Universidade de Lisboa

Faculdade de Medicina de Lisboa

Brain-derived neurotrophic factor and adenosine signalling on amyloid-β peptide induced toxicity: impact on hippocampal function

André Jerónimo Santos

Doutoramento em Ciências Biomédicas

Especialidade em Neurociências

2014

Universidade de Lisboa

Faculdade de Medicina de Lisboa

Brain-derived neurotrophic factor and adenosine signalling on amyloid-β peptide induced toxicity: impact on hippocampal function

André Jerónimo Santos

Tese orientada por Professora Maria José Diógenes

Doutoramento em Ciências Biomédicas

Especialidade em Neurociências

A impressão desta dissertação foi aprovada pelo Conselho Cientifico da Faculdade de Medicina de Lisboa em reunião de 24 de Junho de 2014.

Publications

The scientific content of this thesis was included in the publications of the following original articles:

- Kemppainen S, Rantamaki T, **Jerónimo-Santos A**, Lavasseur G, Autio H, Karpova N, Karkkainen E, Staven S, Miranda HV, Outeiro TF, Diogenes MJ, Laroche S, Davis S, Sebastiao AM, Castren E, Tanila H (2012). Impaired TrkB receptor signaling contributes to memory impairment in APP/PS1 mice. Neurobiol Aging 33:1122 e1123-1139.

- **Jerónimo-Santos A**, Vaz SH, Parreira S, Rapaz-Lérias S, Caetano AP, Buée-Scherrer V, Castrén E, Valente CA, Blum D, Sebastião AM, Diógenes MJ (2014). Dysregulation of TrkB receptors and BDNF function by amyloid-β peptide is mediated by calpain. Cereb Cortex. pii: bhu105. (Epub ahead of print).

- **Jerónimo-Santos A**, Batalha VL, Muller C, Hockemeyer J, Sebastião AM, Lopes LV and Diógenes MJ (2014) Chronic blockade of adenosine A2A receptors prevents BDNF effect on hippocampal LTP. Neuropharmacology. 83: p. 99-106.

Other publications closely related to the content of this thesis:

- Diogenes MJ, Costenla AR, Lopes LV, **Jerónimo-Santos A**, Sousa VC, Fontinha BM, Ribeiro JA, Sebastiao AM (2011). Enhancement of LTP in aged rats is dependent on endogenous BDNF. Neuropsychopharmacology 36:1823-1836.

- Rodrigues TM* and **Jerónimo-Santos A*.,** Outeiro TF, Sebastião AM, Diógenes MJ (2014). Challenges and promises in the development of neurotrophic factor-based therapies for Parkinson´s disease. Drugs Aging. 31: 239-261. *co-first authors

- Rodrigues TM, **Jerónimo-Santos A,** Sebastião AM, Diógenes MJ (2014). Adenosine A_{2A} Receptors as novel upstream regulators of BDNF-mediated attenuation of hippocampal Long-Term Depression (LTD). Neuropharmacology 79:389-98.

Index

Figure index

Acknowledgments

Em primeiro lugar gostaria de agradecer à minha orientadora, Maria José Diógenes, por todos os ensinamentos e sugestões que me deu, pela sua enorme simpatia e boa disposição constante e pela amizade e motivação que me transmitiu.

Gostaria também de mostrar o meu agradecimento à Professora Ana Sebastião e ao Professor Joaquim Alexandre Ribeiro pela receptividade e partilha de conhecimentos e pelas sugestões e discussão científica que me foram bastante úteis.

A todos os colegas do laboratório pelas ajudas em experiências, pela discussão científica e por proporcionarem um bom ambiente de trabalho com boa disposição.

Uma palavra especial de agradecimento à Rita por toda a sua paciência, pelos inúmeros conselhos, ajuda, carinho e apoio.

Um agradecimento ao Instituto de Medicina Molecular pelo acolhimento e bom ambiente, e não menos importante, gostaria de agradecer também à Fundação para a Ciência e Tecnologia por me ter financiado a bolsa de doutoramento (SFRH / BD / 62828 / 2009).

E finalmente, à minha família, em especial aos meus pais, pelo apoio incondicional e incentivo dado, nos bons e maus momentos.

Abbreviations list

5-FU	5-Fluoracil
Aβ	Amyloid-beta
A_1R	Adenosine A1 Receptor
$A_{2A}R$	Adenosine 2A Rreceptor
$A_{2B}R$	Adenosine 2B Receptor
A_3R	Adenosine 3 Receptor
aCSF	Artificial Cerebrospinal Fluid
AD	Alzheimer's Disease
ADA	Adenosine Deaminase
ADDLs	Aβ-derived Diffusible Ligands
ADK	Adenosine Kinase
AFM	Atomic Force Microscopy
AICD	APP Intracellular Domain
AIF	Apoptosis-Inducing Factor
ALLN	N-Acetyl-Leu-Leu-Nle-CHO
AMP	Adenosine 5'-Monophosphate
AMPA	Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
AMPAR	AMPA Receptor
ANOVA	Analysis Of Variation
AOAA	Aminooxyacetic Acid
Apaf-1	Apoptotic protease activating factor 1
APH1	Anterior Pharynx-defective 1
APOE	Apolipoprotein E
APP	Amyloid Precursor Protein
ATP	Adenosine Triphosphate
BACE1	Beta-site APP Cleaving Enzyme 1
BAD	Bcl-2-Associated Death promoter
BAK	Bcl-2 homologous Antagonist/Killer
BBB	Blood-Brain Barrier
Bcl-XL	B-cell lymphoma-extra large
BDNF	Brain-Derived Neurotrophic Factor
CA1	Cornu Ammonis area 1
CaMK	Ca^{2+}-Calmodulin-regulated Kinase
CaMKK	Calcium/calmodulin-dependent protein kinase kinase
cAMP	Cyclic Adenosine Monophosphate
CaMPDB	Calpain for Modulatory Proteolysis Database
CANP	Calcium-activated Neutral Proteinase
CAPN1	Calpain-1 catalytic subunit
CAPN2	Calpain-2 catalytic subunit
CAPNS1	Calpain small subunit 1
CAST	Calpastatin
CDK5	Cyclin-dependent kinase 5
cDNA	Complementary DNA
CNS	Central Nervous System

CRE	cAmp-Responsive Element
CREB	CRE binding protein
CRF	Corticotropin-Releasing Factor
CSF	Cerebrospinal Fluid
CTR	Control
CypD	Cyclophilin D
DAG	Diacylglycerol
DEVD-pNA	Ac-Asp-Glu-Val-Asp-pNA
DIV	Days *In-Vitro*
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DTT	1,4-dithiothreitol
EC	Enzime Classification
EDTA	Ethylenediaminetetraacetic Acid
EF-hand	Helix-loop-helix structural domain
EGF	Epidermal Growth Factor
eIF4E	Initiation Factor 4E
E-LTP	Early LTP
EphB2	Ephrin type B receptor 2
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
FDG	Fluorodeoxyglucose
fEPSP	Field Excitatory Postsynaptic Potential
FL	Full-length
G0/Gi	Gi alpha subunit G protein
Gab1/2	Grb-associated binding protein
GABA	Gamma-Aminobutyric Acid
GAP-43	Growth Associated Protein 43
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GDP	Guanosine 5'-Diphosphate
GFAP	Glial Fibrillary Acidic Protein
GluR	Glutamate receptor
GPCR	G-Protein-Coupled Receptor
Grb2	Growth factor receptor-bound protein
GSK3	Glycogen Synthase Kinase 3
GTPase	Guanosine-5'-triphosphate hydrolase
HBSS	Hanks' Balanced Salt Solution
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HFS	High Frequency Stimulation
HRP	Horseradish Peroxidase
IAPs	Inhibitors of Apoptosis
ICD	Intracellular Domain
IgG	Immunoglobulin G
IP_3	Inositol 1,4,5-triphosphate
IP3R	IP_3 Receptor
ISF	Interstitial Fluid
IκB	Inhibitor of kappa B
JNK	C-Jun N-Terminal Kinase

KHR	Krebs-Henseleit Rinsing
KW6002	Istradefylline
LDH	Lactate Dehydrogenase
LilrB2	Leukocyte immunoglobulin-like receptor B2
LL-37	Human Cathelicidin
L-LTP	Late phase of LTP
LTD	Long Term Depression
LTP	Long-Term Potentiation
MAP	Microtubule-Associated Protein
MAPK	Mitogen-Activated Protein Kinase
MCI	Mild Cognitive Impairment
MDL28170	N-[N-[(Phenylmethoxy)carbonyl]-L-valyl]-phenylalaninal
MEK	Mitogen-activated protein kinase kinase
MG132	Z-Leu-Leu-Leu-CHO
mGluR	Metabotropic Glutamate Receptor
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mPTP	Mitochondrial Permeability Transition Pore
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
NAIP-1	Neuronal Inhibitors of Apoptosis Protein 1
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NGF	Nerve Growth Factor
NMDAR	N-Methyl-D-Aspartate Receptor
NT	Neurotrophin
NTRK2	Neurotrophin Tyrosine Kinase 2
p75NTR	P75 Neurotrophin Receptor
PBS	Phosphate Buffered Saline
PC12	Pheochromocytoma cell line
PCR	Polymerase Chain Reaction
PEN2	Presenilin enhancer 2
PET	Positron Emission Tomography
PI3K	Phosphatidylinositol 3-Kinase
PiB	Pittsburgh compound B
PIP_2	Phosphatidylinositol-4,5-bifosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PKMζ	Atypical Protein Kinase C Isoform
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl Fluoride
pNA	p-nitroaniline
PPF	Pair-pulse facilitation
ProBDNF	Precursor of BDNF
PrPc	Cellular Prion Protein
PS1	Presenilin-1
PSD	Postsynaptic Density
PSEN	Presenilin gene
p-Tau	Phosphorylated tau protein

PTB	Phosphotyrosine Binding
PVDF	Polyvinylidene Difluoride
qPCR	quantitative Polymerase Chain Reaction
RAGE	Receptor For Advanced Glycation End products
RhoGDI	Rho GDP-dissociation inhibitor
RIPA	Radioimmunoprecipitation Assay Buffer
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RSK	Ribosomal S6 Kinase
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SBDP	Spectrin Breakdown Product
SCH-58261	$A_{2A}R$ antagonist
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis
SEM	Standard Error Of The Mean
SH2	Src-Homology 2
siRNA	small interfering RNA
SOS	Son of Sevenless
TBS	Theta-burst Stimulation
TBS-T	Tris-buffered Saline-Tween 20
ThT	Thioflavin T
TLR2	Toll-like Receptor 2
TM	Transmembrane
TNF	Tumor Necrosis Factor
tPA	Tissue Plasminogen Activator
Trk	Tropomyosin-related Kinase
TrkB-FL	TrkB full-length
TrkB-T'	TrkB truncated (calpain-generated)
TrkB-T1	TrkB truncated isoform 1
TrkB-Tc	TrkB truncated (total pool)
VGCC	Voltage-gated Calcium Channels
zVAD-FMK	Z-Val-Ala-Asp(OMe)-FMK

Resumo

O factor neurotrófico derivado do cérebro (*Brain-derived neurotrophic factor*- BDNF) e o seu receptor de alta afinidade, TrkB-FL, desempenham um papel central no sistema nervoso, dado que promovem suporte trófico aos neurónios e que regulam a transmissão e plasticidade sinápticas.

A sinalização mediada pelo BDNF encontra-se diminuída na doença de Alzheimer (*Alzheimer's disease -AD*), uma doença neurodegenerativa na qual ocorre acumulação do péptido beta amilóide (*amyloid-beta* -Aβ). Apesar dos mecanismos envolvidos na redução da sinalização mediada pelo BDNF na AD não serem totalmente conhecidos, o restabelecimento das acções do BDNF tem sido considerado como uma estratégia promissora para a terapêutica desta doença.

Na última década tornou-se claro que a maioria das acções sinápticas do BDNF, incluindo as acções na transmissão e plasticidade sinápticas e também na libertação de neurotransmissores, é dependente da activação dos receptores A_{2A} da adenosina ($A_{2A}R$). Contudo, o uso de antagonistas dos $A_{2A}R$ tem sido apontado como uma possível estratégia terapêutica para o tratamento da AD.

Dada a falta de evidências que clarifiquem os mecanismos envolvidos nas alterações da sinalização mediada pelo BDNF e o conhecimento de que a activação dos $A_{2A}R$ facilita a maioria das acções sinápticas do BDNF, o objectivo principal desta tese foi estudar o impacto dos péptidos Aβ e dos $A_{2A}R$ na sinalização mediada pelo BDNF.

Este trabalho revelou que, em culturas primárias de neurónios corticais, o Aβ aumenta os níveis de mRNA dos receptores TrkB truncados, TrkB-T1 e TrkB-T2, sem afectar os níveis de mRNA dos receptores TrkB completos, TrkB-FL. Por outro lado, verificou-se que o Aβ aumenta os níveis proteicos do conjunto de receptores TrkB truncados e que diminui os níveis proteicos dos receptores TrkB-FL, por um mecanismo independente da proliferação glial e da activação de caspases. Foi ainda possível concluir que o Aβ induz a clivagem, mediada por calpaínas, dos receptores TrkB-FL, esta clivagem dá-se após o local de ligação da Shc e antes do início do domínio de cinase de tirosina, pelo que origina um novo receptor TrkB truncado (TrkB-T'), contendo o local de ligação à Shc, e um novo fragmento intracelular (TrkB-*intracellular domain*- ICD), contendo a totalidade do domínio da cinase. No entanto, a presença destes fragmentos, não mostrou afectar a fosforilação do receptor TrkB-FL induzida pela exposição ao BDNF. Interessantemente, foi possível detectar o fragmento TrkB-ICD em uma amostra, *post-mortem*, de cérebro humano. Mostrou-se também que a inibição das calpaínas previne as alterações dos níveis proteicos das isoformas do TrkB, induzidas pelo Aβ, sem afectar as alterações ao nível do mRNA do TrkB. Por outro lado, este trabalho revelou que o

BDNF exógeno reduz a activação da caspase-3 e das calpaínas induzida pelo Aβ, de uma forma independentemente dos $A_{2A}R$.

Em fatias de hipocampo de ratos adultos, este trabalho mostrou que o Aβ diminui as acções do BDNF na plasticidade sináptica, nomeadamente na potenciação de longa duração (*Long-term potentiation*, LTP) na área CA1 do hipocampo, bem como no seu efeito sobre libertação de neurotransmissores (GABA e glutamato) de sinaptosomas. Notavelmente, o inibidor das calpaínas, MDL28170, mostrou restabelecer os efeitos do BDNF, na presença do péptido Aβ, tanto na plasticidade sináptica como na libertação de neurotransmissores.

Este trabalho permitiu ainda concluir que o bloqueio crónico dos $A_{2A}R$, *in-vivo*, através da administração de um antagonista selectivo (KW-6002), previne o efeito potenciador do BDNF na LTP, registada *ex-vivo* na área CA1 do hipocampo, e que diminui os níveis de mRNA e de proteína do receptor TrkB-FL, no hipocampo de rato.

Em suma, o presente trabalho revelou que o péptido Aβ induz a clivagem dos receptores TrkB-FL, mediada pelas calpaínas, e que bloqueia as acções mediadas pelo BDNF na plasticidade sináptica e na libertação de GABA e glutamato por um mecanismo dependente da actividade das calpaínas. Se por um lado, o efeito do BDNF na plasticidade sináptica é perdido aquando da inibição crónica dos $A_{2A}R$, o efeito protector desta neurotrofina contra a toxicidade induzida pelo Aβ mostrou-se independente da activação dos $A_{2A}R$.

Palavras-Chave:

Doença de Alzheimer; neurodegeneração, neurotrofinas, potenciação de longa duração, libertação de neurotransmissores, TrkB, KW-6002, istradefylline, neuroprotecção

Abstract

Brain-derived neurotrophic factor (BDNF) and its high-affinity full-length receptor, TrkB-FL, play a central role in the nervous system by providing trophic support to neurons and by regulating synaptic transmission and plasticity.

BDNF signalling is impaired in Alzheimer's disease (AD), a neurodegenerative disorder characterized, among other features, by the accumulation of the amyloid-β (Aβ) peptide. Although the mechanisms implicated in the reduction of BDNF signalling in AD were not clarified, the reestablishment of BDNF actions is considered as a promising strategy for AD treatment.

In last decade it became clear that most of synaptic actions of BDNF, including the ones upon synaptic transmission, plasticity or upon neurotransmitter release, are fully dependent on adenosine A_{2A} receptors ($A_{2A}R$) activation. However, evidences indicate that $A_{2A}R$ antagonists can prevent the deficits in AD animal models.

Given the lack of data clarifying the mechanisms behind the changes on BDNF signalling, namely changes on TrkB receptors, and the knowledge that $A_{2A}R$ activation facilitates most of BDNF synaptic actions, the main goal of this project was to study the impact of Aβ peptides and $A_{2A}R$ on BDNF signalling.

This work revealed that in rat primary neuronal cultures Aβ selectively increases mRNA levels for the truncated TrkB-T1 and TrkB-T2 isoforms without affecting TrkB full-length (TrkB-FL) mRNA levels. Moreover, Aβ increases protein levels of total pool of truncated TrkB receptors (TrkB-Tc) and decreases TrkB-FL protein levels. This effect is explained by the Aβ-induced calpain-mediated cleavage on TrkB-FL receptors, downstream of Shc binding site, which results in the formation of a new truncated TrkB receptor (TrkB-T') and a new intracellular fragment (TrkB-ICD), which is also detected in *post-mortem* human brain samples. In hippocampal slices it was observed that Aβ impairs BDNF function in a calpain-dependent way, upon modulation of GABA and glutamate release from hippocampal nerve terminals, and upon modulation of long-term potentiation (LTP). Finally, the exogenous BDNF strongly reduces the Aβ-induced activation of caspase-3 and calpain in neuronal cultures, an effect not affected by $A_{2A}R$ agonist or antagonist.

Moreover, for the first time it was shown that chronic *in vivo* blockade of $A_{2A}R$ by a selective antagonist, prevents the facilitatory action of BDNF upon *ex-vivo* CA1 hippocampal LTP and decreases both mRNA and protein levels of the TrkB-FL receptor in rat hippocampus.

In conclusion, the present work shows that Aβ induces a TrkB-FL cleavage mediated by calpain and impairs BDNF-mediated effects in synaptic plasticity and neurotransmitter release in a calpain-dependent way. While the BDNF action upon synaptic plasticity is abolished under

chronic *in vivo* $A_{2A}R$ blocking conditions, the protective actions of this neurotrophin against Aβ toxicity were found to be dependent on $A_{2A}R$ activation.

Keywords:
Alzheimer´s disease, neurodegeneration, neurotrophins, long-term potentiation, neurotransmitter release, TrkB, KW-6002, istradefylline, neuroprotection

1. Introduction

1.1. Neurotrophins

Neurotrophins (NTs) are a closely related group of secreted proteins that promote growth, survival and differentiation of developing neurons and provide trophic support and regulate synaptic plasticity in mature neurons [1]. The first neurotrophin was discovered in 1949, by Rita Levi-Montalcini. After a transplantation of a rat sarcoma tumour into chicken embryos she observed an increased growth and a hypertrophy of sensory and sympathetic neurons [2]. This observation led to the postulation that the tumour was able to release a soluble factor which induced the abnormal neuronal growth and differentiation. Later, with the collaboration of Stanley Cohen, the soluble factor was isolated and named as nerve growth factor (NGF). These findings were rewarded with Nobel Prize in physiology and medicine in 1986. After the discovery of NGF, more neurotrophins were identified in vertebrates, namely the Brain-derived neurotrophic factor (BDNF), Neurotrophin-3 (NT3) and NT-4 [3-5].

1.1.1. Neurotrophin release

Neurotrophins are initially synthesised as a precursor form (pro-neurotrophin) and secreted as homodimeric proteins [6, 7]. Pro-neurotrophins can be subsequently cleaved intracellularly by furin, or extracellularly by plasmin to produce the mature form (neurotrophins). Intracellular pro-neurotrophins can be released after the cleavage of the pro-domain (released as a mature neurotrophin), or can be released as an unprocessed pro-neurotrophin [6]. The pro-neurotrophins and mature neurotrophins preferentially activate different type of receptors, p75NTR and Trk, respectively, which triggers different signalling pathways producing opposite cellular responses. Neurotrophins can be constitutively released, due to the spontaneous fusing of vesicles with plasma membrane, or can be released in a regulated-way dependent on neuronal activity. In particular, high frequency synaptic activity, such as theta-burst stimulation (TBS), increases the synaptic levels of mature BDNF by either increasing its release and the extracellular plasmin-dependent cleavage of pro-BDNF into mature BDNF. In opposition, low frequency stimulation, which induces synaptic depression, increases the release of pro-BDNF which remains uncleaved at the synapse (see Figure 1.1) [6, 8].

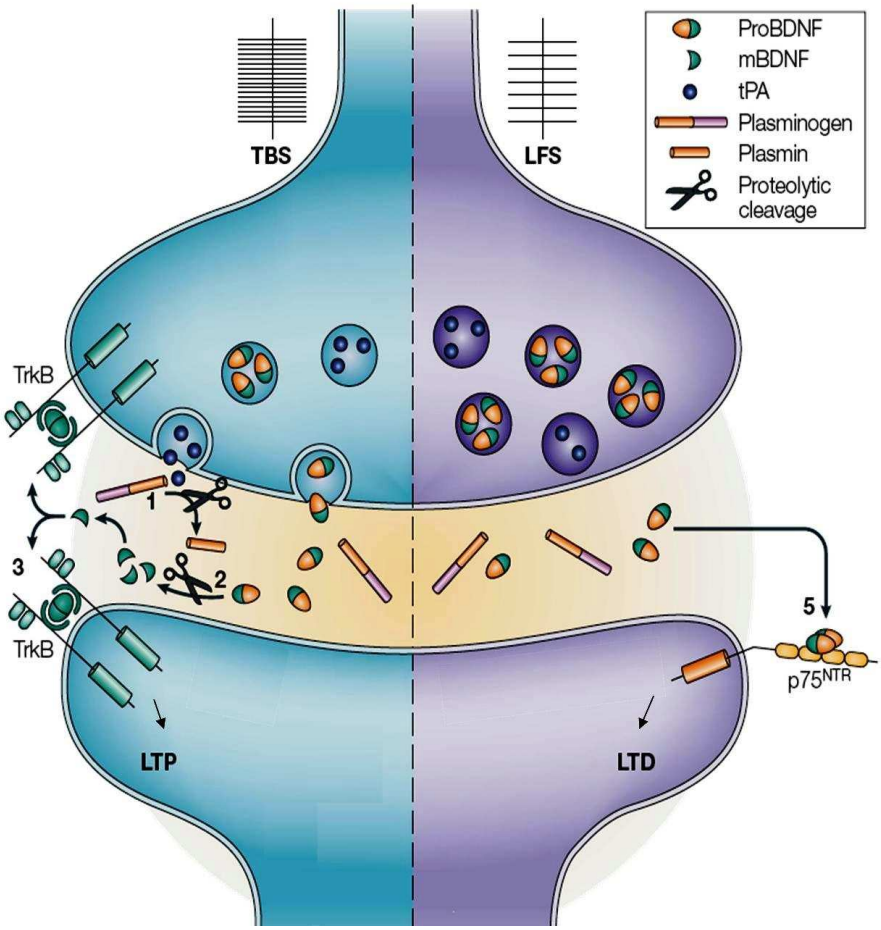


Figure 1.1 – Regulation of BDNF release upon synaptic activity.

Left: In response to theta-burst stimulation (TBS), tissue plasminogen activator (tPA) and proBDNF are released into synaptic cleft. 1) Then, tPA cleaves plasminogen producing the active protease plasmin. 2) Plasmin cleaves proBDNF producing the mature BDNF. 3) BDNF then binds to its high-affinity receptor (TrkB-FL) triggering multiple intracellular signalling pathways which in turn contribute to long-term potentiation (LTP). Right: During low-frequency stimulation (LFS) proBDNF is secreted into the synapse and remains uncleaved in the synapse. Uncleaved proBDNF binds to its high-affinity receptor ($p75^{NTR}$) and facilitates the induction of long-term depression (LTD). Figure adapted from [8].

1.1.2. Neurotrophin receptors

The biological actions of neurotrophins are mediated by the activation of their cognate tropomyosin-related kinase receptor (TrkA, TrkB or TrkC) and by the activation of the common p75 neurotrophin receptor ($p75^{NTR}$), which has been shown to modulate the affinity and selectivity of Trk activation [9]. Trk receptors are members of receptor tyrosine kinase (RTK) superfamily and promote neuronal survival and plasticity, while $p75^{NTR}$ is a member of tumour necrosis factor (TNF) receptor superfamily and can promote neuronal death under certain circumstances [1]. TrkA is the cognate receptor for NGF [10, 11], while TrkB was identified as the receptor for BDNF and NT-4/5 [12, 13], and TrkC as the receptor for NT-3 [14].

The Trk receptors and $p75^{NTR}$ receptor can function synergistically, antagonistically or independently of each other [8]. The mature neurotrophins bind with high affinity to Trk receptors, and $p75^{NTR}$ may act synergistically as a co-receptor [15]. In opposition, the pro-neurotrophins bind with high affinity to the $p75^{NTR}$ with Sortilin acting as a co-receptor. The effects of neurotrophins upon neuronal survival, differentiation and synaptic plasticity are mediated by the Trk receptors system [16], while the opposing effects of pro-neurotrophins, such as cell death and decreased synaptic function, are mediated by $p75^{NTR}$ and Sortilin complex [17-19].

All Trk receptors share a significant sequence homology and a conserved domain organization. The extracellular region of Trk receptors are composed by a leucine rich domain flanked by two cysteine rich regions. Under those domains, and prior to the transmembrane region, there are two immunoglobulin-like domains which define the ligand binding specificities of the receptor [20, 21]. Intracellularly, the Trk receptors are composed by a juxtamembrane sequence that includes the Shc binding site, a tyrosine kinase domain and a C-terminal tail containing the phospholipase C gamma (PLCγ) binding site [21].

Considering the focus of the present thesis, henceforth only BDNF and its receptor TrkB will be explored in more detail.

1.1.3. Truncated TrkB receptors

The TrkB gene (*NTRK2)* can originate a full-length TrkB receptor (TrkB-FL) and, by an alternative splicing mechanism, it also can originate truncated receptors (TrkB-T1, TrkB-T2 and TrkB-T-Shc) [22-24]. The TrkB-T1 and TrkB-T2 have a unique short C-terminal tail (T1 with 11 aminoacid residues and T2 with 9 aminoacid residues) [22], while the TrkB-T-Shc is a human brain-specific isoform which lacks the tyrosine kinase domain but contains the Shc

binding site. The truncated TrkB receptors cannot activate the canonical signalling pathways of full-length receptors, since they lack the intracellular kinase domain [24]. Additionally, truncated TrkB receptors can inhibit the BDNF effects by acting as dominant negative inhibitors of the TrkB-FL receptors [23, 25-27]. Indeed, multiple studies have shown that increased levels of truncated TrkB receptors have a negative impact in neuronal function and survival in both artificial and pathological conditions [28-30].

While the TrkB-FL is the most abundant isoform expressed in the early developmental period, in the post-natal period, and throughout aging, the truncated TrkB-T1 receptor became the most predominant TrkB isoform expressed in forebrain [31, 32]. TrkB-T1 can be expressed by neurons and astrocytes depending on the brain region. Accordingly, TrkB-T1 is highly expressed in astrocytes from pre-frontal cortex and subcortical white matter, but it is not present in astrocytes of the cerebellum and motor and visual cortex [33].

One possible biological role of TrkB-T1 is to regulate extracellular levels and localization of BDNF in the brain. When extracellular BDNF is abundant, TrkB-T1 binds and sequesters the available BNDF, and it is internalized along with its ligand. After BDNF and TrkB-T1 internalization, the BDNF can be degraded in lysosomes or can be sorted to another cellular location and be released by exocytosis [34, 35]. Although the *in-vivo* function of truncated receptors remains unknown, it was shown that TrkB-T1 deficient mice have increased anxiety in association with morphological abnormalities in dendrites of basolateral amygdale neurons. The same study showed that the depletion of TrkB-T1 can also partially rescue the BDNF haploinsufficiency phenotype, further suggesting that TrkB-T1 at physiological levels may regulate and attenuate TrkB-FL signalling [36]. Despite the lack of intracellular kinase domain, some studies have shown that TrkB-T1 receptor activates distinct signalling cascades in astrocytes. In fact, TrkB-T1 alone can promote Ca^{2+} release from the endoplasmic reticulum (ER) in astrocytes, through the activation of G-protein and PLCγ, with consequent inositol-1,4,5-triphosphate (IP3) formation (see Figure 1.2) [37]. Moreover, TrkB-T1 can bind to Rho GDP dissociation inhibitor I (RhoGDI1) and regulate actin cytoskeleton and glial morphology by modulating RhoGTPase activity [38].

1.1.4. TrkB-mediated signalling cascades

The binding of BDNF to TrkB receptor homodimmers, activates the intrinsic tyrosine kinase domain of the receptor promoting an auto-phosphorylation of specific tyrosine residues located in the intracellular domain of TrkB [39, 40]. In particular, the binding of BDNF to TrkB results in a fast phosphorylation of 5 tyrosine residues of the receptor, within seconds to minutes. These phosphorylated residues include 3 tyrosines in the kinase activation loop of

TrkB (Y701, Y705 and Y706) which regulate the kinase activity, and 2 tyrosines residues (Y515 and Y816) responsible for the activation of signalling cascades [41]. Phosphorylation of Y515 of TrkB (or equivalent residues in other Trks receptors) forms an adaptor binding site that couples the receptor to phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinases (MAPK) signalling pathways, while phosphorylation of Y816 recruits and initiates PLCγ signalling pathway (see Figure 1.2) [42].

PI3K / Akt signalling pathway:

When phosphorylated at Y515, TrkB receptors recruit Src homology 2 domain containing (Shc) adaptor protein through its phosphotyrosine-binding (PTB) domain [43]. In turn, Shc protein associates with Growth factor receptor-bound protein 2 (Grb2), Grb-associated binding protein (Gab1/2) and son of sevenless (SOS), culminating in the transient activation of small GTPases, such as Ras. Active Ras stimulates signalling through c-Raf/MEK/ERK (MAPK pathway) and class I PI3 kinase (PI3K) pathway (see Figure 1.2) [44]. Activated PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2), producing the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn stimulates the serine-threonine kinase Akt.

The signalling through the PI3K and Akt pathway are necessary and sufficient for the survival of certain populations of neurons [45]. The Akt kinase modulates the function of several substrates involved in the regulation of cell survival and growth. Akt phosphorylates and inactivates several pro-apoptotic proteins, such as procaspase-9 and Bcl2-associated death promoter (BAD), as well as Forkhead 1 transcription factor [46, 47]. Activated Akt can also inactivate GSK-3β, a kinase which has been implicated in neuronal apoptosis and inhibition of axon growth [48, 49]. On the other hand, Akt activates transcription factors that regulate the expression of anti-apoptotic proteins, such as cyclic AMP response element-binding protein (CREB) and nuclear factor-κB (NFκB) [50].

Ras/MAPK pathway:

As mentioned above, the binding of Shc adaptor protein to the phosphorylated Y515 of TrkB activates the Ras protein. Activated Ras stimulates the MAPK/ERK kinase (MEK) which in turn activates the extracellular signal regulated-kinases (ERK). The ERK/MAPK signalling cascade activates transcription factors such as CREB [51], which in turn control the expression of several proteins implicated in survival, growth and differentiation of neurons. ERK activates, by phosphorylation, the ribosomal s6 kinase (RSK), which in turn further activates transcription factors, such as CREB, c-Fos and NF-κB [52-54]. Additionally, BDNF enhances protein translation in neurons through the Erk/MAPK pathway, by phosphorylating eukaryote initiation factor 4E (eIF4E) and its binding protein (eIF4E-binding protein-1) [55].

PLCy pathway:

Phosphorylated Y816 of TrkB directly recruits PLCγ1 which in turn is phosphorylated and activated by the TrkB kinase domain. Activated PLCγ1 hydrolyses PIP_2 and generates inositol triphosphate (IP3) and diacylglycerol (DAG) [56]. While DAG activates DAG-regulated protein kinase C (PKC) isoforms, the IP3 promotes the release of Ca^{2+} from intracellular stores, such as ER, through activation of IP3 receptor (IP3R). The increase in cytosolic Ca^{2+} activates diverse enzymes, including Ca^{2+}-regulated PKC isoforms and Ca^{2+}-calmodulin-dependent kinases (CaMKII, CaMKK and CaMKIV). PLCγ pathway is crucial for synaptic plasticity, since mice with point mutations on Y816 of TrkB, but not on Y515, have impaired long-term potentiation. The PLCγ also promotes the activation of CREB transcription factor through CaMKIV, and point mutation on Y816 strongly impairs CREB activation [57].

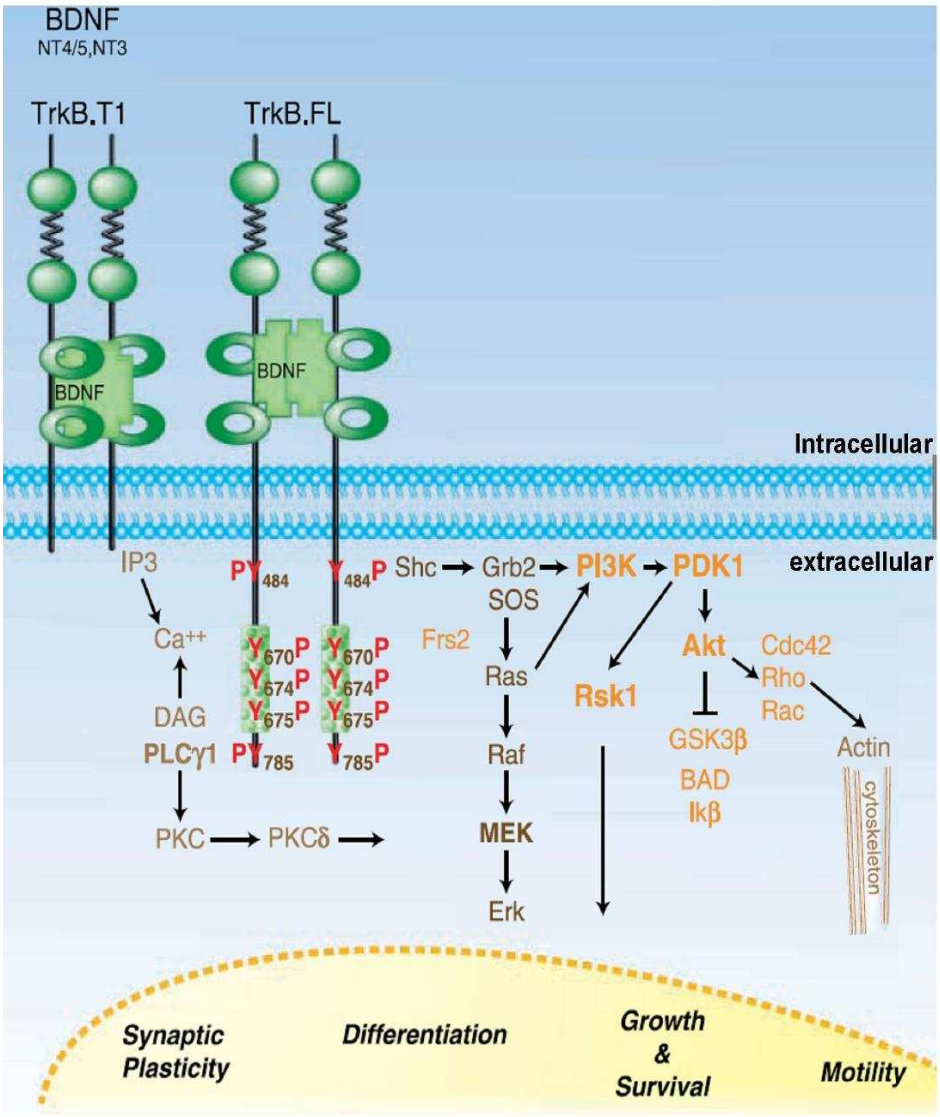


Figure 1.2 – Schematic representation of TrkB receptors signalling pathways.

BDNF binds to the extracellular region of TrkB receptor, which is equal in both TrkB truncated T1 and full-length (FL) receptor. After the binding of homodimeric BDNF to homodimeric TrkB-FL, the kinase domain of the receptor became active and trans-phosphorylates the receptor in the tyrosine 515 and 816 (also named as Y484 and Y785, respectively, when the TrkB signal peptide is ignored for the aminoacid counting). Phosphorylated tyrosine 515 recruits the binding of Shc adaptor protein, which in turn lead to the activation of Ras/MEK/ERK (MAPK pathway) and PI3K/Akt signalling pathway. PLCγ signalling pathway is triggered after the binding of PLCγ to phosphorylated tyrosine 816 of TrkB. The signalling mediated by TrkB-FL promotes transcriptional programs that regulate synaptic plasticity, neuronal differentiation, growth and survival and motility. TrkB-T1 lacks the tyrosine kinase domain, however, evidences show that its activation can trigger Ca^{2+} waves in astrocytes. Figure adapted from [58]

1.1.5. BDNF and long-term potentiation

Long-term potentiation (LTP) is a long-lasting increase in synaptic strength induced by some patterns of synaptic stimulation, such as high-frequency stimulation (HFS) or TBS, and it is commonly accepted as the neurophysiological basis for learning and memory [59, 60]. In opposition, long-term depression (LTD) is a long-lasting depression on synaptic transmission after a period of low-frequency stimulation of the synapse. Thus, synaptic plasticity is the ability of synapses to change their efficacy depending on their activity.

The LTP on Cornu Ammonis 1 (CA1) area in the hippocampus is dependent on NMDAR, a glutamate receptor permeable to Ca^{2+} ions, in which the pore is usually occluded by Mg^{2+} at a resting membrane potential. During high-frequency stimulation the synaptic release of glutamate depolarizes the post-synaptic terminal, through an AMPAR-dependent Na^{+} influx, and allows the removal of Mg^{2+} and the consequent activation of the NMDAR by the glutamate in the synapse. The NMDAR-mediated influx of Ca^{2+} at the post-synaptic terminal activates Ca^{2+}-dependent proteins, such as CaMKII, which trigger intracellular cascades necessary for LTP induction. The increased cytosolic Ca^{2+} on post-synaptic terminal also triggers the AMPARs trafficking to post-synaptic density (PSD), resulting in a greater post-synaptic response to glutamate [61]. LTP is classically 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 *de-novo* proteins synthesis and structural modifications on synapses [62]. Despite years of intensive investigation on hippocampal LTP induced by HFS, only recently it was proven that learning actually induces LTP in the hippocampus of behaving animals [63, 64].

The first evidence that neurotrophins are important for synaptic function arise in early 1990s, when it was discovered that exogenous BDNF or NT-3, but not NGF, enhances synaptic activity on neuromuscular synapses [65]. Later, it was found that BDNF or NT-3, but not NGF, increases the basal synaptic transmission in hippocampal CA1 area [66]. The finding that BDNF might also have a role in hippocampal LTP came from experiments preformed in a BDNF-deficient mice. In this mice model, a significant impairment on hippocampal LTP magnitude was detected [67]. Interestingly, LTP was restored after reintroduction of BDNF gene in CA1 area by a virus-mediated gene transfer [68], or by exogenous addition of BDNF [69]. Several other works have been demonstrating the central role of BDNF in synaptic plasticity. Accordingly, intrahippocampal infusion of BDNF in living rats was shown to elicit long-term synaptic potentiation [70]; the application of exogenous BDNF to hippocampal slices from young mice enhanced the L-LTP induced by tetanic stimulation, which in the absence of BDNF only elicit a short-term potentiation (E-LTP) [71]; the scavenger of endogenous BDNF by

soluble TrkB-IgG fusion protein or by specific antibodies resulted in a reduced hippocampal LTP magnitude [71, 72] not seen for NT-3 or NT-4 [72]. The TrkB or BDNF null mice have severe phenotype and die between birth and weaning age, hampering the study of these proteins in LTP in an adult stage. However, a conditional TrkB knockout mouse, where the gene deletion was restricted to the forebrain neurons in the post-natal period, showed impaired LTP on CA1 hippocampal synapses, and impaired learning behaviour in the adult stage, without gross phenotypical aberrations [73]. Together, these evidences clearly showed that endogenous BDNF is required for normal LTP and learning, and that exogenous BDNF can induce or facilitate the LTP expression.

Other studies have provided mechanistic clues through which BDNF and TrkB activation facilitates LTP at glutamatergic hippocampal synapses (see Figure 1.3). Endogenous BDNF is released from glutamatergic synapses, in a Ca^{2+}-dependent way, in response to stimulus used to induce LTP, such as TBS (see Figure 1.1) [74, 75]. Released BDNF can facilitate LTP at excitatory CA1 synapses by increasing presynaptic release of glutamate, and by amplifying the postsynaptic response to this neurotransmitter [76]. In particular, BDNF increases the Ca^{2+}-dependent release of glutamate in cortical and hippocampal nerve terminals (synaptosomes) [77, 78] and in cultured hippocampal neurons [79]. The presynaptic stimulation of glutamate release by BDNF is mediated by a MAPK-dependent phosphorylation of the synaptic vesicle protein synapsin-I. In mice lacking the synapsin the effect of BDNF upon glutamate release is strongly attenuated [80]. On the other hand, BDNF, through its post-synaptic TrkB receptor, stimulates tyrosine kinase Fyn, which in turn phosphorylates the NMDAR and increases its activity [81, 82]. In cultured neurons, BDNF further modulates glutamatergic synapse at postsynaptic level, by increasing the levels and the trafficking of AMPAR to membrane [83, 84]. Moreover, exogenous BDNF, at nanomolar concentration, depolarizes and excites hippocampal and cortical neurons just as quickly and effective as glutamate at a micromolar concentration [85]. In fact, BDNF induces a fast neuronal depolarization, in a TrkB dependent-way, by activating Na^+ channel $Na_V1.9$ allowing the influx of sodium ions [85, 86]. Consequently, the BDNF-induced depolarization activates voltage-gated Ca^{2+} channels (VGCC) evoking Ca^{2+} transients which are detectable in the dendrites and spines of hippocampal neurons, but not at presynaptic sites [87]. In this way, BDNF cooperates with NMDAR during LTP induction by promoting an additional influx of Ca^{2+} in the post-synaptic terminal. Thus, pairing a brief application of BDNF in dendrites and a weak burst of synaptic stimulation, elicit a fast and robust induction of LTP [87].

Interestingly, a recent study showed that acute or gradual increases in BDNF elicit distinct signalling and neuronal effects. While a gradual increase in BDNF concentration (slow perfusion rate) selectively facilitates LTP in hippocampal slices, a rapid increase in BDNF concentration (fast perfusion rate) increases the synaptic basal transmission instead [88]. This

study highlighted the importance of the kinetics of TrkB activation, and explained, in part, some conflicting results in the literature, regarding the pre- and post-synaptic effects of BDNF.

On top of BDNF-mediated fast changes in synaptic efficacy, BDNF has also a crucial role in maintenance of late-phase LTP (L-LTP), synaptic consolidation and long-term memory storage [89, 90]. BDNF synthesis is found to be increased in hippocampal neurons 2-4 hours after L-LTP-inducing stimuli, such as strong TBS [91, 92]. Unlike E-LTP, the L-LTP expression is dependent on protein synthesis. Surprisingly, application of exogenous BDNF is able to rescue L-LTP in the presence of protein synthesis inhibitors [93]. This perplexing result was recently demonstrated to be dependent on PKMζ, an atypical PKC isoform present in brain [94]. Weak TBS normally induce an E-LTP, which last less than 2 hours, and fail to elicit an L-LTP. However, when pairing BDNF perfusion and weak TBS, it produces a reliable L-LTP in CA1 area of hippocampus [93]. Moreover, mice lacking tissue plasminogen activator (tPA), a protease involved in the conversion of pro-BDNF into BDNF, have a selective deficit in L-LTP expression without affecting E-LTP in hippocampus [95]. Moreover, perfusion of BDNF in tPA null mice prevented the L-LTP impairment [93]. Indeed, evidences suggest that BDNF may trigger L-LTP by regulating local dendritic protein translation and concomitantly increasing synthesis of LTP-associated proteins, such as Arc, GluR1, CaMKII, PSD-95 among others. BDNF can also regulate actin cytoskeletal dynamics which are required for structural changes of synapses and L-LTP formation [96].

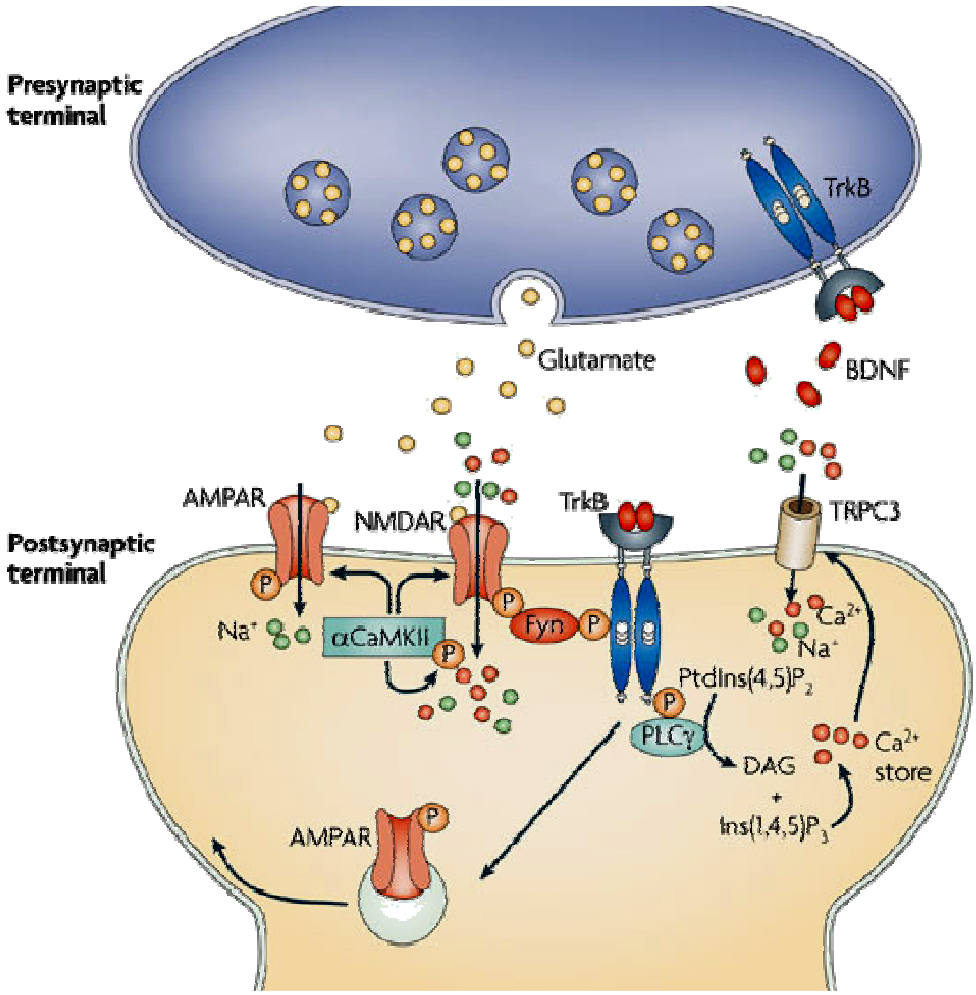


Figure 1.3 – Modulation of glutamatergic synapse by BNDF.

BDNF can enhance the transmission and plasticity of glutamatergic synapses. Presynaptic activation of TrkB-FL by BDNF increases the glutamate release. Postsynaptic activation of TrkB-FL increases the postsynaptic response to glutamate by distinct mechanisms: 1) TrkB induces the phosphorylation of NMDAR, through Fyn kinase, increasing its activity; 2) TrkB depolarizes the postsynaptic terminal by promoting the influx of cations through transient receptor potential channels (TRPC) which might facilitate the Ca2+ entry through NMDAR and voltage-gated channels and 3) TrkB modulates AMPAR expression and trafficking to the postsynaptic membrane. Figure adapted from [97].

1.1.6. Facilitation of BDNF synaptic actions by adenosine

Adenosine is a ubiquitous nucleotide that acts as an extracellular signalling molecule. Adenosine is a neuromodulator that regulates synaptic activity, by modulating the presynaptic neurotransmitter release, by depolarizing or hyperpolarizing the postsynaptic neuron or even by regulating glial cells activity. Overall, adenosine acts as a depressant of excitatory glutamatergic transmission and reduces excitability [98]. Extracellular adenosine can derive from the direct release of intracellular adenosine by equilibrative nucleoside transporters (ENT), or from catabolism of extracellular adenosine triphosphate (ATP) [99]. Adenosine can be released constitutively, or in an active-dependent manner through a calcium-dependent presynaptic release [100]. Extracellular adenosine has a short half-life time, since it is quickly reuptaked or converted to inosine or to adenosine monophosphate (AMP) by adenosine deaminase (ADA) or by adenosine kinase (ADK), respectively [99].

Extracellular adenosine exerts its actions through activation of four distinct G protein-coupled receptors (GPCR) namely, A_1, A_{2A}, A_{2B} and A_3 receptors. Adenosine A_1 receptors (A_1R) are prevalent in the brain, being highly expressed in the cortex, cerebellum, hippocampus and spinal cord. A_1R and $A_{2A}R$ are high affinity receptors, with a K_d of 70 and 150nM, respectively [101]. A_1R are coupled to inhibitory G-proteins (G_0/G_i) which inhibit synaptic transmission by inhibiting cyclic adenosine monophosphate (cAMP) production [102]. In opposition, the $A_{2A}R$ are coupled to stimulatory Gs-proteins and potentiate synaptic transmission by increasing cAMP production [101, 102]. $A_{2A}R$ are mainly expressed in olfactory bulb and striatum, being also present in hippocampus at lower levels [103, 104]. Both $A_{2A}R$ and A_1R can be present in the same synapse, and activated simultaneously by adenosine [99]. Regarding the adenosine $A_{2B}R$ and A_3R, both have low affinity for adenosine (K_d = 5 and 6μM, respectively) and are weakly expressed in CNS [98].

In addition to the modulatory actions of adenosine upon neurotransmitter release and synaptic plasticity, adenosine also modulates the actions of other modulators, such as neurotrophins [99]. The first direct evidence for the cross-talk between adenosine and neurotrophins, arose from the observation that adenosine, or a $A_{2A}R$ agonist, trans-activates TrkA or TrkB receptors in PC12 cells or hippocampal neurons, respectively, in the absence of neurotrophins [105]. Nevertheless, the $A_{2A}R$-mediated transactivation of Trk receptors has different aspects when comparing to the conventional Trk activation by the respective neurotrophins. In particular, $A_{2A}R$ activation promotes the phosphorylation of an immature, non-glycosylated, sub-population of Trk receptors associated mainly with Golgi membranes. In addition, this Trk transactivation is only detectable after 3 hours of $A_{2A}R$ activation, while the

classical phosphorylation of Trk receptors by the cognate neurotrophin, occurs in the mature, fully-glycosylated, receptors within seconds to minutes [106, 107].

