this work (data not published). Therefore, with the increase in the number of experiments, we will probably reach the same results.

Importantly, LTP is correlated with the formation of new spines (Toni et al., 1999), since spine remodeling and the formation of new synapses are activity-dependent processes that provide a basis for memory formation (Knobloch and Mansuy, 2008). In addition, BDNF signaling, in the presence of synaptic activity, can facilitate spine morphogenesis, and that is known to occur in LTP (Pang et al., 2004).

Since we evaluate both spine density and LTP to assess if, in our study, BDNF loss of function induced by Aβ upon synapses was prevented by eNMDAR inhibition, we expected that the results would be correlated, given the well known link between LTP and spines. In fact, we observed that the inhibition of eNMDARs, in the presence of Aβ, totally rescue the ability of BDNF to increase the number of protusions in neurons from primary cultures. However, in hippocampal slices, the BDNF function upon LTP was only partially rescued. On the other hand, in primary neuronal cultures, when cells were incubated with Aβ and BDNF, BDNF exerted an effect, although not its maximum effect, by increasing spine density when compared to neurons that were incubated only with Aβ. Although TrkB-FL receptors are significantly cleaved by activated calpains, there are still other functional TrkB-FL receptors which probably could trigger BDNF signaling. Thus, we can speculate that this effect is due to the TrkB-FL response upon exogenous BDNF application. However, the same did not happen in LTP experiences. When preincubated slices with Aβ were perfused with BDNF, BDNF completely loses its ability to facilitate LTP. Accordingly to the data obtained in primary neuronal cultures, where it was possible to observe an effect, although impaired, of BDNF, even in the presence of Aβ, it would be interesting to understand why is BDNF function totally lost upon LTP. This could be related to: i) the subcellular localization of TrkB receptors that are cleaved, which could have a different impact on BDNF function upon spine density and LTP. In fact, it is not clear what is the localization of TrkB-FL that are cleaved; ii) in hippocampal slices, contrary to primary neuronal cultures, a θ-burst stimulation was delivered to induce LTP. After LTP induction, there is a significant increase on glutamate release, which binds to AMPARs, leading to cell depolarization and allowing the removal of Mg^{2+} from NMDAR channel. Thus, sNMDARs are activated and, due to the Ca^{2+} influx through these channels, a rapid rise of intracellular Ca^{2+} occurs within the dendritic spines of the postsynaptic cell (Purves D, 2001). This increase in intracellular Ca^{2+} levels can further contribute to TrkB-FL cleavage, dramatically affecting BDNF signaling.

Given the discrepancies between our systems (primary neuronal cultures *vs.* hippocampal slices and basal conditions *vs.* electrical stimulation), the results obtained might not be exactly the same. Thus, it would be of great interest to evaluate the alterations in spine number in hippocampal slices before and after LTP induction in our experimental conditions, which could be performed by biocytin labeling, using patch clamp and immunohistochemistry techniques.

In summary, our results suggest that TrkB-FL cleavage occurs by calpains that are activated, at least partially, by a Ca^{2+} influx through Aβ-induced eNMDAR activation. Here, we propose that the inhibition of Aβ-induced eNMDAR activation is capable of i) restoring the ability of BDNF to increase the number of neuronal spines and ii) rescuing BDNF facilitatory action upon LTP.

5.3|eNMDAR activation in AD: The initial trigger of calcium dyshomeostasis?

The role of Ca^{2+} in brain has been widely accepted as crucial in the control of synaptic activity and memory formation, implicating specific Ca^{2+}-dependent protein effectors, such as CaMKs, MAPK/ERKs, and CREB and the activation of different signal transduction pathways. Properly controlled Ca^{2+} homeostasis not only supports normal brain physiology but also maintains the neuronal integrity and cell survival. However, emerging knowledge indicates that Ca^{2+} homeostasis is not only important for cell physiology and health, but also, when deregulated, it can lead to neurodegeneration via complex and diverse mechanisms involved in selective neuronal impairments and death (Marambaud et al., 2009).