In functional experiments, multiple evidences have been shown that $A_{2A}R$ activation is necessary for synaptic effects of BDNF in hippocampus. Indeed, in hippocampal slices from young rats, exogenous application of BDNF increases basal synaptic transmission only when a previous depolarization stimulus is made, an effect blocked by an $A_{2A}R$ antagonist. Moreover, pre-synaptic stimulation, or activation of $A_{2A}R$ by a selective agonist or by adenosine, triggered the excitatory action of BDNF upon synaptic transmission, in a process dependent on PKA signalling [108, 109]. Thus, it is concluded that BDNF effects upon synaptic transmission require an activity-dependent presynaptic release of adenosine and consequent $A_{2A}R$ activation. In addition, in hippocampal slices from adult rats, where both levels and actions of $A_{2A}R$ are increased, the addition of exogenous BDNF spontaneously increases basal synaptic transmission, an effect fully blocked by an $A_{2A}R$ antagonist [109]. Interestingly, in ADK-deficient mice, which have increased levels of extracellular adenosine, the spontaneous increase in hippocampal synaptic transmission induced by BDNF is observed even in young animals, an effect not present in age-matched wild-type mice. In opposition, the BDNF-induced spontaneous increase in hippocampal synaptic transmission present in adult wild-type mice, is not detected in age-matched ADK-overexpressing mice, which have lower adenosine levels [110]. Furthermore, the genetic deletion of $A_{2A}R$ abolished the excitatory effects of BDNF upon synaptic transmission in mice [111]. Similarly to hippocampus, it was also found that $A_{2A}R$ and PKA activation are required for the excitatory BDNF effects upon transmission in neuromuscular junction [112]. In addition to synaptic transmission, the effects of BDNF upon synaptic plasticity, both in LTP and LTD, are also fully dependent on $A_{2A}R$ activation, since both effects are lost when extracellular adenosine is removed or when PKA or $A_{2A}R$ are inhibited [108, 111, 113-116]. Together, these results indicate that $A_{2A}R$ activation, and subsequent PKA signalling, are essential to trigger the BDNF excitatory effects upon synaptic plasticity and transmission. Since that exogenous BDNF spontaneously facilitates LTP induction and expression in hippocampal slices from young animals, it suggests that the release of adenosine and its precursor ATP induced by the high-frequency stimulation is enough to activate $A_{2A}R$ and trigger the BDNF actions [99].

Recently, it was shown that $A_{2A}R$ activation promotes the translocation of TrkB-FL to lipid rafts domains in the membrane. Importantly, high-frequency stimulation resulted in increased levels of TrkB in lipid rafts, an effect abolished by the removal of endogenous extracellular adenosine. Thus, adenosine, through $A_{2A}R$ activation, promotes an activity-dependent insertion of TrkB in lipid rafts , facilitating the phosphorylation of TrkB-FL and the BDNF-mediated actions [117].

Furthermore, the effects of BDNF upon GABA and glutamate release in synaptosomes are also dependent on $A_{2A}R$ activation (*unpublished data from our lab*). Conversely, the effects of BDNF upon neuronal branching [118], or upon GABA uptake at nerve endings [119], do not depend on tonic activation of $A_{2A}R$ (see Figure 1.4). However, pharmacological activation of $A_{2A}R$ in synaptosomes enhances the inhibitory effect of BDNF upon GABA uptake [119].

The findings that many synaptic actions of BDNF are dependent on $A_{2A}R$ activation may open new therapeutic possibilities to boost the BDNF effects in neurodegenerative diseases where its signalling is known to be impaired. Multiple evidences showed that BDNF administration into the brain produces substantial benefits in *in-vitro* and *in-vivo* models of neurodegeneration. However, the translation of this approach to patients has been hampered by difficulty of BDNF to cross the blood-brain barrier (BBB) and by the poor bioavailability and stability of BDNF, which has a short half-life time in biological fluids [120]. Thus, small-molecules that modulate $A_{2A}R$ activity, or its signalling, might constitute a way to promote BDNF synaptic effects in situations where BDNF signalling is compromised. Moreover, in opposition to the indiscriminate administration of BDNF into the brain, the approach of $A_{2A}R$ modulation has the advantage of only stimulating BDNF effects on neuronal subpopulations which co-express $A_{2A}R$, TrkB and BDNF, such as hippocampus or cerebral cortex, both regions affected in Alzheimer's disease (AD) [105, 121].

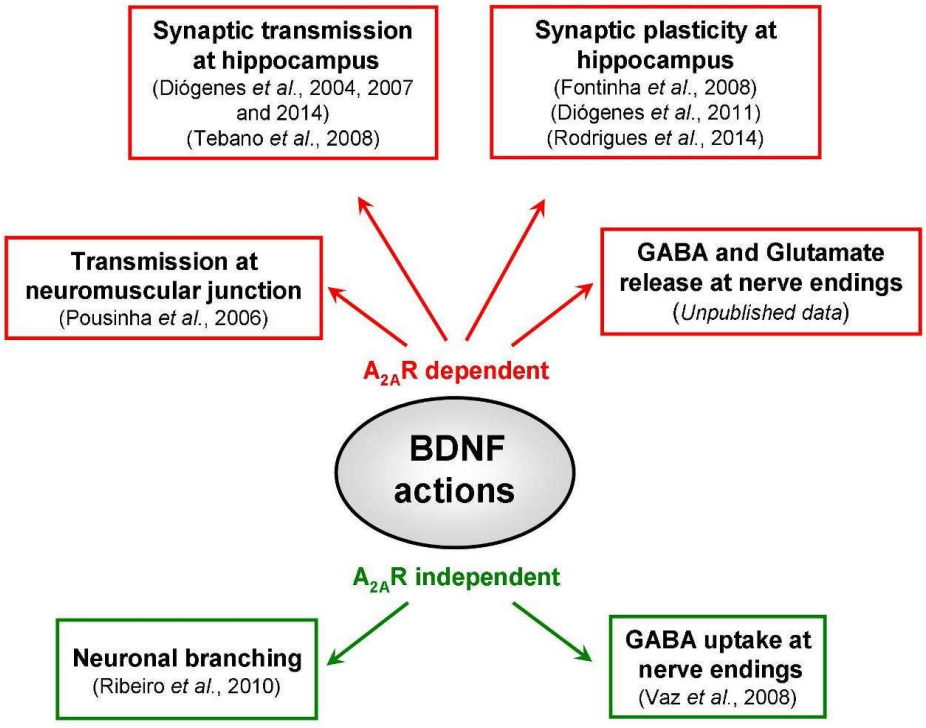


Figure 1.4 – Cross-talk between TrkB and A2A receptors.

BDNF actions upon synaptic transmission in hippocampus and neuromuscular junction, as well as, synaptic plasticity in hippocampus are fully dependent on $A_{2A}R$ activation. Figure adapted from [99]

1.2. Calpain system

Proteases are a class of enzymes that catalyse the hydrolysis of peptide bounds. Depending on mechanism of catalysis, proteases are currently classified into 6 subgroups: aspartic, glutamic, cysteine, metalloproteases, serine, and threonine proteases. Cysteine proteases, also known as thiol proteases, use a nucleophilic cysteine thiol in the active site to attack the carbonyl-carbon of the amide bond and hydrolyse the peptide bond [122]. In the protease classification system of MEROPS database [123], the pure cysteine proteases are currently divided into 10 clans accordingly to their evolutionary relationship, tertiary structure and sequence motifs around catalytic site. Each clan of proteases can be further divided into several families based on their sequence homology. Calpains, papain and cathepsins are all members of clan CA, however, papain and cathepsins belong to the C1 family and are synthetized as inactive proenzymes with N-terminal propeptides, while calpains belong to the family C2 and are not synthetized as classical proenzymes [124].

1.2.1. Calpains

Calpains (EC 3.4.22.17), previously named as Ca^{2+}-activated neutral protease, are cytosolic proteases ubiquitously expressed in mammals and have the peculiarity of being activated by a Ca^{2+}-induced conformational change [125]. Calpains were discovered 50 years ago, in 1964, when a Ca^{2+}-dependent protease activity was found in soluble fractions of rat brain at neutral pH [126]. The prototypical members of calpain family are the µ-calpain and m-calpain. These proteases differ in the Ca^{2+} concentration required for their activation *in-vitro*. The µ-calpain requires 3-50µM of Ca^{2+} for half-maximal activity, while m-calpain requires 400-800µM of Ca^{2+} [127]. Both proteases are heterodimers consisting in an 80 kDa catalytic large subunit (calpain-1 in µ-calpain and calpain-2 in m-calpain), associated with a common 30-kDa small regulatory subunit, CAPNS1 (see Figure 1.5). In 1984 the catalytic large subunit of µ-calpain (calpain-1) was cloned, and its primary sequence revealed that it contained 4 domains. The Domain II of calpain-1 is the catalytic domain and is similar to papain-like thiol proteases, while the domain IV is similar to calmodulin-like Ca^{2+}-binding proteins. These findings suggest that calpains are evolutionary derived from the fusion of a thiol protease with a Ca^{2+}-binding protein [128].

Until now, it was found 15 human isoforms of calpain large subunit based on human genome sequence, being these homologs classified as ubiquitous or tissue-specific [129]. For instance, the mRNA of calpain-3a and calpain-8a are mainly found in muscle, while calpain-6 is found in placenta and calpain-11 in testis [127]. Although the protease domain (domain II) is

conserved in calpain family, there are members that lack, or have different, domains resulting in atypical calpains that may not dependent on Ca^{2+} (eg: Calpain-3) or may not have regulatory subunits. Moreover, there are also a calpain member (Calpain-6), that lacks the essential cysteine residue of the catalytic triad and may not even have proteolytic activity [129]. Nevertheless, atypical calpains have a biological function, and recent evidences showed that the non-catalytic calpain-6 regulates microtubule dynamics in cultured cells and regulates skeletal muscle development in mice [130].

The prototypical calpain-1 and calpain-2 (the large subunits of μ- and m-calpain, respectively) are ubiquitously expressed in mammalian cells and are the most abundant and characterized isoforms in the brain [131]. The calpain small regulatory subunit (CAPNS1) are also expressed in the brain [132]. It was shown that calpain-2 mRNA levels were 15-fold higher than calpain-1 levels in whole mice brain homogenate, whereas the distribution of calpain-1 mRNA was uniform throughout the brain, calpain-2 mRNA was enriched in certain neuronal populations including hippocampal and cortical pyramidal neurons [133]. Subcellular localization of calpains is widely attributed to soluble (cytosolic) fraction; however, recent studies showed that calpains are also associated with different subcellular compartments. In fact, evidences obtained from neuroblastoma cells, neuronal cultures and rat cortex, showed that μ-calpain is present at mitochondrial intermembrane space, placing it in proximity to its mitochondrial substrates and to Ca^{2+} released from mitochondrial stores [134]. During ischemic neuronal injury, the intra-mitochondrial μ-calpain can cleave and activate mitochondrial pro-apoptotic proteins, such as apoptosis inducing factor (AIF), which in turn mediate neuronal death signalling [135]. The m-calpain was also found in the nucleus of cultured neurons, where this nuclear calpain regulates Ca^{2+}-dependent signalling by cleaving the CaMKIV [136].

The crystal structure of Ca^{2+}-free m-calpain revealed that the catalytic site located in domain II (in large subunit), is not assembled in the absence of Ca^{2+}, suggesting that Ca^{2+} may trigger conformational changes necessary to form a functional active site. Moreover, in opposition to classical proenzymes (eg: papain) whereas the N-terminal propeptide blocks the active site, the structure of calpain-2 revealed that the N-terminal anchor (Domain I) does not occupy the active site but inhibits its assembly. In addition, the structure indicated that the N-terminal anchor regulates the calpain affinity to Ca^{2+} by interacting with the small regulatory subunit (CAPNS1) [137]. The N-terminus of Domain I is autolysed during initial Ca^{2+}-induced calpain activation. Although calpain autolysis reduces the requirement for Ca^{2+}, it is not a prerequisite for its activation [124, 127, 138]. Furthermore, the domain III of the large subunit contains C2 subdomains that are implicated in conformational changes during Ca^{2+} binding and may be involved in binding to membrane phospholipids [139, 140]. The carboxy-terminal domain IV of calpain large subunit is a calmodulin-like domain and contains five Ca^{2+}-binding EF-hand motifs, in which the fifth motif promotes the dimerization and binding to the small

subunit CAPNS1 [141]. In addition, the 30-kDa CAPNS1 has two distinct domains. The N-terminal domain (domain V) contains a hydrophobic sequence of aminoacids that may interact with plasma membrane [142, 143]. During calpain activation this domain is autolysed [144]. The Domain VI of CAPNS1 also contains a penta EF-hand domain and is very similar to the domain IV of the large subunit (see Figure 1.5). The biological function of CAPNS1 is not fully understood, and early evidences show that this small subunit is not required for catalytic activity of the large subunit [136]. Nevertheless, *in-vitro* experiments showed the small subunit acts as a chaperone and assists in the folding of the catalytic large subunit [136].

Either the large subunit of m-calpain (calpain-2) or the small subunit CAPNS1 are essential for mammalian life, since the genetic deletion of these proteins cause early embryonic lethality in mice [145, 146]. Interestingly, knockout mice for the large subunit of μ-calpain (calpain-1) are viable and fertile, despite showing a reduced platelet function [147].

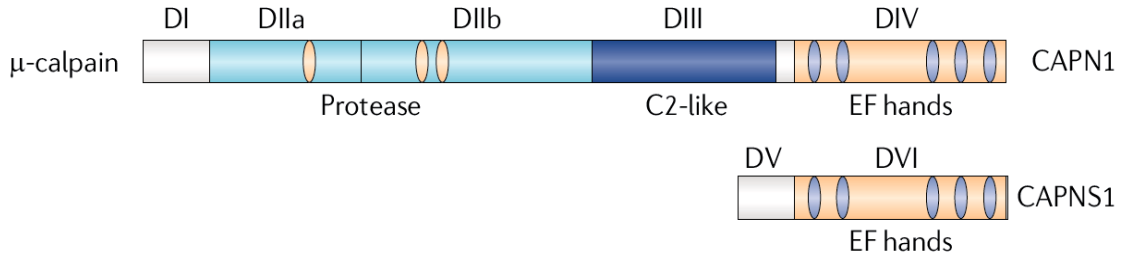


Figure 1.5 – Schematic representation of μ-calpain structure.

The 80-kDa large subunit of μ-calpain (CAPN1 or calpain-1), or m-calpain (CAPN2 or calpain-2), contains four domains (DI to DIV), while the 30-kDa small subunit (CAPNS1) contains two domains (DV and DVI). CAPNS1 associates with CAPN1, or with CAPN2, to form a heterodimer (μ-calpain and m-calpain, respectively). Domain I is autolysed upon calpain activation. The Domain IIa and IIb constitute the protease core, and contain the catalytic triad residues (shown with ovals). C2-like domain (DIII) is involved in binding to phospholipids and Ca2+, and influences the calcium-induced activation of calpain. The domain IV and VI contain five calcium-binding EF hands motifs (shown with ovals), and associate to form the heterodimeric calpain. Domain V is a glycine-rich domain and may interact with cell membranes. The m-calpain large subunit, CAPN2, is structurally similar to CAPN1. The figure was adapted from [148].

1.2.2. Calpain substrates and cleavage specificity

The substrate specificities of the conventional calpains (m- and μ-calpain) are very similar. Calpains usually cleaves the substrates in inter-domain regions, producing large fragments rather than small peptides [127]. Thus, in opposition to digestive enzymes, the limited hydrolysis of calpains indicates their function as modulatory proteases [149]. The crystallography structure of Ca^{2+}-bound m-calpain showed that the active site of this protease is deeper and narrower than others papain-like proteases. The constrains imposed by the cleft size indicate that the substrate should be in an extended conformation to fit the cleft, and may explain why calpains usually cleave unstructured inter-domain regions [150]. Although the protease domain (domain II) is highly conserved in calpain family, the substrate specificities of non-conventional calpains differ from the conventional μ- and m-calpain [151]. These differences suggest that the cleavage in the active site may depend on interactions between the substrate and other calpain interfaces. In opposition to other cysteine proteases, such as caspases, the cleavage mediated by calpains is not merely determined by the aminoacids sequence in the substrate. For calpains, the cleavage site is strongly determined by the conformation of the substrate rather than its primary aminoacid sequence [127]. Studies of bioinformatics have been attempted to predict calpain cleavage sites based on known cleavage sites determined experimentally. The most advanced predicting tool uses a machine learning process instead of the standard sequence analysis algorithms, such as the position-specific scoring-matrix method [152]. However, the accuracy of calpain cleavage site prediction still needs further improvements [153].

Calpains cleave a wide range of substrates. Accordingly to Calpain for Modulatory Proteolysis Database (CaMPDB – www.calpain.org) there are currently 97 experimentally confirmed mammalian calpain substrates and more than 1.000 computationally predicted mammalian substrates [154]. The confirmed calpain substrates include several proteins, such as: **1)** cytoskeletal proteins (eg: integrins, cadherin, microtubule-associated proteins MAP1 and MAP2, neurofilament 1 and 2, glial fibrillary acidic protein - GFAP, spectrin, tau); **2)** signal transduction proteins (CaMKIV, epidermal growth factor receptor kinase, protein kinase A and C, GSK3β, IP3R, calcineurin, IκB, protein tyrosine phosphatase 1B); **3)** apoptotic controllers (Apaf-1, AIF, Bax, Bid, Bcl-XL, BAK, caspase-3, 7, 8, 9, 12 and 14); **4)** transcription factors (p53, c-jun, c-fos) and **5)** synaptic proteins (APP, metabotropic and ionotropic glutamate receptors, dynamin-1, GAP-43, PSD-95), among others [155]. Interestingly, calpains often produce fragments with a reduced, enhanced or even different activity than the original substrate. For instance, a recent study showed that, in hippocampal neurons, an NMDAR-

dependent activation of calpain induces a cleavage in β-catenin at the membrane, producing a stable fragment that translocates to the nucleus and induces gene transcription [156].

The prototypical substrate of calpains is the αII-spectrin, a structural protein that maintains cell shape and links the cytoskeleton to plasma membrane. The cleavage of αII-spectrin by calpain was firstly described in neurons. Calpain initially cleaves αII-spectrin (280kDa) producing two products with identical electrophoretic mobility (150kDa), and then it subsequently cleaves one product producing a 145 kDa spectrin breakdown product (SBDP 145). In addition, caspase-3 cleaves the full-length αII-spectrin or the SBDP145, to produce an apoptosis-specific SBDP120 [157].

1.2.3. Regulation of calpain activity

Calpain activity is regulated by its endogenous inhibitor calpastatin, and by other mechanisms including phosphorylation, Ca^{2+} requirements modifications and most likely subcellular localization [127].

Calpastatin

Calpastatin is a ubiquitous endogenous protein that specifically inhibits the activity of μ-calpain and m-calpain. Calpastatin is encoded by *CAST* gene in humans, and due to multiple promoter usage and alternative splicing, it can originate distinct isoforms with N-terminal variation [148].

The prototypical calpastatin (~80kDa) contains an N-terminal domain (L Domain), which function is not completely known, and four repetitive inhibitory domains (I-IV) in which all four domains have a similar and very high inhibitory activity against μ- and m-calpain [158]. Calpastatin is an intrinsically unstructured protein capable of simultaneously binding to four molecules of calpain, in the presence of Ca^{2+} [159]. Each inhibitory domain of calpastatin contains three conserved subdomains (A, B and C). In the presence of Ca^{2+}, subdomain A and C binds to the calmodulin-like domains of calpain (to domain IV in the large subunit and to domain VI in the small subunit, respectively) [160], while a peptide derived from subdomain B inhibits the catalytic activity, indicating that this region interacts with domain II [161]. Recently, two groups simultaneously resolved the crystallographic structure of calpastatin bonded to m-calpain in the presence of Ca^{2+} [139, 162]. This structure showed that m-calpain binds to ten Ca^{2+} atoms, and that the inhibitory domains of calpastatin recognize the Ca^{2+}-bound m-calpain conformation wrapping around the protease in a tight and specific way. The subdomain B of calpastatin inhibitory domain occludes the catalytic cleft, but it avoids its own

cleavage by looping out and around the active cysteine residue (see Figure 1.6) [139, 162]. Taken together, multiple crystallographic structures of calpains have been shown that Ca^{2+} switches calpain from the inactive to the active state, allowing the binding of calpastatin and consequent calpain inhibition [137, 139, 162].

Calpastatin can be cleaved by calpains in its inter-domains regions, but the proteolytic fragments retain their inhibitory activity [163]. Nevertheless, calpastatin is cleaved by caspases during apoptosis, and in this case, it loses its inhibitory activity allowing the activation of calpains [164-166]. Studies have shown that calpastatin is usually found in an aggregated and phosphorylated state inside the cell, and upon an increase in intracellular Ca^{2+}, calpastatin is dephosphorylated and becomes progressively soluble. The aggregation of calpastatin is regulated by a PKA-mediated phosphorylation [167, 168]. In addition, PKC phosphorylates calpastatin, in a different site than PKA, reducing its inhibitory activity [169]. The regulation of calpastatin availability and activity through PKA and PKC-mediated phosphorylation, respectively, can constitute a way in which active calpains escape from the endogenous calpastatin inhibition.

Interestingly, mice deficient for calpastatin are fertile and viable, and do not show detectable calpain activation during normal conditions. Similarly, mice overexpressing calpastatin display no adverse phenotype. However, the degenerative changes upon neurotoxic and traumatic brain insults were limited in the calpastatin overexpressing mice and exacerbated in calpastatin-deficient mice [170-172].

Calcium requirements

Probably the major unresolved question in the calpain field is how calpains are activated intracellularly. Calpains in *in-vitro* conditions require high Ca^{2+} concentration to become active (~30µM or ~500µM of Ca^{2+} for half-maximal activity for µ-calpain and m-calpain, respectively). However, physiological intracellular Ca^{2+} levels is within nanomolar range, and reaches, at most, very low micromolar concentrations in stimulated cells [173]. This non-physiological high Ca^{2+} demand raised questions regarding the conditions under calpains could be activated *in-vivo*. Thus, it has been suggested several mechanisms that lowers the Ca^{2+} requirement of calpains, in particular the m-calpain, inside the cell. These include autolysis [138], phosphorylation [174], interaction with plasma membrane phospholipids [138, 175] or binding to an activator protein [176].

Interestingly a study has shown that m-calpain can be activated independently of Ca^{2+}, by a Erk/MAPK-mediated phosphorylation [177]. Recently it was showed that BDNF and EGF rapidly activate m-calpain in cultured neurons, via a MAPK-dependent phosphorylation, confirming the previous observations [178]. In addition, m-calpain is inhibited by PKA

phosphorylation [179]. Recent evidences showed that PKA-mediated phosphorylation on m-calpain reduces the binding of phosphatidylinositol-4,5-bisphosphate (PIP_2) to the domain III of m-calpain. In opposition, ERK-mediated phosphorylation increases the binding of PIP_2 to m-calpain. The PIP_2 acts as a co-factor to m-calpain and it promotes anchorage of m-calpain to the plasma membrane. In this way, PKA or ERK control the activity and cellular distribution of m-calpain by regulating its anchorage to the membranes [180, 181].

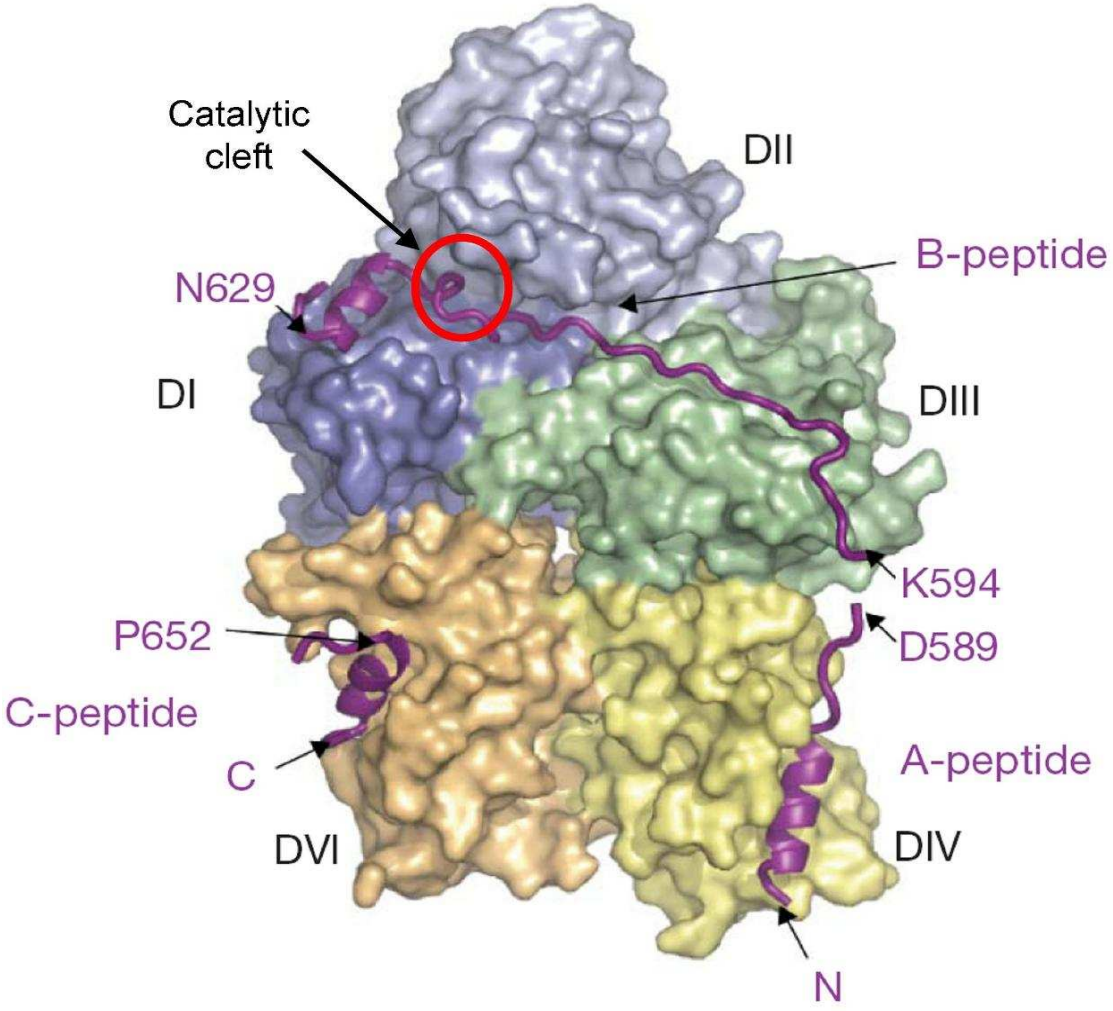


Figure 1.6 – Crystallographic structure of calcium-bound m-calpain enclosed by calpastatin.
Overall 2.4 Å-resolution crystal structure of calcium-bound m-calpain (composed by domains DI to DIV) associated with the inhibitory domain IV of calpastatin (CAST4). CAST4 (in purple) contains the subdomains A, B and C (as shown in the figure). CAST4 is unstructured in the absence of calpain, however, the subdomains of CAST4 form α-helices when associated with calcium-bound m-calpain. Helices of subdomain A and C interact with calpain domain DIV (yellow) and DVI (orange), respectively. The helix of the subdomain B, which is essential for the inhibitory activity of CAST, contacts with the protease core DII (light blue), but escapes from cleavage by looping out and around the catalytic site (red circle).Gaps in the structure of CAST4 are indicated by missing residues. Figure was adapted from [139].

1.2.4. Function in physiology and pathophysiology

At physiological conditions, calpains are involved in multiple biological functions including cell development, proliferation, differentiation, motility, apoptosis, growth cone guidance, LTP and memory [127]. Dysregulation of calpain activity is implicated in several diseases, including acute and chronic neurological disorders, muscle disorders, diabetes, cancer, among others [127, 150, 182]. For instance, calpain activity is increased in many types of cancer, and it contributes to survival, migration and invasion of tumor cells by cleaving oncogenes (eg: c-Fos, c-Jun, Myc), tumor suppressor genes (p53) and focal adhesion proteins [148]. In neurons, when Ca^{2+} homeostasis is disrupted, for example during excitotoxicity, epileptic seizures and acute ischemic and hypoxia injury, the overactivation of μ-calpain and m-calpain may occur, leading to neuronal damage and death [182, 183]. Altered calpain activity has also been found in chronic neurodegenerative disorders such as Alzheimer's, Huntington's and Parkinson's diseases and multiple sclerosis [184]. While physiological calpain activation seems to be essential in many biological processes, the excessive calpain activation contributes to disease and pathology. Considering the focus of this work, here it will be discussed in more detail the importance of calpains on physiology, in particular upon LTP and memory, and in pathology, in particular upon AD.

Calpains in LTP

The hypothesis that calpains are implicated in memory started in 1984. This hypothesis postulated that calpains contribute to LTP by promoting synaptic remodeling through cleavage of structural proteins, including spectrin, and by increasing the number of glutamate receptors in the post-synaptic membrane [185, 186]. Multiple evidences collected until now have reinforced this initial hypothesis. Indeed, TBS in CA1 hippocampal area, induces Ca^{2+} influx and calpain activation in PSDs as determined by the formation of calpain-specific spectrin breakdown products [187]. Additionally, multiple studies have shown that the inhibition or downregulation of calpains, by synthetic inhibitors or siRNA, respectively, greatly reduced the induction and magnitude of hippocampal LTP [188-190]. Recently, a study showed that conditional deletion of calpain-1 and calpain-2 in the CNS does not critically impair brain development, but reduces spine density and dendritic branching complexity of CA1 pyramidal neurons and impairs hippocampal LTP and spatial memory of the mice [191]. Interestingly, BDNF, which it is released during TBS and importantly contributes to LTP induction and memory encoding, promotes m-calpain activation through ERK activation [178]. On the other hand, genetic deficiency of calpastatin enhances hippocampal LTP [192]. Another recent study showed that μ-

calpain is necessary for synaptic potentiation during E-LTP, while m-calpain activation limits the magnitude of the potentiation during L-LTP consolidation [193].

Calpains in Alzheimer's disease

Growing evidence has shown that calpains are overactivated in AD, and this excessive activation may contribute to the progression of the disease [194-199]. In association to calpain overactivation, in AD brains, there is a markedly depletion on calpastatin. The depletion of calpastatin in the dendrites of AD neurons is mediated by caspase-1 and 3 cleavages, and coincides topographically with m-calpain activation and tau phosphorylation [200]. Another recent study also observed a correlation between calpain activation and tau phosphorylation in close proximity to amyloid plaques in *post-mortem* AD brain and in APP transgenic mice (an AD mice model) [199]. The same study showed that genetic deficiency of calpastatin increased Aβ amyloidosis, tau phosphorylation, microgliosis, neuronal dystrophy and increased mortality in APP transgenic mice. In opposition, the overexpression of calpastatin in APP mice had the opposite effect [199]. A similar conclusion was obtained in another study, using transgenic APP mice and a synthetic calpain inhibitor [201]. Furthermore, a third study showed that calpain inhibition reestablishes normal synaptic function and plasticity, and improves spatial-working memory in the APP transgenic mice [202]. Together, these findings highlighted the importance of the calpain-calpastatin system in AD.

1.3. Alzheimer's disease

AD is the most common chronic progressive neurodegenerative disease which affects about 24 million people worldwide and it increases in incidence with age [203]. In an early stage of AD the symptoms may begin as a short term memory loss and incapacity to make new memories, a process which depends on hippocampus and its cholinergic inputs from basal forebrain nuclei [204]. As the disease progresses other symptoms may occur, such as cognitive dysfunction, psychiatric symptoms, behavioural disturbances and long-term memory loss.

AD was firstly described in 1906 by Alois Alzheimer, a German psychiatrist, who examined the brain tissues of a woman who died from an unusual mental illness. He described a general atrophy and neuronal loss in cortical regions, and the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles in about 1/4 to 1/3 of all cortical neurons [205]. In fact, in AD there is a widespread loss of neurons and synapses in cortical areas, being the temporal lobes (hippocampus, parahippocampus and amygdala) the most affected areas [206].

For instance, while the number of neurons in temporal lobe remains constant between non-demented subjects, in AD patients more than 50% of these neurons are lost [207]. As a consequence of the substantial neuronal loss, the grey matter thickness in cerebral cortex is diminished and ventricles are enlarged (see Figure 1.7). In addition, multiple evidences indicate that synapse loss in AD is an early event that precedes neuronal loss [208], and is a major correlate of cognitive impairment [209, 210].

After 80 years of the initial description of AD, it was discovered that Aβ peptides are the main component of amyloid plaques [211] and that neurofibrillary tangles are composed by hyperphosphorylated tau (p-Tau) protein [212]. While the distribution pattern of amyloid plaques varies throughout the brain between AD patients, the tau pathology progresses in a highly regular pattern. Indeed, the neurofibrillary tangles occur first in the transentorhinal cortex, spreading sequentially to entorhinal cortex, to hippocampus and then to cerebral cortex [213].

Currently, several evidences obtained with biomarkers in AD patients, indicate that the initiating event in AD disease is the abnormal processing of Aβ and accumulation of amyloid plaques, which occurs while the individuals are still cognitively normal. Indeed, the first alterations in biomarkers detected are the decrease of Aβ42 levels in cerebrospinal fluid (CSF) and shortly after, amyloid accumulation in the brain measured by PET amyloid imaging. After a latency period that varies between individuals, markers of taupathology appear, with increased levels of total and phosphorylated tau detected in CSF. Alterations in tau biomarkers precede synaptic dysfunction and brain atrophy, which are indicated, respectively, by decreased ^{18}F-fluorodeoxyglucose uptake in PET and structural alterations in magnetic resonance imaging (MRI). Initial brain atrophy correlates with neuronal loss, and initiates the early memory symptoms present in mild-cognitive impairment (MCI) stage. Acceleration in hippocampal atrophy rates in MCI stage lead to the progression to clinical AD stage (see Figure 1.7A and B) [214-216].

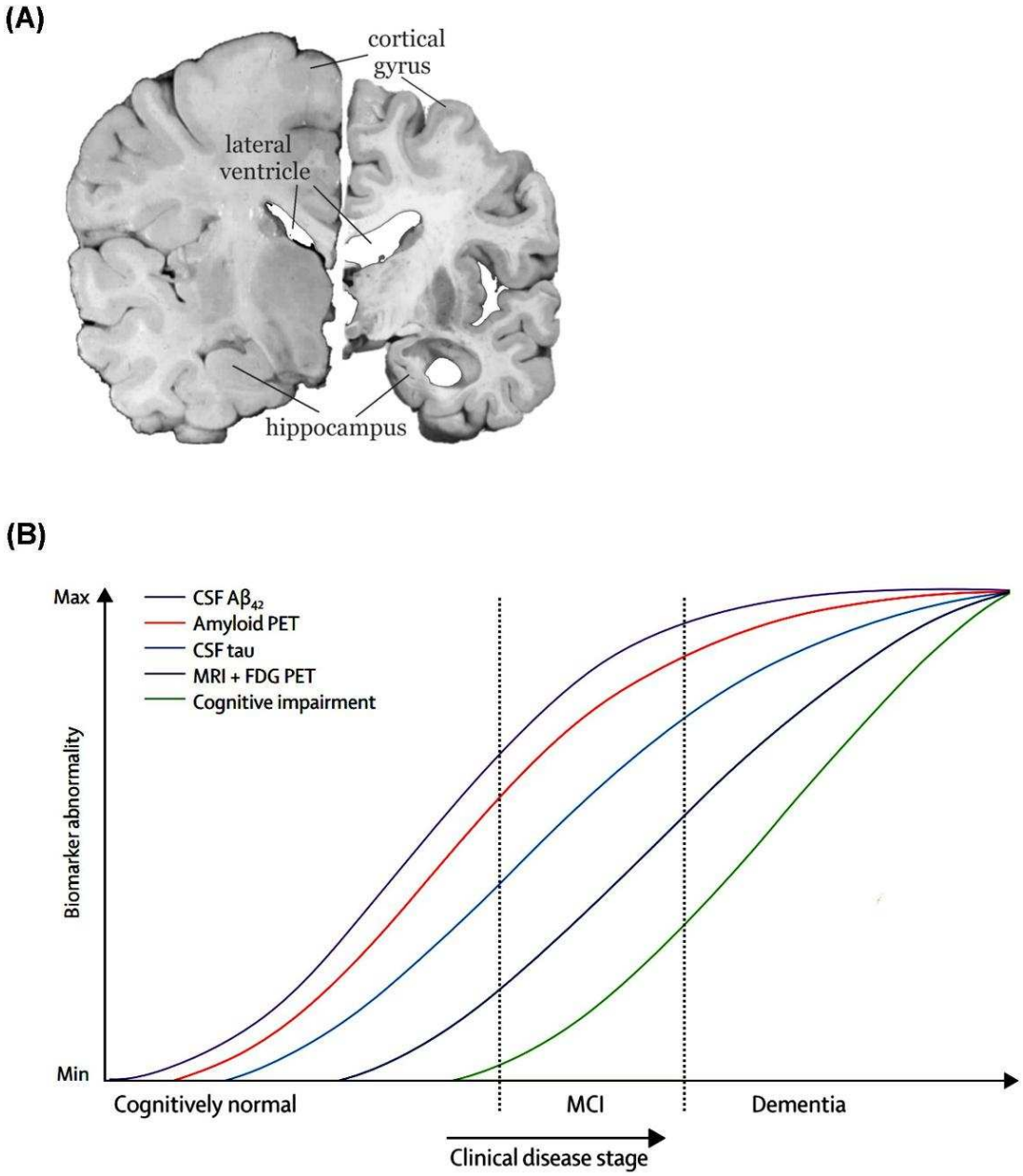


Figure 1.7 – Brain atrophy and hypothetical progression model in AD.

(A) Severe atrophy of human brain in late stage of AD (right), compared with normal brain (left). In advanced stage of AD, the neuronal loss results in the enlargement of lateral ventricles, hippocampal shrinkage and thinning of cortical gyrus. **(B)** Hypothetical model of AD progression showing the temporal order of biomarkers abnormalities across the progression of the disease. In this model, biomarkers of Aβ accumulation become abnormal early before tau accumulation, neurodegeneration and clinical symptoms occur. Aβ accumulation biomarkers include with decreased levels of Aβ in CSF and increased Aβ levels in brain measured by PiB-PET. Tauopathy is indicated by increased levels of total and phosphorylated tau in CSF. Neurodegeneration is measured by FDG-PET and structural MRI. MCI=mild cognitive impairment. Figure adapted from [217] and [215]

1.3.1. Amyloid-β peptides

Based on Aβ peptide sequence, it was found that Aβ derived from another protein, the amyloid-precursor protein (APP) [218]. Shortly after APP identification, it was detected an autosomal dominant mutation in the *APP* gene in some families with early-onset familial AD [219]. This point mutation, which was closely located to the carboxy-terminus of Aβ peptide, provided an important support to the amyloid cascade hypothesis of AD. Then, new findings showed that familial AD could also be caused by mutations in presenilin-1 (*PSEN1*) [220] and presenilin-2 (*PSEN2*) genes [221]. Both presenilins are members of the γ-secretase protease complex. The sequential cleavage of APP by β-secretase (BACE1) and γ-secretase produces the Aβ peptide (see Figure 1.8A and B) [222, 223]. Until now, dozens mutations were found to be associated with early-onset familial AD in the *APP*, *PSEN1* and *PSEN2* genes [224]. The majority of those mutations are associated with increased Aβ production or with increased Aβ42/Aβ40 peptides ratio [225-227]. For instance, some mutations in APP near γ-secretase cleavage site affect the cleavage by γ-secretase and shift the amyloid production to Aβ42, instead of Aβ40 [228]. The critical factor for the rate of amyloidogenesis is the relative concentration of Aβ42 rather than the total Aβ concentration [227, 229, 230]. Aβ42 is the most hydrophobic and amyloidogenic form of the peptide, and it is also more neurotoxic than Aβ40 [231, 232]. Aβ is present in plasma, CSF and brain interstitial fluid (ISF) mainly as soluble Aβ40 peptide [233], however, the major form present in the parenchymal amyloid plaques in AD is the Aβ42 [234, 235].

In sporadic AD, which constitutes more than 95% of all AD cases, there is no known associated mutation in APP or presenilins [236]. Unlike APP and presenilins, no known mutations in BACE are linked to familial early-onset AD. Interestingly, a recent work identified a mutation in APP gene that protects against AD and against cognitive decline in non-demented elderly subjects. This mutation (A673T) is located close to β-secretase cleavage site and reduces cleavage efficacy, lowering Aβ production up to 40% *in-vitro* [237]. In opposition, in the AD brain the β-secretase activity and protein levels were found to be increased [238, 239]. Thus, all the above evidences imply a crucial role of Aβ in AD, and sustain the hypothesis that Aβ is the leading cause of AD.

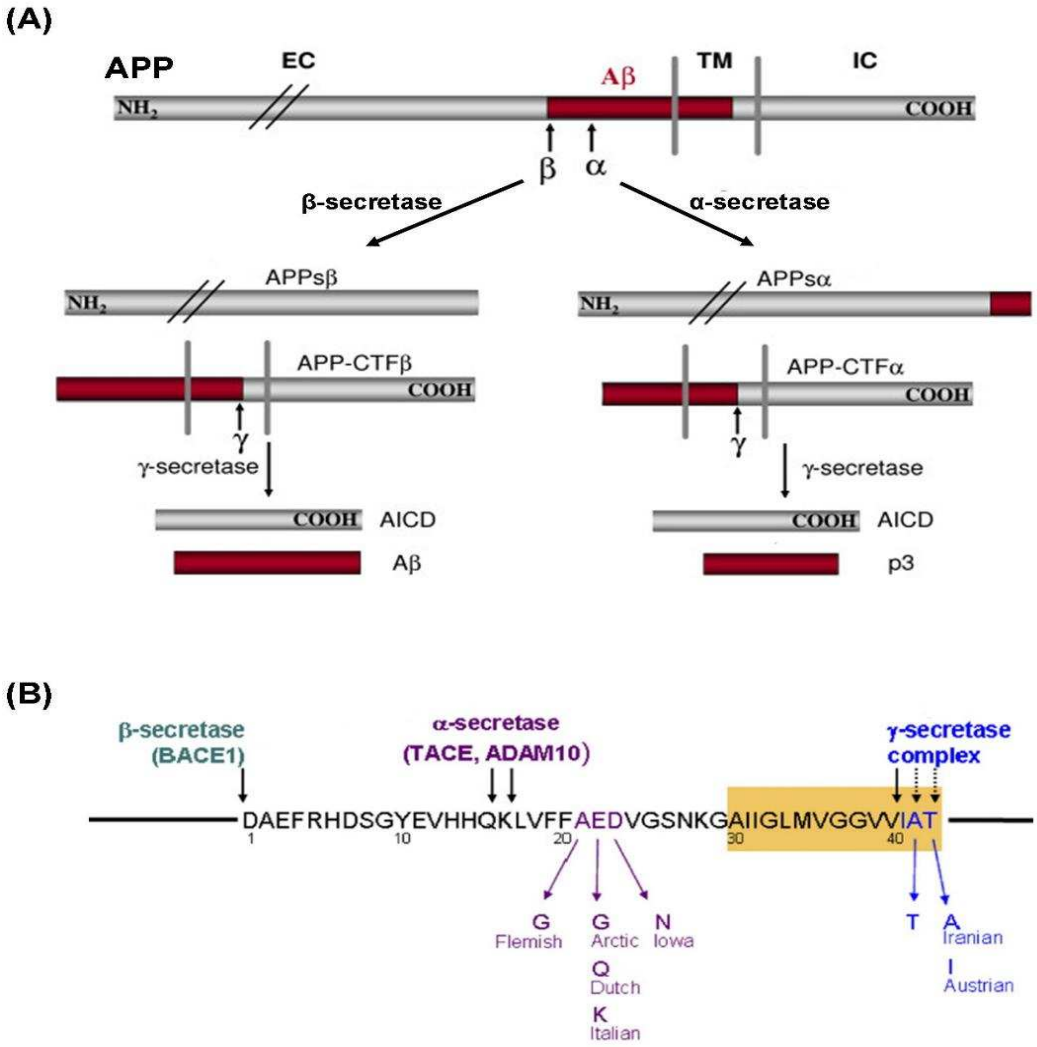


Figure 1.8 – APP processing and Aβ formation.

(A) APP is a transmembrane protein composed by 770 aminoacid residues. APP can be processed by the non-amyloidogenic α-secretase pathway, or by the amyloidogenic β-secretase pathway. In the non-amyloidogenic pathway, α-secretase cleaves APP in an extracellular (EC) position (aa. 687), releasing a large extracellular soluble fragment (sAPP-α). The remaining c-terminal membrane-bound APP fragment (named APP-CTFα, or C83) is subsequently cleaved by γ-secretase complex in the transmembrane region (TM), producing the p3 fragment and the AICD. In the amyloidogenic pathway, APP is firstly cleaved by β-secretase (BACE1) in the extracellular portion (aa. 671) generating the sAPP-β fragment and the membrane-bound APP-CTFβ (also named as C99). The APP-CTFβ is then cleaved by γ-secretase complex generating the AICD and the Aβ peptide. The γ-secretase complex, which is composed by presenilin 1 and 2, nicastrin, APH1 and PEN2, cleaves APP in distinct positions, producing Aβ peptides ranging from 38 to 43 aminoacids length, being the longer peptides (Aβ-42 and Aβ-43) the main pathogenic species. **(B)** Aminoacid sequence of Aβ region within APP, showing some APP point mutations associated with familial AD. The transmembrane region of APP is highlighted in orange and the secretase cleavage sites are indicated. The figure was adapted from [240, 241].

1.3.2. Neurotoxicity mediated by Amyloid-β peptides

After the discovery of Aβ peptide as the main component of amyloid plaques in AD, soon it was discovered that the peptide had neurotoxic properties. Indeed, the incubation of neuronal cells with synthetic Aβ produces significant neuronal death [30, 242, 243]. Moreover the *in-vivo* administration of Aβ into the mice brain also produces significant neurodegeneration and cognitive deficits [244, 245]. In addition, genetically engineered mice that over produce Aβ, either by overexpression of APP and/or presenilin containing mutations linked to familial AD, also recapitulate some key features of AD, including synaptic loss and cognitive impairments [236]. Nevertheless, many AD mice models fail to recapitulate a robust neuronal loss and an evident presence of neurofibrillary tangles [236].

Mechanisms underlying the neurotoxic actions of Aβ peptides are not fully understood, but the existing data suggests the involvement of multiple mechanisms such as oxidative stress, interaction of Aβ with receptors and ion channels, excitotoxicity, synaptic dysfunction, inflammation, mitochondrial dysregulation, membrane permeability alterations and activation of caspases and calpains (Figure 1.9) [246-256]. Here, it will be discussed in more detail the main molecular mechanisms that may contribute for Aβ toxicity.

Oxidative stress

An increase in markers for DNA, RNA, lipids and protein oxidation have been found in AD brain, suggesting an involvement of oxidative stress is the disease [246]. The Aβ peptide found in amyloid plaques, can itself bind to transition metals with high affinity, such as iron, zinc or cupper, and possess the ability to reduce them to a lower oxidation state. This redox reaction produces reactive oxygen species (ROS) which will react and damage cellular components contributing to Aβ neurotoxicity [241].

Calcium homeostasis disruption

Aβ disrupts Ca^{2+} homeostasis and synaptic function by interacting with several neurotransmitters receptors and ion channels. For instance, Aβ binds with high affinity to the nicotinic receptor α7-nAChR, and this interaction leads to an inhibition in acetylcholine release and in α7-nAChR-dependent Ca^{2+} influx, leading to neuronal demise [257]. In addition, Aβ peptide affect glutamatergic synapses by interacting with AMPA and NMDA glutamate receptors, two major players involved in synaptic plasticity and memory formation. In particular, studies have shown that Aβ alters the kinetics of AMPAR and it reduces its surface

membrane expression by increasing intracellular Ca^{2+} and phosphorylation of GluR2 subunit [258]. However, one of the most studied targets of Aβ is the Ca^{2+}-permeable NMDA receptor.

The ionotropic receptor NMDAR plays a central role in synaptic plasticity, however when over-activated it contributes to excitotoxic cell-death by causing a persistent influx of Ca^{2+}. Studies have shown that Aβ oligomers can trigger an increase of Ca^{2+} influx mediated by NMDAR, resulting in mitochondrial and synaptic dysfunction, excitotoxicity and ROS production [259-262]. Aβ can also inhibit astrocytic glutamate uptake, leading to an accumulation of extracellular glutamate and an enhanced NMDAR activation [261, 263]. By exacerbating the influx of Ca^{2+}, Aβ leads to an exacerbated calpain activation, which in turn mediates pathogenic effects by cleaving synaptic substrates. Calpain modulates synaptic function by cleavage of membrane receptors (such as NMDAR), kinases, cytoskeletal proteins and post-synaptic density (PSD) proteins, leading to changes in synaptic organization and stability [264]. By increasing Ca^{2+} influx through NMDAR, Aβ also induces a calpain-mediated cleavage of dynamin-1, a protein essential for recycling of synaptic vesicles [265]. Calpain over-activation also reduces the activity of protein kinase A (PKA), which in turn contributes to a down-regulation of cAMP response element-binding protein (CREB), a key molecule for synaptic plasticity, learning and memory [266]. Additionally, the toxic effects of Aβ are attenuated by NMDAR antagonists further supporting the role of NMDAR in Aβ toxicity [261, 267, 268]. Besides the ionotropic receptors, Aβ can also disturb intracellular Ca^{2+} homeostasis by changing the activity of VGCCs [269] and by evoking the release of Ca^{2+} from intracellular stores, such as ER [270, 271]. Moreover, studies have shown that Aβ can associate with lipid bilayers and spontaneously form novel Ca^{2+}-permeable pores by which uncontrolled Ca^{2+} influx may perturb intracellular Ca^{2+} homeostasis [272]. Although it has been difficult to observe this pores in cell membranes, a recent study used a new single-channel Ca^{2+} imaging technique and provided evidences for an intrinsic Ca^{2+}-permeable pore formed by Aβ oligomers in cell membrane [273]. Membrane permeabilization and pores formation are features also observed in many antimicrobial peptides, such as human LL-37 [274]. Interestingly, a recent study showed that Aβ42 binds to bacterial membranes and has high antimicrobial activity against several microorganisms, suggesting that Aβ belongs to innate immune system and may be a defence mechanism to infections in CNS [275].

Calpain activation

As briefly mentioned above, Aβ perturbs Ca^{2+} homeostasis in neurons and promotes calpain activation [199, 276, 277], an outcome also observed in *post-mortem* human AD brains [194, 195, 197-199, 266, 278]. Interestingly, recent data have suggested a bidirectional link between calpain activation and Aβ deposition. Particularly, in APP overexpressing mice, the genetic deficiency in calpastatin (an endogenous calpain-specific inhibitor) not only increased calpain activity, but it also increased Aβ amyloidosis and Tau phosphorylation, with additional increased somatodendritic dystrophy and mice mortality [199]. This study suggested that calpain activation can also contribute to Aβ production and Tau phosphorylation (two major hallmarks of AD). Actually, both Aβ peptides and calpain activation have already been linked with tau hyperphosphorylation. In one hand, it has been shown that aggregated Aβ peptides can significantly increase tau phosphorylation levels in primary septal neuronal cultures [279]. This study also showed that Aβ activates kinases involved in phosphorylation of tau, such as GSK3 and MAPK [279]. On the other hand, it has been shown that calpain activation enhances the activity of several kinases that mediate tau phosphorylation. For instance, calpains can cleave the inhibitory domain of GSK3, enhancing the kinase activity [280]. Calpain also cleave the CDK5-regulator p35, generating a truncated product, the p25, which causes a constitutive activation of CDK5 and a consequent hyperphosphorylation of Tau [196, 281-283]. The p25 fragment was also detected in higher levels in AD brains than age-matched controls [196, 284]. To evaluate the role of the calpain cleavage fragment p25, it was developed a transgenic mouse that overexpresses the p25 protein under the CaMKII promoter. The overexpression of this single fragment was able to recapitulate many hallmarks of AD, such as progressive neuronal loss in cortex and hippocampus, forebrain atrophy, tau pathology, amyloid plaques, intraneuronal Aβ accumulation, impaired synaptic plasticity and cognitive dysfunction [285-287]. Inhibition of CDK5, as well as inhibition of c-Jun N-terminal Kinase (JNK) and p38 mitogen-protein activated kinase (p38 MAPK), is sufficient to prevent the impairment of LTP induced by Aβ [288]. Moreover, inhibition of CDK5 by a synthetic inhibitor, or an antisense oligonucleotide, prevents Aβ-induced death in cultured hippocampal neurons [289]. Recently, it was shown that calpain-deficient neurons do not convert p35 to p25 and are more resistant to excitotoxicity and mitochondrial toxicity. Interestingly, these calpain-deficient neurons became sensitive to the same toxic stimulus after the infection with a p25 expressing adeno-associated virus [191].

In addition, calpain can also cleave tau protein generating a neurotoxic ~17kDa fragment which has been detected in hippocampal neurons treated with Aβ oligomers [290] and in cortex of AD brain [195]. The expression of this toxic fragment induces neuronal death in hippocampal cultures [290]. Taken together, the evidences suggest that Aβ triggers calpain

activation, by perturbing Ca^{2+} homeostasis, and consequently it activates several kinases, such as CDK5, which can mediate hyperphosphorylation of Tau, Aβ production and neurodegeneration (Figure 1.9).

Receptors interaction

Several studies have shown that Aβ can interact with many receptors. There is evidence that Aβ oligomers can bind to cellular prion protein (PrP^c) and Ephrin type B receptor 2 (EphB2) and the downstream signalling from both receptors alters NMDAR function contributing to decreased synaptic plasticity [291, 292]. Aβ oligomers can also interact with human leukocyte immunoglobulin-like receptor B2 (LilrB2) engaging signalling pathways for neuronal actin organization that results in synapse elimination [293]. Additionally, it has been shown that the neurotrophin receptor $p75^{NTR}$ is a receptor for Aβ and it is required for Aβ-induced neuronal death [294]. Aβ oligomers also compete with insulin for binding to insulin receptor [295] and disrupt insulin signalling, which suggests that Aβ may contribute to insulin resistance observed in AD brain [296]. Previous experiments shown that the receptor for advanced glycation end products (RAGE) also binds to Aβ [297] and mediates Aβ-induced neuronal toxicity [298, 299]. Additionally, aggregated Aβ42 can trigger neuroinflammatory activation in microglia by binding and activating the innate immune receptor Toll-like receptor 2 (TLR2) [300].

Mitochondrial dysfunction

Challenging the classical view that Aβ accumulates extracellularly, emerging evidence have shown that Aβ also accumulates intracellularly, further contributing to AD progression [301]. In fact, the amyloidogenic cleavage of APP also occurs in intracellular membranes of Golgi and ER, whereas the Aβ peptide is released to the intracellular space (cytosol). In addition, extracellular Aβ can bind to several receptors, producing Aβ-receptor complexes that can also be internalized into early endosomes [301-303].

It has been shown that intracellular Aβ accumulates in mitochondria from the brain of transgenic mice expressing mutant human APP and also accumulates in mitochondria from cultured cortical neurons from the transgenic mice, and most importantly, in the brains of AD patients [304, 305]. Interestingly, exogenous Aβ applied to human neuroblastoma cells can be internalized and accumulate within mitochondria [306]. The accumulation of Aβ within mitochondria correlates with lower enzymatic activity of respiratory chain complexes III and IV, lower oxygen consumption and higher levels of hydrogen peroxide and oxidative damage

[304, 305, 307]. In addition to the increased ROS production, mitochondrial Aβ also promotes the opening of the mitochondrial permeability transition pore (mPTP) by directly interacting with Cyclophilin D (CypD), a modulator of mPTP formation [308]. Genetic deficiency of CypD reduces Aβ-induced mitochondrial stress as well as it improves behavioural and synaptic function in the transgenic APP mice [308]. The opening of the mPTP releases pro-apoptotic proteins, such as Cytochrome c, which activates caspase-3 and triggers the intrinsic apoptotic pathway. Activation of caspase-3 in synapses promotes early synaptic dysfunction and behavioural impairment in transgenic APP mice [309].

Complexity of Aβ effects

Aβ toxicity is a complex process, involving multiple and distinct mechanisms. The study of Aβ toxicity mechanisms is further hampered by the fact that distinct Aβ preparations (monomers, oligomers or fibrils) evoke different pathways. For instance, naturally secreted Aβ oligomers, but not fibrils or monomers, potently inhibit *in-vivo* hippocampal LTP [310]. Although initial studies described insoluble Aβ fibrils as neurotoxic, most recent evidences have shown that soluble Aβ oligomers are also neurotoxic and most probably are the main responsible for neurodegeneration, and particularly for synaptic failure in AD [311].

In top of Aβ species complexity, Aβ may also trigger different effects depending on its concentration. For example, it has been shown that oligomeric and monomeric Aβ preparations can markedly increase hippocampal LTP at low picomolar concentration, while at high nanomolar concentrations the same preparations lead to the well-established reduction in LTP [312]. These evidences suggest a dual role for Aβ, whereas at sub or low-nanomolar levels it may have a physiological role and beneficial effects, while at high nanomolar or micromolar concentration it can trigger synaptic dysfunction and neurodegeneration.

The effects of Aβ may also differ depending on the length of the peptide. For instance, the Aβ25-35 peptide, which is the shortest peptide sequence that retains the biological activity of Aβ42, showed enhanced toxicity and enhanced aggregation rate in aqueous solution, when compared to Aβ42 [243, 313].

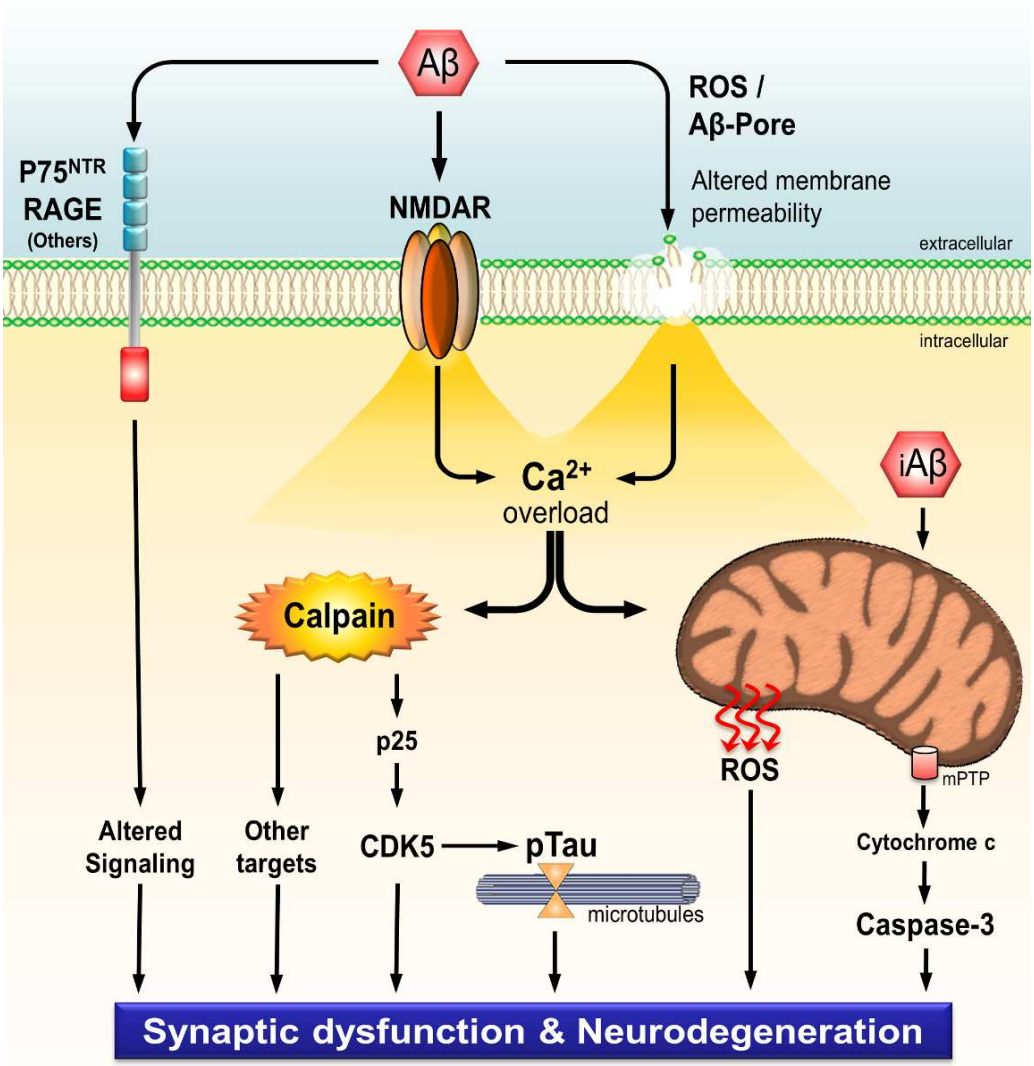


Figure 1.9 – Simplified schematic representation of Aβ toxicity mechanisms.

Extracellular Aβ peptides induce synaptic dysfunction and neurodegeneration by multiple mechanisms. Evidences indicate that Aβ dysregulates signalling pathways by interacting with several receptors (eg: p75NTR, RAGE, PrPc, α7-nAChR, mGluR, insulin receptor, among others) and induces a sustained Ca2+ influx by either interacting with ionic receptors, such as NMDA receptor, or by promoting cell membrane leakage through a pore formation or reactive oxygen species (ROS) production. The prolonged Ca^{2+} influx induces calpain overactivation and mitochondrial calcium overload. Intracellular Aβ (iAβ) further impairs mitochondrial function by interacting with mitochondrial respiratory chain complexes, which decreases ATP production and increased ROS formation. In addition, the iAβ triggers mitochondrial permeability transition pore (mPTP) formation, with consequent release of pro-apoptotic proteins, and caspase-3 activation. On the other hand, calpain overactivation cleaves and changes the function of several proteins (eg: synaptic proteins, structural proteins, signalling proteins, among others). Importantly, calpains activate cyclin-dependent kinase 5 (CDK5) by cleaving the CDK5-modulator p35 into p25. Increased CDK5 activity leads to neurogeneration, Aβ formation, and tau hyperphosphorylation.

1.3.3. BDNF signalling in AD

The neurotrophin BDNF prevents the neuronal death induced by a wide variety of *in-vitro* insults, including serum-deprivation, oxidative stress, glutamate excitotoxicity, ischemia, and Aβ peptides [120, 243]. BDNF is required for the maintenance of dopaminergic neurons and it confers protection against neurotoxins, such as MPTP and 6-OHDA, in both *in-vitro* and *in-vivo* Parkinson's disease models [314]. In addition, BDNF delivery into entorhinal cortex or hippocampus reverses the neurodegeneration and improves cognitive performance in rodent and primate models of AD [243, 315-317]. Notably, the BDNF administration does not reduce amyloid plaques number in transgenic AD models, indicating that amyloid load reduction is not necessary to achieve neuroprotective effects [315-317]. In addition to neuroprotective effects, BDNF also rescues the impairment in the HFS-induced LTP in hippocampal slices treated with oligomeric Aβ [318].

In addition to the benefits of increasing BDNF actions in AD models, it is noteworthy that multiple evidences support that BDNF signalling is reduced in AD patients. In fact, it was reported that in *post-mortem* brain samples from end-stage AD patients, the mRNA levels of BDNF are substantially reduced in hippocampus and parietal cortex [319-321], whereas the levels of BDNF protein are reduced in hippocampus and in multiple areas of cortex [322-324]. In addition, in pre-clinical stages of AD it was also detected, in parietal cortex, a reduction in protein levels of both BDNF and its precursor pro-BDNF [325, 326]. This reduction of BDNF and pro-BDNF levels positively correlates with disease progression and the loss of cognitive function [325]. The importance of BDNF in AD is further enhanced by a number of studies that associated BDNF polymorphisms with the disease. The BDNF Val66Met polymorphism is probably the most investigated one, and influences intracellular trafficking and activity-dependent secretion of BDNF [327]. Multiple studies have shown that the synergetic interaction between APOE ε4 and BDNF Val66Met polymorphisms is associated with increased risk and progression to AD [328-331]. Interestingly, in non-demented elderly individuals, BDNF Val66Met polymorphism increases amyloid load in brains from the APOE ε4 carriers, but not in APOE ε4 noncarriers, and this Aβ deposition negatively correlates with episodic memory encoding [332].

Beyond the reduction on BDNF levels, alterations in the levels of TrkB receptors have also been found in AD brain. In particular, it was reported that TrkB-FL is decreased in hippocampus and in temporal and frontal cortex of patients with advanced AD [322, 333]. A substantial decrease in TrkB, but also in TrkA and TrkC, was also found in AD patients in the cholinergic neurons from the nucleus basalis, an area severely affected during the disease [334, 335]. Similarly to what was observed for BDNF, the downregulation in Trk receptors levels is

progressive, starting in pre-clinical stages, and it correlates with the cognitive decline of patients [335]. Conversely, the levels of truncated TrkB receptor, a negative regulator of the full-length isoform, have been found to be increased in association with senile plaques in frontal cortex [322] and hippocampus [336] of AD patients. Moreover, in a recent study, it was reported an increase in protein levels of truncated TrkB receptors in hippocampus and temporal cortex of AD brains. The same study also found a selective increase in the mRNA levels of the neuron-specific TrkB-T-Shc isoform, without detecting differences in TrkB-T1 and TrkB-FL transcripts [337]. Although the overall evidences suggest that levels of truncated TrkB are increased in AD, in a previous study, no alterations on truncated TrkB levels were detected in temporal and frontal cortex of AD brain [333]. On the other hand, it was found that TrkB-T1 expression is increased in the APP/PS1 mice model, and its expression increases with age and amyloid load in frontal and parietal cortex, but not in hippocampus [30]. Interestingly, overexpression of TrkB-T1 in the APP/PS1 mice exacerbated their spatial memory deficits, while the overexpression of TrkB-FL ameliorates the deficits [30]. Finally, a recent family-based study observed a genetic association between polymorphisms in *NTRK2* (TrkB gene) and AD, enhancing the importance of TrkB in the susceptibility to this disease [338].

Interestingly, recent works suggested that BDNF and TrkB may be mechanistically involved in the pathogenesis of AD. Indeed, in a neuroblastoma cell line, it was shown that BDNF increases APP transcription and shifts the APP processing towards the non-amyloidogenic α-secretase cleavage, promoting the accumulation of AICD and sAPP-α [339, 340]. Conversely, blockade of BDNF signalling rapidly activates the amyloidogenic pathway and causes apoptotic death in cultured hippocampal neurons [341]. The role of BDNF in APP processing might be related with the observation that certain BDNF polymorphisms are associated with increased Aβ production in the brain [332]. In addition to the effects of BDNF in APP processing, it has been showed that acute activation of TrkB, by BDNF stimulation, induces a rapid decrease in levels of phosphorylated tau in cultured neurons, by a mechanism dependent on PI3K and GSK3 activity [342]. However, recent evidences showed that genetic reduction in BDNF levels (heterozygous BDNF knockout) did not exacerbate the amyloid load and tau pathology in aged transgenic AD mice models [343, 344]. Nevertheless, similarly to what was described for TrkB-FL, the genetic down-regulation of BDNF also exacerbated the spatial learning impairment in the APP/PS1 AD mice model [344].

In summary, the observations that 1) BDNF and TrkB-FL receptor are required for synaptic plasticity and neuronal survival on CNS [345], 2) Increased TrkB and BDNF signalling ameliorate the neurodegeneration and cognitive impairment in multiple AD models and 3) BDNF and TrkB levels are reduced in AD brain; raised the hypothesis that the loss of BDNF signalling contribute to the progression of AD. Whether the loss of neurotrophic support is a cause or a consequence of the disease, is a question far from being resolved.

2. Aims

The main goal of this project was to study the impact of Aβ peptides and adenosine A_{2A} receptors on BDNF signalling.

Multiple evidences suggest that BDNF signalling is impaired in Alzheimer´s disease (AD). Indeed, both BDNF and its TrkB-FL receptor levels are decreased in AD *post-mortem* brain samples, while the truncated TrkB-T1 levels are increased. In the first part of this work, we aimed to test whether Aβ peptide, by itself, could induce similar alterations on TrkB receptor isoforms, and to study the mechanisms involved and the functional impact (Chapter 4).

BDNF synaptic actions are dependent or potentiated by adenosine A_{2A} receptors ($A_{2A}R$) activation. Therefore, in the second part of the work, we evaluated whether the *in-vivo* chronic blockade of $A_{2A}R$ receptors, a therapeutic strategy already stated for AD, would affect BDNF effect upon CA1 hippocampal LTP (Chapter 5) and whether $A_{2A}R$ inhibition would also affect the neuroprotective effects of BDNF upon Aβ-induced toxicity (Chapter 6).

3. Methods

The methods presented in this chapter were published in Kemppainen et al., 2012 [30], Jerónimo-Santos et al., 2014a [116] and Jerónimo-Santos et al., 2014b [346]. Experiments in Figure 3.1 were performed by H.V. Miranda. The methods described in chapters 3.14, 3.15 and 3.16 were used by S.H. Vaz, S. Parreira and S. Rapaz-Lérias to perform the experiments in chapter 4.8. The KW-6002 administration to animals (chapter 3.17) was in part performed by V.Batalha.

3.1. Materials

Unless stated otherwise all reagents were purchased from Sigma (St. Louis, MO, USA). Culture reagents and supplements were from Gibco (Paisley, UK). Recombinant human BDNF was a gift from Regeneron Pharm. (Tarrytown, NY). Rat recombinant m-calpain was from Calbiochem (MA, USA) and the N-terminal His6 tagged recombinant human TrkB active (aa.455-end) was from Millipore (Billerica, MA, USA). MG132, ALLN, MDL28170 and Pepstatin A were from Tocris Bioscience (Bristol, UK). $A\beta_{25-35}$ and $A\beta_{35-25}$ peptide and zVAD(OMe)-FMK were purchased from Bachem (Bubendorf, Switzerland), and $A\beta_{1-42}$ peptide was purchased from rPeptide (Georgia, USA). [^{3}H]GABA (4-amino-n-[2,3–3H]butyric acid, specific activity 92.0 Ci/mmol) and [^{3}H]glutamic acid (L-[3,4-3H] glutamic acid, specific activity 49.6Ci/mmol) were purchased from PerkinElmer Life Sciences.

3.2. Amyloid-β peptides

Most of the experiments with Aβ were performed using the truncated $A\beta_{25–35}$ form, and in selected key experiments also the full-length $A\beta_{1-42}$ and the reverse $A\beta_{35–25}$ peptide were used. Stock solutions of $A\beta_{25–35}$, $A\beta_{1-42}$ and $A\beta_{35–25}$ peptides were performed in MilliQ water to a final concentration of 1mg/mL. To analyse the structural properties of the different species applied to neuronal cultures, both ThT binding assays and atomic force microscopy (AFM) were performed. Both $A\beta_{1-42}$ and $A\beta_{25-35}$ peptides showed a ThT emission wavelength shift followed by a fluorescence intensity enhancement, typical of β-sheet amyloid structures interaction (Figure 3.1A). By AFM the $A\beta_{1-42}$ and $A\beta_{25-35}$ peptides show similar structures as a heterogeneous population exhibiting protofibrilar and fibrilar ones (Figure 3.1B) [30].

The concentration of Aβ42 present in interstitial fluid (ISF) of human AD brain parenchyma is not known and difficult to predict. Nevertheless, it is estimated that Aβ42 is

present in ISF at a concentration within nanomolar range [230]. Aβ toxicity may differ according to the duration of Aβ exposure, the species present (monomeric, oligomeric or fibrillar ones) and the length of Aβ peptide (e.g. 25-35, 1-42). It is, therefore, difficult to use conditions that mimic exactly what happen in human AD patients. The concentrations of Aβ used in our work were 25μM for $A\beta_{25–35}$ and 20μM for $A\beta_{1–42}$, similar to used by others [243]. It is worthwhile to note that, although the Aβ concentration used is relatively high, the exposure period to the peptide was much smaller (24 hours for the cultures, 3 hours in the acute slice preparations) than what happen in human AD patients (several years). Importantly, as a control, reverse $A\beta_{35–25}$ peptide was used, also at a concentration of 25μM.

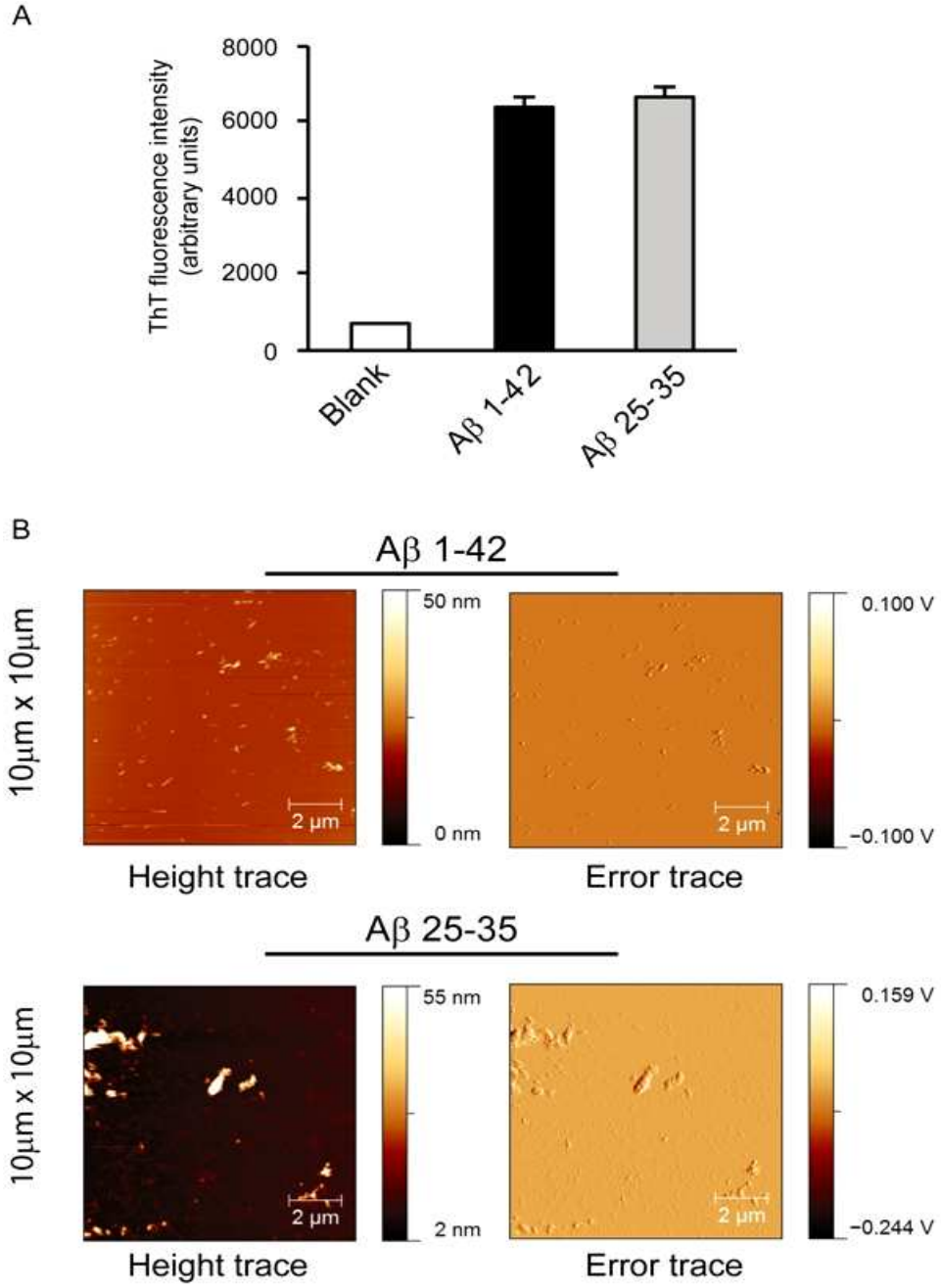


Figure 3.1 – $A\beta_{1-42}$ and $A\beta_{25-35}$ peptides have fibrillary structures.

(**A**) ThT fluorescence assay with excitation and emission wavelengths of 450 nm and 490 nm, respectively. Both peptides interacted with ThT, suggesting that they display an increased β-sheet structure, typical of fibrillary species. (**B**) AFM analysis of the $A\beta_{1-42}$ and $A\beta_{25-35}$. Both peptides appear as heterogeneous population with protofibrillar and fibrilar structures.

3.3. BDNF

BDNF used was a recombinant human-met-BDNF (supplied by Regeneron pharmaceuticals and manufactured by Amgen Inc.). The stock formulation of BDNF was: recombinant Human-met-BDNF 1.0mg/ml diluted in 150mM NaCl, 10mM sodium phosphate buffer, pH 7.2 and 0.004% Tween-20. The stock solution was diluted by a factor of 50.000, to achieve a final concentration of BDNF 20ng/ml (corresponding to 750 pM). To confirm the purity, a western-blot using 20ng of supplied BDNF was performed and probed with an antibody selective for BDNF (Abcam ab46176). It was only detected a band at ~14kDa, corresponding to mature BDNF (Figure 3.2).

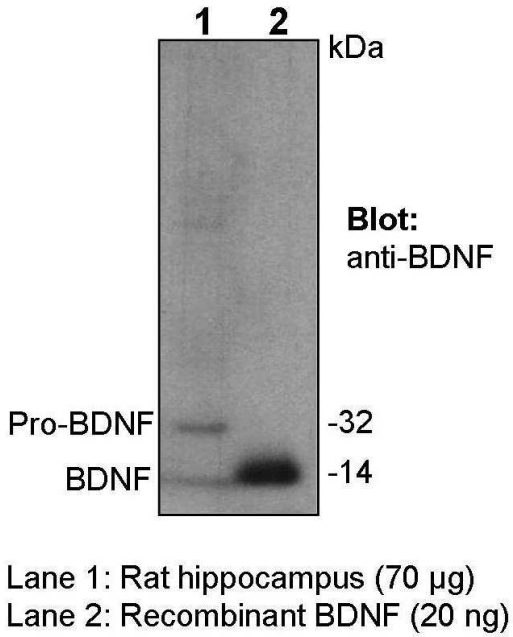


Figure 3.2 – Recombinant BDNF purity.

Western-blot probed with the anti-BDNF (Abcam ab46176) antibody (raised against N-terminal of mature BDNF) with [lane 1] 70µg of protein from rat hippocampal homogenate (8week-old) and [lane 2] 20ng of recombinant Human-Met-BDNF. Pro-BDNF (~32kDa) was detected only on hippocampal homogenate.

3.4. Animals and brain areas used

Sprague-Dawley and Wistar rats were purchased from Harlan Interfauna Iberica, SL (Barcelona, Spain) and were housed in a temperature (21 ± 1ºC) and humidity-controlled (55 ± 10%) room with a 12:12 hour light/dark cycle with food and water *ad libitum*. All animals were handled according with the current Portuguese Laws and with the European Union Directive (86/609/EEC) on the protection of Animals used for Experimental and other scientific purposes. All efforts were made to minimize animal suffering. Rats were deeply anesthetized with halothane before decapitation and tissue preparation. For functional studies we used the hippocampus, which is a brain area severely affected in AD, and where the effects of BDNF are extensively caracterized. Since Aβ-induced TrkB alterations are similar in cortical and hippocampal cultures [30], we used cortical cultures for the molecular studies in order to increase the culture yeld and reduce the number of animals.

3.5. Primary Neuronal cultures and drug treatments

Neurons were isolated from foetuses of 18-day pregnant females. The foetuses were collected in Hanks' balanced salt solution (HBSS-1) and brains were rapidly removed. The cerebral cortices were isolated and mechanically fragmented. Further tissue digestion was performed with 0.025% (wt/vol) trypsin solution in HBSS without Ca^{2+} and Mg^{2+} (HBSS-2) for 15 min at 37°C. After trypsinization, cells were washed and resuspended in Neurobasal medium supplemented with 0.5mM L-glutamine, 25mM glutamic acid, 2% B-27 and 25U/mL penicillin/streptomycin. Cells were plated at a density of $7x10^4$ cells/cm^2, on 10µg/ml poly-D-lysine-coated dishes, and maintained at 37°C in a humidified atmosphere of 5% CO_2. Incubations with Aβ peptides were performed at 7 or 14 DIV for 24 hours. In the experiments where protease inhibitors were used, the inhibitors were incubated 20 min before Aβ treatment.

3.6. Human brain sample

Frontal cortex from a control case was obtained from the Lille Neurobank, France (Male, 41 years old, *Post-mortem* Interval: 11hours) after scientific committee agreement.

3.7. Western-blot

For neuronal cultures, cells were washed with ice cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4.2H_2O$ and 1.5 mM KH_2PO_4, pH 7.4) and lysed with 1% NP-40 lysis buffer containing (in mM): 50 Tris-HCl (pH 7.5), 150 NaCl, 5 ethylenediamine tetra-acetic acid (EDTA), 2 dithiothreitol (DTT) and protease inhibitors (Roche, Penzberg, Germany). In experiments were rat hippocampi were homogenized, it was used a Radio-Immunoprecipitation Assay (RIPA) buffer containing: 50mM Tris-HCl (pH 7.5), 150mM NaCl, 5mM ethylenediamine tetra-acetic acid (EDTA), 0.1% SDS and 1%Triton X-100 and protease inhibitors cocktail (Roche, Penzberg, Germany). Cell lysates or homogenates were clarified by centrifugation (16.000g, 10min) and the amount of protein in the supernatant was determined by Bio-Rad DC reagent. All samples were applied with same amount of total protein and separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (GE Healthcare, Buckinghamshire, UK). Membranes were stained with Ponceau S solution to check for protein transference efficacy. After blocking with a 5% non-fat dry milk solution in TBS-T (20 mM Tris base, 137 mM NaCl and 0.1% Tween-20), membranes were incubated with the primary (overnight at 4ºC) and secondary antibodies (1hour at room temperature). Finally, immunoreactivity was visualized using ECL chemiluminescence detection system (Amersham-ECL Western Blotting Detection Reagents from GE Healthcare, Buckinghamshire, UK) and bands intensities were quantified by digital densitometry (ImageJ 1.45 software). The intensities of α-tubulin or Ponceau S bands were used as loading control.

The pan-TrkB mouse monoclonal antibody (1:1500), raised against the extracellular domain of human TrkB (aa. 156-322), was purchased from BD Bioscience (Franklin Lakes, NJ, USA) and its specificity was confirmed in Figure 3.3. The C-terminal of Trk-FL rabbit polyclonal antibody (1:2000), raised against the C-terminus (C-14), the αII-spectrin (C-3) mouse monoclonal antibody (1:2500), raised against human αII-spectrin (aa. 2368-2472), and the pan-Caspase-3 (H-277) rabbit polyclonal antibody (1:1000) were purchased from Santa Cruz, inc (CA, USA). The phospho-TrkA (Tyr 490) rabbit polyclonal antibody (1:1500), which detects TrkB receptor when phosphorylated on the corresponding residue (Tyr^{515}), was from Cell Signaling Technology (MA, USA). Phospho-TrkB (Tyr 816) antibody (1:1500) specifically detects TrkB when phosphorylated on Tyr^{816}. Anti-BDNF [ab46176] antibody (1:1000) and α-tubulin rabbit polyclonal antibody (1:5000) were purchased from Abcam (Cambridge, UK). The specificity of anti-BDNF antibody was confirmed (Figure 3.2). The IgG-horseradish peroxidase conjugated secondary antibodies used were goat anti-mouse and goat anti-rabbit (Santa Cruz,

CA, USA). Mouse anti-rabbit IgG light chain specific (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used in immunoprecipitation experiment.

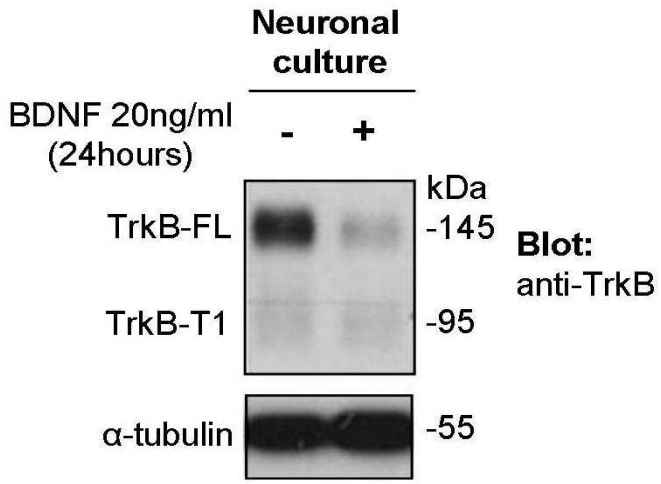


Figure 3.3 – Anti-TrkB antibody specificity

Western-blot probed with the anti-TrkB antibody (raised against extracellular portion of TrkB receptor) using non-treated neuronal cultures (7 days *in-vitro*) [lane 1] or treated with 20ng/mL of BDNF for 24hours [lane 2]. BDNF exposure leads to a selective down-regulation of TrkB-FL receptors without affecting TrkB-T1 levels as described in [347].

3.8. Cell death evaluation

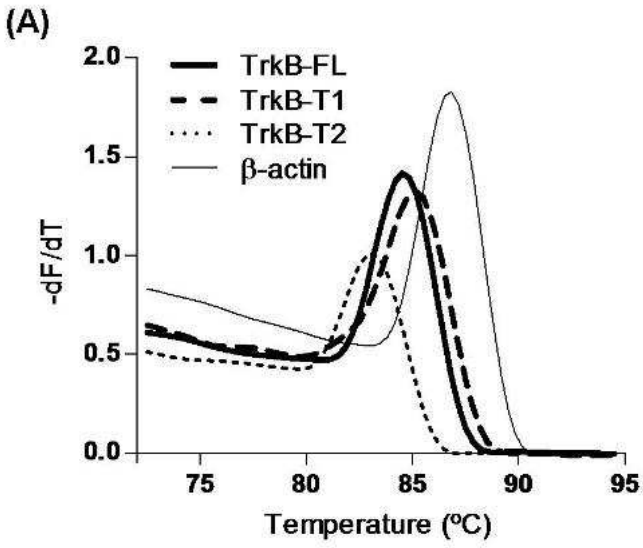
Global cell death in neuronal cultures was evaluated by the lactate dehydrogenase (LDH) assay (Sigma) according to the manufacturer's instructions and using 120 µl of the incubation medium. To specifically evaluate the degree of cell death induced by apoptosis, caspase-3 activation was measured in 50 µg of total protein from cell lysates. General caspase-3-like activity was evaluated by enzymatic cleavage of p-nitroanilide chromophore (pNA) from the substrate N-acetyl-Asp-Glu-Val-Asp (DEVD)-pNA (Sigma). The proteolytic reaction was preceded in lysis buffer containing 50 µM DEVD-pNA. The reaction mixtures were incubated at 37º C for 1 hour, and the release of pNA was determined by measuring absorbance at 405 nm using a 96-well plate reader. Active caspase-3 (17-kDa) levels were also evaluated by western-blot using a pan-caspase3 antibody (*Santa Cruz Biotech.*). Extension of neuronal degeneration was also evaluated by the breakdown of αII-spectrin, a cytoskeletal protein that is cleaved by caspase-3 and calpain.

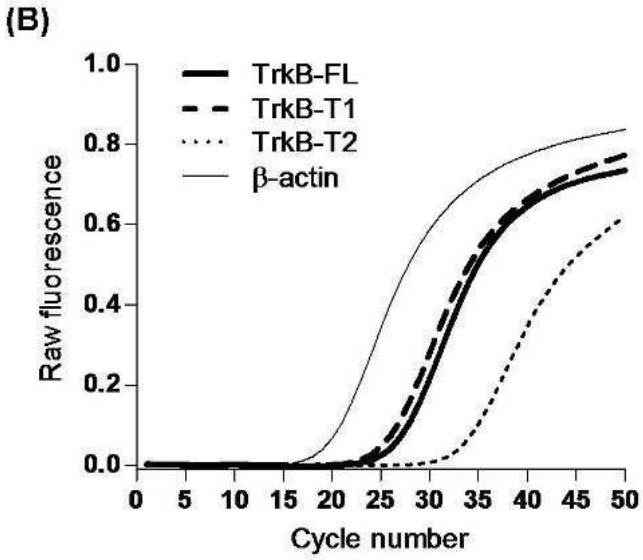
3.9. N-sequencing

Five micrograms of TrkB active (Millipore) were incubated with purified m-calpain (2.5U) and $CaCl_2$ (2mM) in a final volume of 30µl for 30min at 25ºC. The mixture was resolved by SDS-PAGE with 2mM of thioglycolic acid in upper running buffer and transferred to a PVDF membrane. After Ponceau S staining, the band of interest (TrkB-ICD) was cut with a sharp clean blade. The data was provided by the protein sequencing service of Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa. Note that the proline residue is not detected by N-sequencing.

3.10. RNA extraction and qPCR

RNA isolation and qPCR were performed as previously described [348]. Briefly, total RNA was extracted from rat neuronal cultures or hippocampus (GE Healthcare RNAspin Mini RNA Isolation Kit). First-strand cDNA were synthesised from 1µg of total RNA (in 20µL) according to the manufacturer's recommendations (SuperScript First Strand Synthesis Systems for RT-PCR from Invitrogen, NY, USA). cDNA was amplified in Rotor-Gene 6000 real-time rotary analyser thermocycler (Corbett Life Science, Hilden, Germany) in the presence of SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and each specific gene primer (0.2µM for TrkB-FL and TrkB-T2 and 0.5µM for TrkB-T1). Primers specificity was confirmed by melting curves (Figure 3.4A). The threshold cycle (Ct) (Figure 3.4B) and the melting curves required for the relative quantification [349] were acquired with Rotor-Gene 6000 Software 1.7 (Corbett Life Science). β-actin was used as reference internal standard. Replica reactions were always performed. The primers used were: 5´-GTGATGCTGCTTCTGCTCAA-3' and 5´-CCTCCGAAG AAGACGGAGTG-3' for TrkB FL; 5´-TAAGATCCCCCTGGATGGGTAG-3' and 5´-AAGCAGCACTTCCTGGGATA-3' for TrkB T1; 5'-CGGGAGCATCTCTCGGTCT-3' and 5'-TCCACTTAAGAAGCAAAATAAGC-3' for TrkB T2; 5'-AGCCATGTACGTAGC CATCC-3' and 5'-CTCTCAGCTGTGGTGGTGAA-3' for β-actin. The primers for TrkB-T2 were designed using the OligoAnalyzer 3.1 tool, provided by Integrated DNA Technologies (Coralville, IA, USA). The TrkB-T2 mRNA sequence from Rattus norvegicus was obtained from the GenBank sequence database of the National Center for Biotechnology Information (*http://www.ncbi.nlm.nih.gov/*). The primers were synthesized by Invitrogen.





	Ct number
TrkB-FL	24.6 ± 0.3
TrkB-T1	23.5 ± 0.3
TrkB-T2	30.8 ± 1.2
β-actin	18.0 ± 0.3

Figure 3.4 – Melting curves, amplification curves and Ct numbers from qPCR.

(A) Melting curves of TrkB isoforms (FL, T1 and T2) and β-actin transcripts obtained by qPCR in a control 8 DIV culture. The graph represents the first derivate of raw fluorescence plotted against the temperature. The single melting peak obtained for each curve indicates that a single PCR product is being amplified. **(B)** Raw data obtained in a representative qPCR from a 8 DIV control neuronal culture for TrkB-FL, T1, T2 and β-actin transcripts. The graph represents raw fluorescence plotted against cycle number. The table represents the cycle threshold (Ct) values for TrkB-FL, T1, T2 and β-actin (values are mean ± SEM of 8 independent control cultures).

3.11. Immunoprecipitation

Trk-FL receptors were immunoprecipitated from 500µl of neuronal cultures lysates (~1mg total protein) using 2µg of C-terminal Trk-FL (sc14) antibody. After overnight incubation at 4ºC, 30µl of packed G-protein agarose beads were added for 24 hours at 4ºC and then the tube was centrifuged and the supernatant (wash-flow) was collected. The remaining pellet of beads was washed 5 times with lysis buffer and resuspended in 100µL of calpain lysis buffer containing purified m-calpain and $CaCl_2$ (30min at 25ºC, as described above). The reactions were boiled at 95ºC in the presence of denaturing SDS-sample buffer.

3.12. Calpain in-vitro digestion

In calpain digestion assays, the cultured cells or brain tissue were homogenized on ice in 1% NP-40 buffer containing in (mM): 50 Tris-HCl (pH 7.5), 150 NaCl, 0.1 EDTA, 2 DTT, 1 phenylmethylsulfonyl fluoride (PMSF) and Aprotinin 5µg/ml. The homogenates were clarified by centrifugation (16.000g, 10min) and protein concentration was determined. In exogenous calpain digestion assays, the purified rat m-calpain (Calbiochem) was incubated for 30min at 25ºC in a 100µl final volume of lysis buffer containing 100µg of homogenate protein and 2mM of $CaCl_2$ (unless stated otherwise). In endogenous calpain digestion assays, 5mM of $CaCl_2$ and/or MDL28170 were added to the homogenates for 4 hours at 25ºC. In calpain digestion assays (exogenous or endogenous), each condition have the same amount of protein and total volume. For endogenous calpain activation in synaptosomes, $CaCl_2$ and/or MDL28170 (20µM) were added to the intact isolated synaptosomes suspended in KHR buffer, for 30min at 37ºC. All reactions were stopped by boiling the samples at 95ºC in the presence of the denaturing SDS-sample buffer.

3.13. Acutely prepared hippocampal slices

Male Wistar rats (8-10 weeks old) were deeply anesthetized with halothane before decapitation. The brain was quickly removed into ice-cold continuously oxygenated (O_2/CO_2: 95%/5%) artificial cerebrospinal fluid (aCSF) (124 mM NaCl, 3 mM KCl, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgSO_4$ and 10 mM glucose, pH 7.40) and the hippocampi were dissected out. The hippocampal slices were cut perpendicularly to the long axis of the hippocampus (400µm thick) and were allowed to recover functionally and energetically for at least 1 hour in a resting chamber filled with continuously oxygenated aCSF,

at room temperature (22–25°C). In the experiments were the effects of KW-6002 were tested, the slices were used for electrophysiology recordings after the resting period. In the experiments where the effects of Aβ were tested, the slices were then incubated for 3 hours (the minimum time required to observe initial Aβ-induced changes in TrkB receptor) with oxygenated aCSF (control), or with aCSF containing $A\beta_{25-35}$ peptide (25μM) or with aCSF containing $A\beta_{25-35}$ and MDL28170 (calpain inhibitor, 20μM), or with $A\beta_{1-42}$ (20μM), or with the inverted $A\beta_{35-25}$. After the incubation period the slices were used for electrophysiology recordings or for synaptosomal isolation to evaluate GABA and glutamate release.

3.14. Isolation of synaptosomes

Hippocampal slices were homogenized in ice-cold isosmotic sucrose solution (0.32 M, containing 1mM EDTA, 1 mg/ml bovine serum albumin, and 10 mM HEPES, pH 7.4), and centrifuged at 3,000*g* for 10 min; the supernatant was centrifuged again at 14,000*g* for 12 min. The whole procedure was conducted at 4°C. The pellet was resuspended in 45% Percoll in KHR solution consisting of (in mM) NaCl 140, EDTA 1, HEPES 10, KCl 5, and glucose 5, and was centrifuged at 14,000g for 2 min. The synaptosomal fraction corresponds to the top buoyant layer and was collected from the tube. Percoll was removed by two washes with a KHR solution; synaptosomes were then kept on ice and used within 3 hours.

3.15. [^{3}H] Neurotransmitter release from hippocampal synaptosomes

The [^{3}H]GABA release experiments were performed as previously [350]. For each experiment, synaptosomes were prepared from approximately 60 hippocampal slices (30 per condition) from 6 hippocampi of 3 animals. Synaptosomes (protein concentration 1-2mg/ml) were resuspended in 2ml of oxygenated Krebs medium (in mM: NaCl 125, KCl 3, NaH_2PO_4 1, glucose 10 $NaHCO_3$ 25, $CaCl_2$ 1.5 and $MgSO_4$ 1.2) and allowed to equilibrate for 5 min at 37°C. From this time onward, all solutions applied to the synaptosomes were kept at 37°C and continuously gassed with O_2/CO_2 (95%/5%). Aminooxyacetic acid (AOAA, 0.1 mM) was present in all solutions up the end of the experiments to prevent GABA catabolism by inhibition of GABA transaminase. The synaptosomes were loaded for 20 min at 37ºC, with [^{3}H]GABA (1.5 μCi/ml, 18.5 nM), together with 0.625 μM unlabelled GABA to decrease specific activity of the [^{3}H]GABA solutions to 2.3 μCi/nmol) and equally layered onto perfusion chambers over Whatman GF/C filters (flow rate, 0.8 ml/min; chamber volume, 90 μl).

The [^{3}H]glutamate release assays were performed as routinely in our laboratory [350]. All procedures were similar to [^{3}H]GABA release experiments with the necessary modifications. Synaptosomes were loaded with 0.2 µM [^{3}H]glutamate (specific activity was 30–60 Ci/mmol) for 5 min and equally layered onto perfusion chambers over Whatman GF/C filters (flow rate, 0.6 ml/min; chamber volume, 90µl).

After a 20 min washout period, the effluent was collected for 40 min in 2 min intervals. The GABA or glutamate release from synaptosomes was stimulated during 2 min with a high-K^+ solution (15 mM, isomolar substitution of Na^+ with K^+ in the perfusion buffer) at the 5th [first stimulation period (S_1)] and 29th [second stimulation period (S_2)] minute after starting sample collection. BDNF (30ng/ml) was added to the superfusion medium at the 9th minute, therefore before S2, and remained in the bath up to the end of the experiments, and its effect was quantified as percentage changes of the S_2/S_1 ratio compared with the S_2/S_1 ratio in the absence of BDNF in the same synaptosomal batch and under similar drug conditions. Thus BDNF effect upon S_2/S_1 ratio was determined from synaptosomes prepared from slices incubated without any drug, incubated with Aβ or incubated with both Aβ and MDL28170.

3.16. Calculation of drug effects on GABA and glutamate release

At the end of each experiment, aliquots (500 µl) of each sample as well as the filters from each superfusion chamber were analysed by liquid scintillation counting. The fractional release was expressed in terms of the percentage of total radioactivity present in the preparation at the beginning of the collection of each sample. The amount of radioactivity released by each pulse of K^+ (S1 and S2) was calculated by integration of the area of the peak upon subtraction of the estimated basal tritium release. In each experiment, two synaptosome-loaded chambers were used as control chambers, the others being used as test chambers. In the test chambers, the test drug was added to the perfusion solution before S2 and the S2/S1 ratios in control and test conditions were calculated. The effect of the drug on the K^+-evoked tritium release was expressed as percentage of change of the S2/ S1 ratios in test conditions compared to the S2/S1 ratios in control conditions, in the same experiments (i.e., with the same pool of synaptosomes).

3.17. KW-6002 treatment

KW-6002 was synthesized according to described procedures [351]. The drug and the dose were selected according to described in [352]. Four to six week-old male rats were treated with an orally active $A_{2A}R$ antagonist, istradefylline (KW-6002), 3 mg/kg/day for 30 days in

drinking water available *ad libitum* in light protected bottles. The vehicle (control) group were treated only with vehicle (0.025% methylcellulose in water) as described before [353]. The weight of the animals and the volume intake were assessed twice a week in both control and treated animals and the concentration of the solution adjusted so that the drug intake was constant. No differences in body weight or water intake were detected between the KW-6002 and vehicle group. The animals were sacrificed either immediately or after 24 h of KW-6002 withdrawal.

3.18. Ex-vivo electrophysiology recordings

Long-term potentiation (LTP) induction and quantification were performed as described previously [114]. Briefly, the hippocampal slices from 8-10 week-old Wistar rats were transferred to a recording chamber continuously superfused with oxygenated aCSF at 32°C (flow rate of 3ml/min in open system). The stimulation pulses were delivered every 10s alternately to two independent pathways through electrodes placed on Shaffer collateral/commissural fibres in stratum radiatum, and the fEPSPs were recorded in stratum radiatum of CA1 area (Figure 3.5A). LTP was induced by theta-burst protocol consisting of four trains of 100Hz, 4 stimuli, separated by 200ms (Figure 3.5B). We used theta-burst stimulation to induce LTP, since this pattern of stimulation is considered closer to what occurs physiologically in the hippocampus during episodes of learning and memory in living animals [354]. Furthermore, the facilitatory action of BDNF upon LTP is mostly seen under θ-burst [72]. In addition, we previously showed that the effect of BDNF upon CA1 LTP is more evident under weak (as the used in this work) than under strong θ-burst paradigms [113]. Therefore, we selected the optimal stimulation paradigm to observe an effect of BDNF upon LTP, so that we could evaluate the influence of Aβ upon the effect of BDNF.