The Ca^{2+} hypothesis of AD was first proposed a few decades ago, it postulates that sustained disturbances in Ca^{2+} homeostasis are a proximal cause of neurodegeneration in AD (Khachaturian, 1989) and targeting this process might be therapeutically beneficial (LaFerla, 2002). In fact, although the molecular mechanism is still not fully understood, it is generally believed that Aβ-induced synaptic dysfunction is dependent on NMDAR-mediated activity. In particular, Aβ oligomers at pathological concentrations are thought to trigger overstimulation of eNMDARs, leading to, among other mechanisms, an elevation of intracellular Ca^{2+} and activation of downstream pathways leading to synaptic disruption and neuronal loss (Tu et al., 2014, Palop and Mucke, 2010, Selkoe 2008).

Besides leading to the impairment of BDNF/TrkB-FL signaling, which was the focus of the present work, increased levels of intracellular Ca^{2+} due to eNMDARs activation by Aβ (De Felice et al., 2007, Ferreira et al., 2012b) also culminates in other important mechanisms that lead to neurodegeneration, which might be important to briefly clarify to get a better understanding of these mechanisms. For example, studies reported that high levels of intracellular Ca^{2+} induced by Aβ oligomers stimulate a prominent increase in ROS formation in mature hippocampal neurons in culture (De Felice et al., 2007). Given the important roles of low levels of ROS in physiological mechanisms related to synaptic plasticity, the dysregulation of NMDAR activity that leads to oxidative stress may have a dual deleterious role in AD. In an early phase, oxidative stress impairs memory by interfering with molecular mechanisms of plasticity, whereas in later stages of the disease, oxidative stress is mainly related to neuronal degeneration and death (Pratico and Delanty, 2000). Thus, the blockade of excessive NMDAR activity induced by Aβ is helpfull by protecting synapses against Aβ-induced neuronal oxidative stress. Furthermore, Aβ oligomers (5 μM) were reported to cause mitochondrial Ca^{2+} overload in response to Ca^{2+} entry in neurons through NMDARs activation (Alberdi et al., 2010, Sanz-Blasco et al. 2008) leading to mitochondrial dysfunction (Nicholls, 2009). Recent reports have been indicating that Aβ-induced intracellular Ca^{2+} and mitochondrial Ca^{2+} rise are, specifically, mediated by GluN2B-containing NMDARs activation (Ferreira et al., 2015). It was also seen that GluN2B antagonists can block other effects of exogenous Aβ application to neurons, including endoplasmic reticulum (ER) oxidative stress. ER oxidative stress is known to activate different cascades that culminate in the impairment of ER itself and cytosolic Ca^{2+} homeostasis. These events precede changes in cell viability and activation of the ER stress-mediated apoptotic pathway (Costa et al., 2012).

In fact, pathological eNMDAR activation can be the trigger to an abnormal Ca^{2+} entry into the cell that not only can activate calpains and, consequently, contribute to BDNF loss of function, but also can promote dysregulations in cell organelles which will lead to synaptic dysfunction and, eventually, neuronal death.

6 I CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, our results showed that, in primary neuronal cultures and hippocampal slices, Aβ severely impairs BDNF/TrkB-FL signaling, affecting the synaptic actions of BDNF by a mechanism mediated by eNMDARs activation (Figure 24). These findings highlight the functional consequence of the Aβ-induced cleavage of TrkB receptors and propose eNMDAR blockade to prevent disruption of Ca^{2+} homeostasis and, consequently, the loss of physiological mechanisms that depend on BDNF.