One hour after LTP induction in one group of synapses, BDNF (20ng/ml) was added to the superfusion solution and LTP was induced in the other group of synapses, no less than 20min after BDNF perfusion. In experiments where CGS21680 was used, it was applied 15min before BDNF (which corresponds to 35min prior to LTP induction). Whenever an increase on the slope of fEPSP was detected in the presence of BDNF, the intensity of stimulation was adjusted before LTP induction for similar values recorded before BDNF application. LTP was quantified as percentual change in the average slope of the fEPSP taken from 46-60min after LTP induction in relation to the average slope of the fEPSP measured during the 14min before the induction of LTP. The effect of BDNF upon LTP was evaluated by comparing the magnitude of LTP in the first pathway in the absence of BDNF (control pathway), with the magnitude of LTP in the second pathway in the presence of BDNF (test pathway). The

independence of the two pathways was tested in the end of experiments by studying the pair-pulse facilitation (PPF) across both pathways, less than 10% facilitation being usually observed. In the absence of drugs, the LTP magnitude was similar in both independent pathways (Figure 3.5C).

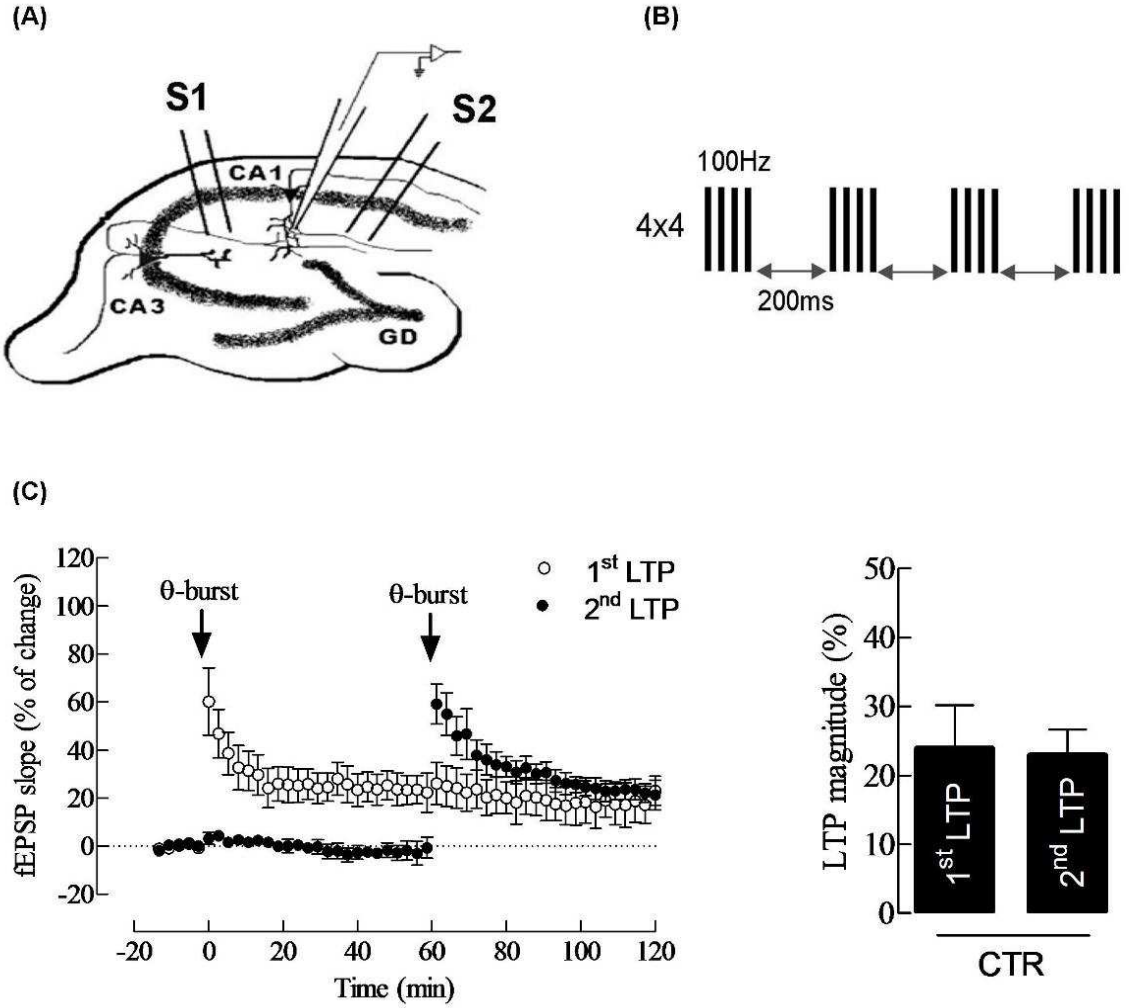


Figure 3.5 – Electrophysiology recording configuration and LTP magnitude on both pathways.
(A) Schematic representation of a hippocampal transverse slice showing the recording configuration used to obtain extracellular responses in the CA1 dendritic layer (stratum radiatum) evoked by stimulation of two separate sets of the Schaffer pathway (S1 and S2). **(B)** Schematic representation of the θ-burst stimulus paradigm used to induce LTP. (**C**) Left: Averaged time course changes in fEPSP slope on both independent pathways upon sequential θ-Burst stimulation in the first pathway (○) and 60 min after in the second pathway (●) in rat hippocampal slices (n=5). Right: LTP magnitude (change in fEPSP slope at 46-60 minutes after θ-Burst stimulation) in relation to pre-θ-Burst values (0%) (n=5).

3.19. Input / Output curves

Input/output curves were performed after a stable baseline of at least 15 min. The stimulus delivered to the slice was decreased until no fEPSPs evoked and subsequently increased by steps of 20 μA. Data from three consecutive fEPSPs were collected for each stimulation intensity. The range of all input delivered to the slice was typically from 60 μA to a supramaximum stimulation amplitude of 360 μA. The input/output curve was plotted as the relationship of fEPSP slope versus stimulus intensity.

3.20. Statistical analysis

The data are expressed as mean ± SEM of the *n* number of independent experiments. The significance of differences between the means of two conditions was evaluated by Student's t-test. To perform multiple comparisons between the means of more than two conditions a one-way ANOVA followed by a Bonferroni post-test was performed. To perform comparisons on LTP magnitude in the presence or absence of BDNF between different slice treatments, a two-way ANOVA followed by a Bonferroni post-test was performed. Values of $p<0.05$ were considered to represent statistically significant differences. *Prism GraphPad* software was used for statistical analysis.

4. Dysregulation of TrkB receptors and BDNF function by Aβ peptide is mediated by calpain

The work presented in subchapter 4.3 was published in Kemppainen et al., 2012 [30].
The remaining work described in chapter 4 was published in Jerónimo-Santos et al., 2014b [346].
The experiments described in subchapter 4.8 were performed by S. Vaz, S. Parreira and S.Rapaz-Lerias.

4.1. Summary

Brain-derived neurotrophic factor (BDNF) and its high-affinity full-length receptor, TrkB-FL, play a central role in the nervous system by providing trophic support to neurons and regulating synaptic plasticity and memory. TrkB and BDNF signalling are impaired in Alzheimer's disease (AD), a neurodegenerative disease involving accumulation of amyloid-β (Aβ) peptide. In the present study, we found that 1) Aβ selectively increases mRNA and protein levels of truncated TrkB isoforms, and strongly decreases TrkB-FL protein levels without affecting its mRNA levels; 2) Aβ induces a calpain-mediated cleavage on TrkB-FL receptors, downstream of Shc binding site, originating a new truncated TrkB receptor (TrkB-T') and an intracellular fragment (TrkB-ICD), which is also detected in *post-mortem* human brain samples; 3) Aβ impairs BDNF function in a calpain-dependent way, as assessed by the inability of BDNF to modulate neurotransmitter (GABA and glutamate) release from hippocampal nerve terminals, and long-term potentiation (LTP) in hippocampal slices. It is concluded that Aβ-induced calpain activation leads to TrkB cleavage and impairment of BDNF neuromodulatory actions.

4.2. Rational

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that promotes neuronal survival, differentiation and synaptic plasticity through activation of its full-length receptor, TrkB-FL. Besides encoding for this receptor, the TrkB gene (*NTRK2*) also encodes for truncated isoforms [355], which may act as negative modulators of TrkB-FL signalling [25, 29]. Both decreases in the ratio between full-length (FL) and truncated (Tc) receptors and reduced BDNF signaling have been detected in several neurodegenerative disorders. Particularly, hippocampal and cortical *post-mortem* samples from AD patients revealed a decrease in both BDNF and TrkB-FL and an increase in TrkB-Tc levels [319, 321-323, 333]. These changes are thought to

be involved in spatial memory impairments and, accordingly, the activation or overexpression of TrkB-FL has been associated to spatial memory improvements [30, 316, 356].

AD is characterized not only by the accumulation of intracellular neurofibrillary tangles made of hyperphosphorylated tau proteins, but also of extracellular plaques composed by amyloid-β peptides (Aβ). Aβ plaques are largely composed by $A\beta_{40}$ and $A\beta_{42}$, but also by Aβ fragments including the $A\beta_{25–35}$ [357], which has been proposed to be the active region of the full-length Aβ peptide responsible for its neurotoxic effects [242]. Mechanisms underlying the neurotoxic actions of Aβ peptides are not fully understood, but the existing data suggests that oxidative stress, perturbation of Ca^{2+} homeostasis, mitochondrial dysfunction, synaptic loss and caspases and calpains activation are strongly involved [253-256]. Calpains are Ca^{2+}-dependent proteases that play a physiologic role by the cleavage of several substrates, changing their function or localization. Abnormal activation of calpains and downregulation of its endogenous inhibitor (calpastatin) have been linked to AD [194, 358, 359]. In addition, calpain overactivation contributes to tau hyperphosphorylation, a hallmark of AD, through the activation of cyclin-dependent kinase 5 (CDK5), followed by the cleavage of its regulatory protein – p35 [282, 360, 361]. Moreover, calpain also contributes to the formation and accumulation of Aβ peptides and its inhibition prevents neurodegeneration and restores normal synaptic function and spatial memory in AD animal models [201, 202, 362].

4.3. Aβ increases truncated TrkB protein levels

Since in AD brain decreased TrkB-FL and increased truncated TrkB receptor levels have been reported, we hypothesized that amyloid-beta peptide (Aβ), by itself, could induce similar changes in TrkB receptor isoforms. To test this hypothesis, cortical and hippocampal cells were cultured for 7 DIV and treated with Aβ peptides and TrkB receptor immunoreactivity was evaluated by western-blotting. Incubation of cortical cells with $A\beta_{25-35}$ (25µM) induced a dramatic increase in truncated TrkB receptor levels compared to control cells (100 ± 2.3 % vs. 223 ± 19.9 %; n = 8, $p<0.01$, Student's-test, Figure 4.1A), whereas TrkB-FL receptor levels decreased (100 ± 1.7% vs. 61 ± 6.3%; n=8, $p<0.01$, Student's-test, Figure 4.1A). The same pattern of alteration in TrkB receptor isoforms was also observed in hippocampal cultures (Figure 4.1A). The effects on TrkB receptors upon $A\beta_{25-35}$ exposure were time and concentration-dependent (Figure 4.1B and C), so that longer incubation times with Aβ or higher concentrations of Aβ produced a more robust effect, with increasing changes on TrkB isoforms levels.

Since glial cells are enriched in truncated TrkB.T1 isoform [37], we tested the effect of the $A\beta_{25-35}$ peptide on truncated TrkB in neuronal cultures previously treated with the antimitotic drug 5-Fluorouracil (5-FU). In spite of the marked reduction in the astrocytic marker, the glial fibrillary acidic protein (GFAP), observed in the cultures treated with 5-FU (Figure 4.2A, lower left panel), Aβ25-35 treatment still increased truncated TrkB receptor levels in a similar magnitude as that observed in cells from the same culture but not treated with 5-FU (Figure 4.2A, right panel). In cortical cultures (7 DIV) treated with the most frequent Aβ peptide in AD, the Aβ1-42 (10-20µM for 24h), also caused a concentration-dependent increase (≈28% for 20µM) of the truncated TrkB and a concentration-dependent decrease (≈40% for 20µM) in TrkB-FL levels (Figure 4.2B). We can therefore conclude that in primary neuronal cultures the Aβ peptide simultaneously increases the levels of truncated TrkB receptors and decreases levels of full-length TrkB receptors, producing a similar pattern of alterations as reported in the brain of AD patients.

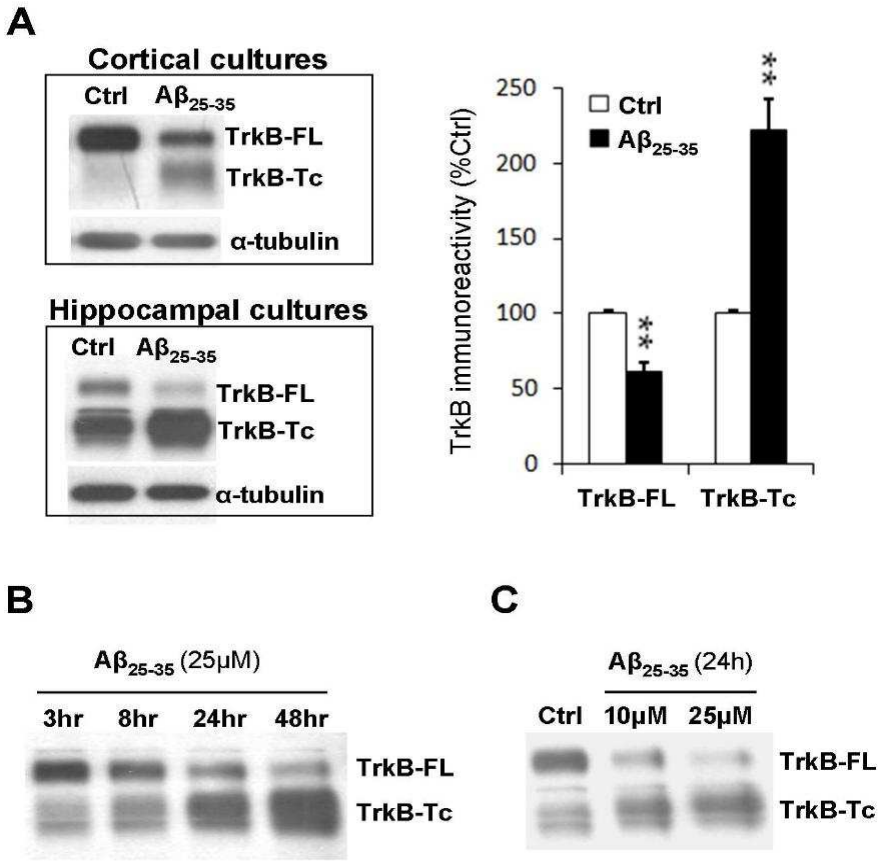


Figure 4.1 – Effect of Aβ 25-35 upon truncated and full-length TrkB receptors protein levels.

(A) Primary cultures of cortical cells (upper left panel) and hippocampal cells (lower left panel) were incubated at 7 DIV with $A\beta_{25-35}$ (25μM) for 24h and levels of full-length (TrkB-FL) and truncated TrkB (TrkB-Tc) were determined by western-blotting. Average data from 8 independent cortical cultures is shown in right panel (**p<0.01 compared to control (Ctrl), Student's *t*-test). **(B)** Time-dependent changes in TrkB-FL and TrkB-Tc densities after 3, 8, 24 and 48 hours of incubation of 7 DIV cortical cultures with $A\beta_{25-35}$ (25μM). **(C)** Dose-dependent changes in TrkB-FL and TrkB-Tc levels after 24 hours of incubation of 7 DIV cortical cultures with $A\beta_{25-35}$.

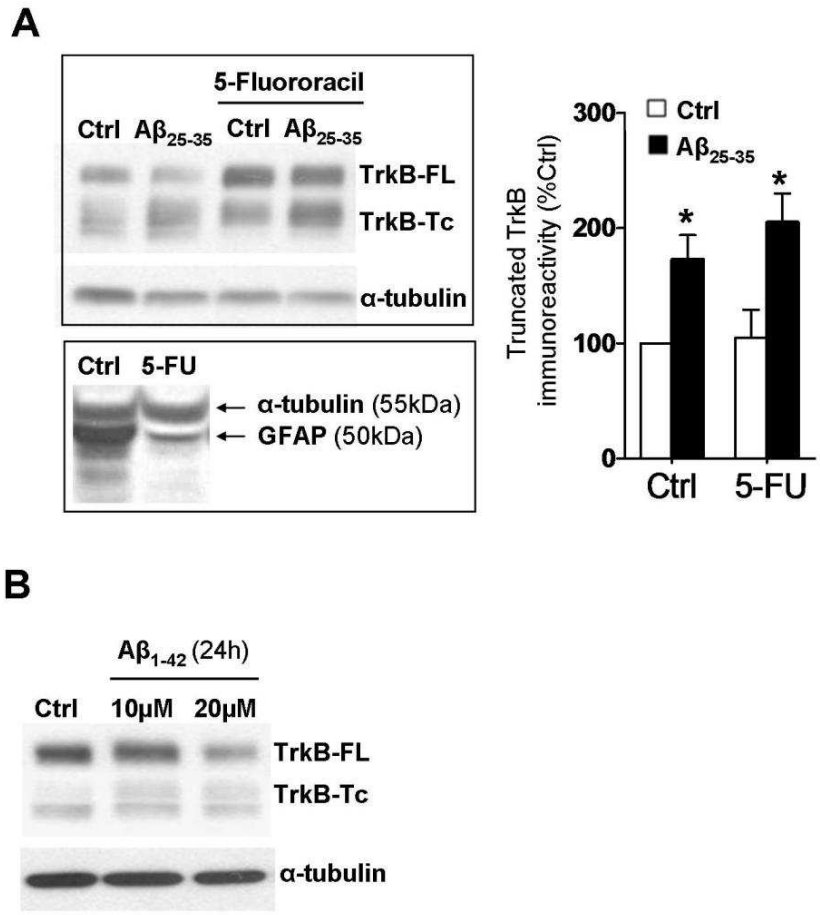


Figure 4.2 – Effect of glial cells upon Aβ-induced changes in TrkB isoforms levels.

(A) Comparison of the influence of $A\beta_{25-35}$ (25μM) exposure in non-treated cortical cultures and in cultures treated with 5-Fluorouracil (5-FU) to markedly decrease glial cells number as confirmed by glial fibrillary acidic protein levels (GFAP). Average effect of $A\beta_{25-35}$ (25μM) on truncated TrkB levels in 3 independent cultures both conditions were shown in left graph (*$p<0.05$ as compared with non-treated control, Student's *t*-test). **(B)** Dose-dependent changes on TrkB-FL and TrkB-Tc levels on 7 DIV cortical cultures exposed for 24 hours to $A\beta_{1-42}$ peptide.

Moderate cell death is expected to occur after Aβ peptide incubation. Indeed, 24h after incubating the neurons with $A\beta_{25-35}$ (25μM) there was an increase (21±5%, $p<0.05$, n=6) in activity of lactate dehydrogenase (LDH), a soluble cytosolic enzyme that is released following loss of membrane integrity resulting from either apoptosis or necrosis [363], into incubation medium. The intracellular caspase-3 activity, a central mediator of apoptotic cell death, was even more markedly increased (3 fold increase, Figure 4.3B, $p<0.01$, n=4). The cell-permeable pan-caspase inhibitor, Z-VAD(OMe)-FMK, which is known to inhibit apoptotic cellular death [364], fully blocked the $A\beta_{25-35}$-induced activation of caspase-3 (Figure 4.3B, $p<0.01$, n=4).

However, the pan-caspase inhibitor failed to influence the $A\beta_{25-35}$-induced increase in TrkB-Tc and decrease in TrkB-FL levels (Figure 4.3C and D, $p<0.05$, n=4). These results indicate that Aβ-induced alterations on TrkB receptors isoforms are not a direct consequence of apoptotic cellular death.

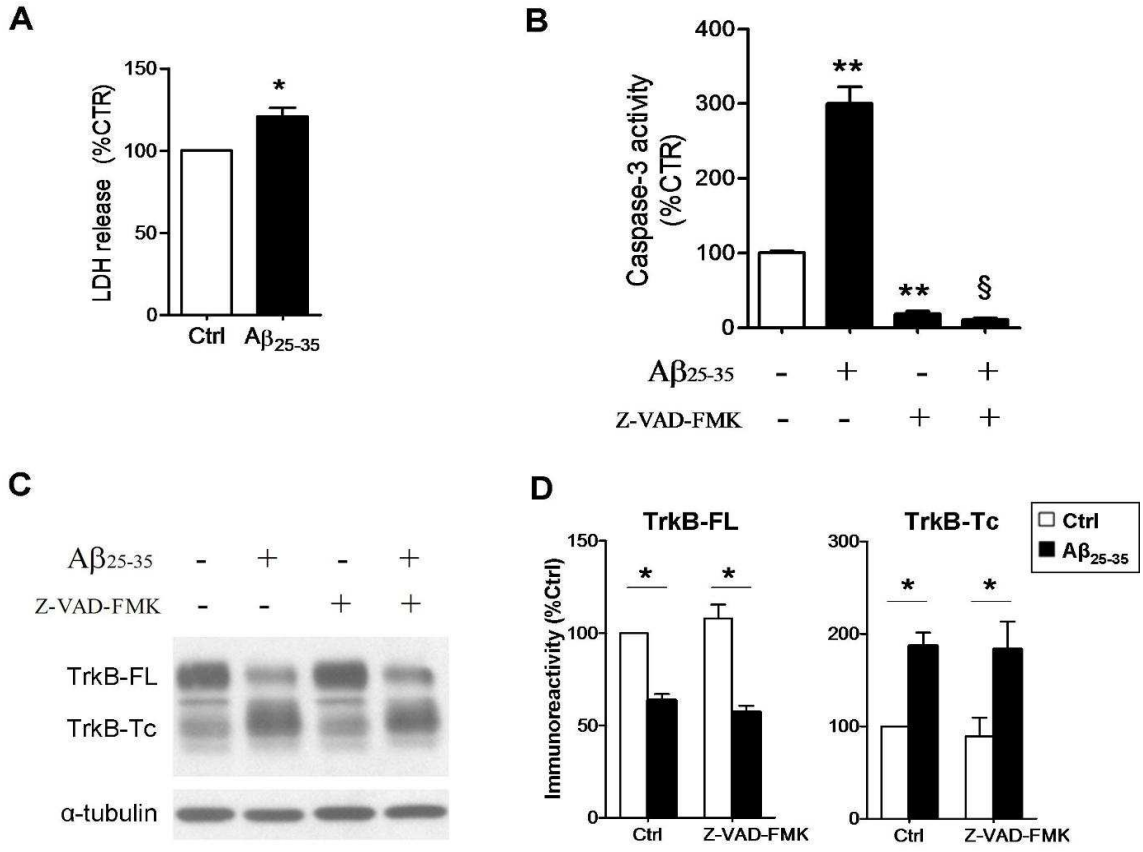


Figure 4.3 – Effect of caspases inhibition upon Aβ-induced changes in TrkB isoforms levels.

(A) Primary cultures of cortical cells were incubated at 7 DIV with $A\beta_{25-35}$ (25μM) for 24h and levels of released lactate dehydrogenase (LDH) were measured. Average data from 6 independent cortical cultures is represented (*$p<0.05$ compared to control (Ctrl), Student's *t*-test). (B) Caspase3-like activity of cell lysates measured after 24hours of $A\beta_{25-35}$ (25μM) incubation of 7 DIV cortical cultures, in the presence and absence of the pan-caspase inhibitor Z-VAD(OMe)-FMK (20μM). (**$p<0.01$ compared to control (Ctrl); §$p<0.01$ compared to $A\beta_{25-35}$ alone; ANOVA with Bonferroni's correction). (C) Representative western-blot image of TrkB-FL and TrkB-Tc levels detected in neuronal cultures from 7 DIV with $A\beta_{25-35}$ (25μM) in presence or absence of Z-VAD(OMe)-FMK (20μM). (D) Average data from densitometry quantification of TrkB-FL (left) and TrkB-Tc (right) immunoreactivity (C) of 4 independent cortical cultures (*$p<0.05$, Student's *t*-test).

4.4. Aβ up-regulates TrkB-T1 mRNA levels

To evaluate the effects of Aβ upon TrkB receptors expression, we determined the mRNA levels of the main TrkB isoforms produced by alternative splicing (FL, T1 and T2). qPCR data showed that neuronal cultures incubated with $A\beta_{25-35}$ (25µM) for 24 hours displayed a significant increase of truncated TrkB-T1 (45 ± 19%, n=8, $p<0.05$, Figure 4.4) and truncated TrkB-T2 (58 ± 17%, n=8, $p<0.05$, Figure 4.4) mRNA levels as compared to non-treated control cultures. Conversely, no significant change of TrkB-FL mRNA levels was detected upon $A\beta_{25-35}$ incubation (Figure 4.4, n=8). As shown in Figure 3.4B, the TrkB-FL and TrkB-T1 are the main TrkB isoforms present in the cultures, while TrkB-T2 was expressed at lower levels. Thus, only TrkB-T1 will be mentioned henceforward, since it is the main spliced truncated isoform.

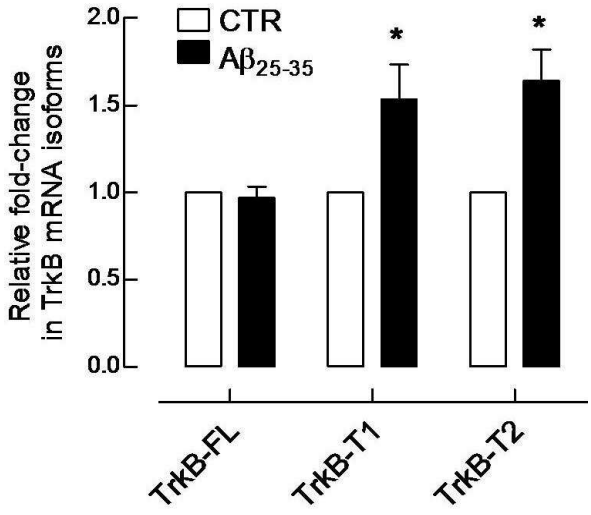


Figure 4.4 – Aβ peptide up-regulates mRNA levels of truncated TrkB-T1 and T2.

(A) Analysis of mRNA levels by relative qPCR of TrkB full-length (TrkB-FL) and truncated isoforms (TrkB-T1 and TrkB-T2) on 8 DIV cortical cultures treated with (black bars) or without (white bars-CTR) $A\beta_{25-35}$ (25µM) for 24hours. β-actin was used as an internal loading control. *$p<0.05$ comparing to control (CTR) of the respective isoform (n=8, student's *t*-test). Values presented are mean ± SEM.

4.5. Aβ induces a cleavage on TrkB-FL receptors

Although the above results clearly show that TrkB-FL mRNA levels were not significantly affected by $A\beta_{25-35}$, a strong decrease of 40 ± 5% in TrkB-FL protein levels was observed in $A\beta_{25-35}$-treated cells when compared to control (Figure 4.5A, n=10, $p<0.01$), as we previously observed for both $A\beta_{25-35}$ and $A\beta_{1-42}$ (Figure 4.1) [30]. Thus, in order to assess whether Aβ could also promote TrkB-FL cleavage, an antibody recognizing the intracellular C-terminal of TrkB-FL was used to detect a possible product of such cleavage. The results show that the decrease on TrkB-FL receptors, in $A\beta_{25-35}$-treated cultures, is concomitant with the formation of a ~32kDa band (Figure 4.5A, n=10, $p<0.01$ compared to control), indicating that Aβ induces a cleavage of TrkB-FL receptor, whereby it generates an intracellular domain (ICD) fragment (designated for now on as TrkB-ICD). Moreover, in cells treated with the full-length $A\beta_{1-42}$ (20 µM) there is also an increase in the formation of TrkB-ICD (Figure 4.6C).

Given that the cytosolic domain of rat TrkB-FL (starting at Lys^{454} until Gly^{821}) has a predicted molecular weight of 41.6 kDa, and since TrkB-ICD fragment migrates in SDS-PAGE with a relative molecular weight of ~32kDa, the cleavage site might be located ~10kDa downstream the transmembrane domain of the receptor. We thus anticipated that the Aβ-induced cleavage would lead to the generation of a new membrane-bound truncated TrkB receptor ~10kDa heavier than the natural truncated TrkB-T1 (which lacks the whole intracellular domain). To directly evaluate this possibility, we used a pan-TrkB antibody that recognizes an extracellular epitope and we increased the separation in SDS-PAGE electrophoresis, which allowed us to identify two distinct truncated TrkB bands: one broad band at ~90kDa corresponding to the natural truncated TrkB-T1 receptor, and another broad band around ~100kDa band corresponding to the new truncated receptor produced by the cleavage of TrkB-FL (henceforth designated as TrkB-T', Figure 4.5B). The TrkB-T' levels were very low in control neuronal cultures (Figure 4.5B and Figure 4.7C), being also negligible in control rat brain homogenates (Figure 4.7E), indicating that this fragment is only formed under conditions that trigger robust cleavage of TrkB-FL receptor.

Taken together, the data show that Aβ induces a selective up-regulation of truncated TrkB-T1 and T2 transcripts, while it simultaneously promotes TrkB-FL protein cleavage, thus producing a new truncated receptor (TrkB-T') and an intracellular fragment containing the C-terminal of the receptor (TrkB-ICD). The natural truncated receptors produced by alternative splicing (TrkB-T1 and -T2) and the cleavage-generated truncated receptor (TrkB-T'), all contribute to the total pool of truncated TrkB receptors, which will be referred in this work as TrkB-Tc.

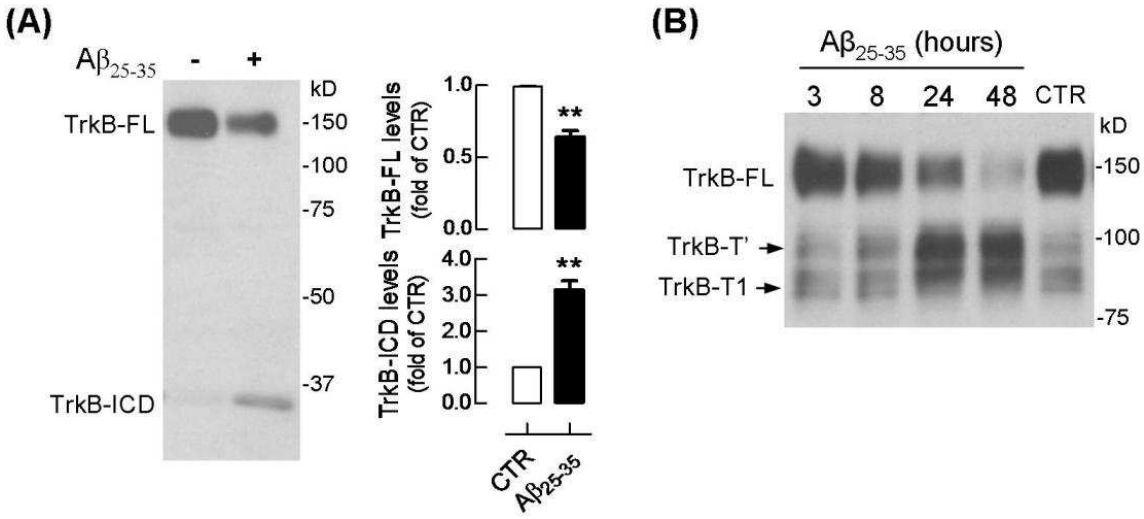


Figure 4.5 – Aβ peptide induces a TrkB-FL receptor cleavage.

(A) In left panel is a representative western-blot of 8 DIV neuronal cultures showing TrkB-FL receptor levels and the TrkB cleavage fragment (TrkB-ICD: ~32 kDa) after 24hours of $A\beta_{25-35}$ incubation. The primary antibody used recognizes the C-terminal of Trk-FL. Right panels show the average band intensity of TrkB-FL (upper histogram) and TrkB-ICD (lower histogram). **p<0.01 comparing to control (n=10, student's *t*-test). **(B)** Western-blot using a pan-TrkB antibody (extracellular TrkB epitope), which recognizes simultaneously the full-length (~145 kDa) and the truncated TrkB species: TrkB-T' (TrkB-FL cleavage product, ~100 kDa) and TrkB-T1 (natural truncated TrkB originated by alternative splicing, ~90 kDa). These bands were detected in neuronal cultures extracts prepared after the exposure to $A\beta_{25-35}$ (25μM) for 3, 8, 24 and 48 hours or from control cultures (CTR) as indicated above each lane. All values presented are mean ± SEM.

4.6. Calpain mediates the Aβ-induced TrkB-FL cleavage

The next series of experiments were designed to identify the enzyme involved in the cleavage of TrkB-FL by Aβ. The cell-permeable thiol proteases inhibitor, E-64d, caused a concentration-dependent inhibition of the TrkB-FL cleavage induced by $A\beta_{25-35}$, with a maximal effect achieved at the concentration of 100μM (Figure 4.6A). To identify which thiol proteases were involved in the cleavage, several inhibitors were tested, including the inhibitors of proteases with calpain-like activity, *N*-acetyl-Leu-Leu-norleucinal (ALLN 20μM), *N*-acetyl-Leu-Leu-methional (MG132 2μM) and MDL28170 (20μM), and the cell permeable pan-caspases inhibitor (z-VAD-FMK 20μM). In addition, a potent aspartyl proteases inhibitor, pepstatin A (1μM) was also tested. Neither the caspase inhibitor zVAD-FMK, nor pepstatin A,

mitigated Aβ-induced cleavage of TrkB-FL receptors (Figure 4.6B). Conversely, the inhibitors of calpain-like activity ALLN, MG132 and MDL28170 significantly prevented Aβ-induced cleavage of TrkB-FL and the subsequent formation of TrkB-T' and TrkB-ICD fragments (Figure 4.6B, n=5, $p<0.01$).

In parallel, we observed that the exposure of neuronal cultures to $A\beta_{25-35}$ resulted in a strong activation of calpain, as confirmed by the 6-fold increase (Figure 4.6C, n=4, $p<0.001$) in the formation of the calpain-specific αII-Spectrin breakdown products (SBDP 150/145kDa), a standard assay for monitoring calpain activity. The $A\beta_{1-42}$ peptide (20μM) also induced robust calpain activation on neuronal cultures (see Figure 4.6C).

Since calpains are calcium-dependent proteases, we next evaluated whether the activation of endogenous calpains by Ca^{2+} would induce TrkB-FL cleavage by itself, in the absence of Aβ. Therefore, cell lysates of neuronal cultures were incubated with 5mM of CaCl2 for 4 hours at 25ºC and, in these conditions, the characteristic TrkB-ICD band was detected, an effect fully blocked by the calpain inhibitor MDL28170 (Figure 4.7A, n=3, $p<0.05$). The cleavage of TrkB-FL by endogenous calpains was also detected in isolated nerve terminals (synaptosomes) prepared from adult rat hippocampus (Figure 4.7B). In addition, the cleavage of TrkB-FL and subsequent production of TrkB fragments was observed following the incubation of neuronal cell lysates, or cortical homogenates from adult rat, with purified recombinant rat m-calpain in presence of 2mM Ca^{2+} (~100kDa TrkB-T' and ~32kDa TrkB-ICD; Figure 4.7C). To confirm the specificity of the fragments detected, the full-length TrkB receptors were immunopurified from neuronal cultures and incubated with purified rat m-calpain. The results showed that rat m-calpain cleaved the immunopurified receptors producing the characteristic TrkB-T' and TrkB-ICD fragments, allowing to conclude that the fragments detected arise specifically from TrkB-FL receptors (Figure 4.7D).

Human TrkB-FL and rat TrkB-FL share the same amino-acid sequence in the region of the calpain-cleavage site (Fig.3B). Thus, it is expected that human TrkB-FL could also be cleaved by human calpains. Indeed, basal levels of TrkB-ICD were detected in a parietal cortex homogenate from a human control case (Figure 4.7E). Addition of purified m-calpain completely cleaved human TrkB-FL, further enhancing the levels of TrkB-ICD fragment (Figure 4.7E). This result clearly showed that human TrkB-FL is also prone to be cleaved by calpains, leading to the production of TrkB-ICD. Contrary to the toxic $A\beta_{25-35}$, the reverse peptide $A\beta_{35-25}$ (25μM) did not induce calpain activation and TrkB cleavage on neuronal cultures (Figure 4.8A and B, n=3).

As previously shown, Aβ selectively up-regulates TrkB-T1 mRNA levels (Figure 4.4). However, in opposition to what was observed for protein levels, the inhibition of calpains by MDL28170 did not affect the $A\beta_{25}$-35-induced changes upon mRNA levels of TrkB receptors (Figure 4.8, n=4).

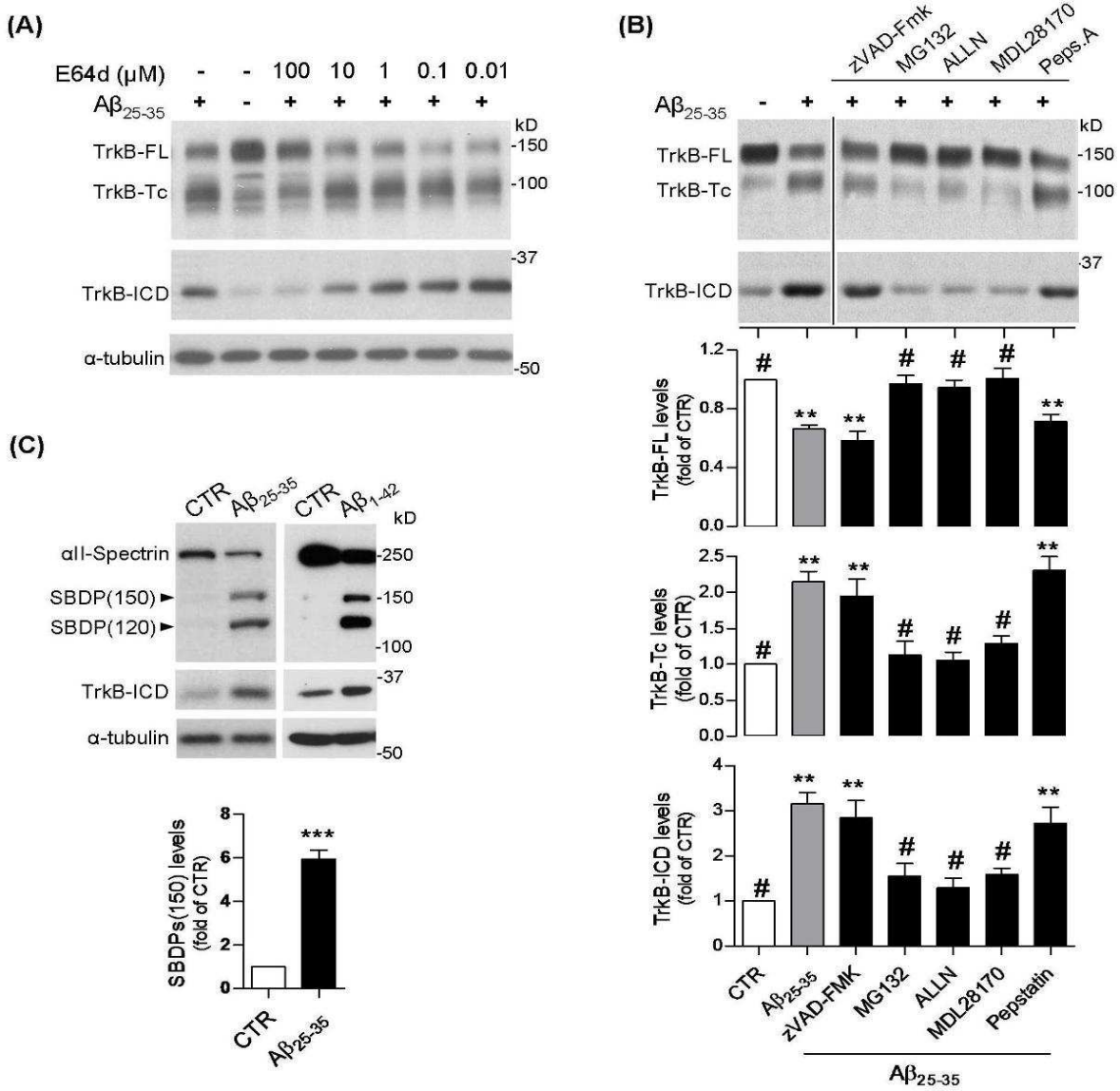

(A)
E64d (μM) - - 100 10 1 0.1 0.01
Aβ25-35 + - + + + + +
kD
TrkB-FL
TrkB-Tc
TrkB-ICD
α-tubulin
150
100
37
50
(B)
zVAD-Fmk
MG132
ALLN
MDL28170
Peps.A
Aβ25-35
TrkB-FL levels (fold of CTR)
TrkB-Tc levels (fold of CTR)
TrkB-ICD levels (fold of CTR)
CTR
Aβ25-35
zVAD-FMK
MG132
ALLN
MDL28170
Pepstatin
Aβ25-35
(C)
CTR
Aβ25-35
CTR
Aβ1-42
αII-Spectrin
SBDP(150)
SBDP(120)
250
SBDPs(150) levels (fold of CTR)
CTR
Aβ25-35

Figure 4.6 – Aβ-induced cleavage of TrkB-FL is repressed by inhibitors of calpain-like activity.
(A) Western-blot of 8 DIV neuronal cultures showing a concentration-dependent inhibition of $A\beta_{25-35}$-induced TrkB-FL cleavage and TrkB fragments production by E64-d (a general thiol proteases inhibitor). **(B)** Upper image: Representative western-blot of 8 DIV neuronal cultures showing the impact of several protease inhibitors on the Aβ-induced TrkB-FL cleavage and TrkB fragments production. The protease inhibitors tested were: zVAD-FMK 20μM (pan-caspase inhibitor); MG132 2μM, ALLN 20μM, MDL28170 20μM (inhibitors of calpain-like activity) and Pepstatin A 1μM (aspartyl protease inhibitor). All bands represented in the image are from the same gel and the order of the first two lanes was rearranged. Lower panel: Average immunoreactive band intensity of TrkB-FL, TrkB-Tc and TrkB-ICD bands (upper, center and lower histogram, respectively). The order of the histogram bars is the same as the above lanes of the western-blot (**p<0.01 compared to CTR, #p<0.05 compared to $A\beta_{25-35}$, n=5, one-way ANOVA with Bonferroni's multiple comparison test). **(C)** Left image: Representative western-blot showing the effect of 24 hours of $A\beta_{25-35}$ (25μM) and $A\beta_{1-42}$ (20μM) incubation on 8 DIV neuronal cultures upon brain αII-Spectrin levels and the formation of the calpain-specific spectrin breakdown products (SBDPs 145/150), caspase-3 specific SBDP (120) and TrkB-ICD fragment. Right histogram: Analysis of calpain-specific SBDPs (145/150) immunoreactive band intensity of control (white bar) and $A\beta_{25-35}$ treatment (black bar), ***p<0.001 comparing to control (n=4, student´s *t*-Test). All values presented are mean ± SEM.

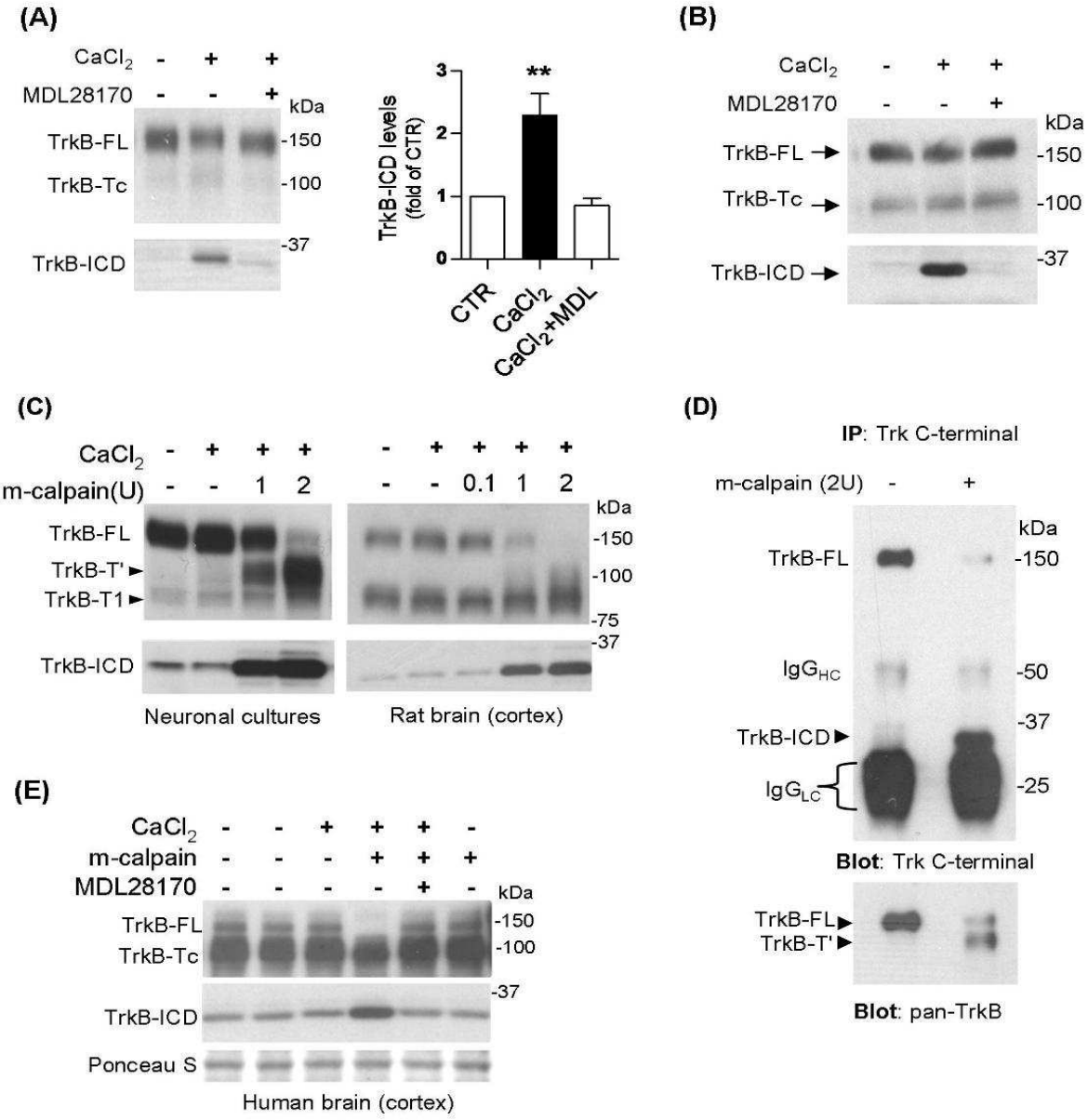

(A)
CaCl2 - + +
MDL28170 - - +
kDa
TrkB-FL
TrkB-Tc
TrkB-ICD
-150
-100
-37
TrkB-ICD levels (fold of CTR)
**
CTR
CaCl2
CaCl2+MDL
(B)
CaCl2 - + +
MDL28170 - - +
kDa
TrkB-FL
TrkB-Tc
TrkB-ICD
-150
-100
-37
(C)
CaCl2 - + + + - + + + +
m-calpain(U) - - 1 2 - - 0.1 1 2
kDa
TrkB-FL
TrkB-T'
TrkB-T1
TrkB-ICD
-150
-100
-75
-37
Neuronal cultures
Rat brain (cortex)
(D)
IP: Trk C-terminal
m-calpain (2U) - +
kDa
TrkB-FL
IgGHC
TrkB-ICD
IgGLC
-150
-50
-37
-25
Blot: Trk C-terminal
TrkB-FL
TrkB-T'
Blot: pan-TrkB
(E)
CaCl2 - - + + + -
m-calpain - - - + + +
MDL28170 - - - - + -
kDa
TrkB-FL
TrkB-Tc
TrkB-ICD
Ponceau S
-150
-100
-37
Human brain (cortex)

Figure 4.7 – Calpain cleaves rat and human TrkB-FL receptor.

(A) Left: Representative western-blot showing the production of TrkB-ICD upon $CaCl_2$ (5mM) incubation on cell lysates for 4h at 25ºC. Right: Analysis of TrkB-ICD immunoreactive band intensity (**p<0.01, n=3, ANOVA). **(B)** Western-blot showing levels of TrkB-FL, truncated TrkB-Tc and TrkB-ICD fragment of isolated synaptosomes incubated in the absence or presence of $CaCl_2$ (5mM) or $CaCl_2$ with calpain inhibitor MDL28170 (20μM). **(C)** Cleavage of rat TrkB by exogenous m-calpain, with consequent production of TrkB-T' and TrkB-ICD in neuronal cultures lysates (left) and in adult rat brain homogenates (right). **(D)** Western-blot performed using anti- C-terminal of Trk-FL antibody (upper panel) and the pan-TrkB antibody (lower panel). First lane: Immunopurified TrkB-FL receptors from neuronal culture lysates using the anti- C-terminal TrkB-FL antibody. Second lane: immunopurified TrkB-FL after an *In-vitro* digestion with m-calpain. IgG_{HC} (50kDa) and IgG_{IC} (20-30kDa) bands correspond, respectively, to the Heavy and Light chain of Immunoglobulin G of the antibody used for the immunoprecipitation. **(E)** Western-blot of *post-mortem* human cortical sample, showing endogenous levels of TrkB and TrkB-ICD (in control condition) and depletion of TrkB-FL with concomitant formation of TrkB-ICD when both exogenous m-calpain and Ca^{2+} were added in the absence of calpain inhibitor MDL28170. Ponceau S staining was used for loading control. All values presented are mean ± SEM.

4.7. TrkB-FL calpain cleavage site is located downstream the Shc binding site

Given the molecular weight (~32kDa) of the TrkB-ICD band detected by western-blot, we hypothesized that the calpain cleavage site would be located close to the Shc binding site (Tyr^{515}). To clarify this possibility, 5μg of recombinant cytosolic domain of TrkB-FL (Human TrkB active, aa.455-end, Millipore) were digested by purified m-calpain. Following membrane staining after SDS-PAGE electrophoresis, we observed that the recombinant cytosolic domain of human TrkB-FL (~42kDa) were cleaved by m-calpain producing the same characteristic ~32kDa TrkB-ICD fragment band (Figure 4.9A), as detected in neuronal cultures exposed to Aβ. The TrkB-ICD band was then cut (as depicted in Figure 4.9A) and analysed by N-terminal sequencing (Edman degradation) in order to identify the first five N-terminal aminoacids, hence revealing the calpain cleavage site position. The five N-terminal aminoacids detected were Ser-Gln-Leu-Lys-Asp (S-Q-L-K-D), which allows to conclude that the TrkB-FL is cleaved between the $Asn(N)^{520}$ and $Ser(S)^{521}$ residues, considering the rat TrkB sequence (Figure 4.9B). This cleavage site is located between the Shc binding site (Tyr^{515}) and the TrkB kinase domain (Figure 4.9B and C). Therefore, these data indicate that the truncated TrkB-T' receptor contains the Shc binding site (Tyr^{515}), whereas the TrkB-ICD fragment contains the complete tyrosine

kinase domain of TrkB-FL receptor (Ile^{537}-Leu^{806}), as well as the C-terminal tail of TrkB (Gln^{807}-Gly^{821}), since the fragment is recognized by the antibody specific for the C-terminal tail of Trk-FL, as shown above (Figure 4.5A).

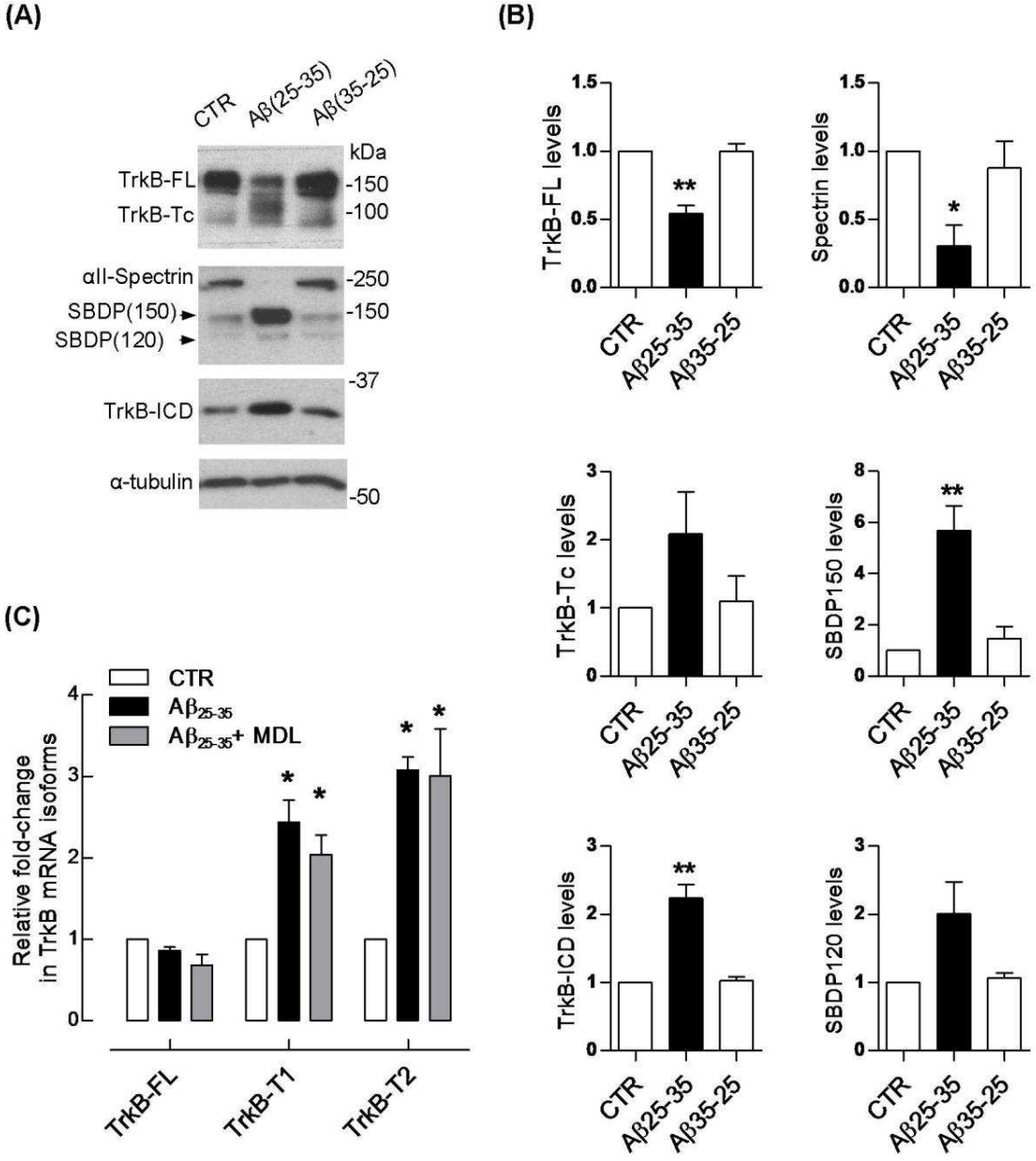


Figure 4.8 – Calpain inhibition does not block the Aβ-induced changes on TrkB mRNA levels.

(A) Western-blot showing the effect of $Aβ_{25-35}$ and reverse $Aβ_{35-25}$ peptide (both at 25μM) upon the levels of TrkB-FL, TrkB-Tc, TrkB-ICD and αII-spectrin along with its fragments (SBDP150 and 120), on 8DIV cortical neurons. **(B)** Analysis of bands intensities represented in (A) (*p<0.05, **p<0.01, n=3, ANOVA). **(C)** mRNA levels obtained by qPCR of TrkB-FL, TrkB-T1 and TrkB-T2 on 8DIV cortical cultures treated with $Aβ_{25-35}$ (25μM) (black bars) or with both $Aβ_{25-35}$ (25μM) and MDL28170 (20μM) (gray bars) for 24hours. β-actin was used as an internal loading control. *p<0.05 comparing to control (white bars) of the respective isoform (n=4, ANOVA with Bonferroni's multiple comparison test).

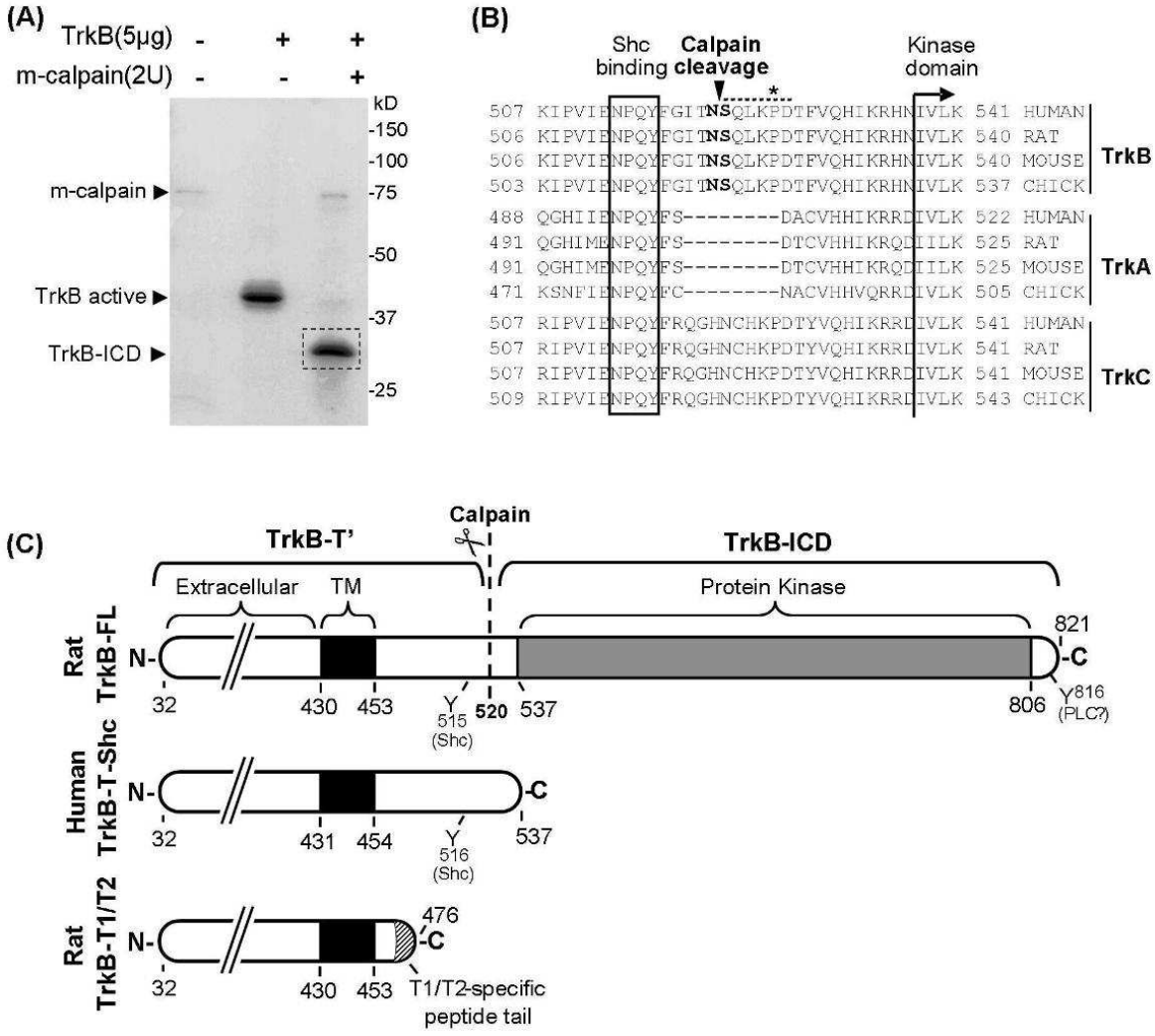


Figure 4.9 – Calpain cleaves TrkB downstream Shc binding site (Tyr 515).

(A) Ponceau S staining of a PVDF membrane after transfer from SDS-PAGE. Purified cytosolic domain of human TrkB (TrkB-active aa.450-end, Millipore) was digested with purified m-calpain, producing TrkB-ICD. TrkB-ICD band was cut (dashed square) and submitted for N-terminal sequencing (Edman degradation). $CaCl_2$ (2mM) was present in all conditions. **(B)** Multiple alignment of TrkA, TrkB and TrkC protein sequences for different species. The Shc binding motif, the Trk kinase domain, and the calpain-cleavage site identified by N-sequencing are identified in the sequence. Protein sequences were obtained in UniProtKD, and the multiple alignments were performed using the Clustal Omega tool. The proline residue (P) present in the TrkB sequence (*) was not detected by N-sequencing. **(C)** Schematic representation of mature rat TrkB-FL, TrkB-T1, T2, and human TrkB-T-Shc isoforms showing the relevant domains and aminoacid residues positions. Calpain-cleavage site (aa. 520) is represented based on N-sequencing data. Y^{515} and Y^{816} represent the phospho-tyrosine residues able to recruit Shc and PLCγ, respectively. Note that the first 31 aminoacid residues of TrkB compose the signal peptide and are not present in the mature protein. Protein sequences were obtained from UniProtKD database (Accession number: Q63604 for Rat and Q16620 for Human).

Upon BDNF binding to TrkB-FL receptor, both Tyr^{515} and Tyr^{816} residues of the receptor are phosphorylated, allowing the binding of Shc adaptor protein and Phospholipase C-γ (PLCγ), respectively, with subsequent activation of signalling cascades [see 365]. By using specific antibodies against phosphorylated Tyr^{515} and Tyr^{816} of TrkB, we evaluated whether TrkB-T' or TrkB-ICD fragments could undergo phosphorylation on Tyr^{515} or Tyr^{816}, respectively. In a first attempt, neurons were incubated with Aβ for 24hours to produce the TrkB fragments, and then, BDNF (20ng/mL) was briefly applied (10 min) to induce TrkB phosphorylation. Whereas BDNF incubation induced robust phosphorylation in Tyr^{515} of TrkB-FL, such phosphorylation was not detected in the truncated TrkB-T' fragment (Figure 4.10A). The levels of phosphorylated (Tyr^{515}) TrkB-FL upon BDNF exposure were 40 ± 9% lower in the Aβ-treated cultures than in control cultures (Figure 4.10A, n=4, $p<0.01$), a reduction similar to that observed in total levels of TrkB-FL induced by Aβ (Figure 4.5A). Thus, the ratio between the levels of total TrkB-FL and BDNF-induced phosphorylated TrkB-FL does not differ when comparing control cultures with Aβ-treated cultures (Figure 4.10A, n=4), suggesting that TrkB-FL phosphorylation efficacy remained similar regardless Aβ treatment. A similar result was also obtained in more mature cortical cultures with 15 DIV (Figure 4.10B).

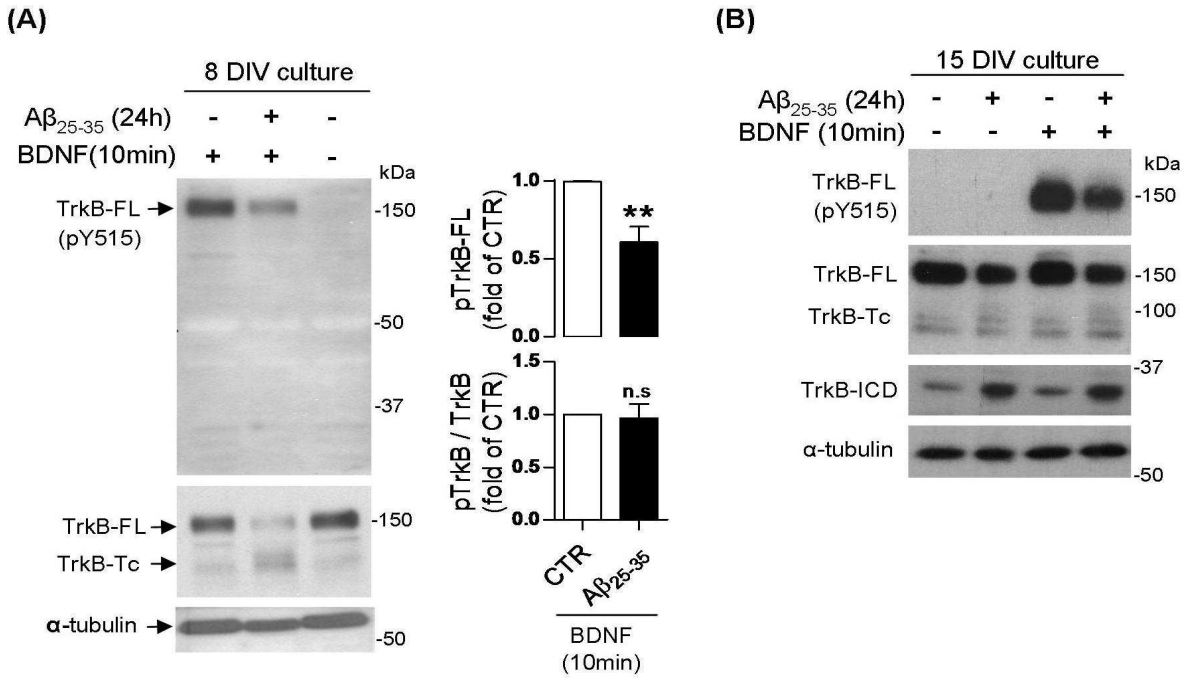


Figure 4.10 – Effect of Aβ upon BDNF-induced TrkB phosphorylation on Tyr 515.

(A) Left: Representative western-blot showing phosphorylated Tyr^{515} of TrkB-FL (upper image), pan-TrkB (middle image) and α-tubulin (lower image) on 8 DIV neurons incubated firstly with (or without) 24hours of $Aβ_{25-35}$ (25μM) and with (or without) 10min of BDNF (20ng/ml). Right: Levels of phosphorylated TrkB-FL (Tyr^{515}) normalized for α-tubulin (upper histogram) and ratio between phosphorylated and total TrkB-FL levels (lower graph) for control (white bars) and $Aβ_{25-35}$ treatment (black bars). Values are mean ± SEM (**p<0.01, n=4, student´s *t*-Test, compared to CTR). **(B)** Western-blot showing phosphorylated Tyr^{515} of TrkB-FL, pan-TrkB, TrkB-ICD and α-tubulin on a 15 DIV cortical culture incubated firstly with (or without) $Aβ_{25-35}$ (25μM, 24hours) and then incubated with (or without) BDNF (20ng/ml) for 10min.

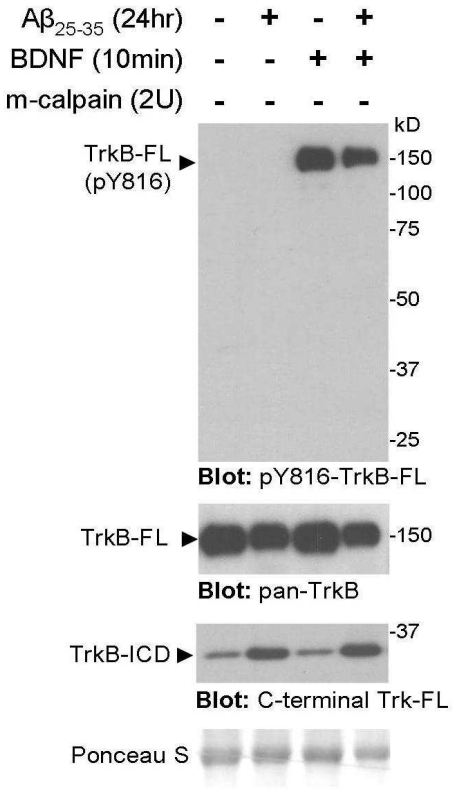


Figure 4.11 – Effect of Aβ upon BDNF-induced TrkB phosphorylation on Tyr 816.

Western-blot of 8 DIV neuronal cultures showing the levels of phosphorylated Tyr^{816} of TrkB-FL; the total TrkB-FL; and total TrkB-ICD. The neurons were firstly incubated with or without Aβ (25μM) during 24hours, and then, briefly exposed for 10 min to vehicle or BDNF (20ng/ml) to induce TrkB phosphorylation. Ponceau S staining is shown as a protein loading control.

4.8. Calpain mediates detrimental effects of Aβ upon BDNF actions on GABA and Glutamate release.

The influence of Aβ upon the modulatory action of BDNF on glutamate and GABA release was evaluated on synaptosomes, which were prepared from rat hippocampal slices pre-treated with $A\beta_{25-35}$ (25μM) or $A\beta_{1-42}$ (20μM) for 3hours. The hippocampal synaptosomes were loaded with [3H]GABA or [3H]glutamate as previously described [350] and neurotransmitter release was evoked twice (S1 and S2) by perfusion with 15 mM KCl for 2 min.

In [3H]glutamate release assays, the S2/S1 ratio in control conditions was 0.75 ± 0.03 and it was increased up to 0.93 ± 0.04 when BDNF (20 ng/mL) was added before S2, corresponding to an enhancement of 27 ± 7 % in the evoked release of glutamate (Figure 4.12A, n=5, $p<0.05$). In [3H]GABA release assays, the S2/S1 ratio was, in control conditions, 1.06±0.03 and it was significantly decreased to 0.82±0.07 when BDNF (20ng/ml) was added before S2, corresponding to a decrease of 26 ± 6 % in the evoked release of GABA (Figure 4.12B, n=7, $p<0.05$). BDNF-induced inhibition of GABA and facilitation of glutamate release from hippocampal synaptosomes was expected based on our previous studies [77]. When synaptosomes were prepared from hippocampal slices that had been exposed for 3 hours to $A\beta_{25-35}$ (25μM) or to $A\beta_{1-42}$ (20μM), the S2/S1 ratios in GABA release assays (S2/S1 $A\beta_{25-35}$: 1.04 ± 0.06, n=13; S2/S1 $A\beta_{1-42}$: 1.16 ± 0.02, n=3, Figure 4.13A, E and Figure 4.12D) or glutamate release assays (S2/S1 $A\beta_{25-35}$: 0.85±0.08, n=10; S2/S1 $A\beta_{1-42}$: 0.85 ± 0.03, n=3, Figure 4.13B, F and Figure 4.12C) were not significantly altered ($p>0.05$) as compared with control conditions. However, in synaptosomes prepared from $A\beta_{25-35}$- and $A\beta_{1-42}$-treated slices, BDNF lost its ability to decrease GABA release (S2/S1 $A\beta_{25-35}$+BDNF 0.96 ± 0.06, n=13; S2/S1 $A\beta_{1-42}$+BDNF: 0.95 ± 0.17, n=3 Figure 4.13A, E and Figure 4.12D) and to increase glutamate release (S2/S1$A\beta_{25-35}$+BDNF: 0.78 ± 0.07, n=10; S2/S1$A\beta_{1-42}$+BDNF: 0.86 ± 0.07, n=3 Figure 4.13B, F and Figure 4.12C). These results suggest that Aβ causes a functional impairment of BDNF modulatory actions upon glutamate and GABA release in the hippocampus.

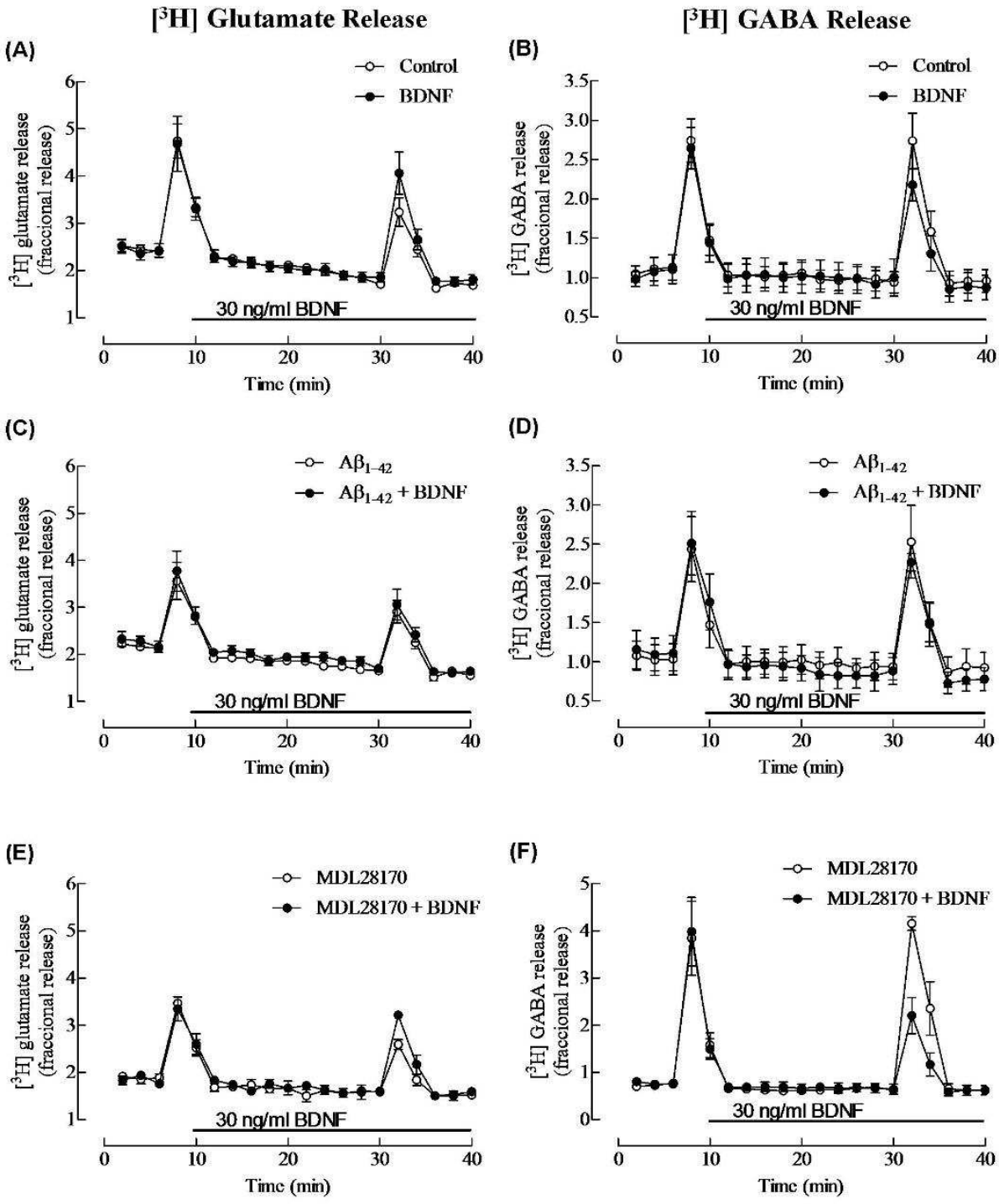

[3H] Glutamate Release
[3H] GABA Release
(A)
(B)
(C)
(D)
(E)
(F)
Control
BDNF
Aβ1–42
Aβ1-42 + BDNF
MDL28170
MDL28170 + BDNF
30 ng/ml BDNF
[3H] glutamate release (fraccional release)
[3H] GABA release (fraccional release)
Time (min)

Figure 4.12 – Modulation of GABA and glutamate release by BDNF, $A\beta_{1-42}$ and MDL28170.
Fractional release of [^{3}H]glutamate (**A, C and E**) and [^{3}H]GABA (**B, D and F**) evoked by two 15mM K^+ stimuli of 2-minuts duration, at 5–7 min (S1) and 29–31 min (S2). BDNF (30 ng/ml) was added at 9 min and remained in the perfusion solution until the end of the experiments (closed circles), as indicated by the horizontal bar. Control curves in absence or in presence of $A\beta_{1-42}$ (20 μM) and in the presence of MDL (20μM), performed in parallel with the same synaptosomal batch, are represented by the open circles. BDNF effect upon [^{3}H]glutamate fractional release (**A**), modulation of BDNF (30ng/ml) effect upon fractional release of [^{3}H] glutamate by $A\beta_{1-42}$ (20 μM) (**C**) and by MDL (20 μM) (**E**). BDNF effect upon [^{3}H]GABA fractional release (**B**), and modulation of BDNF (30ng/ml) effect upon fractional release of [^{3}H] GABA by $A\beta_{1-42}$ (20 μM) (**D**) and by MDL(20 μM) (**F**). In each experiment, the S2/S1 ratio obtained while BDNF was present during S2 was normalized, taking as 100% the S2/S1 ratio obtained in parallel chambers under the same drug conditions but in the absence of BDNF. Data are represented as mean ± SEM of three to ten independent experiments.

In order to determine the contribution of calpains towards the Aβ-dependent impairment of BDNF modulation of neurotransmitters release, hippocampal slices were incubated simultaneously with both $A\beta_{25-35}$ (25μM) and MDL28170 (20μM) for 3 hours. In the absence of BDNF, MDL28170 did not impact S2/S1 ratios for both GABA (S2/S1$A\beta_{25-35}$+MDL: 1.13 ± 0.05, n=4, Figure 4.13C,E) and glutamate release (S2/S1$A\beta_{25-35}$+MDL: 0.78 ± 0.07, n=4, Figure 4.13D,F). On the contrary, in synaptosomes prepared from hippocampal slices incubated under similar conditions ($A\beta_{25-35}$ and MDL28170), the addition of BDNF before S2 affected the S2/S1 ratio of GABA (S2/S1 $A\beta_{25-35}$+MDL+BDNF: 0.78 ± 0.15, $p<0.05$ vs S2/S1 $A\beta_{25-35}$+MDL, n=4, Figure 4.13C,E) and glutamate (S2/S1 $A\beta_{25-35}$+MDL+BDNF 0.96 ± 0.08, $p<0.05$, vs S2/S1 $A\beta_{25-35}$+MDL, n=4, Figure 4.13D,F) release similar to what had been observed when BDNF was added alone (Figure 4.13D,F and Figure 4.12A,B). The incubation of hippocampal slices with MDL28170 alone for 3 hours did not affect BDNF actions upon glutamate and GABA release (for glutamate: S2/S1 MDL+BDNF: 0.95 ± 0.01, n=3, Figure 4.12E; for GABA: S2/S1 MDL+BDNF: 0.69 ± 0.28, n=3, Figure 4.12F). These results strongly suggest that the impairment caused by Aβ upon the modulatory action of BDNF on neurotransmitter release is rescued by calpain inhibition.

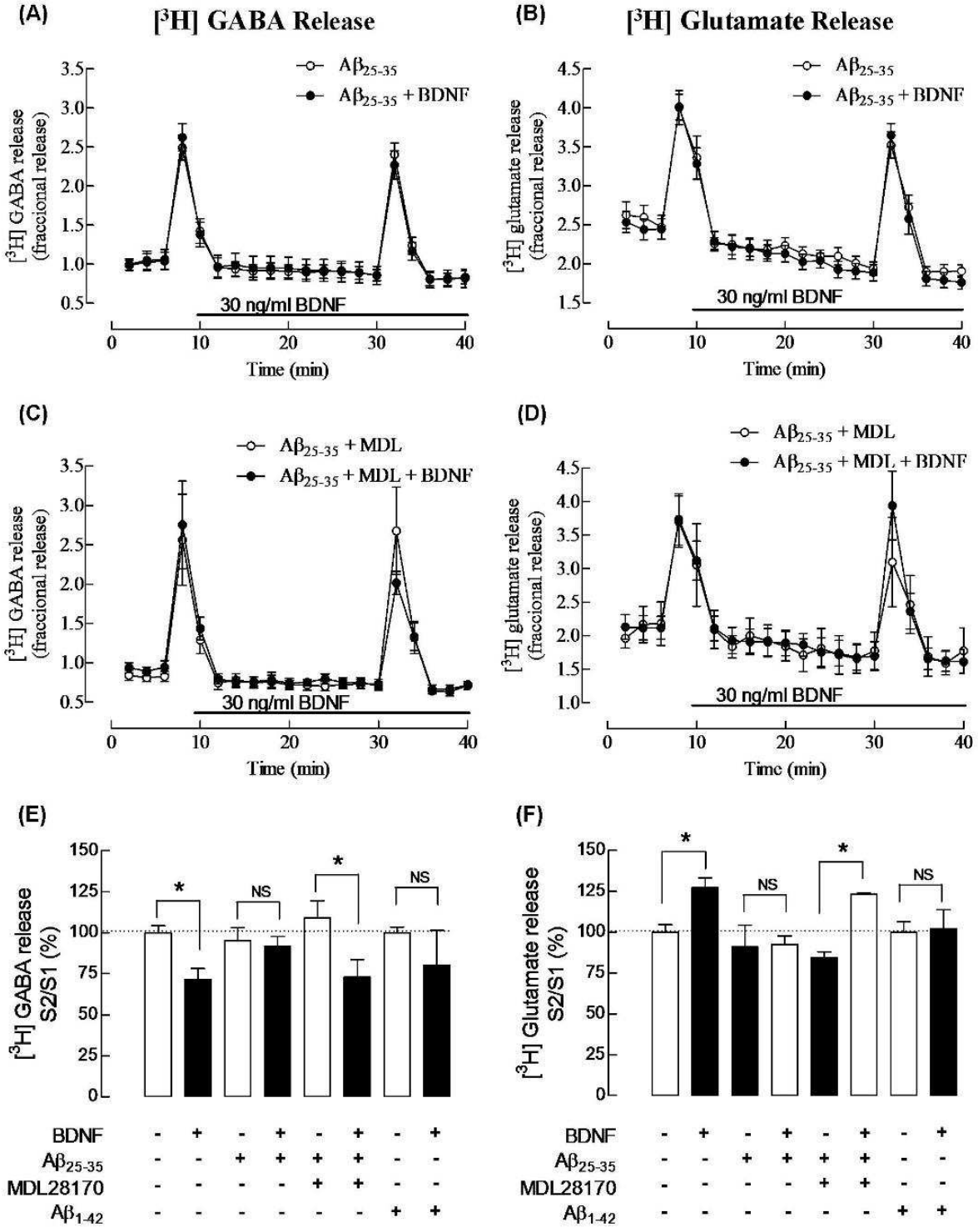

(A)
[3H] GABA Release
Aβ25-35
Aβ25-35 + BDNF
[3H] GABA release (fraccional release)
30 ng/ml BDNF
Time (min)
(B)
[3H] Glutamate Release
Aβ25-35
Aβ25-35 + BDNF
[3H] glutamate release (fraccional release)
30 ng/ml BDNF
Time (min)
(C)
Aβ25-35 + MDL
Aβ25-35 + MDL + BDNF
[3H] GABA release (fraccional release)
30 ng/ml BDNF
Time (min)
(D)
Aβ25-35 + MDL
Aβ25-35 + MDL + BDNF
[3H] glutamate release (fraccional release)
30 ng/ml BDNF
Time (min)
(E)
[3H] GABA release S2/S1 (%)
*
NS
*
NS
BDNF
Aβ25-35
MDL28170
Aβ1-42
(F)
[3H] Glutamate release S2/S1 (%)
*
NS
*
NS
BDNF
Aβ25-35
MDL28170
Aβ1-42

Figure 4.13 – Aβ inhibits BDNF effect upon neurotransmitter release in a calpain-dependent way. Fractional release of [^{3}H] GABA (**A** and **C**) and [^{3}H] glutamate (**B** and **D**) evoked by two 15mM K^+ stimuli of 2min duration, at 5–7 min (S1) and 29–31 min (S2). BDNF (30 ng/ml) was added at 9 min and remained in the perfusion solution until the end of the experiments (closed circles), as indicated by the horizontal bar. Control curves in the presence of $A\beta_{25-35}$ (25μM) or $A\beta_{25-35}$ (25μM) and MDL28170 (20μM), performed in parallel with the same synaptosomal batch, are represented by the open circles. Modulation of BDNF (30ng/ml) effect upon fractional release of [^{3}H] GABA by $A\beta_{25-35}$ (25μM) (**A**), or by simultaneous treatment with $A\beta_{25-35}$ (25μM) and MDL28170 (20μM) (**C**). Modulation of BDNF (30ng/ml) effect upon fractional release of [^{3}H]glutamate by $A\beta_{25-35}$ (25μM) (**B**), or by simultaneous treatment with $A\beta_{25-35}$ (25μM) and MDL28170 (20μM) (**D**). **E** and **F**, S2/S1 ratios (%), calculated in each experiment from the fractional release curves, as described in Materials and Methods. BDNF (30 ng/ml) was tested in synaptosomes prepared from hippocampal slices treated or non-treated with $A\beta_{25-35}$ (25μM) or $A\beta_{1-42}$ (20μM), or treated simultaneously with $A\beta_{25-35}$ (25μM) and MDL28170 (20μM), as indicated below each bar. In each experiment, the S2/S1 ratio obtained while BDNF was present during S2 was normalized, taking as 100% the S2/S1 ratio obtained in parallel chambers under the same drug conditions but in the absence of BDNF. Data are represented as mean ± SEM of five to ten independent experiments. *p<0.05, compared with 100%, except when otherwise indicated (one-way ANOVA followed by Bonferroni's multiple comparison test).

4.9. Calpain mediates detrimental effects of Aβ upon BDNF actions on CA1 long-term potentiation

BDNF has a well-documented ability to increase LTP on hippocampal CA1 area through TrkB-FL activation [67, 71]. To evaluate the impact of Aβ upon BDNF effects on LTP, hippocampal slices were exposed for 3 hours to oxygenated aCSF with or without $A\beta_{25-35}$ (25μM) or $A\beta_{1-40}$ (20μM) or even the inverted $A\beta_{35-25}$ (25μM) peptide. As mentioned in the methods section, the experiments were conducted using two independent stimulation pathways, being each pathway used as control or test in alternate days, in order to compare LTP magnitude in the absence and in the presence of BDNF, within the same slice. The LTP was firstly induced by θ-burst stimulation in one 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 expected [113], the θ-burst stimulus applied in the presence of BDNF (20 ng/mL) induced a robust LTP (LTP_{BDNF}: 40.0±1.7% increase in fEPSP slope), which was significantly

higher (P<0.01) than that obtained in the absence of BDNF (LTP_{CTR}: 22.1±3.9% increase in fEPSP slope; n= 11, Figure 4.14A, E). Pre-treatment of hippocampal slices with Aβ for 3 hours did not affect LTP magnitude when compared to untreated slices ($LTP_{Aβ25-35}$: 23.5±3.5%, n=10 or $LTP_{Aβ1-42}$: 17.6±7%, n=5 vs LTP_{CTR}: 22.1±3.9%, n= 11, Figure 4.14E). However, in Aβ-treated slices, BDNF (20ng/mL) failed to enhance LTP magnitude ($LTP_{Aβ25-35}$: 23.5±3.5% vs $LTP_{Aβ25-35+BDNF}$: 24.3 ±4.7%, n= 10, p>0.05, Figure 4.14B, E and $LTP_{Aβ1-42}$: 17.6±7% vs $LTP_{Aβ1-42+BDNF}$: 21.4 ±4.0%, n= 5, p>0.05, Figure 4.14C, E).

In slices treated with 25µM of inverted $Aβ_{35-25}$ (control peptide), the facilitation of BDNF upon LTP was not lost (n=4, p<0.05; Figure 4.15). To evaluate whether Aβ peptides could affect basal synaptic efficiency, input/output curves were performed and no significant differences were detected between control slices, and $Aβ_{25-35}$ or $Aβ_{1-42}$-treated slices (n=4, Figure 4.14F).

To explore if calpains played a role in the Aβ-induced loss of BDNF effect upon LTP, hippocampal slices were pre-treated simultaneously with the $Aβ_{25-35}$ and the calpain inhibitor MDL28170 (20µM), for 3 hours. As shown in Figure 4.14D, pre-treatment with MDL28170 rescued the facilitatory effect of BDNF upon LTP ($LTP_{Aβ25-35+MDL}$: 15.6±3.9% vs $LTP_{Aβ25-35+MDL+BDNF}$: 32.5±3.3%, n=6, p<0.05, Figure 4.14D, E). MDL28170 by itself did not significantly affect LTP magnitude in slices treated with $Aβ_{25-35}$ (Figure 4.14D).

Taken together, these data demonstrate that Aβ severely hampers BDNF action on hippocampal LTP and neurotransmitter (GABA and glutamate) release and that these impairments are dependent on calpain activation. These functional results correlate with the results obtained in neuronal cultures treated with Aβ showing a calpain-mediated cleavage of the TrkB-FL BDNF receptor. Therefore, the data strongly suggest that Aβ impairs BDNF/TrkB mediated actions in hippocampal slices through a mechanism that involves calpain activation.

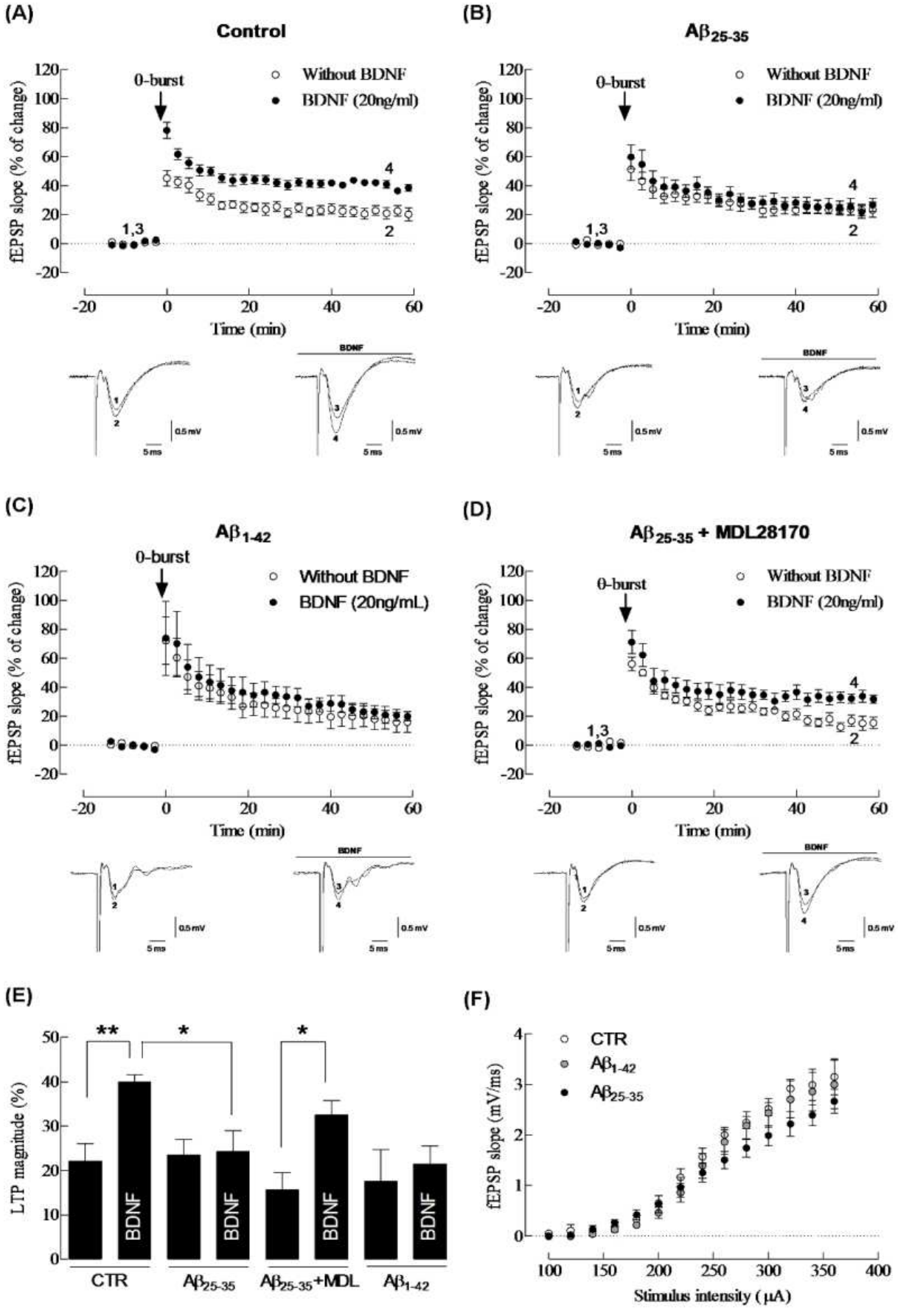

(A)
Control
0-burst
Without BDNF
BDNF (20ng/ml)
fEPSP slope (% of change)
Time (min)
1,3
2
4
BDNF
0.5 mV
5 ms
(B)
Aβ25-35
(C)
Aβ1-42
BDNF (20ng/mL)
(D)
Aβ25-35 + MDL28170
(E)
LTP magnitude (%)
**
*
*
CTR
Aβ25-35
Aβ25-35+MDL
Aβ1-42
(F)
fEPSP slope (mV/ms)
Stimulus intensity (μA)

Figure 4.14 – Aβ decreases the effect of BDNF upon LTP, in a calpain-dependent way.

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 20ng/ml (●) in the second stimulation pathway in rat hippocampal slices without (**A**, n=11) or with a pre-exposure for 3 hours to aCSF solution containing 25μM $A\beta_{25-35}$ (**B,** n=10); 20μM $A\beta_{1-42}$ (**C**, n=5); or 25μM $A\beta_{25-35}$ in the presence of 20μM MDL28170 (**D**, n=6). The traces from representative experiments are shown below panels (**A**-**D**); each trace is the average of eight 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. (**E**) LTP magnitude (change in fEPSP slope at 46-60min) induced by θ-Burst stimulation in relation to pre-θ-Burst values (0%), for each group of pre-treated slices (Control, $A\beta_{25-35}$, $A\beta_{25-35}$ + MDL28170, and $A\beta_{1-42}$). **p*<0.05; ***p*<0.01, one-way ANOVA followed by Bonferroni's multiple comparison test. Data are represented as mean ± SEM. (**F)** Input/output curves corresponding to fEPSP slope evoked by various stimulation intensities (100–360 μA) in non-treated hippocampal slices or treated for 3hours with $A\beta_{25-35}$ (25μM) or $A\beta_{1-42}$ (20μM) (n=4).

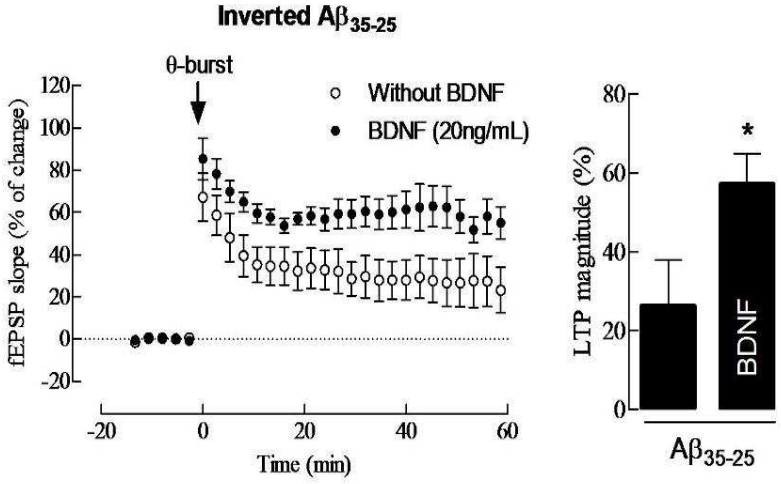


Figure 4.15 – Reverse Aβ35-25 peptide does not affect BDNF effect upon LTP

Left: 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 20ng/ml (●) in the second stimulation pathway in rat hippocampal slices pre-exposed for 3hours to inverted $A\beta_{35-25}$ peptide (25μM) (n=4). Right: LTP magnitude (change in fEPSP slope at 46-60 minutes after θ-Burst stimulation) in relation to pre-θ-Burst values (0%) (n=4, *p<0.05, student's t-test). Data are represented as mean ± SEM.

4.10. Discussion

In this chapter, it was demonstrated that Aβ promotes a calpain-mediated cleavage of TrkB-FL receptor and that impairs, in a calpain-dependent manner, BDNF modulation of neurotransmitter release and synaptic plasticity. Moreover, we found that, in primary cortical cultures, Aβ significantly increases mRNA levels of truncated TrkB isoforms without affecting mRNA levels of TrkB-FL, in a mechanism independent of calpains.

The alterations at the transcriptional level, namely the increase in mRNA levels of truncated TrkB isoforms is in line with previous data showing a correlation between amyloid-load and up-regulation of TrkB-T1 mRNA levels in cortical regions of a transgenic AD mice model without affecting TrkB-FL mRNA levels [30]. A selective up-regulation of truncated TrkB mRNA levels (TrkB-T1 and TrkB-T-Shc), without changes in TrkB-FL mRNA levels, was also reported in the hippocampus of AD *post-mortem* human brain [337]. As we now clearly show, the influence of Aβ upon TrkB-FL occurs at the post-translational level, rather than at the transcriptional level since Aβ strongly reduces TrkB-FL protein levels. Remarkably this occurs through calpain-mediated cleavage of TrkB-FL receptor protein, leading to a truncated receptor with a different molecular weight than the known isoforms of the truncated TrkB receptors that we named as TrkB-T'. Interestingly, Aβ did not affect the proportion of BDNF-induced phosphorylated TrkB-FL over total TrkB-FL levels, in accordance with previous data showing that sub-lethal Aβ concentrations do not interfere with BDNF-induced phosphorylation of TrkB-FL [366]. Thus, Aβ may impair BDNF signalling through a decrease in the TrkB-FL/TrkB-Tc ratio (Tc referring to all isoforms of truncated receptors), rather than by affecting the phosphorylation of the remaining TrkB-FL. In addition, Aβ could also affect downstream mediators of TrkB signalling, such as the docking proteins of TrkB, as already described for sub-lethal concentrations of Aβ [366].

By performing a detailed characterization of TrkB-FL cleavage, it was possible to show that calpain cleavage of TrkB-FL occurs between the Asn^{520} and Ser^{521} residues, producing two TrkB cleavage products: 1) the new truncated TrkB receptor (TrkB-T') which is heavier than the natural truncated TrkB-T1 and -T2 splicing products and 2) a fragment of ~32kDa which corresponds to the intracellular domain of TrkB-FL (TrkB-ICD). Moreover, we found that the calpain cleavage site of TrkB-FL is located downstream to the Shc binding site (Tyr^{515}), indicating that the new truncated TrkB-T' generated by calpain contains the Shc-binding site (Tyr^{515}). Thus, the TrkB-T' is only 16 aminoacid residues shorter than the described truncated TrkB-T-Shc isoform, a neuron-specific alternative splicing product of human TrkB gene [23]. Although TrkB-T-Shc function is poorly understood, it cannot be tyrosine phosphorylated [23], as the now described TrkB-T', and it could act as a negative regulator of BDNF function. Our results suggest that TrkB-T' may not act as a negative modulator of BDNF signalling since, in

spite of its presence, the efficiency of TrkB-FL phosphorylation by BDNF, assessed as the ratio between pTrkB-FL/TrkB-FL, was not appreciably affected.

The second Aβ-induced TrkB-FL cleavage product, the TrkB-ICD, corresponds to the remaining intracellular domain (ICD) of TrkB-FL upstream to the cleavage site (Ser^{521}-Gly^{821}). The theoretical molecular weight of TrkB-ICD is 34.6kDa, which is similar to the relative molecular weight observed in SDS-PAGE (~32kDa). TrkB-ICD was also detected in *post-mortem* human brain samples showing that human endogenous calpains could also cleave human TrkB-FL receptor. However, we cannot exclude the possibility that the presence of basal levels of TrkB-ICD detected in the human brain sample could be exacerbated due to calpain activation during the *post-mortem* period. Nevertheless, in freshly prepared rat cortex homogenates, and therefore without a significant *post-mortem* delay, it was also possible to detect small amounts of TrkB-ICD, suggesting that TrkB-FL cleavage could also occur in the alive healthy brain.

It is known that some members of the receptor tyrosine kinase family can undergo proteolytic cleavage by caspases, metalloproteases or secretases, producing intracellular domain (ICDs) fragments that may possess a biological function [see 367], as it is the case of the pro-apoptotic fragment that results from caspase-mediated TrkC cleavage upon NT-3 deprivation [368]. Calpain-mediated proteolytic cleavage usually occurs between two domains of the substrate, releasing big stable fragments that can also have biological activity [see 127]. As an example, the calpain-generated fragment p25 from p35 cleavage constitutively activates the cyclin-dependent kinase 5 (CDK5), contributing to tau hyperphosphorylation, morphological degeneration and neuronal death [196]. Indeed, overexpression of p25 fragment in mice forebrain is sufficient to recapitulate the major hallmarks of AD, including hippocampal neuronal loss, aggregation of hyperphosphorylated tau, accumulation of Aβ and impairments on synaptic plasticity and cognition [285, 286]. Therefore, one may propose that calpains are a key element of a vicious cycle, since its activation leads to β-amyloid generation [361], which in turn leads to enhanced calpain activity with subsequent impaired signalling of key neurotrophic molecules such as BDNF (present work). Whether the resulting fragment, TrkB-ICD, also contributes to exacerbate neuronal damage, awaits further investigation.