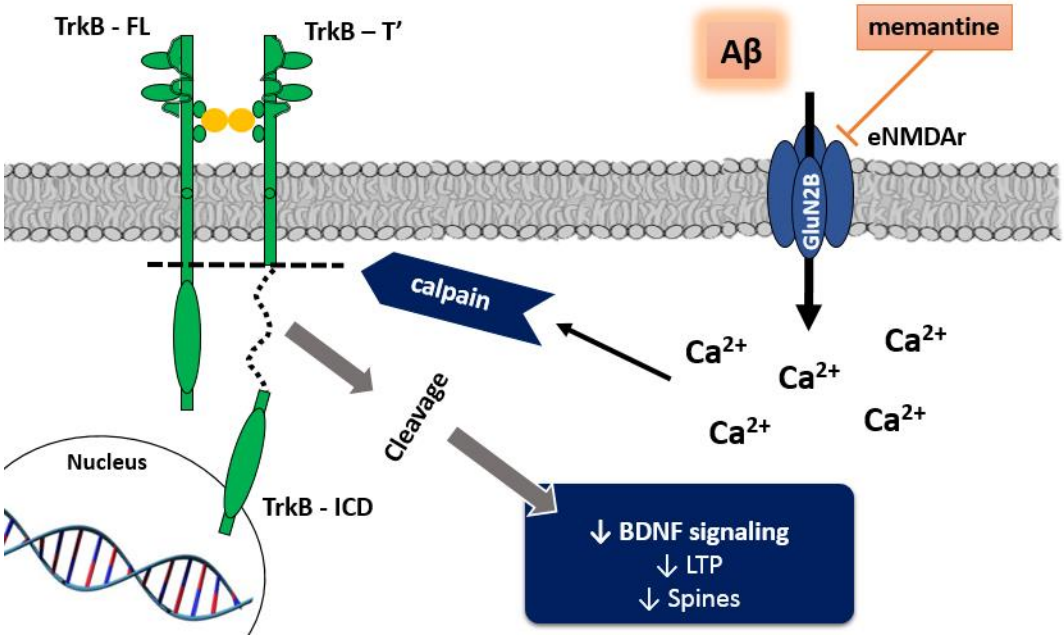


Figure 24 – **Schematic representation of our hypothesis.** Aβ severely impairs BDNF/TrkB-FL signaling affecting the synaptic actions of BDNF by a mechanism involving intracellular Ca^{2+} raise through eNMDARs activation.

Therefore, since calpains have several physiological roles and there is a need to develop selective inhibitors of calpains to be used in humans (Saez et al., 2006), the modulation of eNMDAR, which are receptors known to be exclusively detrimental in pathological conditions, could have a promising role as a therapeutic strategy. In fact, preclinical research in a variety of neurodegenerative disease models, AD included, raise hopes for neuroprotection with GluN2B antagonists (Beinat et al., 2010, Gogas, 2006, Mony et al., 2009), the subunit by which eNMDAR are mostly composed.

By detailing the mechanisms involved in the dysregulation of TrkB receptors induced by Aβ, as well as the early functional consequences of this dysregulation, this

work reinforces the idea that a better understanding of the mechanisms underlying synapse loss may enable us to find an appropriate therapy to halt or even reverse the progress of AD. In fact, although memantine, which has already been administrated to AD patients, helps controlling the symptoms of this pathology for a limited period of time, probably because it modulates NMDAR activation, it does not treat the underlying disease or delay its progression. When patients start to develop the symptoms, such as memory loss, the pathophysiological mechanisms that caused the disease have been triggered for several years. For example, synaptic failure, and probably the TrkB truncation, precedes AD's symptoms. Thus, when patients start their medication, it is probably too late to prevent TrkB-FL cleavage and BDNF loss of function upon synapses, since TrkB receptors have possibly already been truncated and the downstream effects of BDNF signaling impairment have become permanent. Given that synapse deterioration begins early in AD, it is needed to develop better diagnostics and investigate the neurological changes that take place during early phases of the disease, because this stage is likely the most opportune time for intervention.

Thus, and given that synaptic density and synaptic plasticity are highly affected in AD pathology, the modulation of eNMDAR to prevent the disruption of Ca^{2+} homeostasis and, consequently, the loss of physiological mechanisms that depend on BDNF would be of total interest to maintain the neuroprotection in early phases of AD.

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