The present work clearly demonstrates that Aβ impairs the facilitatory effects of BDNF upon glutamate release and the inhibitory effect of BDNF upon GABA release from isolated nerve terminals (synaptosomes). GABAergic and Glutamatergic hippocampal synaptosomes represent each one approximately 40% of total synaptophysin-positive nerve terminals, less than 5% of nerve terminals being cholinergic [369]. Interestingly, in spite of the greater vulnerability of the glutamatergic terminals to Aβ toxicity, as compared with the GABAergic ones [369, 370], the effect BDNF upon GABA release was also impaired by Aβ. Moreover, Aβ impairs BDNF-mediated effects upon LTP. In all cases it was possible to rescue the effect of BDNF by

adding a calpain inhibitor (MDL28170), indicating that the Aβ-induced loss of function of BDNF at the synapses is mediated by calpains. The finding that the calpain inhibitor prevents the molecular changes in TrkB receptors as well as prevents the loss of synaptic BDNF modulatory action, strongly suggests that a single mechanism underlies both phenomena, highlighting the functional consequence of the Aβ-induced cleavage of TrkB receptors.

Interestingly, Aβ did not affect LTP magnitude (without BDNF), or basal synaptic transmission, as evaluated by input/output curves. This provides evidence that Aβ may impair BDNF signalling, even before impairment of synaptic transmission and plasticity, suggesting that loss of neuromodulation by neurotrophins is a very early sign of synaptic impairments induced by Aβ. Nevertheless, there are evidences that Aβ peptides could impair LTP [eg: 371]. In our experimental conditions, we did not detect any significant change in LTP magnitude induced by a very-weak θ-burst in hippocampal slices exposed to Aβ. This absence of Aβ effect upon LTP, already seen by others [372], could be due to several factors such as the stimulation protocol, the Aβ preparation, the developmental age or genetic background of the animals used [372]. Interestingly, Smith and collaborators (2009) showed that soluble oligomeric $A\beta_{1\text{-}42}$ significantly blocked hippocampal LTP when induced by high-frequency stimulation but not by θ-burst, the type of stimulation used in this work.

Our findings provide a possible biochemical mechanism for previous observations that TrkB-FL receptors are decreased in pathological situations, including AD [322, 333], where calpains are found overactivated [194]. Calpain-dependent down-regulation of TrkB-FL protein also occurs after acute insults, such as excitotoxicity and ischemia [373, 374]. It is not known whether calpains can also cleave other Trk receptors, such as TrkA or TrkC. However, by comparing the sequences of Trk receptors, we can predict that TrkA and TrkC are probably not cleaved by calpains, since they both lack the calpain cleavage site present in TrkB, which is conserved within species. Calpain overactivation has been associated with several neuropathological conditions, including prion-like diseases, muscular dystrophies, Huntington's disease, Parkinson's disease, Alzheimer's disease, multiple sclerosis, ischemia, stroke, and brain trauma. Calpain inhibition is therefore a promising therapeutic strategy with demonstrated efficacy in animal models. However, translation to clinical trials waits for the development of selective inhibitors of calpains to be used in humans [see 184].

In summary we highlighted the mechanisms responsible for Aβ-induced TrkB receptor dysregulation. Namely, we found that Aβ selectively increases the mRNA levels of truncated TrkB-T1 and T2 receptors and it induces a calpain-mediated cleavage of TrkB-FL protein. The cleavage of TrkB-FL occurs between Asn^{520} and Ser^{521} and produces a new truncated receptor, containing the Shc-binding site (TrkB-T'), and a new intracellular cleavage product (TrkB-ICD), containing the complete kinase domain of TrkB-FL (see Figure 4.16). At a functional level, Aβ severely impairs BDNF effects upon GABA and glutamate release and upon synaptic

plasticity, in a calpain-dependent way. Taken together, the data demonstrate that calpain overactivation induced by Aβ severely impairs BDNF/TrkB-FL signalling, affecting the synaptic actions of BDNF. By detailing the mechanisms involved in the endogenous dysregulation of TrkB receptors induced by Aβ, as well as the early functional consequences of this dysregulation, this work reinforces the rational for the use of calpain inhibitors as a therapeutic tool in AD.

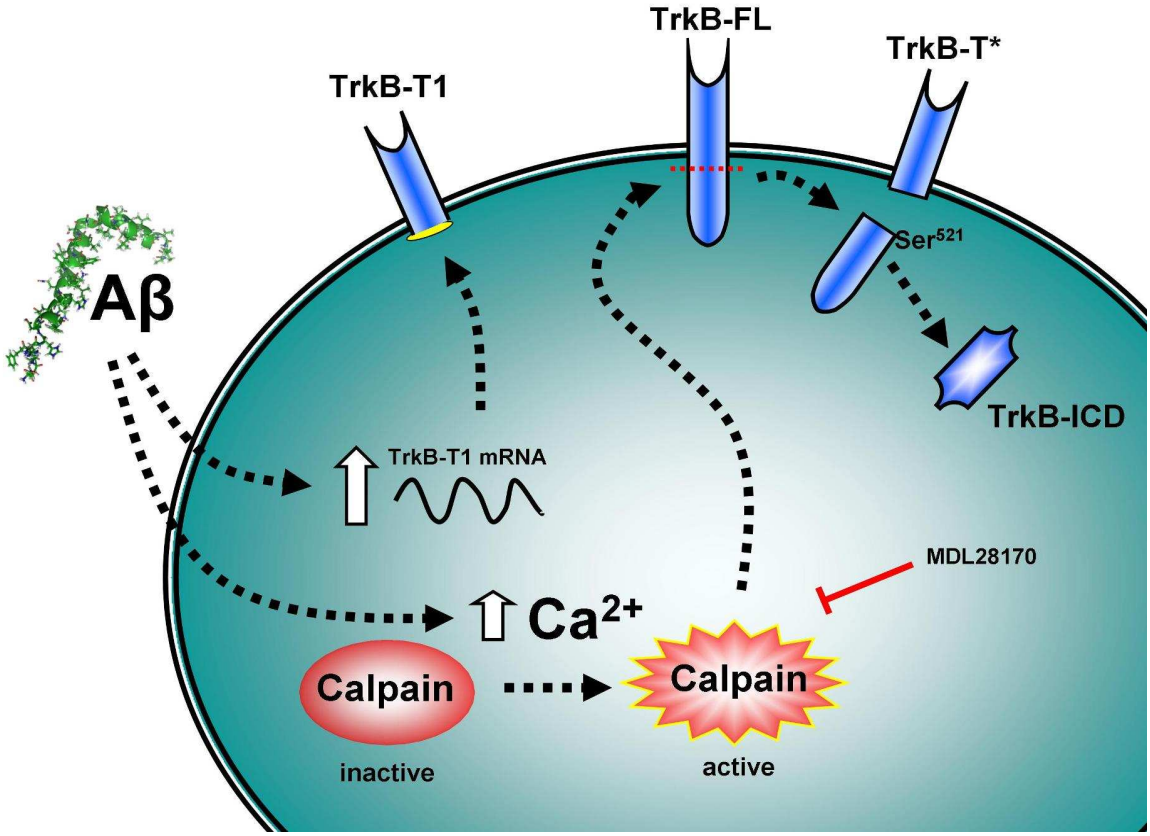


Figure 4.16 – Schematic representation of Aβ-induced dysregulation of TrkB receptors.
Aβ peptide selectively increases TrkB-T1 mRNA levels by an unknown mechanism and activates calpains by perturbing the intracellular Ca^{2+} homeostasis. Activated calpain cleaves TrkB-FL receptor in the intracellular domain (in the Asn^{520}-Ser^{521} peptide bond), producing a membrane-bound truncated TrkB-T' receptor and an intracellular fragment (TrkB-ICD). TrkB-ICD may have a putative biological function and may have a different structure when comparing to the original intracellular domain of TrkB-FL. The calpain inhibitor, MDL28170, inhibits calpain-mediated cleavage of TrkB-FL, and restores BDNF function upon synaptic plasticity and neurotransmitter release in Aβ-treated hippocampal slices (not shown).

5. Impact of *in-vivo* chronic blockade of $A_{2A}R$ upon BDNF-mediated facilitation on LTP

The work presented in this chapter was published in Jerónimo-Santos et al., 2014a [116].

5.1. Summary

Brain-derived neurotrophic factor (BDNF) through the activation of its receptor (TrkB-FL) exert well-described neuroprotective effects playing a major role in hippocampal synaptic transmission and plasticity such as long-term potentiation (LTP), a molecular surrogate for learning and memory. Impairments in BDNF signalling have been associated to several neurodegenerative disorders such as Alzheimer disease (AD). Therefore, the reestablishment of BDNF actions is considered a promising strategy for AD treatment. While, most of BDNF synaptic actions, namely on LTP, require the activation of adenosine A_{2A} receptor ($A_{2A}R$), the antagonists of $A_{2A}R$ have been proven to prevent AD induced deficits in several animal models. Therefore in this work we aimed to evaluate the impact of the chronic *in vivo* oral administration of the $A_{2A}R$ antagonist, KW-6002, in the BDNF actions upon hippocampal CA1 LTP. The results showed that chronic blockade of $A_{2A}R$ in male Wistar rats inhibits the facilitatory action of BDNF upon LTP and decreases both mRNA and protein levels of the TrkB-FL receptor in hippocampus. These findings imply that BDNF signalling may be affected in chronic $A_{2A}R$ blocking conditions.

5.2. Rational

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that, through activation of it high affinity full-length TrkB receptor (TrkB-FL), exerts well-described neuroprotective effects and plays major roles in hippocampal synaptic transmission and plasticity, such as LTP. LTP is a form of synaptic plasticity classically accepted as the neurophysiological correlate of learning and memory encoding [59]. In fact, in TrkB-FL or BDNF knockout mice, LTP induction is severely compromised [57, 67, 69, 71].

There are evidences that, in neurodegenerative disorders such as in AD, BDNF signalling is drastically impaired. In several regions of AD patient *post-mortem* brains, both BDNF and TrkB-FL receptor levels are decreased while the dominant-negative truncated TrkB receptors are increased [319, 321-323, 333]. Indeed, in AD animal models, where LTP

induction is strongly impaired [see 375], the potentiation of BDNF actions ameliorate the observed LTP deficits [356]. The loss of endogenous BDNF neuroprotection in AD has been seen as part of the pathophysiology of AD. In fact, the administration of BDNF exerts neuroprotective actions in rodent and primate models of AD [315]. However the clinical use of BDNF has been hampered due to its inability to reach the brain and to its short half-life [120]. Therefore, the identification of molecules able to potentiate endogenous BDNF actions gained emphasis in recent years. One of the drugs attracting much attention is adenosine due to its ability to modulate the actions of BDNF [see 99]. Indeed, evidence has been accumulating that $A_{2A}R$ activation is required for BDNF effects upon hippocampal synaptic transmission [108] and plasticity, namely facilitation of LTP [113, 114]. Moreover, it has been shown that $A_{2A}R$ activation can induce phosphorylation of an intracellular pool of TrkB receptors (associated with Golgi membranes) without the involvement of neurotrophins [105, 107].

However, despite the requirement of $A_{2A}R$ activation to promote BDNF actions upon LTP [113, 114], the administration of $A_{2A}R$ antagonists has been proven to prevent AD induced deficits in different animal models [376-378]. Given this apparent paradox, it is crucial to understand whether *in vivo* $A_{2A}R$ blockade impacts on BNDF effects upon synaptic plasticity. Therefore, in the present study we chronically administered an orally active $A_{2A}R$ antagonist (istradefylline; KW-6002; 3mg/kg/day) for one month to 4-6 week-old male Wistar rats and assessed the impact on BDNF action upon hippocampal CA1 LTP.

5.3. Chronic blockade of adenosine $A_{2A}R$ prevents BDNF-induced facilitation of CA1 LTP which is not restored by acute $A_{2A}R$ activation

The θ-burst stimulation delivered to CA1 area of hippocampal acute slices prepared from hippocampus taken from 8-10 week old rats induced a statistically significant LTP (LTP_{CTR}: 22±3%, n=5; $p<0.01$ as compared with baseline, Student's t-test, Figure 5.1A, C). As expected [113], the θ-burst stimulus applied in the presence of BDNF (20 ng/mL) caused a marked facilitation of LTP which was significantly higher than that obtained in the absence of BDNF (LTP_{BDNF}: 42.0± 2%, n=5, $p<0.01$, Student's t-test, Figure 5.1A, C). To evaluate the ability of BDNF to potentiate LTP in hippocampal slices taken from rats under chronic $A_{2A}R$ blockade, KW-6002, an $A_{2A}R$ oral antagonist, was administered for 1 month (3 mg/kg/day). In the KW-6002 treated group, the θ-burst stimulation increased the slope of the fEPSP by 24± 2% (n=5, Figure 5.1B, C), which was not different from the LTP magnitude obtained in hippocampal slices of animals not treated with the $A_{2A}R$ antagonist (LTP_{CTR}: 22± 3%, n=5, $p>0.05$, 2-way ANOVA, Figure 5.1A, C). However, in KW-6002 group, BDNF (20 ng/ml) lost

its ability to further increase LTP magnitude ($LTP_{KW+BDNF}$: 26± 3%; $p<0.05$ when compared with $LTP_{CTR+BDNF:}$ 42± 2%, n=5, 2-way ANOVA, Figure 5.1B, C). No differences were observed in input-output curves obtained in slices from vehicle or KW-6002 treated group (n=4 and n=6, respectively, $p>0.05$, 2-Way ANOVA, Figure 5.1D, E), indicating that KW-6002 treatment does not account for significant differences in basal synaptic efficiency.

To test if the loss of BDNF effect upon KW-6002 treatment was due to the presence of the $A_{2A}R$ antagonist in the tissues, the animals were withdrawn from the drug prior to the experiment. Given that the maximum plasmatic concentration of KW-6002 decreases to half in around 2 hours [352] the treatment was interrupted 24 hours before sacrificing the animals to assure minimum plasmatic concentration of KW-6002. In these conditions BDNF was still not able to enhance LTP ($LTP_{KW(-24h):}$ 28± 6% ; $LTP_{KW(-24h)+BDNF}$: 18± 6%, n=3, Student's t-test, $p>0.05$, Figure 5.2A, C).

Given that $A_{2A}R$ activation is required for the BDNF facilitatory actions on synaptic transmission [108, 109, 111] and synaptic plasticity [113, 114] we tested if acute $A_{2A}R$ activation could rescue the BDNF facilitatory effect upon LTP, in the KW-6002 treated animals. Acute *ex-vivo* activation of $A_{2A}R$ with the selective agonist, CGS21680 [379, 380] added 35 min before θ-burst stimulation, did not unravel the facilitatory effect of BDNF (20ng/ml) upon LTP (LTP_{KW}: 29± 8% vs $LTP_{KW+BDNF+CGS}$: 34± 7%, n=5, Student's t-test, $p>0.05$, Figure 5.2B, D). When applied alone, CGS21680 (30μM) was virtually devoid of effect upon LTP magnitude on KW-6002 treated animals (LTP_{KW}: 30± 11% vs LTP_{KW+CGS}: 30± 7%, n=3, Student's t-test, $p>0.05$, not shown).

Together these data show that chronic administration of KW-6002 *per se* did not affect the magnitude of θ-burst induced LTP comparing to vehicle animals, however, it prevented the facilitatory effect of BDNF upon LTP even if the pharmacological treatment had been stopped for 24 hours or if acute activation of $A_{2A}R$ had been provided. Therefore, the result suggests that chronic blockade of $A_{2A}R$ could induce irreversible or long-lasting changes in molecules involved in BDNF signaling.

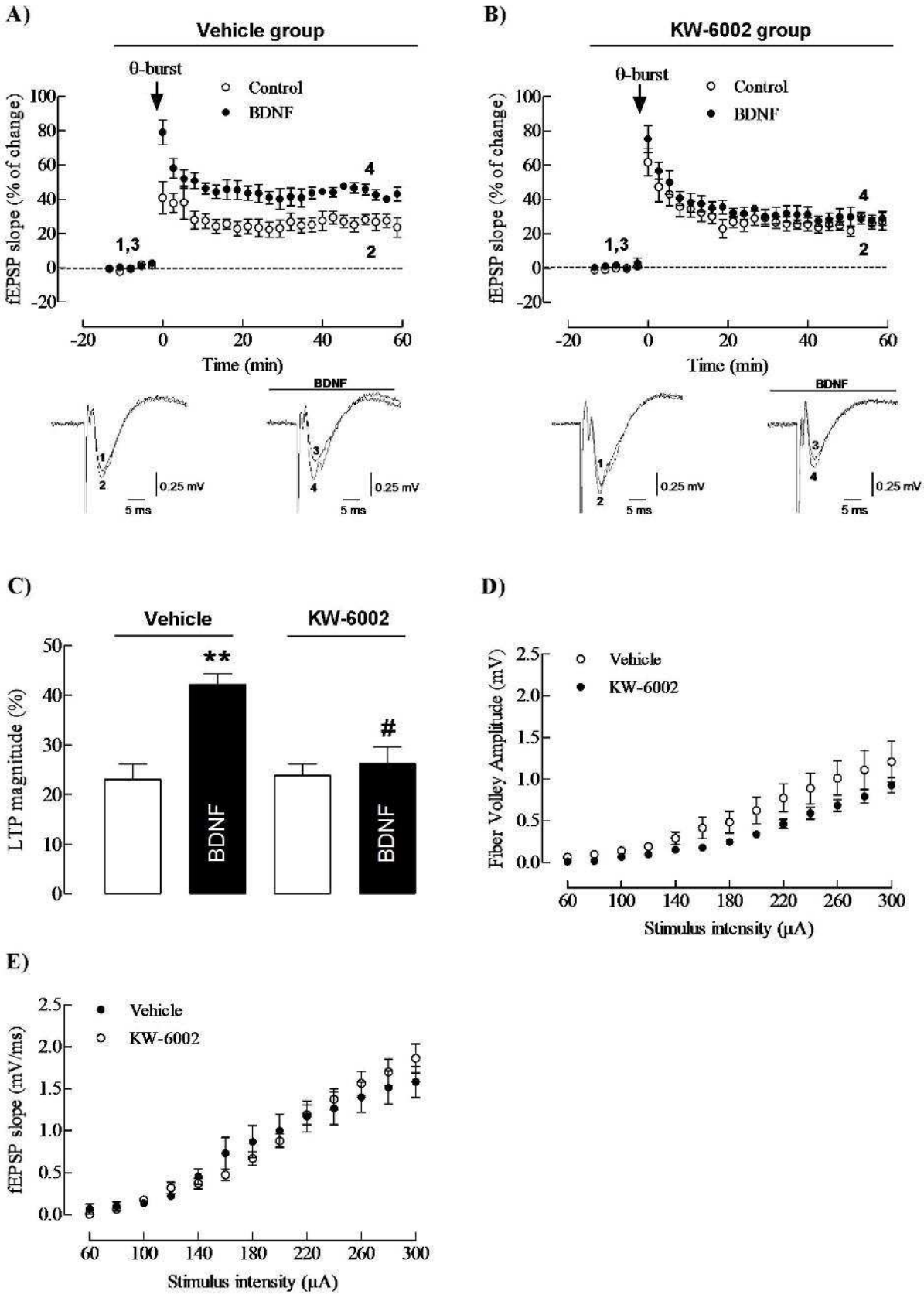

A)
Vehicle group
θ-burst
Control
BDNF
fEPSP slope (% of change)
Time (min)
1,3
2
4
BDNF
0.25 mV
5 ms
B)
KW-6002 group
C)
Vehicle
KW-6002
LTP magnitude (%)
**
#
D)
Fiber Volley Amplitude (mV)
Stimulus intensity (μA)
E)
fEPSP slope (mV/ms)
Stimulus intensity (μA)

Figure 5.1 – Chronic treatment with KW-6002 abolishes the facilitatory effect of BDNF upon LTP. Panels **(A, B)** 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 20ng/ml (●) in hippocampal slices obtained from: **(A)** animals treated with vehicle for 1 month (n=5); **(B)** animals treated with KW-6002 (3mg/kg/day) for 1 month (n=5). The traces from representative experiments are shown below the respective panels (**A, B**); each trace is the average of eight 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 left traces (1 and 2) and right traces (3 and 4) were obtained in the absence or in the presence of BDNF, respectively. **(C)** LTP magnitudes (change in fEPSP slope at 46-60min) induced by θ-burst stimulation in relation to pre-θ-burst values (0%), for the panels A-D. **p<0.01 compared to vehicle-control, #p<0.05 compared to vehicle-BDNF (ANOVA followed by Bonferroni post-test). **(D)** Input/output curves corresponding to responses generated between amplitude of pre-synaptic fibre volley and **(E)** fEPSP slope evoked by various stimulation intensities (60–300 μA) in hippocampal slices taken from vehicle group (○, n=6) and KW-6002 treated group (●, n=4). All values in the figure are represented as the mean ± SEM of *n* independent experiments with different animals.

5.4. Chronic $A_{2A}R$ blockade reduces protein and mRNA levels of TrkB-FL receptor without affecting BDNF levels.

Since the prolonged administration of KW-6002 compromised BDNF effects upon LTP, which are known to be mediated by TrkB-FL receptor activation, we evaluated if the lack of effect observed after the pharmacologic treatment could be related to changes in the levels of TrkB-FL receptors. Using a specific pan-TrkB antibody that recognizes the extracellular portion of TrkB receptor, a significant reduction in protein levels of TrkB-FL in the hippocampus of KW-6002 treated rats was observed (68 ± 12% vs 100%, n=5, Student's t-test, $p<0.05$, Figure 5.3A, B). Moreover, by relative qPCR, we found a significant reduction on TrkB-FL mRNA levels in hippocampus (64 ± 7% vs 100%, n=4, Student's t-test, $p<0.05$, Figure 5.3C). No significant differences were observed on truncated TrkB-T1 mRNA and protein levels between vehicle and KW-6002 treated rats (n=4 and 5, respectively, $p>0.05$, Student's t-test, Figure 5.3B, C). Moreover, total levels of mature BDNF and Pro-BDNF were also analyzed by western-blot, using an antibody raised against BDNF, and no significant differences were detected between vehicle and KW-6002 treated group (Figure 5.3A and D, n=5, Student's t-test).

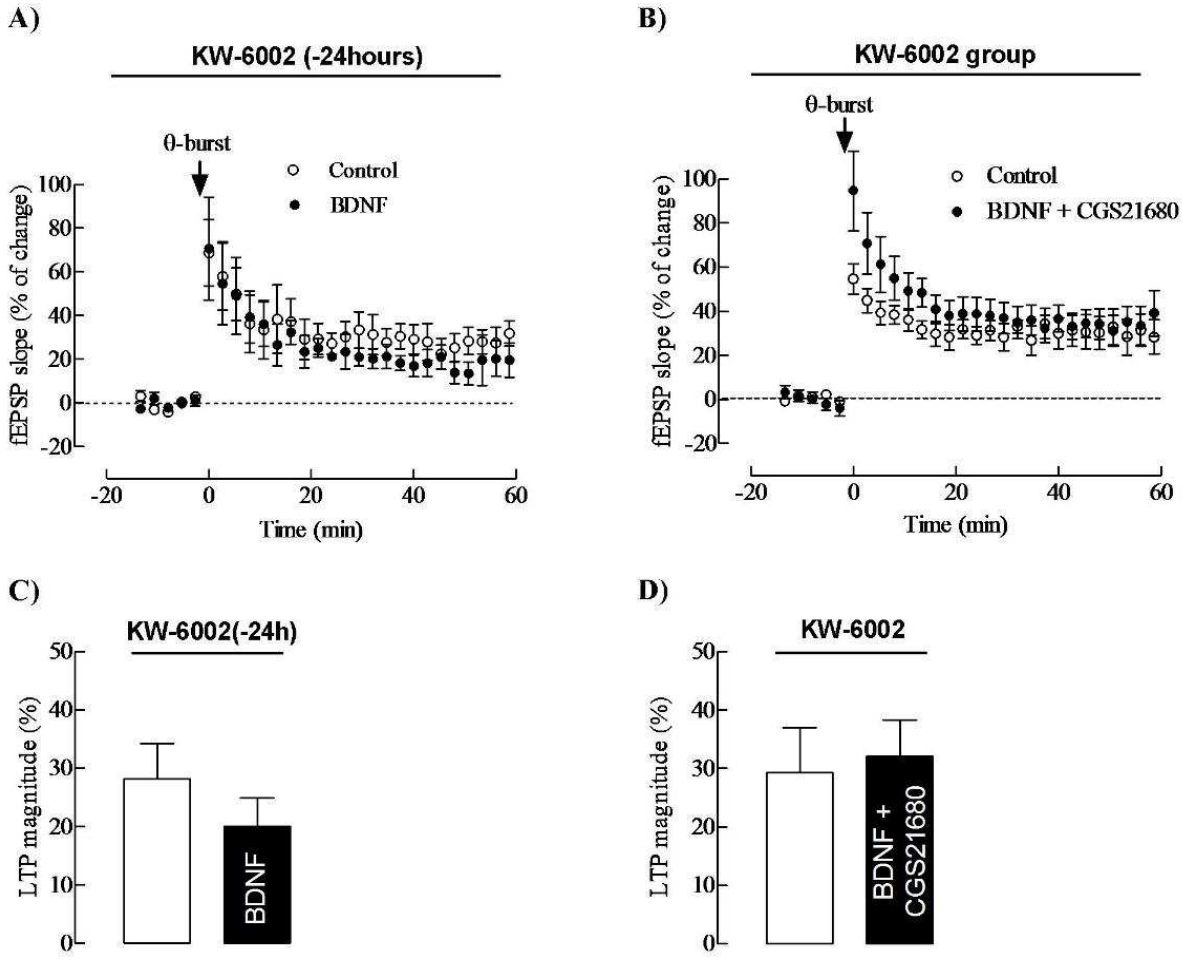


Figure 5.2 – Effects of KW-6002 are not reverted by treatment withdrawn or acute $A_{2A}R$ activation.
Panels **(A, B)** show the averaged time courses changes in field excitatory post-synaptic potential (fEPSP) slope induced by a θ-burst stimulation in the absence (○) or in the presence of BDNF 20ng/ml **(●, A)** or in the presence of BDNF (20ng/mL) and CGS21680 (30nM) **(●, B)** in hippocampal slices obtained from animals: **(B)** treated with KW-6002 (3mg/kg/day) for 1 month (n=5); or **(A)** animals treated with KW-6002 (3mg/kg/day) for 1 month and withdrawn from the drug 24 hours prior to the experiment (n=3). **(C, D)** LTP magnitudes (change in fEPSP slope at 46-60min) induced by θ-burst stimulation in relation to pre-θ-burst values (0%), for the panels A and B, respectively. All values in the figure are represented as the mean ± SEM of *n* independent experiments.

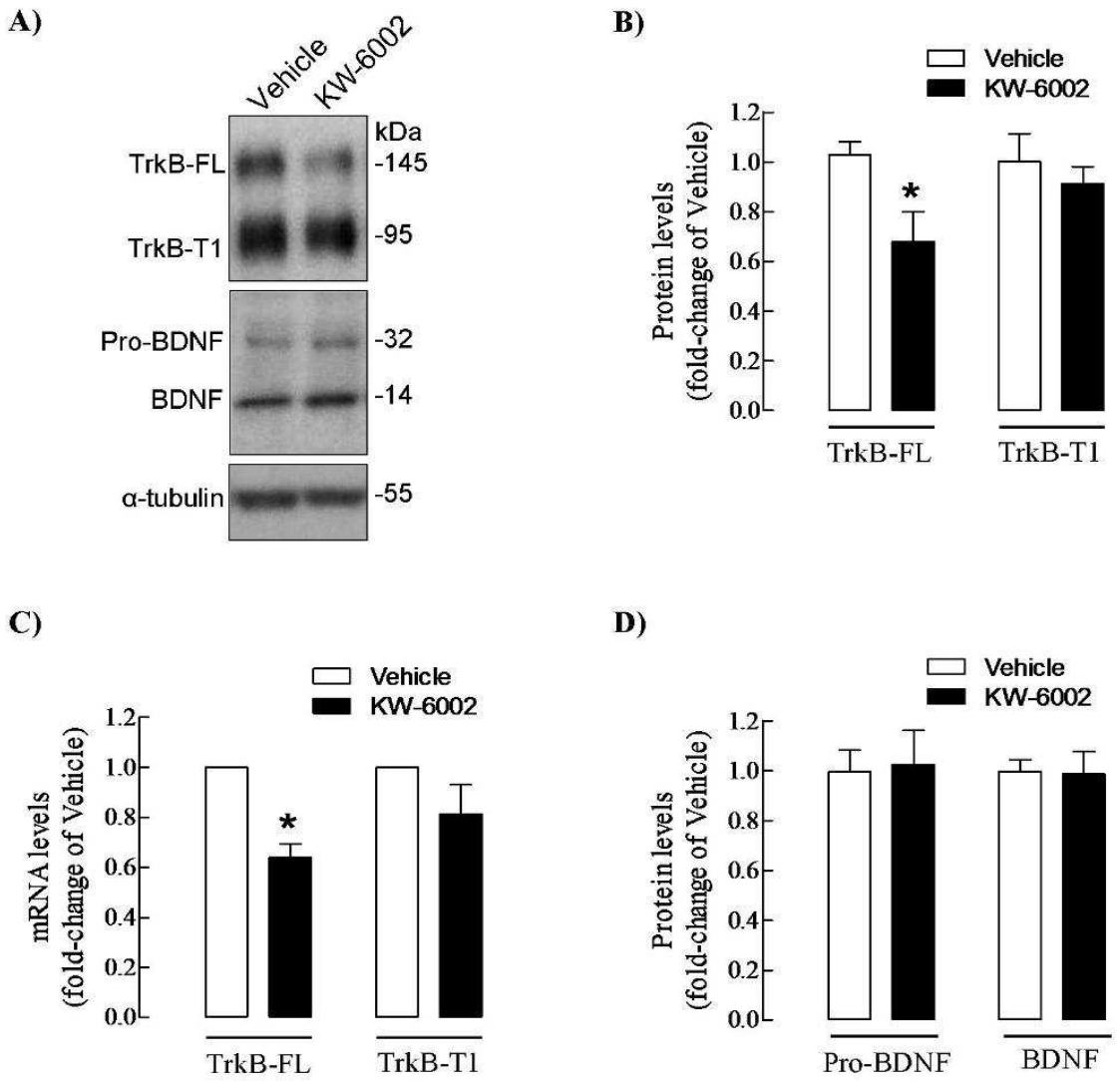


Figure 5.3 – Protein and mRNA levels of TrkB-FL receptor are decreased in KW-6002 treated rats.

(A) Representative western-blot comparing TrkB-FL, TrkB-T1, Pro-BDNF and mature BDNF levels in vehicle- and KW-6002-treated rats. The pan-TrkB antibody used recognizes both the TrkB-FL (~145 kDa) and truncated TrkB (TrkB-T1: ~95 kDa). The anti-BDNF antibody used in middle panel recognizes both pro-BDNF (~32kDa) and mature BDNF (~14kDa). α-tubulin was used as a loading control. **(B)** Quantification of TrkB-FL and TrkB-T1 band intensities obtained in western-blot for vehicle-treated (white bars) and KW-6002-treated (black bars) animals. Values were normalised with α-tubulin levels and represented as fold-change of vehicle (n=5). **(C)** Relative qPCR data showing mRNA levels of TrkB-FL and truncated TrkB-T1 from vehicle-treated animals (white bars) and KW-6002 treated animals (black bars). β-actin was used as an internal loading control. **(D)** Quantification of pro-BDNF and mature BDNF band intensities obtained by western-blot for vehicle-treated (white bars) and KW-6002 treated (black bars) animals. Values were normalised with α-tubulin levels and represented as fold-change of vehicle (n=5). Data are represented as the mean ± SEM of *n* independent experiments. *p<0.05 when compared to vehicle (Student's *t*-test).

5.5. Discussion

The results presented in this work reveal that *in vivo* chronic treatment with the $A_{2A}R$ antagonist, KW-6002, impairs BDNF actions upon LTP. Moreover, the data shows that this loss of BDNF effect is probably a consequence of the down-regulation of its full-length receptor (TrkB-FL) detected after the treatment with KW-6002.

Evidences show that the levels of BDNF and TrkB-FL are both decreased in several neurodegenerative disorders, particularly in AD [319, 321-323, 333]. Moreover, activation or overexpression of TrkB-FL in AD mice models improves cognitive function, suggesting that BDNF signalling deficits are an important contributor to the pathogenesis of AD [30, 316, 356]. Previous studies showed that the facilitatory effects mediated by BDNF upon synaptic transmission and plasticity are dependent or potentiated by $A_{2A}R$ activation [108, 113, 114]. However evidence is accumulating towards the beneficial effects of $A_{2A}R$ antagonists as a chronic pharmacologic treatment for neurodegenerative disorders such as AD [see 381]. Indeed, the neuronal death and memory impairments induced by amyloid-β peptide are prevented by $A_{2A}R$ antagonists and in the $A_{2A}R$ knock-out mice [382, 383]. Nevertheless $A_{2A}R$ activation or its downstream molecules such as cAMP were shown to also have putative beneficial effects on AD. Actually, the activation of $A_{2A}R$ is known to facilitate acetylcholine release [384, 385] promoting cholinergic transmission which is decreased in AD. Moreover, adenosine, acting at $A_{2A}R$, is an effective endogenous anti-inflammatory agent that can modulate inflammation both in the periphery and in the brain [386]. Neuronal maturation mediated by BDNF [see 387], is regulated by cAMP [388]; therefore, one could anticipate that this action of BDNF could also be influenced by activation of $A_{2A}R$. In addition, $A_{2A}Rs$ activation can rescue neurite outgrowth impairment caused by interference with the NGF signalling cascade in PC12 cells [389] as well as to promote PC12 cell survival upon NGF withdrawal [105]. All this data together with the fact that synaptic actions of BDNF are fully dependent on $A_{2A}R$ activation [108, 109, 111-113] highlighted the need for cautious blockade of $A_{2A}R$ whenever aiming to protect neurons from excitotoxicity while aiming to exacerbate neuroprotection of BDNF.

In the present study we evaluated the effect of the chronic administration of KW-6002 for 1 month, which is known to be effective in blocking $A_{2A}R$ mediated actions [353], in the BDNF action upon CA1 hippocampal LTP. In previous studies the same dose regimen was used and it had no impact in behavioural tasks and in synaptic plasticity or neuronal branching in healthy animals [353]. However the consequences of this treatment for BDNF signalling were not evaluated. Here we show that chronic blockade of $A_{2A}R$ in living animals, avoids exogenous BDNF facilitatory effect upon CA1 hippocampal LTP, without affecting the magnitude of LTP in absence of BDNF. As previously reported [353] the KW-6002 treatment induces an up-

regulation on $A_{2A}R$, which could suggest that KW-6002 treatment may potentiate the $A_{2A}R$/TrkB crosstalk. However the consequences of the pharmacological activation of $A_{2A}R$ upon synaptic transmission are similar between control and KW-6002 treated animals, suggesting that the increased levels of $A_{2A}R$ do not result in a gain of function at the synaptic level [390]. In addition, KW-6002 treated animals preserve hippocampal adenosine levels [390]. This implies that chronic KW-6002 treatment may not affect the expression of synaptic $A_{2A}R$, therefore explaining why the increase in $A_{2A}R$ levels does not impact into amplification of BDNF signalling in plasticity. Indeed it was already reported that different $A_{2A}R$ antagonists have different pharmacological profiles for specific populations of adenosine $A_{2A}R$ receptors [391, 392]. This would explain the increase in $A_{2A}R$ levels with no effect in their ability to modulate BDNF actions as well as the differential effect of the drug depending on the readout.

We previously reported [114] that θ-burst induced-LTP in hippocampal slices taken from young animals (the same age as that used in the present work), is independent on the endogenous BDNF since it is not altered by the prevention of TrkB-FL signaling. This might explain why KW-6002 treatment did not affect the basal LTP *per se*, in comparison with control slices. However one cannot preclude that KW-6002 treatment may reduce endogenous BDNF dependent LTP upon ageing [114].

The loss of BDNF effect upon CA1 hippocampal LTP in KW-6002 treated animals, even after 24hours of KW-6002 withdrawal, might be related to the decreased levels of TrkB-FL observed in these animals. One might speculate that the levels of TrkB-FL receptor could be decreased due to increased levels of endogenous BDNF, with the consequent over-activation and downregulation of TrkB-FL receptor as previously observed in [347] and Figure 3.3. However, our data do not support this hypothesis since no significant difference was detected on total BDNF or pro-BDNF levels in hippocampus from vehicle or KW-6002 treated animals. Moreover, it is known that cAMP regulates TrkB gene transcription because of its CRE promoter [393, 394]. Accordingly, since $A_{2A}R$ activation leads to cAMP formation [395], the chronic treatment with KW-6002 could result in a decrease of cAMP levels and a concomitant decrease in TrkB expression. This may explain the down-regulation of TrkB-FL receptors detected after $A_{2A}R$ antagonist treatment. Although the levels of the truncated TrkB-T1 receptor are not affected by KW-6002 treatment, the ratio TrkB-FL/TrkB-T1 is decreased given the significant decrease of TrkB-FL receptor levels. Given that TrkB-T1 receptors are dominant negative modulators of TrkB-FL receptors [25, 29], the decrease on TrkB-FL/TrkB-T1 ratio might aggravate the loss TrkB-FL signalling.

It is classically established that this particular type of synaptic plasticity, LTP, in the hippocampus is a prototypical experimental model that translates into forms of learning and memory associated with that brain area [396]. Although hippocampal dependent spatial memory was not affected in KW-6002 treated animals [353], which do not have any neurodegenerative

disorder. The question remains whether in AD mice models, in which BDNF and TrkB-FL receptors are severely decreased, other forms of hippocampal dependent memory that require BDNF signalling [397, 398] could be affected.

The present work shows that *in-vivo* chronic blockade of $A_{2A}R$, by an orally active antagonist (KW-6002), ablated the facilitatory effect of exogenous BDNF upon hippocampal CA1 LTP and it promotes a reduction on mRNA and protein levels of the BDNF receptor (TrkB full-length).

6. BDNF mediates neuroprotection against Aβ-induced toxicity in a mechanism independent on A_{2A}R activation

The work presented in this chapter is in preparation for submission.

6.1. Summary

Brain-derived neurotrophic factor (BDNF) promotes neuronal survival through the activation of its TrkB-FL receptor.

Evidences have shown that activation of adenosine A_{2A} receptors (A_{2A}R) is essential for most of BDNF-mediated synaptic actions, such upon synaptic plasticity, transmission and neurotransmitter release. In this chapter we evaluated the influence of A_{2A}R upon BDNF-mediated neuroprotection against neuronal death induced by Aβ25-35 (25μM) peptide. By measuring caspase-3 activity and protein levels, and αII-spectrin breakdown, we showed that BDNF reduces the activation of caspase-3 and calpain induced by Aβ peptide, in cortical cultures. This BNDF-mediated neuroprotection was not affected by A_{2A}R activation or inhibition. Moreover neither activation nor inhibition of A_{2A}R, *per se*, significantly influenced the Aβ-induced neuronal death and the calpain-mediated cleavage of TrkB induced by Aβ. In conclusion, these results suggest that, in opposition to the fast synaptic actions mediated by BDNF, the neuroprotection mediated by this neurotrophin against a strong Aβ insult, does not require A_{2A}R activation.

6.2. Rational

Since the identification of Aβ peptide as the main component of amyloid plaques present in the brain of AD patients, multiple studies were developed in order to clarify the involvement of Aβ in the AD neurodegenerative process. Indeed, data show that Aβ is neurotoxic and promotes cell death and synaptic dysfunction.

In the brain of AD patients, BDNF and TrkB-FL levels are decreased comparing to age matched controls, and in opposition, the truncated TrkB, which is a dominant-negative modulator of TrkB-FL, is increased. Given the impairment on BDNF signalling in AD, the administration of this neurotrophin direct into the brain was considered a possible therapeutic approach. Indeed, *in-vivo* administration of BDNF to animals models of AD and also Parkinson´s disease (PD), produced beneficial effects with improved synaptic and cognitive function and reduced neurodegeneration [120, 314]. However, despite the encouraging results from pre-clinical studies, the results from the BDNF-based clinical trials conducted so far (four in amyotrophic lateral sclerosis and one in diabetic neuropathy) have shown inconclusive results. However, there are no evidence that BDNF reached its target during the treatment [120]. Indeed, BDNF-based therapies have been hampered by multiple technical difficulties, such as the low penetrance of BDNF through blood-brain barrier and its rapid *in-vivo* clearance with consequent low half-life time in plasma or CSF [120]. Given these issues, an effort has been made to find small molecules that can activate directly the TrkB-FL, or that can boost BDNF actions in the brain, by an indirect mechanism. Molecularly, it has been shown that activation of A_{2A}R is able to transactivate a pool of immature TrkB receptors in the absence of BNDF [105, 107], and to induce the translocation of TrkB into lipid rafts microdomains in the membrane [117]. Functionally, it has been shown that most BDNF-mediated synaptic actions, such as in synaptic plasticity, transmission and neurotransmitter release, are fully dependent on A_{2A}R activation. However, some BDNF-mediated actions, such as in neuronal branching and GABA uptake, are not dependent on A_{2A}R activation [99].

Thus, agonists of A_{2A}R or activators of A_{2A}R-signalling pathways might be therapeutically relevant in neurodegenerative diseases where BDNF actions are decreased. In this chapter it was evaluated whether the neuroprotective effect of BDNF upon Aβ-induced neuronal death was also dependent, or potentiated, on A_{2A}R activation.

6.3. BDNF reduces cellular death induced by Aβ

It has been shown, in general cell viability assays, that BDNF protects neurons from Aβ toxicity [243]. Therefore, to reproduce this, 8 DIV neuronal cultures were incubated with $A\beta_{25-35}$ peptide (25µM) and BDNF (20ng/mL), and neuronal death was evaluated by analysing caspase-3 and calpain activation. Caspase-3 is a central mediator of apoptotic cell death, while calpains have been seen as a central player in necrotic cell death [399].Indeed, calpains rather than caspases have a prominent role in *in-vivo* excitotoxic neuronal death [171]. However, evidences indicate that calpains also play a central role in the execution of apoptosis [400, 401]. Accordingly, the calpain inhibition has shown protective effects in several models, including the *in-vivo* Aβ-induced neurodegeneration in rats [362]. Thus, both proteases, caspases and calpains, contribute to cellular architecture derangement and functional loss in neurons under degenerative conditions [399].

Incubation of 8 DIV cortical neurons with $A\beta_{25-35}$ (25µM) for 24 hours induced robust caspase-3 activation, as evaluated by enzymatic activity assay and active caspase-3 formation by western blot (2.5-fold increase in caspase-3 activity and 5-fold increase in active 17-kDa caspase-3 protein levels, when compared to control, $p<0.001$, n=5, Figure 6.1). Conversely, $A\beta_{25-35}$ induced a 4-fold increase in the levels of the caspase-3 specific αII-spectrin breakdown product SBDP120 ($p<0.001$, n=6, Figure 6.2C and D). As expected, Aβ also induced a very robust increase in SBDP150 levels indicating the activation of calpains ($p<0.001$, n=6, Figure 6.2B and D). When BDNF (20ng/mL) was simultaneously incubated with $A\beta_{25-35}$ (25µM), the levels of caspase-3 activity and 17-kDa caspase-3 formation were significantly reduced by 39 ±7% and 41 ±8%, respectively, when compared to $A\beta_{25-35}$ ($p<0.01$, n=5, Figure 6.1). Moreover, BDNF reduced the Aβ-induced formation of SBDP120 and SBDP150, by 61 ±18% and 58 ±26%, respectively ($p<0.01$ when compared to Aβ, n=6, Figure 6.2B-D). As a consequence of the inhibition of both calpain and caspase-3, BDNF also reduced the Aβ-induced cleavage of αII-spectrin by 59 ± 11% ($p<0.01$, n=6, Figure 6.2A and D).

Taken together this data show that BDNF significantly reduces the Aβ-induced activation of caspase-3 and calpain.

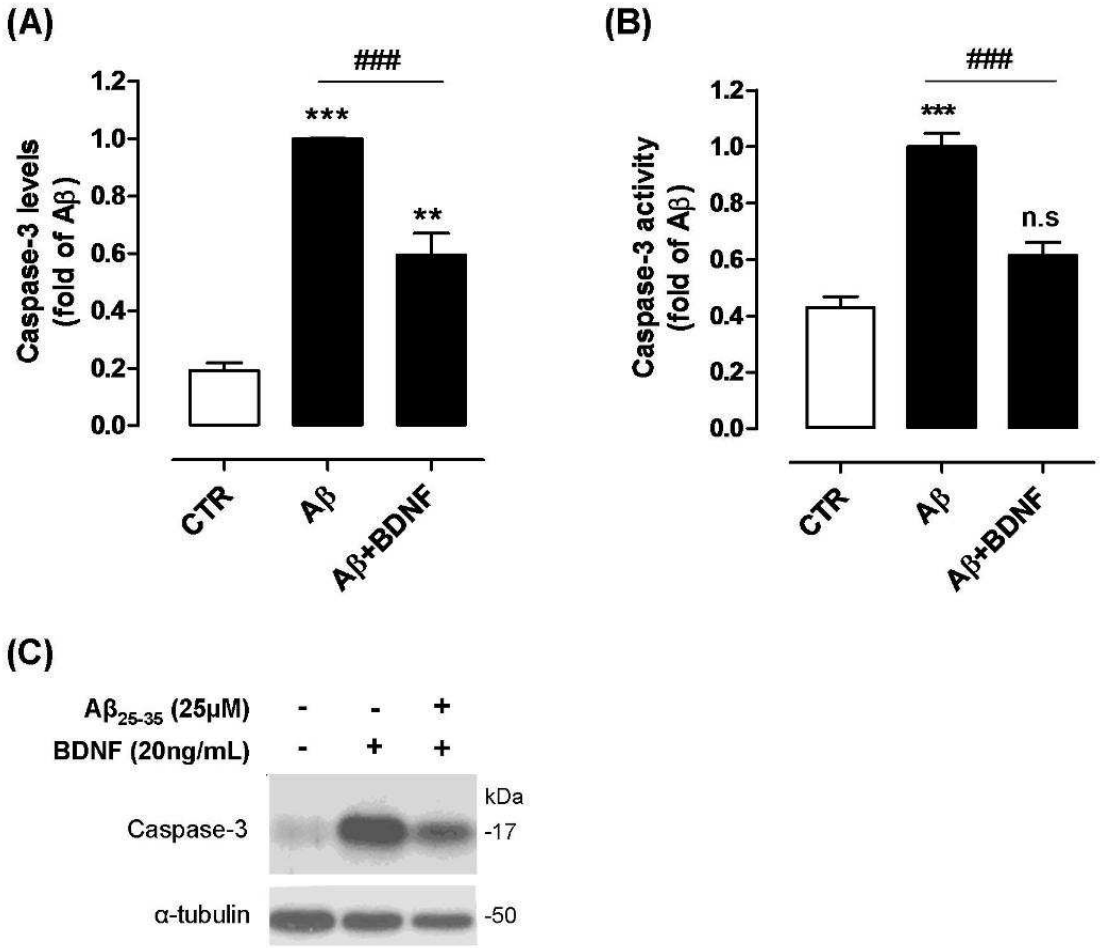


Figure 6.1 – BDNF reduces the caspase-3 activation induced by Aβ.

(A) Quantification of active Caspase-3 protein levels and **(B)** Caspase-3 activity, in 8 DIV cortical neurons non-treated or treated with $A\beta_{25-35}$ (25μM) for 24hours in the absence or presence of BDNF (20ng/mL). Data is normalized to Aβ condition given the very low, almost undetectable, caspase activation in control condition. ***p<0.001, **p<0.01, n.s (not significant) when comparing to control and ###p<0.001 when compared to Aβ (n=5, ANOVA followed by Bonferroni post-test) **(C)** Representative western-blot used in (A), showing the protein levels of active Caspase-3 (17-kDa). α-tubulin was used as a loading control. Data represented are mean ± SEM of *n* independent experiments.

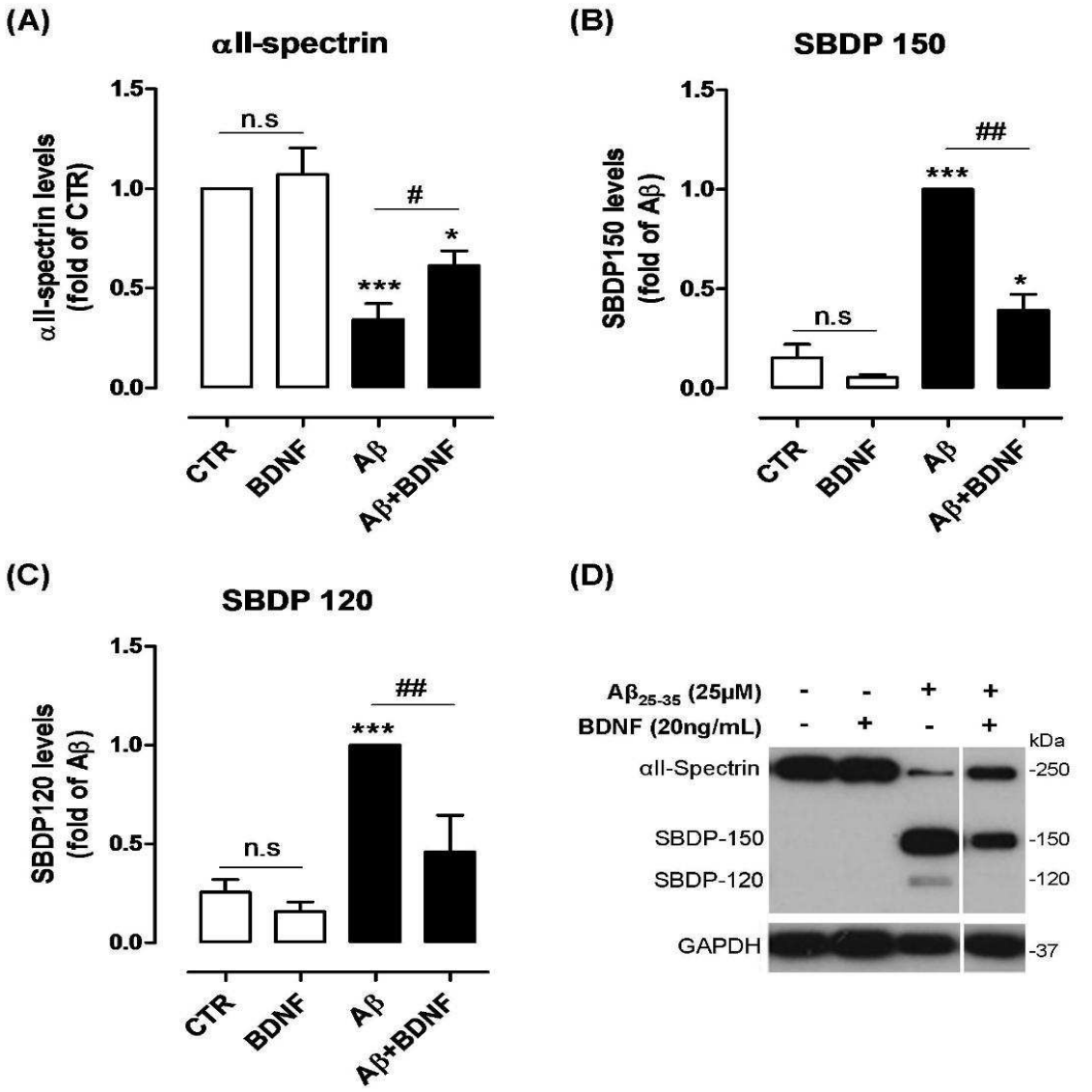


Figure 6.2 – BDNF reduces the αII-spectrin breakdown induced by Aβ.

(A) Quantification of αII-spectrin breakdown with consequent formation of **(B)** SBDP150 and **(C)** SBDP120 breakdown products. SBDP150 and SBDP120 levels are normalized to Aβ condition given their almost undetectable amount in control conditions. ***p<0.001, *p<0.05, n.s (not significant) when comparing to control and #p<0.05 and ##p<0.01 when comparing to Aβ (n=6, ANOVA followed by Bonferroni post-test) **(D)** Representative western-blot used in (A-C), showing the protein levels of αII-spectrin, SBDP150 and SBDP120. GAPDH was used as a loading control. Data represented are mean ± SEM of *n* independent experiments.

6.4. Cellular death prevented by BDNF does not require $A_{2A}R$ activation

To address if the activation of $A_{2A}R$ was required for BDNF-mediated neuroprotection, as it is verified for most synaptic actions mediated by this neurotrophin, 8 DIV cortical cells were incubated with $A\beta_{25-35}$ (25µM) and BDNF (20ng/ml) for 24 hours, in the presence or in the absence of the $A_{2A}R$ antagonist, SCH58261 (100nM), or the $A_{2A}R$ agonist, CGS21680 (10nM). Neither SCH58261 nor CGS21680, added 30 min prior of Aβ and BDNF, influenced the BDNF-mediated reduction of caspase-3 levels (Figure 6.3A, n=6) and activity (Figure 6.3B, n=6) upon Aβ toxicity. Regarding the data from αII-spectrin breakdown, similar results were obtained. Indeed, the reduction of αII-spectrin cleavage (Figure 6.4A) and correspondent reduction on SBDP150 (Figure 6.4B) and SBDP120 (Figure 6.4C) formation (cleavage product mediated by calpains and caspases, respectively) induced by BDNF, in cells incubated simultaneously with Aβ, was not influenced by the presence of both $A_{2A}R$ antagonist and agonist (n=6). *Per se*, the incubation with SCH58261 (100nM) or CGS21680 (10nM), 30 minutes prior Aβ incubation, did not influenced the Aβ-induced activation of both caspase-3 (n=4, $p>0.05$, Figure 6.3 and Figure 6.4C) and calpain (n=6, Figure 6.4A, B and D).

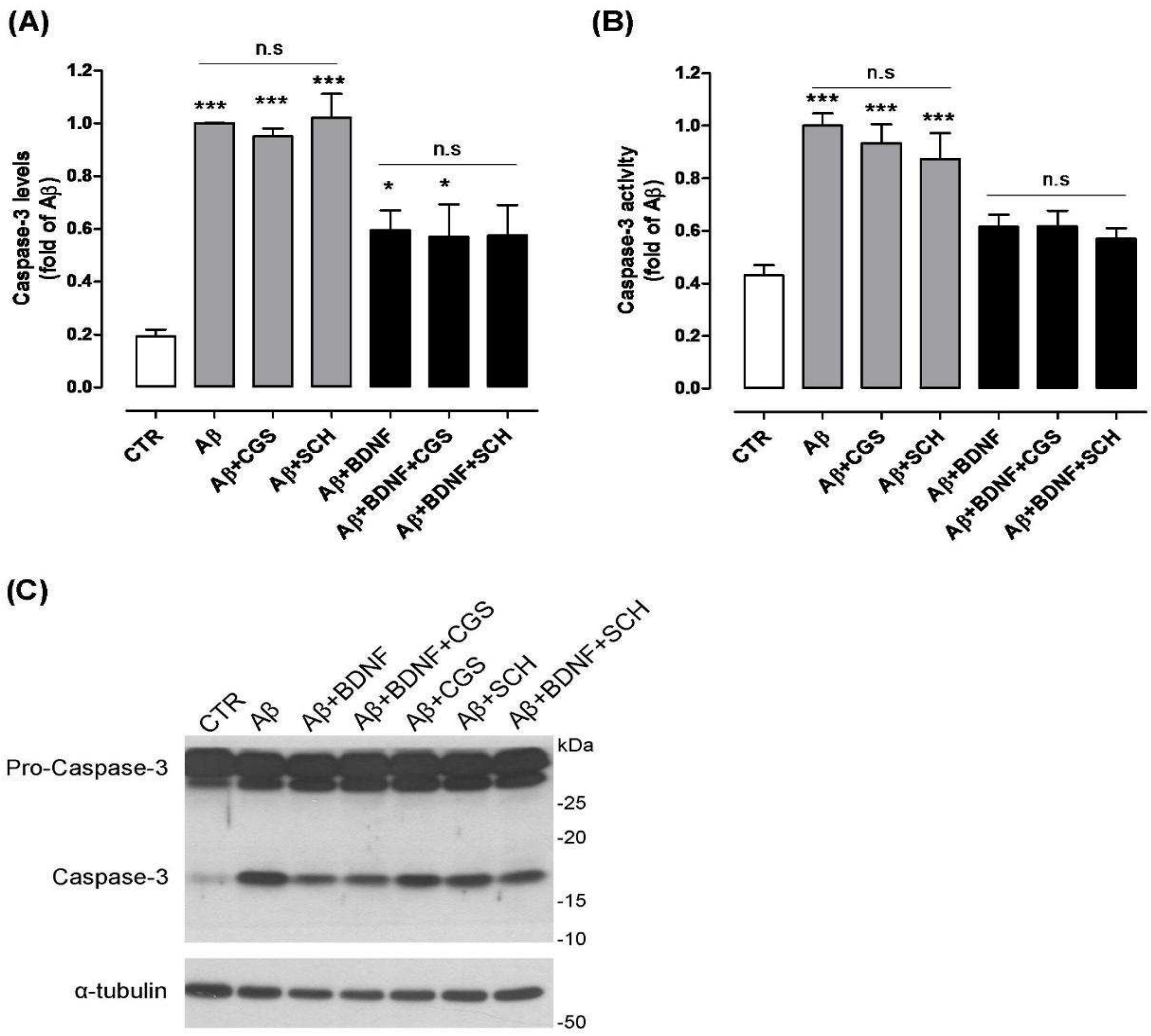


Figure 6.3 – $A_{2A}R$ does not influence BDNF-mediated reduction in Aβ-induced Caspase-3 activation
(A) Quantification of active Caspase-3 protein levels and **(B)** Caspase-3 activity, in 8 DIV cortical neurons non-treated or treated with Aβ25-35 (25μM) for 24hours in the absence or presence of BDNF (20ng/mL) and SCH58261 (100nM) or CGS21680 (10nM). Data is normalized to Aβ condition given the very low, almost undetectable, caspase activation in control condition. ***p<0.001, *p<0.05, when comparing to control and n.s (not significant) when compared between the conditions indicated by the horizontal line (n=6, ANOVA followed by Bonferroni post-test) **(C)** Representative western-blot used in (A), showing the protein levels of the unprocessed Pro-Caspase-3 (32 kDa) and the active Caspase-3 (17-kDa). Note that the order of the conditions is different from (A). α-tubulin was used as a loading control. Data represented are mean ± SEM of *n* independent experiments.

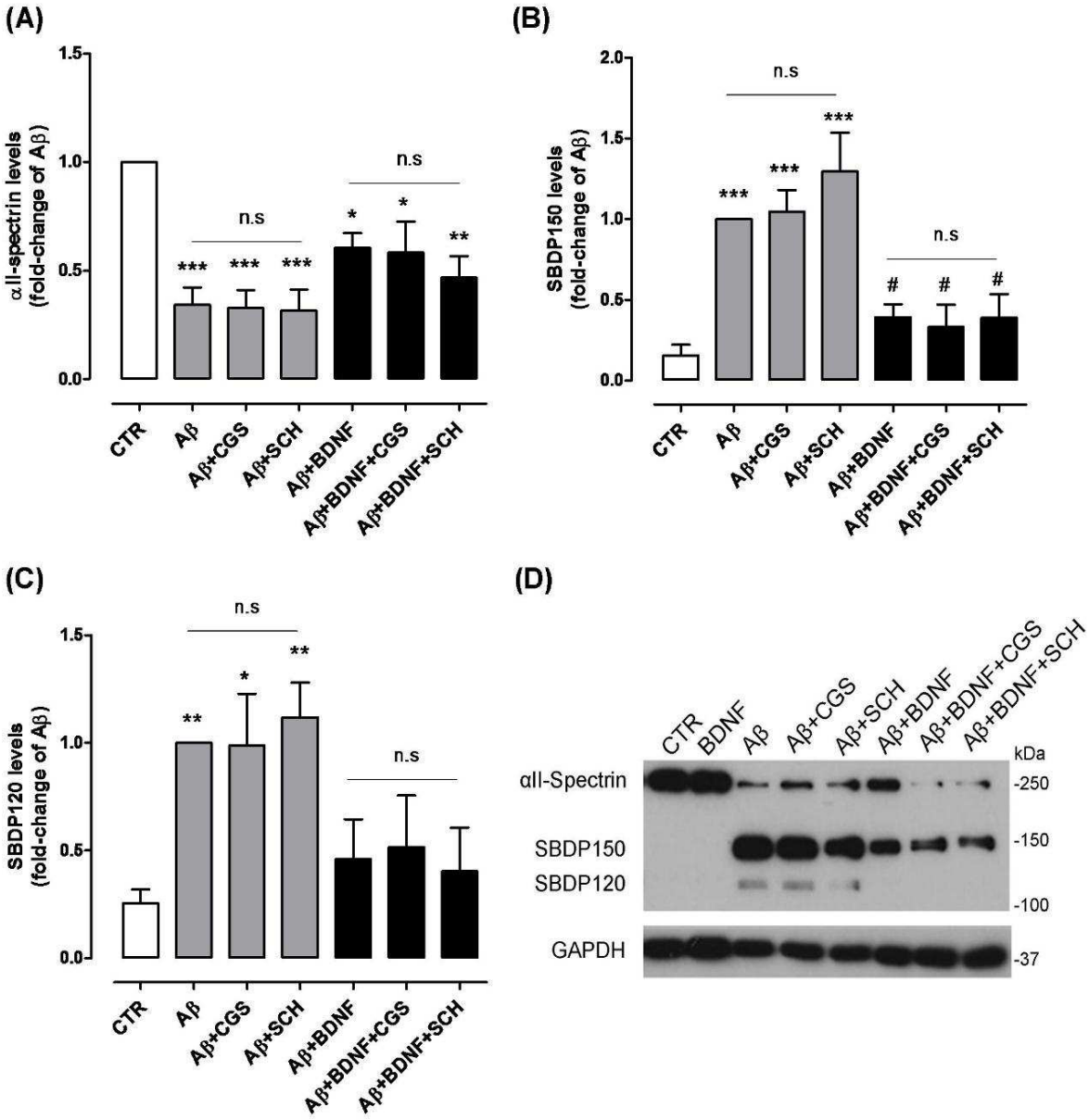


Figure 6.4 – $A_{2A}R$ does not influence BDNF-mediated reduction in Aβ-induced spectrin breakdown. **(A)** Quantification of αII-spectrin breakdown with consequent formation of **(B)** SBDP150 and **(C)** SBDP120 breakdown products in 8 DIV cortical neurons non-treated or treated with Aβ25-35 (25μM) for 24hours in the absence or presence of BDNF (20ng/mL) and SCH58261 (100nM) or CGS21680 (10nM). SBDP150 and SBDP120 levels are normalized to Aβ condition given their almost undetectable amount in control conditions. ***p<0.001, **p<0.01, *p<0.05, when comparing to control, n.s (not significant) when compared between the conditions indicated by the horizontal line and #p<0.05 when comparing to Aβ (n=6, ANOVA followed by Bonferroni post-test) **(D)** Representative western-blot used in (A-C), showing the protein levels of αII-spectrin, SBDP150 and SBDP120. GAPDH was used as a loading control. Data represented are mean ± SEM of *n* independent experiments.

6.5. Aβ-induced TrkB truncation is not influenced by $A_{2A}R$ activation

As previously shown (Chapter 4.6), Aβ induces a calpain-mediated truncation on TrkB-FL receptor, with concomitant formation of an intracellular fragment (TrkB-ICD) and an increase on truncated receptor (TrkB-Tc) levels [346]. As we showed previously, $A_{2A}R$ do not influence calpain activation induced by Aβ. However, given that $A_{2A}R$ activation is able to induce the translocation of TrkB receptors into lipid rafts microdomains [117], we considered noteworthy to test the hypothesis that $A_{2A}R$ might protect TrkB receptors from calpain-mediated cleavage, by allocating them in a different location in the membrane.

To test this hypothesis, the levels of TrkB-FL, TrkB-Tc and TrkB-ICD were analysed in 8 DIV cortical cultures treated with $A\beta_{25-35}$ (25µM), in the presence or absence of SCH58261 (100nM) or CGS21680 (10nM).

The results show that, upon Aβ incubation the $A_{2A}R$ agonist, did not protected the TrkB-FL receptor from the Aβ-induced cleavage ($p<0.05$, n=6, Figure 6.5A-D). Conversely, the $A_{2A}R$ antagonist did not change the magnitude of TrkB cleavage induced by Aβ ($p<0.05$, n=6, Figure 6.5A-D). Moreover, the incubation with the $A_{2A}R$ agonist, or antagonist, for 24 hours did not significantly change TrkB expression ($p>0.05$, n=6, Figure 6.5A and D).

In addition, we also evaluated if BDNF can prevent the Aβ-induced TrkB truncation. The results show that 24 hours of BDNF (20ng/mL) incubation on neuronal cultures prevent the Aβ-induced formation on TrkB-ICD and TrkB-Tc ($p<0.05$, n=6, Figure 6.5B,C and D), an effect unaffected by the presence of the $A_{2A}R$ agonist or antagonist ($p>0.05$, n=6, Figure 6.5B,C and D). However, the BDNF incubation also strongly reduced the levels of TrkB-FL, even in the absence of Aβ ($p<0.001$, n=6, Figure 6.5A and D).

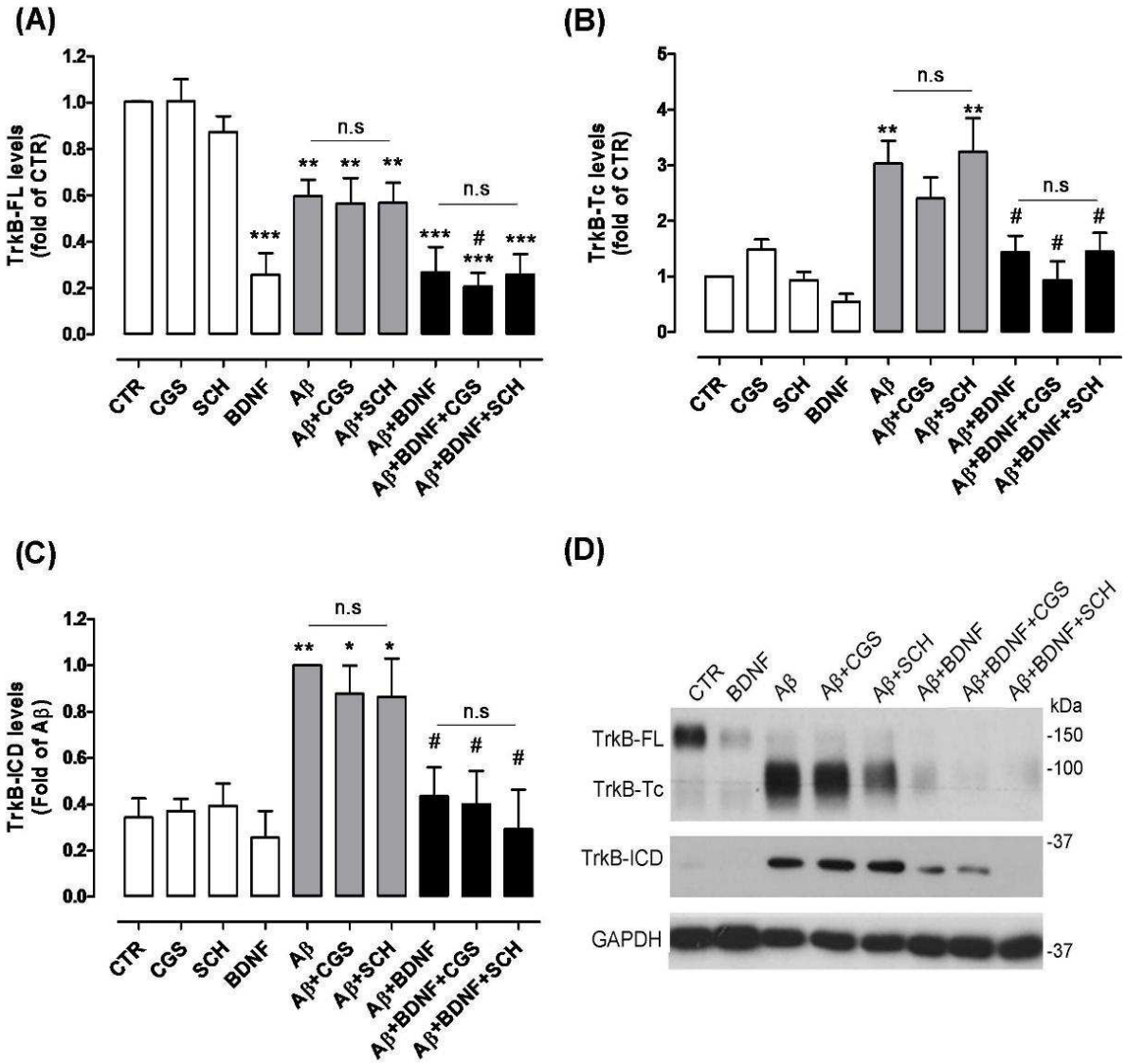


Figure 6.5 – Effect of $A_{2A}R$ and BDNF upon Aβ-induced TrkB truncation

(A) Quantification of TrkB-FL cleavage with consequent formation of **(B)** truncated TrkB and **(C)** TrkB-ICD fragment, in 8 DIV cortical neurons non-treated and treated with Aβ25-35 (25μM) for 24hours in the absence or presence of BDNF (20ng/mL) and SCH58261 (100nM) or CGS21680 (10nM). TrkB-ICD levels are normalized to Aβ condition given their almost undetectable amount in control conditions. ***p<0.001, **p<0.01, *p<0.05, when comparing to control, n.s (not significant) when compared between the conditions indicated by the horizontal line and #p<0.05 when comparing to Aβ (n=6, ANOVA followed by Bonferroni post-test) **(D)** Representative western-blot used in (A-C), showing the protein levels of TrkB-FL, TrkB-Tc and TrkB-ICD. GAPDH was used as a loading control. Data represented are mean ± SEM of *n* independent experiments.

6.6. Discussion

The results described in this chapter show that the BDNF exerts protective effects by reducing the caspase-3 and calpain activation upon $A\beta_{25-35}$ toxicity, an effect independent on A_{2A}R activation.

The BNDF effects are widely described in several *in-vitro* and *in-vivo* models of neurodegenerative disorders, as Parkinson's and Alzheimer's disease [120, 402]. In particular, BDNF is able to increase cell viability of neurons incubated with toxic concentrations of Aβ peptide [243]. By evaluating the levels of caspase-3 and calpain activation, both proteases involved in cellular death, it was confirmed the BDNF neuroprotective effects upon Aβ toxicity. Accordingly, the cortical neurons incubated with Aβ together with BDNF present a robust reduction in conversion of pro-caspase-3 into active caspase-3 and in the caspase-3 activity. Moreover, BDNF reduced the breakdown of αII-spectrin, a neuronal cytoskeletal protein highly susceptible to neurodegeneration, and consequently, reduced the formation of the calpain- and caspase-3-specific spectrin breakdown products (SBDP150 and SBDP120, respectively).

Caspases and calpains may act synergistically to induce neuronal death. It is now known that: 1) both calpains and caspases share multiple common substrates; 2) calpains can cleave a variety of caspases leading to their activation, or inhibition; 3) caspases can cleave calpastatin leading to the activation of calpains [256, 399, 403, 404]. In accordance to the results described in this chapter, others have shown that Aβ triggers the activation of both calpains and caspases in septal cultured neurons [256]. Therefore, caspases or calpains inhibitors can markedly protect cultured neurons against Aβ-induced toxicity. However the effects of calpain and caspases inhibitors are not additive, suggesting that other pathways might be involved [256]. Here we show that BDNF can reduce the Aβ-induced activation of both caspase-3 and calpain. It has been shown that BDNF can also block caspase-3 activation in neurons submitted to different types of insults, such as radiation or hypoxia-ischemia [405, 406]. The signalling pathways implicated in the BDNF-mediated inhibition of both caspase-3 and calpain were not evaluated in this work. Nevertheless, it is known that BDNF can activate PI3K/Akt signalling pathway, which in turn can halt apoptosis through phosphorylation and inhibition of pro-apoptotic proteins such as Bad and caspase-9 [407]. Importantly, other member of neurotrophin family, the neurotrophin-3 (NT-3), also protects cortical neurons from Aβ-induced toxicity by inhibiting caspase-3, -8 and -9, in an Akt-dependent and ERK/MAPK-independent way. In particular, NT-3 incubation activates Akt, which in turn induces the expression of NAIP-1, a member of the inhibitors of apoptosis proteins (IAPs), which can directly inhibit caspase activation [408]. Although the BDNF-mediated inhibition of caspase-3 is most likely Akt-dependent, the mechanism that contributes to calpain inhibition is not known and awaits further elucidation.

Strikingly, BDNF and EGF can activate m-calpain in a MAPK dependant way [178]. However, although calpains could play multiple biological roles upon physiological activation, when overactivated, these enzymes, promote neuronal death and contribute to neurodegeneration. Thus, upon physiological conditions the BDNF-mediated activation of m-calpain might be relevant for some BDNF actions, while upon pathological conditions, the BDNF might normalize calpain activity by reducing its excessive activation, and preventing the neurodegenerative process.

The actions of BDNF upon synaptic transmission and plasticity are well characterized as being dependent on A_{2A}R receptor activation [108-116]. However, the results presented in this work indicate that A_{2A}R activation is not required for BDNF neuroprotective actions upon Aβ insult to 8 DIV primary cortical cultures. Similarly to the results obtained here, A_{2A}R activation is not required for the effect of BDNF upon neuronal branching [99, 118]. Thus, taken together this data might indicate that the trophic and survival actions of BDNF do not depend on A_{2A}R activation. However, it is not clear why the BDNF actions could be dependent or independent on A_{2A}R activation, in distinct situations. Nevertheless, most A_{2A}R-dependent actions of BDNF are synaptic and fast-mediated actions, while the trophic and neuroprotective actions of BDNF are slow and long-lasting events that require protein synthesis. Thus, one possibility that might explain the discrepancy in A_{2A}R dependence is the fundamental difference between the slow and fast-mediated actions of BDNF, which in turn involve different signalling pathways.

One might speculate whether the lack of A_{2A}R expression, in the cultures, could explain the absence of A_{2A}R effects. However, this is not the case since although A_{2A}R are not abundantly expressed in the hippocampus or neocortex [98], the A_{2A}R is detected in 6 DIV cortical cultures by immunocytochemistry [409], and also by western-blot and RT-PCR in 9 DIV cortical cultures [410]. Controversial data have shown protective effects against insults either by blocking or activating A_{2A}R. Accordingly, it has been shown that A_{2A}R activation, by CGS21680, reduces kainite-induced excitotoxicity by 40%, in 6DIV cortical cultures [409]. In opposition, the blockade of A_{2A}R by SCH58261 improves cell viability against the glutamate (20-1000μM, 24h) insult, whereas A_{2A}R activation does not protect neurons, in 9 DIV cortical cultures [410]. In addition, both caffeine (a non-selective adenosine receptor antagonist) and A_{2A}R antagonists prevent the toxicity induced by 48h of $A\beta_{25-35}$ (25μM) incubation on cultured cerebellar granule neurons [411]. Moreover, A_{2A}R blockade, or genetic deletion of A_{2A}R, prevents synaptotoxicity and memory dysfunction caused by intracerebroventricular administration of 2nmol of $A\beta_{1-42}$ in mice [383, 412]. Finally, blockade of A_{2A}R, by SCH58261, prevented the reduction in cell viability induced by the incubation of oligomeric-enriched $A\beta_{1-42}$ (500nM), in hippocampal cultures [383]. In this work, using cortical neurons, no significant protective effect of the A_{2A}R agonist, or antagonist, against the toxicity induced by the

incubation of fibrillary-enriched $A\beta_{25-35}$ (25µM) [30] was seen. Considering that A_{2A}R inhibition exerts protective actions, probably it was not sufficient to prevent the strong toxic insult used in this work. Indeed, previous results obtained showed that the same preparation of $A\beta_{25-35}$ (25µM, 24hours) decreased cell viability by around 45%, while glutamate (1000µM, 24h) only decreased viability by around 30% [409]. Moreover, the same study showed that the neuroprotection afforded by A_{2A}R blockade against glutamate insult (100 µM, 24h) requires the activation of the corticotrophin-releasing factor (CRF) receptor subtype 2. Interestingly, the agonist of the CRF receptors was able to protect cortical neurons when glutamate was present at lower concentrations (50 and 100µM, 24hour), but failed to protect neurons at a higher concentration of glutamate (500 and 1000µM) [409]. Thus, this results support the hypothesis that A_{2A}R inhibition might be not able to revert the toxicity of a strong insult such as $A\beta_{25-35}$ (25µM). Nevertheless, it is noteworthy to highlight that BDNF was able to reduce, very significantly, both caspase-3 activation and αII-spectrin breakdown, upon the $A\beta_{25-35}$ (25µM) insult.

Despite that TrkB gene transcription can be regulated by Ca^{2+} or cAMP [393, 394], the results obtained here show that the activation of A_{2A}R, which is known to increase cAMP levels, does not significantly change TrkB-FL expression. Although incubation of cortical neurons with forskolin, which stimulates cAMP production, increased mRNA transcripts of both full-length and truncated TrkB within 1 hour, the increase on protein only appear to be evident after 16 hours [393]. Thus, it is possible that A_{2A}R activation had not been sufficient to significantly increase TrkB protein levels after 24hours. In addition, the results also show that acute blockade of A_{2A}R for 24hours does not changed TrkB expression. However, the *in-vivo* chronic blockade of A_{2A}R for one month decreased both protein and mRNA levels of TrkB-FL [116].

Finally, we evaluated whether BDNF was able to prevent the Aβ-induced truncation of TrkB, which is known to be mediated by calpains, as we previously described in [346]. We found that BDNF significantly reduces the levels of TrkB-FL cleavage fragments (TrkB-Tc and TrkB-ICD). However, in accordance to what had been initially described [347], the present results confirm that BNDF strongly downregulates TrkB-FL protein levels after 24 hours of incubation. This BDNF-mediated downregulation of TrkB-FL is a fast event, in which total TrkB-FL receptor levels decrease by 80% in just 3 hours after the ligand binding, a value that remain almost constant up to 24 hours, at least [347]. Given that the calpain-mediated cleavage of TrkB-FL has a much slower time course (Figure 4.5B)Figure 4.5 – Aβ peptide induces a TrkB-FL receptor cleavage. [346], it is likely that when calpains became active and begin to cleave TrkB-FL, the levels of the receptor are almost fully reduced due to the BDNF-mediated downregulation. Thus, the observation that BDNF prevent the formation of TrkB-FL cleavage fragments might be explained by both calpain inhibition and by BDNF-mediated downregulation of TrkB-FL.

Together, these results show that BDNF exerts a robust neuroprotective effect upon Aβ-induced toxicity, by reducing calpain and caspase-3 activation. In opposition to most fast synaptic actions of BDNF, the neuroprotective effect of this neurotrophin does not depend on A_{2A}R activation.

7. General discussion and conclusions

Multiple studies have shown the vital importance of BDNF in the brain. Indeed, BDNF is not only essential for the regulation of synaptic plasticity and memory formation, but also confers strong protection to neurons against a wide variety of toxins and insults [120]. Interestingly, a growing number of studies have shown that BDNF signalling is impaired in several chronic neurodegenerative disorders, including AD [319-326, 333-337]. Thus, the dysregulation of BDNF signalling might be a consequence of the disease which in turn could aggravate the progression of the degenerative process, or might be one of the leading causes of the pathology. Indeed, BDNF administration or upregulation of TrkB and BDNF signalling on AD animal models have shown strong beneficial effects, including the reduction of neurodegeneration and increased cognitive performance [120, 243, 315-317, 344, 413]. Despite the importance of BDNF on AD, the mechanisms that contribute to the loss of BNDF signalling upon AD remained largely unknown.

In this work, it was found that Aβ induces a dysregulation on TrkB receptors levels and abolishes BDNF actions upon synaptic plasticity and neurotransmitter release, in a calpain-dependent mechanism. In particular, Aβ induces calpain activation on cortical cultures, resulting in the cleavage of TrkB-FL receptor in the juxtamembrane region, producing a new truncated receptor (TrkB-T') which contains the Shc binding site and an intracellular domain fragment (TrkB-ICD) which contains the entire TrkB kinase domain. Interestingly, the presence of TrkB-ICD was detected in human brain sample, indicating that endogenous human calpains are able to cleave human TrkB receptor. Moreover, Aβ selectively increases the mRNA levels of truncated TrkB isoforms, an effect independent on calpains.

In addition, the exposure of hippocampal slices to Aβ results in the abolishment of BDNF action upon LTP and upon glutamate and GABA release, an effect reverted by the inhibition of calpains.

Several studies have demonstrated the benefits of calpain inhibitors in animal models of neurodegenerative disorders. In particular, calpain inhibitors reduce neurodegeneration and improved cognitive performance in AD animal models [199, 201, 202]. The present work shows that calpain inhibition prevents TrkB receptors changes induced by Aβ and also restores BDNF actions reinforcing the rational for the use of calpain inhibitors in the therapeutics AD.

Interestingly, most of BDNF synaptic actions are dependent or facilitated by $A_{2A}R$ activation [108-116]. However, inhibitors of $A_{2A}R$, in particular the orally-active KW-6002 inhibitor, have been proposed as a therapeutical tool against Parkinson's disease and AD [376-378]. Here, we evaluated the effect of the chronic *in-vivo* administration of KW-6002 on the BDNF actions upon synaptic plasticity. It was shown that KW-6002 administration for one

month to young rats, reduces the mRNA and protein levels of TrkB-FL in the hippocampus, and also abolishes the facilitatory effect of exogenous BDNF upon LTP in hippocampal slices. Thus, we conclude that $A_{2A}R$ inhibition might impair BDNF signalling, at least in young rats.

$A_{2A}R$ inhibition has been associated to the reduction of neuronal excitability and to neuroprotection against toxic insults such as Aβ [376-378, 383, 411, 412]. Here, by evaluating the extension of caspase-3 and calpain activation, it was possible to show that acute $A_{2A}R$ inhibition does not protect cortical neurons against a strong Aβ insult. Moreover, the activation of $A_{2A}R$ also does not influence neuronal death induced by Aβ. However, BDNF reduces the activation of caspase-3 and calpain induced by Aβ insult, an effect that it is also observed in the presence of the $A_{2A}R$ agonist or antagonist. Thus, it is possible to conclude that, in opposition to synaptic plasticity, the protective action of BDNF upon caspase-3 and calpain activation, in Aβ toxicity, does not depend on $A_{2A}R$ activation.

Together, the present work shows that Aβ dysregulates TrkB receptors and BDNF actions on synaptic plasticity and neurotransmitter release, by a calpain-dependent mechanism. This data reinforces the rational of using calpain inhibitors for the therapeutics of AD. Moreover, the acute blockade, or activation, of $A_{2A}R$ does not influence the protective effect mediated by BDNF against Aβ peptide. Interestingly the chronic *in vivo* blockade of $A_{2A}R$, which has been considered as a useful therapeutical strategy against neurodegenerative disorders, abolishes BDNF actions upon hippocampal synaptic plasticity and decreases TrkB-FL receptors levels. This data highlight the caution that must be taken whenever studying $A_{2A}R$ antagonist as pharmacological tools to treat neurodegenerative disorders where BDNF signalling is impaired.

8. Future perspectives

One interesting question raised in chapter 4, that would be interesting to address is whether the TrkB intracellular cleavage fragment (TrkB-ICD) has any biological function. Calpains usually cleave proteins in interdomain regions, releasing big stable fragments that can have a distinct or enhanced biological activity [127]. For instance, calpain cleave p35 protein into p25, enhancing its activity and promoting an aberrant activation of CDK5, which in turn contributes to neurodegeneration [196, 282, 285, 361]. In another study closely-related to this work, it was described that calpain cleaves Src kinase upon excitotoxicity, both *in-vivo* and *in-vitro*, releasing a cytosolic 52kDa fragment which contains the Src kinase domain. The expression of this fragment in neuronal cultures was sufficient to induce neuronal death, possibly by inactivating Akt kinase [414]. Our preliminary results suggest that TrkB-ICD fragment released after the cleavage, has a different conformation than the native TrkB-FL protein (see subchapter 10.1). Moreover, TrkB-ICD appears to be enriched in the non-cytosolic fraction (probably located within the nucleus or another organelle, or even associated with membranes). Thus, in future, it would be interesting to confirm the subcellular localization of TrkB-ICD and its putative biological function. To help addressing this issue we already have cloned the TrkB-ICD fragment and generated an expressing vector containing the fragment, the pcDNA-TrkB-ICD-V5 (see subchapter 10.2). As future work, we would like to transfect neurons or neuronal lines and evaluate: 1) the subcellular localization of the fragment by microscopy; 2) cell viability alterations (since many of calpain-generated fragments have a pro-apoptotic role); 3) phosphorylation status of the signalling pathways associated with TrkB-FL (Akt, ERK, PLCγ), since TrkB-ICD might be phosphorylating these proteins, or TrkB-ICD might be not functional and attenuating the phosphorylation of these signalling molecules by competing with native kinase receptors. Additionally, as a major goal, we would like to evaluate if the *in-vivo* administration of calpain inhibitors increases BDNF signalling in a neurodegenerative AD mice model.

Finally, regarding chapter 6, it would be important in future to identify the molecular pathways by which BDNF inhibits the Aβ-induced calpain overactivation.

9. References

1. Arevalo, J.C. and S.H. Wu, *Neurotrophin signaling: many exciting surprises!* Cell Mol Life Sci, 2006. **63**(13): p. 1523-37.
2. Levi-Montalcini, R. and V. Hamburger, *Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo.* J Exp Zool, 1951. **116**(2): p. 321-61.
3. Barde, Y.A., D. Edgar, and H. Thoenen, *Purification of a new neurotrophic factor from mammalian brain.* EMBO J, 1982. **1**(5): p. 549-53.
4. Hohn, A., et al., *Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family.* Nature, 1990. **344**(6264): p. 339-41.
5. Hallbook, F., C.F. Ibanez, and H. Persson, *Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary.* Neuron, 1991. **6**(5): p. 845-58.
6. Lessmann, V., K. Gottmann, and M. Malcangio, *Neurotrophin secretion: current facts and future prospects.* Prog Neurobiol, 2003. **69**(5): p. 341-74.
7. Kolbeck, R., S. Jungbluth, and Y.A. Barde, *Characterisation of neurotrophin dimers and monomers.* Eur J Biochem, 1994. **225**(3): p. 995-1003.
8. Lu, B., P.T. Pang, and N.H. Woo, *The yin and yang of neurotrophin action.* Nat Rev Neurosci, 2005. **6**(8): p. 603-14.
9. Teng, K.K., et al., *Understanding proneurotrophin actions: Recent advances and challenges.* Dev Neurobiol, 2010. **70**(5): p. 350-9.
10. Kaplan, D.R., D. Martin-Zanca, and L.F. Parada, *Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF.* Nature, 1991. **350**(6314): p. 158-60.
11. Klein, R., et al., *The trk proto-oncogene encodes a receptor for nerve growth factor.* Cell, 1991. **65**(1): p. 189-97.
12. Berkemeier, L.R., et al., *Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB.* Neuron, 1991. **7**(5): p. 857-66.
13. Squinto, S.P., et al., *trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor.* Cell, 1991. **65**(5): p. 885-93.
14. Lamballe, F., R. Klein, and M. Barbacid, *trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3.* Cell, 1991. **66**(5): p. 967-79.
15. Barker, P.A., *p75NTR is positively promiscuous: novel partners and new insights.* Neuron, 2004. **42**(4): p. 529-33.
16. Zhang, Y., et al., *Cell surface Trk receptors mediate NGF-induced survival while internalized receptors regulate NGF-induced differentiation.* J Neurosci, 2000. **20**(15): p. 5671-8.
17. Casaccia-Bonnefil, P., et al., *Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75.* Nature, 1996. **383**(6602): p. 716-9.
18. Bamji, S.X., et al., *The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death.* J Cell Biol, 1998. **140**(4): p. 911-23.
19. Teng, H.K., et al., *ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin.* J Neurosci, 2005. **25**(22): p. 5455-63.
20. Holden, P.H., et al., *Immunoglobulin-like domains define the nerve growth factor binding site of the TrkA receptor.* Nat Biotechnol, 1997. **15**(7): p. 668-72.
21. Schneider, R. and M. Schweiger, *A novel modular mosaic of cell adhesion motifs in the extracellular domains of the neurogenic trk and trkB tyrosine kinase receptors.* Oncogene, 1991. **6**(10): p. 1807-11.
22. Middlemas, D.S., R.A. Lindberg, and T. Hunter, *trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors.* Mol Cell Biol, 1991. **11**(1): p. 143-53.
23. Stoilov, P., E. Castren, and S. Stamm, *Analysis of the human TrkB gene genomic organization reveals novel TrkB isoforms, unusual gene length, and splicing mechanism.* Biochem Biophys Res Commun, 2002. **290**(3): p. 1054-65.
24. Allen, S.J., et al., *Cloning of a non-catalytic form of human trkB and distribution of messenger RNA for trkB in human brain.* Neuroscience, 1994. **60**(3): p. 825-34.

25. Eide, F.F., et al., *Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling.* J Neurosci, 1996. **16**(10): p. 3123-9.
26. Haapasalo, A., et al., *Truncated trkB.T1 is dominant negative inhibitor of trkB.TK+-mediated cell survival.* Biochem Biophys Res Commun, 2001. **280**(5): p. 1352-8.
27. Li, Y.X., et al., *Expression of a dominant negative TrkB receptor, T1, reveals a requirement for presynaptic signaling in BDNF-induced synaptic potentiation in cultured hippocampal neurons.* Proc Natl Acad Sci U S A, 1998. **95**(18): p. 10884-9.
28. De Wit, J., et al., *Long-term adeno-associated viral vector-mediated expression of truncated TrkB in the adult rat facial nucleus results in motor neuron degeneration.* J Neurosci, 2006. **26**(5): p. 1516-30.
29. Dorsey, S.G., et al., *In vivo restoration of physiological levels of truncated TrkB.T1 receptor rescues neuronal cell death in a trisomic mouse model.* Neuron, 2006. **51**(1): p. 21-8.
30. Kemppainen, S., et al., *Impaired TrkB receptor signaling contributes to memory impairment in APP/PS1 mice.* Neurobiol Aging, 2012. **33**(6): p. 1122 e23-39.
31. Fryer, R.H., et al., *Developmental and mature expression of full-length and truncated TrkB receptors in the rat forebrain.* J Comp Neurol, 1996. **374**(1): p. 21-40.
32. Silhol, M., et al., *Age-related changes in brain-derived neurotrophic factor and tyrosine kinase receptor isoforms in the hippocampus and hypothalamus in male rats.* Neuroscience, 2005. **132**(3): p. 613-24.
33. Fenner, B.M., *Truncated TrkB: beyond a dominant negative receptor.* Cytokine Growth Factor Rev, 2012. **23**(1-2): p. 15-24.
34. Biffo, S., et al., *Selective binding and internalisation by truncated receptors restrict the availability of BDNF during development.* Development, 1995. **121**(8): p. 2461-70.
35. Alderson, R.F., et al., *Truncated TrkB mediates the endocytosis and release of BDNF and neurotrophin-4/5 by rat astrocytes and schwann cells in vitro.* Brain Res, 2000. **871**(2): p. 210-22.
36. Carim-Todd, L., et al., *Endogenous truncated TrkB.T1 receptor regulates neuronal complexity and TrkB kinase receptor function in vivo.* J Neurosci, 2009. **29**(3): p. 678-85.
37. Rose, C.R., et al., *Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells.* Nature, 2003. **426**(6962): p. 74-8.
38. Ohira, K., et al., *A truncated tropomyosin-related kinase B receptor, T1, regulates glial cell morphology via Rho GDP dissociation inhibitor 1.* J Neurosci, 2005. **25**(6): p. 1343-53.
39. Cunningham, M.E., et al., *Autophosphorylation of activation loop tyrosines regulates signaling by the TRK nerve growth factor receptor.* J Biol Chem, 1997. **272**(16): p. 10957-67.
40. McCarty, J.H. and S.C. Feinstein, *Activation loop tyrosines contribute varying roles to TrkB autophosphorylation and signal transduction.* Oncogene, 1998. **16**(13): p. 1691-700.
41. Middlemas, D.S., J. Meisenhelder, and T. Hunter, *Identification of TrkB autophosphorylation sites and evidence that phospholipase C-gamma 1 is a substrate of the TrkB receptor.* J Biol Chem, 1994. **269**(7): p. 5458-66.
42. !!! INVALID CITATION !!!
43. Kavanaugh, W.M. and L.T. Williams, *An alternative to SH2 domains for binding tyrosine-phosphorylated proteins.* Science, 1994. **266**(5192): p. 1862-5.
44. Castellano, E. and J. Downward, *RAS Interaction with PI3K: More Than Just Another Effector Pathway.* Genes Cancer, 2011. **2**(3): p. 261-74.
45. Crowder, R.J. and R.S. Freeman, *Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons.* J Neurosci, 1998. **18**(8): p. 2933-43.
46. Datta, S.R., et al., *Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery.* Cell, 1997. **91**(2): p. 231-41.
47. Cantley, L.C., *The phosphoinositide 3-kinase pathway.* Science, 2002. **296**(5573): p. 1655-7.
48. Chin, P.C., N. Majdzadeh, and S.R. D'Mello, *Inhibition of GSK3beta is a common event in neuroprotection by different survival factors.* Brain Res Mol Brain Res, 2005. **137**(1-2): p. 193-201.
49. Zhou, F.Q., et al., *NGF-induced axon growth is mediated by localized inactivation of GSK-3beta and functions of the microtubule plus end binding protein APC.* Neuron, 2004. **42**(6): p. 897-912.
50. Fresno Vara, J.A., et al., *PI3K/Akt signalling pathway and cancer.* Cancer Treat Rev, 2004. **30**(2): p. 193-204.

51. Xing, J., et al., *Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation.* Mol Cell Biol, 1998. **18**(4): p. 1946-55.
52. Xing, J., D.D. Ginty, and M.E. Greenberg, *Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase.* Science, 1996. **273**(5277): p. 959-63.
53. Chen, R.H., C. Abate, and J. Blenis, *Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase.* Proc Natl Acad Sci U S A, 1993. **90**(23): p. 10952-6.
54. Schouten, G.J., et al., *IkappaB alpha is a target for the mitogen-activated 90 kDa ribosomal S6 kinase.* EMBO J, 1997. **16**(11): p. 3133-44.
55. Takei, N., et al., *Brain-derived neurotrophic factor enhances neuronal translation by activating multiple initiation processes: comparison with the effects of insulin.* J Biol Chem, 2001. **276**(46): p. 42818-25.
56. Vetter, M.L., et al., *Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C-gamma 1 by a kinase activity associated with the product of the trk protooncogene.* Proc Natl Acad Sci U S A, 1991. **88**(13): p. 5650-4.
57. Minichiello, L., et al., *Mechanism of TrkB-mediated hippocampal long-term potentiation.* Neuron, 2002. **36**(1): p. 121-37.
58. Thiele, C.J., Z. Li, and A.E. McKee, *On Trk--the TrkB signal transduction pathway is an increasingly important target in cancer biology.* Clin Cancer Res, 2009. **15**(19): p. 5962-7.
59. Lynch, M.A., *Long-term potentiation and memory.* Physiol Rev, 2004. **84**(1): p. 87-136.
60. Neves, G., S.F. Cooke, and T.V. Bliss, *Synaptic plasticity, memory and the hippocampus: a neural network approach to causality.* Nat Rev Neurosci, 2008. **9**(1): p. 65-75.
61. Malenka, R.C. and M.F. Bear, *LTP and LTD: an embarrassment of riches.* Neuron, 2004. **44**(1): p. 5-21.
62. Frey, U., et al., *Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region in vitro.* Brain Res, 1988. **452**(1-2): p. 57-65.
63. Whitlock, J.R., et al., *Learning induces long-term potentiation in the hippocampus.* Science, 2006. **313**(5790): p. 1093-7.
64. Gruart, A., M.D. Munoz, and J.M. Delgado-Garcia, *Involvement of the CA3-CA1 synapse in the acquisition of associative learning in behaving mice.* J Neurosci, 2006. **26**(4): p. 1077-87.
65. Lohof, A.M., N.Y. Ip, and M.M. Poo, *Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF.* Nature, 1993. **363**(6427): p. 350-3.
66. Kang, H. and E.M. Schuman, *Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus.* Science, 1995. **267**(5204): p. 1658-62.
67. Korte, M., et al., *Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor.* Proc Natl Acad Sci U S A, 1995. **92**(19): p. 8856-60.
68. Korte, M., et al., *Virus-mediated gene transfer into hippocampal CA1 region restores long-term potentiation in brain-derived neurotrophic factor mutant mice.* Proc Natl Acad Sci U S A, 1996. **93**(22): p. 12547-52.
69. Patterson, S.L., et al., *Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice.* Neuron, 1996. **16**(6): p. 1137-45.
70. Ying, S.W., et al., *Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis.* J Neurosci, 2002. **22**(5): p. 1532-40.
71. Figurov, A., et al., *Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus.* Nature, 1996. **381**(6584): p. 706-9.
72. Chen, G., et al., *Relative contribution of endogenous neurotrophins in hippocampal long-term potentiation.* J Neurosci, 1999. **19**(18): p. 7983-90.
73. Minichiello, L., et al., *Essential role for TrkB receptors in hippocampus-mediated learning.* Neuron, 1999. **24**(2): p. 401-14.
74. Balkowiec, A. and D.M. Katz, *Cellular mechanisms regulating activity-dependent release of native brain-derived neurotrophic factor from hippocampal neurons.* J Neurosci, 2002. **22**(23): p. 10399-407.

75. Aicardi, G., et al., *Induction of long-term potentiation and depression is reflected by corresponding changes in secretion of endogenous brain-derived neurotrophic factor.* Proc Natl Acad Sci U S A, 2004. **101**(44): p. 15788-92.
76. Carvalho, A.L., et al., *Role of the brain-derived neurotrophic factor at glutamatergic synapses.* Br J Pharmacol, 2008. **153 Suppl 1**: p. S310-24.
77. Canas, N., et al., *Brain-derived neurotrophic factor facilitates glutamate and inhibits GABA release from hippocampal synaptosomes through different mechanisms.* Brain Res, 2004. **1016**(1): p. 72-8.
78. Pascual, M., E. Climent, and C. Guerri, *BDNF induces glutamate release in cerebrocortical nerve terminals and in cortical astrocytes.* Neuroreport, 2001. **12**(12): p. 2673-7.
79. Li, Y.X., et al., *Enhancement of neurotransmitter release induced by brain-derived neurotrophic factor in cultured hippocampal neurons.* J Neurosci, 1998. **18**(24): p. 10231-40.
80. Jovanovic, J.N., et al., *Synapsins as mediators of BDNF-enhanced neurotransmitter release.* Nat Neurosci, 2000. **3**(4): p. 323-9.
81. Levine, E.S., et al., *Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing N-methyl-D-aspartic acid receptor activity.* Proc Natl Acad Sci U S A, 1998. **95**(17): p. 10235-9.
82. Mizuno, M., et al., *Involvement of BDNF receptor TrkB in spatial memory formation.* Learn Mem, 2003. **10**(2): p. 108-15.
83. Caldeira, M.V., et al., *Brain-derived neurotrophic factor regulates the expression and synaptic delivery of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunits in hippocampal neurons.* J Biol Chem, 2007. **282**(17): p. 12619-28.
84. Nakata, H. and S. Nakamura, *Brain-derived neurotrophic factor regulates AMPA receptor trafficking to post-synaptic densities via IP3R and TRPC calcium signaling.* FEBS Lett, 2007. **581**(10): p. 2047-54.
85. Kafitz, K.W., et al., *Neurotrophin-evoked rapid excitation through TrkB receptors.* Nature, 1999. **401**(6756): p. 918-21.
86. Blum, R., K.W. Kafitz, and A. Konnerth, *Neurotrophin-evoked depolarization requires the sodium channel Na(V)1.9.* Nature, 2002. **419**(6908): p. 687-93.
87. Kovalchuk, Y., et al., *Postsynaptic Induction of BDNF-Mediated Long-Term Potentiation.* Science, 2002. **295**(5560): p. 1729-34.
88. Ji, Y., et al., *Acute and gradual increases in BDNF concentration elicit distinct signaling and functions in neurons.* Nat Neurosci, 2010. **13**(3): p. 302-9.
89. Bekinschtein, P., et al., *BDNF is essential to promote persistence of long-term memory storage.* Proc Natl Acad Sci U S A, 2008. **105**(7): p. 2711-6.
90. Bekinschtein, P., et al., *Persistence of long-term memory storage requires a late protein synthesis- and BDNF- dependent phase in the hippocampus.* Neuron, 2007. **53**(2): p. 261-77.
91. Castren, E., et al., *The induction of LTP increases BDNF and NGF mRNA but decreases NT-3 mRNA in the dentate gyrus.* Neuroreport, 1993. **4**(7): p. 895-8.
92. Dragunow, M., et al., *Brain-derived neurotrophic factor expression after long-term potentiation.* Neurosci Lett, 1993. **160**(2): p. 232-6.
93. Pang, P.T., et al., *Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity.* Science, 2004. **306**(5695): p. 487-91.
94. Mei, F., et al., *BDNF facilitates L-LTP maintenance in the absence of protein synthesis through PKMzeta.* PLoS One, 2011. **6**(6): p. e21568.
95. Huang, Y.Y., et al., *Mice lacking the gene encoding tissue-type plasminogen activator show a selective interference with late-phase long-term potentiation in both Schaffer collateral and mossy fiber pathways.* Proc Natl Acad Sci U S A, 1996. **93**(16): p. 8699-704.
96. Panja, D. and C.R. Bramham, *BDNF mechanisms in late LTP formation: A synthesis and breakdown.* Neuropharmacology, 2014. **76 Pt C**: p. 664-76.
97. Minichiello, L., *TrkB signalling pathways in LTP and learning.* Nat Rev Neurosci, 2009. **10**(12): p. 850-60.
98. Ribeiro, J.A., A.M. Sebastiao, and A. de Mendonca, *Adenosine receptors in the nervous system: pathophysiological implications.* Prog Neurobiol, 2002. **68**(6): p. 377-92.

99. Sebastiao, A.M., et al., *Modulation of brain-derived neurotrophic factor (BDNF) actions in the nervous system by adenosine A(2A) receptors and the role of lipid rafts.* Biochim Biophys Acta, 2011. **1808**(5): p. 1340-9.
100. Dias, R.B., et al., *Adenosine: setting the stage for plasticity.* Trends Neurosci, 2013. **36**(4): p. 248-57.
101. Dunwiddie, T.V. and S.A. Masino, *The role and regulation of adenosine in the central nervous system.* Annu Rev Neurosci, 2001. **24**: p. 31-55.
102. Linden, J., *Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection.* Annu Rev Pharmacol Toxicol, 2001. **41**: p. 775-87.
103. Sebastiao, A.M. and J.A. Ribeiro, *Evidence for the presence of excitatory A2 adenosine receptors in the rat hippocampus.* Neurosci Lett, 1992. **138**(1): p. 41-4.
104. Rosin, D.L., et al., *Immunohistochemical localization of adenosine A2A receptors in the rat central nervous system.* J Comp Neurol, 1998. **401**(2): p. 163-86.
105. Lee, F.S. and M.V. Chao, *Activation of Trk neurotrophin receptors in the absence of neurotrophins.* Proc Natl Acad Sci U S A, 2001. **98**(6): p. 3555-60.
106. Wiese, S., et al., *Adenosine receptor A2A-R contributes to motoneuron survival by transactivating the tyrosine kinase receptor TrkB.* Proc Natl Acad Sci U S A, 2007. **104**(43): p. 17210-5.
107. Rajagopal, R., et al., *Transactivation of Trk neurotrophin receptors by G-protein-coupled receptor ligands occurs on intracellular membranes.* J Neurosci, 2004. **24**(30): p. 6650-8.
108. Diogenes, M.J., et al., *Activation of adenosine A2A receptor facilitates brain-derived neurotrophic factor modulation of synaptic transmission in hippocampal slices.* J Neurosci, 2004. **24**(12): p. 2905-13.
109. Diogenes, M.J., et al., *Influence of age on BDNF modulation of hippocampal synaptic transmission: interplay with adenosine A2A receptors.* Hippocampus, 2007. **17**(7): p. 577-85.
110. Diogenes, M.J., et al., *Homeostatic control of synaptic activity by endogenous adenosine is mediated by adenosine kinase.* Cereb Cortex, 2014. **24**(1): p. 67-80.
111. Tebano, M.T., et al., *Adenosine A(2A) receptors are required for normal BDNF levels and BDNF-induced potentiation of synaptic transmission in the mouse hippocampus.* J Neurochem, 2008. **104**(1): p. 279-86.
112. Pousinha, P.A., et al., *Triggering of BDNF facilitatory action on neuromuscular transmission by adenosine A2A receptors.* Neurosci Lett, 2006. **404**(1-2): p. 143-7.
113. Fontinha, B.M., et al., *Enhancement of long-term potentiation by brain-derived neurotrophic factor requires adenosine A2A receptor activation by endogenous adenosine.* Neuropharmacology, 2008. **54**(6): p. 924-33.
114. Diogenes, M.J., et al., *Enhancement of LTP in aged rats is dependent on endogenous BDNF.* Neuropsychopharmacology, 2011. **36**(9): p. 1823-36.
115. Rodrigues, T.M., et al., *Adenosine A(2A) Receptors as novel upstream regulators of BDNF-mediated attenuation of hippocampal Long-Term Depression (LTD).* Neuropharmacology, 2014. **79**: p. 389-98.
116. Jeronimo-Santos, A., et al., *Impact of in vivo chronic blockade of adenosine A2A receptors on the BDNF-mediated facilitation of LTP.* Neuropharmacology, 2014. **83**: p. 99-106.
117. Assaife-Lopes, N., et al., *Regulation of TrkB receptor translocation to lipid rafts by adenosine A receptors and its functional implications for BDNF-induced regulation of synaptic plasticity.* Purinergic Signal, 2013.
118. Ribeiro, F.F., et al., *Adenosine A2A and BDNF TrkB receptors have independent roles upon neuronal maturation,.* Purine Meeting, Spain, 2010.
119. Vaz, S.H., et al., *Brain-derived neurotrophic factor inhibits GABA uptake by the rat hippocampal nerve terminals.* Brain Res, 2008. **1219**: p. 19-25.
120. Lu, B., et al., *BDNF-based synaptic repair as a disease-modifying strategy for neurodegenerative diseases.* Nat Rev Neurosci, 2013. **14**(6): p. 401-16.
121. Chao, M.V., R. Rajagopal, and F.S. Lee, *Neurotrophin signalling in health and disease.* Clin Sci (Lond), 2006. **110**(2): p. 167-73.
122. Chapman, H.A., R.J. Riese, and G.P. Shi, *Emerging roles for cysteine proteases in human biology.* Annu Rev Physiol, 1997. **59**: p. 63-88.

123. Rawlings, N.D., et al., *MEROPS: the database of proteolytic enzymes, their substrates and inhibitors.* Nucleic Acids Res, 2014. **42**(Database issue): p. D503-9.
124. Molinari, M., J. Anagli, and E. Carafoli, *Ca(2+)-activated neutral protease is active in the erythrocyte membrane in its nonautolyzed 80-kDa form.* J Biol Chem, 1994. **269**(45): p. 27992-5.
125. Strobl, S., et al., *The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium.* Proc Natl Acad Sci U S A, 2000. **97**(2): p. 588-92.
126. Guroff, G., *A Neutral, Calcium-Activated Proteinase from the Soluble Fraction of Rat Brain.* J Biol Chem, 1964. **239**: p. 149-55.
127. Goll, D.E., et al., *The calpain system.* Physiol Rev, 2003. **83**(3): p. 731-801.
128. Ohno, S., et al., *Evolutionary origin of a calcium-dependent protease by fusion of genes for a thiol protease and a calcium-binding protein?* Nature, 1984. **312**(5994): p. 566-70.
129. Sorimachi, H., S. Hata, and Y. Ono, *Expanding members and roles of the calpain superfamily and their genetically modified animals.* Exp Anim, 2010. **59**(5): p. 549-66.
130. Tonami, K., et al., *Calpain-6 deficiency promotes skeletal muscle development and regeneration.* PLoS Genet, 2013. **9**(8): p. e1003668.
131. Bevers, M.B. and R.W. Neumar, *Mechanistic role of calpains in postischemic neurodegeneration.* J Cereb Blood Flow Metab, 2008. **28**(4): p. 655-73.
132. Friedrich, P., et al., *Differential distribution of calpain small subunit 1 and 2 in rat brain.* Eur J Neurosci, 2004. **19**(7): p. 1819-25.
133. Li, J., et al., *Regional differences in gene expression for calcium activated neutral proteases (calpains) and their endogenous inhibitor calpastatin in mouse brain and spinal cord.* J Neurobiol, 1996. **30**(2): p. 177-91.
134. Garcia, M., V. Bondada, and J.W. Geddes, *Mitochondrial localization of mu-calpain.* Biochem Biophys Res Commun, 2005. **338**(2): p. 1241-7.
135. Cao, G., et al., *Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury.* J Neurosci, 2007. **27**(35): p. 9278-93.
136. Tremper-Wells, B. and M.L. Vallano, *Nuclear calpain regulates Ca2+-dependent signaling via proteolysis of nuclear Ca2+/calmodulin-dependent protein kinase type IV in cultured neurons.* J Biol Chem, 2005. **280**(3): p. 2165-75.
137. Hosfield, C.M., et al., *Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation.* EMBO J, 1999. **18**(24): p. 6880-9.
138. Cong, J., et al., *The role of autolysis in activity of the Ca2+-dependent proteinases (mu-calpain and m-calpain).* J Biol Chem, 1989. **264**(17): p. 10096-103.
139. Hanna, R.A., R.L. Campbell, and P.L. Davies, *Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin.* Nature, 2008. **456**(7220): p. 409-12.
140. Tompa, P., et al., *Domain III of calpain is a ca2+-regulated phospholipid-binding domain.* Biochem Biophys Res Commun, 2001. **280**(5): p. 1333-9.
141. Imajoh, S., H. Kawasaki, and K. Suzuki, *The COOH-terminal E-F hand structure of calcium-activated neutral protease (CANP) is important for the association of subunits and resulting proteolytic activity.* J Biochem, 1987. **101**(2): p. 447-52.
142. Brandenburg, K., et al., *Domain V of m-calpain shows the potential to form an oblique-orientated alpha-helix, which may modulate the enzyme's activity via interactions with anionic lipid.* Eur J Biochem, 2002. **269**(22): p. 5414-22.
143. Dennison, S.R., et al., *Investigations into the membrane interactions of m-calpain domain V.* Biophys J, 2005. **88**(4): p. 3008-17.
144. Imajoh, S., H. Kawasaki, and K. Suzuki, *The amino-terminal hydrophobic region of the small subunit of calcium-activated neutral protease (CANP) is essential for its activation by phosphatidylinositol.* J Biochem, 1986. **99**(4): p. 1281-4.
145. Arthur, J.S., et al., *Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division.* Mol Cell Biol, 2000. **20**(12): p. 4474-81.
146. Dutt, P., et al., *m-Calpain is required for preimplantation embryonic development in mice.* BMC Dev Biol, 2006. **6**: p. 3.
147. Azam, M., et al., *Disruption of the mouse mu-calpain gene reveals an essential role in platelet function.* Mol Cell Biol, 2001. **21**(6): p. 2213-20.

148. Storr, S.J., et al., *The calpain system and cancer.* Nat Rev Cancer, 2011. **11**(5): p. 364-74.
149. Sorimachi, H., S. Ishiura, and K. Suzuki, *Structure and physiological function of calpains.* Biochem J, 1997. **328 (Pt 3)**: p. 721-32.
150. Sorimachi, H. and Y. Ono, *Regulation and physiological roles of the calpain system in muscular disorders.* Cardiovasc Res, 2012. **96**(1): p. 11-22.
151. Ono, Y., et al., *Possible regulation of the conventional calpain system by skeletal muscle-specific calpain, p94/calpain 3.* J Biol Chem, 2004. **279**(4): p. 2761-71.
152. DuVerle, D.A., et al., *Calpain cleavage prediction using multiple kernel learning.* PLoS One, 2011. **6**(5): p. e19035.
153. Sorimachi, H., H. Mamitsuka, and Y. Ono, *Understanding the substrate specificity of conventional calpains.* Biol Chem, 2012. **393**(9): p. 853-71.
154. duVerle, D., et al., *CaMPDB: a resource for calpain and modulatory proteolysis.* Genome Inform, 2010. **22**: p. 202-13.
155. Vosler, P.S., C.S. Brennan, and J. Chen, *Calpain-mediated signaling mechanisms in neuronal injury and neurodegeneration.* Mol Neurobiol, 2008. **38**(1): p. 78-100.
156. Abe, K. and M. Takeichi, *NMDA-receptor activation induces calpain-mediated beta-catenin cleavages for triggering gene expression.* Neuron, 2007. **53**(3): p. 387-97.
157. Czogalla, A. and A.F. Sikorski, *Spectrin and calpain: a 'target' and a 'sniper' in the pathology of neuronal cells.* Cell Mol Life Sci, 2005. **62**(17): p. 1913-24.
158. Maki, M., et al., *All four internally repetitive domains of pig calpastatin possess inhibitory activities against calpains I and II.* FEBS Lett, 1987. **223**(1): p. 174-80.
159. Hanna, R.A., B.E. Garcia-Diaz, and P.L. Davies, *Calpastatin simultaneously binds four calpains with different kinetic constants.* FEBS Lett, 2007. **581**(16): p. 2894-8.
160. Takano, E., et al., *Preference of calcium-dependent interactions between calmodulin-like domains of calpain and calpastatin subdomains.* FEBS Lett, 1995. **362**(1): p. 93-7.
161. Maki, M., et al., *Inhibition of calpain by a synthetic oligopeptide corresponding to an exon of the human calpastatin gene.* J Biol Chem, 1989. **264**(32): p. 18866-9.
162. Moldoveanu, T., K. Gehring, and D.R. Green, *Concerted multi-pronged attack by calpastatin to occlude the catalytic cleft of heterodimeric calpains.* Nature, 2008. **456**(7220): p. 404-8.
163. DeMartino, G.N., et al., *Proteolysis of the protein inhibitor of calcium-dependent proteases produces lower molecular weight fragments that retain inhibitory activity.* Arch Biochem Biophys, 1988. **262**(1): p. 189-98.
164. Kato, M., et al., *Caspases cleave the amino-terminal calpain inhibitory unit of calpastatin during apoptosis in human Jurkat T cells.* J Biochem, 2000. **127**(2): p. 297-305.
165. Wang, K.K., et al., *Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis.* Arch Biochem Biophys, 1998. **356**(2): p. 187-96.
166. Porn-Ares, M.I., A. Samali, and S. Orrenius, *Cleavage of the calpain inhibitor, calpastatin, during apoptosis.* Cell Death Differ, 1998. **5**(12): p. 1028-33.
167. De Tullio, R., et al., *Changes in intracellular localization of calpastatin during calpain activation.* Biochem J, 1999. **343 Pt 2**: p. 467-72.
168. Averna, M., et al., *Changes in intracellular calpastatin localization are mediated by reversible phosphorylation.* Biochem J, 2001. **354**(Pt 1): p. 25-30.
169. Averna, M., et al., *Phosphorylation of rat brain calpastatins by protein kinase C.* FEBS Lett, 1999. **450**(1-2): p. 13-6.
170. Schoch, K.M., et al., *Calpastatin overexpression limits calpain-mediated proteolysis and behavioral deficits following traumatic brain injury.* Exp Neurol, 2012. **236**(2): p. 371-82.
171. Higuchi, M., et al., *Distinct mechanistic roles of calpain and caspase activation in neurodegeneration as revealed in mice overexpressing their specific inhibitors.* J Biol Chem, 2005. **280**(15): p. 15229-37.
172. Takano, J., et al., *Calpain mediates excitotoxic DNA fragmentation via mitochondrial pathways in adult brains: evidence from calpastatin mutant mice.* J Biol Chem, 2005. **280**(16): p. 16175-84.
173. Friedrich, P., *The intriguing Ca2+ requirement of calpain activation.* Biochem Biophys Res Commun, 2004. **323**(4): p. 1131-3.

174. Glading, A., et al., *Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation.* Mol Cell Biol, 2004. **24**(6): p. 2499-512.
175. Saido, T.C., et al., *Positive regulation of mu-calpain action by polyphosphoinositides.* J Biol Chem, 1992. **267**(34): p. 24585-90.
176. Melloni, E., et al., *Acyl-CoA-binding protein is a potent m-calpain activator.* J Biol Chem, 2000. **275**(1): p. 82-6.
177. Shiraha, H., et al., *Activation of m-calpain (calpain II) by epidermal growth factor is limited by protein kinase A phosphorylation of m-calpain.* Mol Cell Biol, 2002. **22**(8): p. 2716-27.
178. Zadran, S., et al., *Brain-derived neurotrophic factor and epidermal growth factor activate neuronal m-calpain via mitogen-activated protein kinase-dependent phosphorylation.* J Neurosci, 2010. **30**(3): p. 1086-95.
179. Smith, S.D., et al., *Glutamate substitutions at a PKA consensus site are consistent with inactivation of calpain by phosphorylation.* FEBS Lett, 2003. **542**(1-3): p. 115-8.
180. Leloup, L., et al., *m-Calpain activation is regulated by its membrane localization and by its binding to phosphatidylinositol 4,5-bisphosphate.* J Biol Chem, 2010. **285**(43): p. 33549-66.
181. Shao, H., et al., *Spatial localization of m-calpain to the plasma membrane by phosphoinositide biphosphate binding during epidermal growth factor receptor-mediated activation.* Mol Cell Biol, 2006. **26**(14): p. 5481-96.
182. Huang, Y. and K.K. Wang, *The calpain family and human disease.* Trends Mol Med, 2001. **7**(8): p. 355-62.
183. Bartus, R.T., et al., *Calpain as a novel target for treating acute neurodegenerative disorders.* Neurol Res, 1995. **17**(4): p. 249-58.
184. Saez, M.E., et al., *The therapeutic potential of the calpain family: new aspects.* Drug Discov Today, 2006. **11**(19-20): p. 917-23.
185. Lynch, G. and M. Baudry, *The biochemistry of memory: a new and specific hypothesis.* Science, 1984. **224**(4653): p. 1057-63.
186. Baudry, M., et al., *The biochemistry of memory: The 26year journey of a 'new and specific hypothesis'.* Neurobiol Learn Mem, 2011. **95**(2): p. 125-33.
187. Vanderklish, P., et al., *Proteolysis of spectrin by calpain accompanies theta-burst stimulation in cultured hippocampal slices.* Brain Res Mol Brain Res, 1995. **32**(1): p. 25-35.
188. Vanderklish, P., E. Bednarski, and G. Lynch, *Translational suppression of calpain blocks long-term potentiation.* Learn Mem, 1996. **3**(2-3): p. 209-17.
189. Oliver, M.W., M. Baudry, and G. Lynch, *The protease inhibitor leupeptin interferes with the development of LTP in hippocampal slices.* Brain Res, 1989. **505**(2): p. 233-8.
190. Denny, J.B., et al., *Calpain inhibitors block long-term potentiation.* Brain Res, 1990. **534**(1-2): p. 317-20.
191. Amini, M., et al., *Conditional disruption of calpain in the CNS alters dendrite morphology, impairs LTP, and promotes neuronal survival following injury.* J Neurosci, 2013. **33**(13): p. 5773-84.
192. Muller, D., et al., *A genetic deficiency in calpastatin and isovalerylcarnitine treatment is associated with enhanced hippocampal long-term potentiation.* Synapse, 1995. **19**(1): p. 37-45.
193. Wang, Y., et al., *A molecular brake controls the magnitude of long-term potentiation.* Nat Commun, 2014. **5**: p. 3051.
194. Saito, K., et al., *Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration.* Proc Natl Acad Sci U S A, 1993. **90**(7): p. 2628-32.
195. Ferreira, A. and E.H. Bigio, *Calpain-mediated tau cleavage: a mechanism leading to neurodegeneration shared by multiple tauopathies.* Mol Med, 2011. **17**(7-8): p. 676-85.
196. Patrick, G.N., et al., *Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration.* Nature, 1999. **402**(6762): p. 615-22.
197. Tsuji, T., et al., *m-Calpain (calcium-activated neutral proteinase) in Alzheimer's disease brains.* Neurosci Lett, 1998. **248**(2): p. 109-12.
198. Nixon, R.A., et al., *Calcium-activated neutral proteinase (calpain) system in aging and Alzheimer's disease.* Ann N Y Acad Sci, 1994. **747**: p. 77-91.

199. Higuchi, M., et al., *Mechanistic involvement of the calpain-calpastatin system in Alzheimer neuropathology.* FASEB J, 2012. **26**(3): p. 1204-17.
200. Rao, M.V., et al., *Marked calpastatin (CAST) depletion in Alzheimer's disease accelerates cytoskeleton disruption and neurodegeneration: neuroprotection by CAST overexpression.* J Neurosci, 2008. **28**(47): p. 12241-54.
201. Medeiros, R., et al., *Calpain inhibitor A-705253 mitigates Alzheimer's disease-like pathology and cognitive decline in aged 3xTgAD mice.* Am J Pathol, 2012. **181**(2): p. 616-25.
202. Trinchese, F., et al., *Inhibition of calpains improves memory and synaptic transmission in a mouse model of Alzheimer disease.* J Clin Invest, 2008. **118**(8): p. 2796-807.
203. Ferri, C.P., et al., *Global prevalence of dementia: a Delphi consensus study.* Lancet, 2005. **366**(9503): p. 2112-7.
204. Babic, T., *The cholinergic hypothesis of Alzheimer's disease: a review of progress.* J Neurol Neurosurg Psychiatry, 1999. **67**(4): p. 558.
205. Alzheimer, A., et al., *An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde".* Clin Anat, 1995. **8**(6): p. 429-31.
206. Reitz, C. and R. Mayeux, *Alzheimer disease: Epidemiology, diagnostic criteria, risk factors and biomarkers.* Biochem Pharmacol, 2014. **88**(4): p. 640-651.
207. Gomez-Isla, T., et al., *Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease.* Ann Neurol, 1997. **41**(1): p. 17-24.
208. Coleman, P., H. Federoff, and R. Kurlan, *A focus on the synapse for neuroprotection in Alzheimer disease and other dementias.* Neurology, 2004. **63**(7): p. 1155-62.
209. Terry, R.D., et al., *Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment.* Ann Neurol, 1991. **30**(4): p. 572-80.
210. DeKosky, S.T. and S.W. Scheff, *Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity.* Ann Neurol, 1990. **27**(5): p. 457-64.
211. Glenner, G.G. and C.W. Wong, *Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein.* Biochem Biophys Res Commun, 1984. **120**(3): p. 885-90.
212. Grundke-Iqbal, I., et al., *Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology.* Proc Natl Acad Sci U S A, 1986. **83**(13): p. 4913-7.
213. Braak, H. and E. Braak, *Neuropathological stageing of Alzheimer-related changes.* Acta Neuropathol, 1991. **82**(4): p. 239-59.
214. Jack, C.R., Jr., et al., *Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade.* Lancet Neurol, 2010. **9**(1): p. 119-28.
215. Jack, C.R., Jr., et al., *Tracking pathophysiological processes in Alzheimer's disease: an updated hypothetical model of dynamic biomarkers.* Lancet Neurol, 2013. **12**(2): p. 207-16.
216. Leung, K.K., et al., *Cerebral atrophy in mild cognitive impairment and Alzheimer disease: rates and acceleration.* Neurology, 2013. **80**(7): p. 648-54.
217. Allen, S.J., J.J. Watson, and D. Dawbarn, *The neurotrophins and their role in Alzheimer's disease.* Curr Neuropharmacol, 2011. **9**(4): p. 559-73.
218. Kang, J., et al., *The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor.* Nature, 1987. **325**(6106): p. 733-6.
219. Goate, A., et al., *Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease.* Nature, 1991. **349**(6311): p. 704-6.
220. Sherrington, R., et al., *Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease.* Nature, 1995. **375**(6534): p. 754-60.
221. Levy-Lahad, E., et al., *Candidate gene for the chromosome 1 familial Alzheimer's disease locus.* Science, 1995. **269**(5226): p. 973-7.
222. Wolfe, M.S., et al., *Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity.* Nature, 1999. **398**(6727): p. 513-7.
223. Vassar, R., et al., *Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE.* Science, 1999. **286**(5440): p. 735-41.
224. Ertekin-Taner, N., *Genetics of Alzheimer's disease: a centennial review.* Neurol Clin, 2007. **25**(3): p. 611-67, v.

225. Kumar-Singh, S., et al., *Mean age-of-onset of familial alzheimer disease caused by presenilin mutations correlates with both increased Abeta42 and decreased Abeta40.* Hum Mutat, 2006. **27**(7): p. 686-95.
226. Scheuner, D., et al., *Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease.* Nat Med, 1996. **2**(8): p. 864-70.
227. Wolfe, M.S., *When loss is gain: reduced presenilin proteolytic function leads to increased Abeta42/Abeta40. Talking Point on the role of presenilin mutations in Alzheimer disease.* EMBO Rep, 2007. **8**(2): p. 136-40.
228. De Jonghe, C., et al., *Pathogenic APP mutations near the gamma-secretase cleavage site differentially affect Abeta secretion and APP C-terminal fragment stability.* Hum Mol Genet, 2001. **10**(16): p. 1665-71.
229. Jarrett, J.T., E.P. Berger, and P.T. Lansbury, Jr., *The C-terminus of the beta protein is critical in amyloidogenesis.* Ann N Y Acad Sci, 1993. **695**: p. 144-8.
230. Karran, E., M. Mercken, and B. De Strooper, *The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics.* Nat Rev Drug Discov, 2011. **10**(9): p. 698-712.
231. Jarrett, J.T., E.P. Berger, and P.T. Lansbury, Jr., *The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease.* Biochemistry, 1993. **32**(18): p. 4693-7.
232. Iwatsubo, T., et al., *Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42(43).* Neuron, 1994. **13**(1): p. 45-53.
233. Mehta, P.D., et al., *Plasma and cerebrospinal fluid levels of amyloid beta proteins 1-40 and 1-42 in Alzheimer disease.* Arch Neurol, 2000. **57**(1): p. 100-5.
234. Welander, H., et al., *Abeta43 is more frequent than Abeta40 in amyloid plaque cores from Alzheimer disease brains.* J Neurochem, 2009. **110**(2): p. 697-706.
235. Gravina, S.A., et al., *Amyloid beta protein (A beta) in Alzheimer's disease brain. Biochemical and immunocytochemical analysis with antibodies specific for forms ending at A beta 40 or A beta 42(43).* J Biol Chem, 1995. **270**(13): p. 7013-6.
236. Chin, J., *Selecting a mouse model of Alzheimer's disease.* Methods Mol Biol, 2011. **670**: p. 169-89.
237. Jonsson, T., et al., *A mutation in APP protects against Alzheimer's disease and age-related cognitive decline.* Nature, 2012. **488**(7409): p. 96-9.
238. Yang, L.B., et al., *Elevated beta-secretase expression and enzymatic activity detected in sporadic Alzheimer disease.* Nat Med, 2003. **9**(1): p. 3-4.
239. Fukumoto, H., et al., *Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease.* Arch Neurol, 2002. **59**(9): p. 1381-9.
240. Zheng, H. and E.H. Koo, *The amyloid precursor protein: beyond amyloid.* Mol Neurodegener, 2006. **1**: p. 5.
241. Smith, D.G., R. Cappai, and K.J. Barnham, *The redox chemistry of the Alzheimer's disease amyloid beta peptide.* Biochim Biophys Acta, 2007. **1768**(8): p. 1976-90.
242. Pike, C.J., et al., *Structure-activity analyses of beta-amyloid peptides: contributions of the beta 25-35 region to aggregation and neurotoxicity.* J Neurochem, 1995. **64**(1): p. 253-65.
243. Arancibia, S., et al., *Protective effect of BDNF against beta-amyloid induced neurotoxicity in vitro and in vivo in rats.* Neurobiol Dis, 2008. **31**(3): p. 316-26.
244. Jhoo, J.H., et al., *Beta-amyloid (1-42)-induced learning and memory deficits in mice: involvement of oxidative burdens in the hippocampus and cerebral cortex.* Behav Brain Res, 2004. **155**(2): p. 185-96.
245. Kowall, N.W., et al., *An in vivo model for the neurodegenerative effects of beta amyloid and protection by substance P.* Proc Natl Acad Sci U S A, 1991. **88**(16): p. 7247-51.
246. Butterfield, D.A., et al., *Roles of amyloid beta-peptide-associated oxidative stress and brain protein modifications in the pathogenesis of Alzheimer's disease and mild cognitive impairment.* Free Radic Biol Med, 2007. **43**(5): p. 658-77.
247. Parameshwaran, K., M. Dhanasekaran, and V. Suppiramaniam, *Amyloid beta peptides and glutamatergic synaptic dysregulation.* Exp Neurol, 2008. **210**(1): p. 7-13.

248. Canevari, L., J.B. Clark, and T.E. Bates, *beta-Amyloid fragment 25-35 selectively decreases complex IV activity in isolated mitochondria.* FEBS Lett, 1999. **457**(1): p. 131-4.
249. Rosales-Corral, S., et al., *Kinetics of the neuroinflammation-oxidative stress correlation in rat brain following the injection of fibrillar amyloid-beta onto the hippocampus in vivo.* J Neuroimmunol, 2004. **150**(1-2): p. 20-8.
250. Lin, H., R. Bhatia, and R. Lal, *Amyloid beta protein forms ion channels: implications for Alzheimer's disease pathophysiology.* FASEB J, 2001. **15**(13): p. 2433-44.
251. Varadarajan, S., et al., *Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity.* J Struct Biol, 2000. **130**(2-3): p. 184-208.
252. Carrillo-Mora, P., R. Luna, and L. Colin-Barenque, *Amyloid Beta: Multiple Mechanisms of Toxicity and Only Some Protective Effects?* Oxid Med Cell Longev, 2014. **2014**: p. 795375.
253. Holscher, C., *Development of beta-amyloid-induced neurodegeneration in Alzheimer's disease and novel neuroprotective strategies.* Rev Neurosci, 2005. **16**(3): p. 181-212.
254. Holscher, C., *Possible causes of Alzheimer's disease: amyloid fragments, free radicals, and calcium homeostasis.* Neurobiol Dis, 1998. **5**(3): p. 129-41.
255. Selkoe, D.J., *Alzheimer's disease is a synaptic failure.* Science, 2002. **298**(5594): p. 789-91.
256. Wei, Z., et al., *Role of calpain and caspase in beta-amyloid-induced cell death in rat primary septal cultured neurons.* Neuropharmacology, 2008. **54**(4): p. 721-33.
257. Wang, H.Y., et al., *beta-Amyloid(1-42) binds to alpha7 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer's disease pathology.* J Biol Chem, 2000. **275**(8): p. 5626-32.
258. Liu, S.J., et al., *Amyloid-beta decreases cell-surface AMPA receptors by increasing intracellular calcium and phosphorylation of GluR2.* J Alzheimers Dis, 2010. **21**(2): p. 655-66.
259. Alberdi, E., et al., *Amyloid beta oligomers induce Ca2+ dysregulation and neuronal death through activation of ionotropic glutamate receptors.* Cell Calcium, 2010. **47**(3): p. 264-72.
260. Ferreira, I.L., et al., *Amyloid beta peptide 1-42 disturbs intracellular calcium homeostasis through activation of GluN2B-containing N-methyl-d-aspartate receptors in cortical cultures.* Cell Calcium, 2012. **51**(2): p. 95-106.
261. Harkany, T., et al., *beta-amyloid neurotoxicity is mediated by a glutamate-triggered excitotoxic cascade in rat nucleus basalis.* Eur J Neurosci, 2000. **12**(8): p. 2735-45.
262. Wu, J., R. Anwyl, and M.J. Rowan, *beta-Amyloid selectively augments NMDA receptor-mediated synaptic transmission in rat hippocampus.* Neuroreport, 1995. **6**(17): p. 2409-13.
263. Matos, M., et al., *Amyloid-beta peptide decreases glutamate uptake in cultured astrocytes: involvement of oxidative stress and mitogen-activated protein kinase cascades.* Neuroscience, 2008. **156**(4): p. 898-910.
264. Wu, H.Y. and D.R. Lynch, *Calpain and synaptic function.* Mol Neurobiol, 2006. **33**(3): p. 215-36.
265. Kelly, B.L. and A. Ferreira, *beta-Amyloid-induced dynamin 1 degradation is mediated by N-methyl-D-aspartate receptors in hippocampal neurons.* J Biol Chem, 2006. **281**(38): p. 28079-89.
266. Liang, Z., et al., *Down-regulation of cAMP-dependent protein kinase by over-activated calpain in Alzheimer disease brain.* J Neurochem, 2007. **103**(6): p. 2462-70.
267. Miguel-Hidalgo, J.J., et al., *Neuroprotection by memantine against neurodegeneration induced by beta-amyloid(1-40).* Brain Res, 2002. **958**(1): p. 210-21.
268. Shelat, P.B., et al., *Amyloid beta peptide and NMDA induce ROS from NADPH oxidase and AA release from cytosolic phospholipase A2 in cortical neurons.* J Neurochem, 2008. **106**(1): p. 45-55.
269. Ueda, K., et al., *Amyloid beta protein potentiates Ca2+ influx through L-type voltage-sensitive Ca2+ channels: a possible involvement of free radicals.* J Neurochem, 1997. **68**(1): p. 265-71.
270. Demuro, A. and I. Parker, *Cytotoxicity of intracellular abeta42 amyloid oligomers involves Ca2+ release from the endoplasmic reticulum by stimulated production of inositol trisphosphate.* J Neurosci, 2013. **33**(9): p. 3824-33.
271. Ferreiro, E., C.R. Oliveira, and C. Pereira, *Involvement of endoplasmic reticulum Ca2+ release through ryanodine and inositol 1,4,5-triphosphate receptors in the neurotoxic effects induced by the amyloid-beta peptide.* J Neurosci Res, 2004. **76**(6): p. 872-80.
272. Arispe, N., H.B. Pollard, and E. Rojas, *Giant multilevel cation channels formed by Alzheimer disease amyloid beta-protein [A beta P-(1-40)] in bilayer membranes.* Proc Natl Acad Sci U S A, 1993. **90**(22): p. 10573-7.

273. Demuro, A., M. Smith, and I. Parker, *Single-channel Ca(2+) imaging implicates Abeta1-42 amyloid pores in Alzheimer's disease pathology.* J Cell Biol, 2011. **195**(3): p. 515-24.
274. Lee, C.C., et al., *Transmembrane pores formed by human antimicrobial peptide LL-37.* Biophys J, 2011. **100**(7): p. 1688-96.
275. Soscia, S.J., et al., *The Alzheimer's disease-associated amyloid beta-protein is an antimicrobial peptide.* PLoS One, 2010. **5**(3): p. e9505.
276. Jordan, J., M.F. Galindo, and R.J. Miller, *Role of calpain- and interleukin-1 beta converting enzyme-like proteases in the beta-amyloid-induced death of rat hippocampal neurons in culture.* J Neurochem, 1997. **68**(4): p. 1612-21.
277. Shea, T.B., S. Prabhakar, and F.J. Ekinci, *Beta-amyloid and ionophore A23187 evoke tau hyperphosphorylation by distinct intracellular pathways: differential involvement of the calpain/protein kinase C system.* J Neurosci Res, 1997. **49**(6): p. 759-68.
278. Veeranna, et al., *Calpain mediates calcium-induced activation of the erk1,2 MAPK pathway and cytoskeletal phosphorylation in neurons: relevance to Alzheimer's disease.* Am J Pathol, 2004. **165**(3): p. 795-805.
279. Zheng, W.H., et al., *Amyloid beta peptide induces tau phosphorylation and loss of cholinergic neurons in rat primary septal cultures.* Neuroscience, 2002. **115**(1): p. 201-11.
280. Goni-Oliver, P., et al., *N-terminal cleavage of GSK-3 by calpain: a new form of GSK-3 regulation.* J Biol Chem, 2007. **282**(31): p. 22406-13.
281. Kusakawa, G., et al., *Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25.* J Biol Chem, 2000. **275**(22): p. 17166-72.
282. Lee, M.S., et al., *Neurotoxicity induces cleavage of p35 to p25 by calpain.* Nature, 2000. **405**(6784): p. 360-4.
283. Nath, R., et al., *Processing of cdk5 activator p35 to its truncated form (p25) by calpain in acutely injured neuronal cells.* Biochem Biophys Res Commun, 2000. **274**(1): p. 16-21.
284. Tseng, H.C., et al., *A survey of Cdk5 activator p35 and p25 levels in Alzheimer's disease brains.* FEBS Lett, 2002. **523**(1-3): p. 58-62.
285. Cruz, J.C., et al., *Aberrant Cdk5 activation by p25 triggers pathological events leading to neurodegeneration and neurofibrillary tangles.* Neuron, 2003. **40**(3): p. 471-83.
286. Fischer, A., et al., *Opposing roles of transient and prolonged expression of p25 in synaptic plasticity and hippocampus-dependent memory.* Neuron, 2005. **48**(5): p. 825-38.
287. Cruz, J.C., et al., *p25/cyclin-dependent kinase 5 induces production and intraneuronal accumulation of amyloid beta in vivo.* J Neurosci, 2006. **26**(41): p. 10536-41.
288. Wang, Q., et al., *Block of long-term potentiation by naturally secreted and synthetic amyloid beta-peptide in hippocampal slices is mediated via activation of the kinases c-Jun N-terminal kinase, cyclin-dependent kinase 5, and p38 mitogen-activated protein kinase as well as metabotropic glutamate receptor type 5.* J Neurosci, 2004. **24**(13): p. 3370-8.
289. Alvarez, A., et al., *Inhibition of tau phosphorylating protein kinase cdk5 prevents beta-amyloid-induced neuronal death.* FEBS Lett, 1999. **459**(3): p. 421-6.
290. Park, S.Y. and A. Ferreira, *The generation of a 17 kDa neurotoxic fragment: an alternative mechanism by which tau mediates beta-amyloid-induced neurodegeneration.* J Neurosci, 2005. **25**(22): p. 5365-75.
291. Um, J.W., et al., *Alzheimer amyloid-beta oligomer bound to postsynaptic prion protein activates Fyn to impair neurons.* Nat Neurosci, 2012. **15**(9): p. 1227-35.
292. Cisse, M., et al., *Reversing EphB2 depletion rescues cognitive functions in Alzheimer model.* Nature, 2011. **469**(7328): p. 47-52.
293. Kim, T., et al., *Human LilrB2 is a beta-amyloid receptor and its murine homolog PirB regulates synaptic plasticity in an Alzheimer's model.* Science, 2013. **341**(6152): p. 1399-404.
294. Sotthibundhu, A., et al., *Beta-amyloid(1-42) induces neuronal death through the p75 neurotrophin receptor.* J Neurosci, 2008. **28**(15): p. 3941-6.
295. Xie, L., et al., *Alzheimer's beta-amyloid peptides compete for insulin binding to the insulin receptor.* J Neurosci, 2002. **22**(10): p. RC221.
296. Zhao, W.Q., et al., *Amyloid beta oligomers induce impairment of neuronal insulin receptors.* FASEB J, 2008. **22**(1): p. 246-60.

297. Yan, S.D., et al., *RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease.* Nature, 1996. **382**(6593): p. 685-91.
298. Hadding, A., B. Kaltschmidt, and C. Kaltschmidt, *Overexpression of receptor of advanced glycation end products hypersensitizes cells for amyloid beta peptide-induced cell death.* Biochim Biophys Acta, 2004. **1691**(1): p. 67-72.
299. Sturchler, E., et al., *Site-specific blockade of RAGE-Vd prevents amyloid-beta oligomer neurotoxicity.* J Neurosci, 2008. **28**(20): p. 5149-58.
300. Liu, S., et al., *TLR2 is a primary receptor for Alzheimer's amyloid beta peptide to trigger neuroinflammatory activation.* J Immunol, 2012. **188**(3): p. 1098-107.
301. LaFerla, F.M., K.N. Green, and S. Oddo, *Intracellular amyloid-beta in Alzheimer's disease.* Nat Rev Neurosci, 2007. **8**(7): p. 499-509.
302. Nagele, R.G., et al., *Intracellular accumulation of beta-amyloid(1-42) in neurons is facilitated by the alpha 7 nicotinic acetylcholine receptor in Alzheimer's disease.* Neuroscience, 2002. **110**(2): p. 199-211.
303. Pagani, L. and A. Eckert, *Amyloid-Beta interaction with mitochondria.* Int J Alzheimers Dis, 2011. **2011**: p. 925050.
304. Caspersen, C., et al., *Mitochondrial Abeta: a potential focal point for neuronal metabolic dysfunction in Alzheimer's disease.* FASEB J, 2005. **19**(14): p. 2040-1.
305. Manczak, M., et al., *Mitochondria are a direct site of A beta accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression.* Hum Mol Genet, 2006. **15**(9): p. 1437-49.
306. Hansson Petersen, C.A., et al., *The amyloid beta-peptide is imported into mitochondria via the TOM import machinery and localized to mitochondrial cristae.* Proc Natl Acad Sci U S A, 2008. **105**(35): p. 13145-50.
307. Lustbader, J.W., et al., *ABAD directly links Abeta to mitochondrial toxicity in Alzheimer's disease.* Science, 2004. **304**(5669): p. 448-52.
308. Du, H., et al., *Cyclophilin D deficiency attenuates mitochondrial and neuronal perturbation and ameliorates learning and memory in Alzheimer's disease.* Nat Med, 2008. **14**(10): p. 1097-105.
309. D'Amelio, M., et al., *Caspase-3 triggers early synaptic dysfunction in a mouse model of Alzheimer's disease.* Nat Neurosci, 2011. **14**(1): p. 69-76.
310. Walsh, D.M., et al., *Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo.* Nature, 2002. **416**(6880): p. 535-9.
311. Ferreira, S.T. and W.L. Klein, *The Abeta oligomer hypothesis for synapse failure and memory loss in Alzheimer's disease.* Neurobiol Learn Mem, 2011. **96**(4): p. 529-43.
312. Puzzo, D., et al., *Picomolar amyloid-beta positively modulates synaptic plasticity and memory in hippocampus.* J Neurosci, 2008. **28**(53): p. 14537-45.
313. Millucci, L., et al., *Rapid aggregation and assembly in aqueous solution of A beta (25-35) peptide.* J Biosci, 2009. **34**(2): p. 293-303.
314. Rodrigues, T.M., et al., *Challenges and promises in the development of neurotrophic factor-based therapies for Parkinson's disease.* Drugs Aging, 2014. **31**(4): p. 239-61.
315. Nagahara, A.H., et al., *Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease.* Nat Med, 2009. **15**(3): p. 331-7.
316. Blurton-Jones, M., et al., *Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease.* Proc Natl Acad Sci U S A, 2009. **106**(32): p. 13594-9.
317. Nagahara, A.H., et al., *Early BDNF treatment ameliorates cell loss in the entorhinal cortex of APP transgenic mice.* J Neurosci, 2013. **33**(39): p. 15596-602.
318. Zeng, Y., D. Zhao, and C.W. Xie, *Neurotrophins enhance CaMKII activity and rescue amyloid-beta-induced deficits in hippocampal synaptic plasticity.* J Alzheimers Dis, 2010. **21**(3): p. 823-31.
319. Phillips, H.S., et al., *BDNF mRNA is decreased in the hippocampus of individuals with Alzheimer's disease.* Neuron, 1991. **7**(5): p. 695-702.
320. Garzon, D., G. Yu, and M. Fahnestock, *A new brain-derived neurotrophic factor transcript and decrease in brain-derived neurotrophic factor transcripts 1, 2 and 3 in Alzheimer's disease parietal cortex.* J Neurochem, 2002. **82**(5): p. 1058-64.

321. Holsinger, R.M., et al., *Quantitation of BDNF mRNA in human parietal cortex by competitive reverse transcription-polymerase chain reaction: decreased levels in Alzheimer's disease.* Brain Res Mol Brain Res, 2000. **76**(2): p. 347-54.
322. Ferrer, I., et al., *BDNF and full-length and truncated TrkB expression in Alzheimer disease. Implications in therapeutic strategies.* J Neuropathol Exp Neurol, 1999. **58**(7): p. 729-39.
323. Connor, B., et al., *Brain-derived neurotrophic factor is reduced in Alzheimer's disease.* Brain Res Mol Brain Res, 1997. **49**(1-2): p. 71-81.
324. Narisawa-Saito, M., et al., *Regional specificity of alterations in NGF, BDNF and NT-3 levels in Alzheimer's disease.* Neuroreport, 1996. **7**(18): p. 2925-8.
325. Peng, S., et al., *Precursor form of brain-derived neurotrophic factor and mature brain-derived neurotrophic factor are decreased in the pre-clinical stages of Alzheimer's disease.* J Neurochem, 2005. **93**(6): p. 1412-21.
326. Michalski, B. and M. Fahnestock, *Pro-brain-derived neurotrophic factor is decreased in parietal cortex in Alzheimer's disease.* Brain Res Mol Brain Res, 2003. **111**(1-2): p. 148-54.
327. Egan, M.F., et al., *The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function.* Cell, 2003. **112**(2): p. 257-69.
328. Hashimoto, R., et al., *Effect of the brain-derived neurotrophic factor and the apolipoprotein E polymorphisms on disease progression in preclinical Alzheimer's disease.* Genes Brain Behav, 2009. **8**(1): p. 43-52.
329. Feher, A., et al., *Association between BDNF Val66Met polymorphism and Alzheimer disease, dementia with Lewy bodies, and Pick disease.* Alzheimer Dis Assoc Disord, 2009. **23**(3): p. 224-8.
330. Voineskos, A.N., et al., *The brain-derived neurotrophic factor Val66Met polymorphism and prediction of neural risk for Alzheimer disease.* Arch Gen Psychiatry, 2011. **68**(2): p. 198-206.
331. Ventriglia, M., et al., *Association between the BDNF 196 A/G polymorphism and sporadic Alzheimer's disease.* Mol Psychiatry, 2002. **7**(2): p. 136-7.
332. Adamczuk, K., et al., *Polymorphism of brain derived neurotrophic factor influences beta amyloid load in cognitively intact apolipoprotein E epsilon4 carriers.* Neuroimage Clin, 2013. **2**: p. 512-20.
333. Allen, S.J., G.K. Wilcock, and D. Dawbarn, *Profound and selective loss of catalytic TrkB immunoreactivity in Alzheimer's disease.* Biochem Biophys Res Commun, 1999. **264**(3): p. 648-51.
334. Salehi, A., et al., *Co-localization of high-affinity neurotrophin receptors in nucleus basalis of Meynert neurons and their differential reduction in Alzheimer's disease.* Neuroscience, 1996. **75**(2): p. 373-87.
335. Ginsberg, S.D., et al., *Down regulation of trk but not p75NTR gene expression in single cholinergic basal forebrain neurons mark the progression of Alzheimer's disease.* J Neurochem, 2006. **97**(2): p. 475-87.
336. Connor, B., et al., *Trk receptor alterations in Alzheimer's disease.* Brain Res Mol Brain Res, 1996. **42**(1): p. 1-17.
337. Wong, J., et al., *Amyloid beta selectively modulates neuronal TrkB alternative transcript expression with implications for Alzheimer's disease.* Neuroscience, 2012. **210**: p. 363-74.
338. Chen, Z., et al., *Genetic association of neurotrophic tyrosine kinase receptor type 2 (NTRK2) With Alzheimer's disease.* Am J Med Genet B Neuropsychiatr Genet, 2008. **147**(3): p. 363-9.
339. Ansaloni, S., et al., *TrkB Isoforms Differentially Affect AICD Production through Their Intracellular Functional Domains.* Int J Alzheimers Dis, 2011. **2011**: p. 729382.
340. Holback, S., L. Adlerz, and K. Iverfeldt, *Increased processing of APLP2 and APP with concomitant formation of APP intracellular domains in BDNF and retinoic acid-differentiated human neuroblastoma cells.* J Neurochem, 2005. **95**(4): p. 1059-68.
341. Matrone, C., et al., *NGF and BDNF signaling control amyloidogenic route and Abeta production in hippocampal neurons.* Proc Natl Acad Sci U S A, 2008. **105**(35): p. 13139-44.
342. Elliott, E., et al., *Brain-derived neurotrophic factor induces a rapid dephosphorylation of tau protein through a PI-3 Kinase signalling mechanism.* Eur J Neurosci, 2005. **22**(5): p. 1081-9.
343. Castello, N.A., K.N. Green, and F.M. LaFerla, *Genetic knockdown of brain-derived neurotrophic factor in 3xTg-AD mice does not alter Abeta or tau pathology.* PLoS One, 2012. **7**(8): p. e39566.
344. Rantamaki, T., et al., *The impact of Bdnf gene deficiency to the memory impairment and brain pathology of APPswe/PS1dE9 mouse model of Alzheimer's disease.* PLoS One, 2013. **8**(7): p. e68722.

345. Alcantara, S., et al., *TrkB signaling is required for postnatal survival of CNS neurons and protects hippocampal and motor neurons from axotomy-induced cell death.* J Neurosci, 1997. **17**(10): p. 3623-33.
346. Jeronimo-Santos, A., et al., *Dysregulation of TrkB Receptors and BDNF Function by Amyloid-beta Peptide is Mediated by Calpain.* Cereb Cortex, 2014.
347. Sommerfeld, M.T., et al., *Down-regulation of the neurotrophin receptor TrkB following ligand binding. Evidence for an involvement of the proteasome and differential regulation of TrkA and TrkB.* J Biol Chem, 2000. **275**(12): p. 8982-90.
348. Aroeira, R.I., et al., *Age-related changes of glycine receptor at the rat hippocampus: from the embryo to the adult.* J Neurochem, 2011. **118**(3): p. 339-53.
349. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Res, 2001. **29**(9): p. e45.
350. Sousa, V.C., et al., *Regulation of hippocampal cannabinoid CB1 receptor actions by adenosine A1 receptors and chronic caffeine administration: implications for the effects of Delta9-tetrahydrocannabinol on spatial memory.* Neuropsychopharmacology, 2011. **36**(2): p. 472-87.
351. Hockemeyer, J., J.C. Burbiel, and C.E. Muller, *Multigram-scale syntheses, stability, and photoreactions of A2A adenosine receptor antagonists with 8-styrylxanthine structure: potential drugs for Parkinson's disease.* J Org Chem, 2004. **69**(10): p. 3308-18.
352. Yang, M., et al., *Characterization of the potency, selectivity, and pharmacokinetic profile for six adenosine A2A receptor antagonists.* Naunyn Schmiedebergs Arch Pharmacol, 2007. **375**(2): p. 133-44.
353. Batalha, V.L., et al., *Adenosine A(2A) receptor blockade reverts hippocampal stress-induced deficits and restores corticosterone circadian oscillation.* Mol Psychiatry, 2013. **18**(3): p. 320-31.
354. Albensi, B.C., et al., *Electrical stimulation protocols for hippocampal synaptic plasticity and neuronal hyper-excitability: are they effective or relevant?* Exp Neurol, 2007. **204**(1): p. 1-13.
355. Klein, R., et al., *The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain.* Cell, 1990. **61**(4): p. 647-56.
356. Devi, L. and M. Ohno, *7,8-dihydroxyflavone, a small-molecule TrkB agonist, reverses memory deficits and BACE1 elevation in a mouse model of Alzheimer's disease.* Neuropsychopharmacology, 2012. **37**(2): p. 434-44.
357. Kubo, T., et al., *In vivo conversion of racemized beta-amyloid ([D-Ser 26]A beta 1-40) to truncated and toxic fragments ([D-Ser 26]A beta 25-35/40) and fragment presence in the brains of Alzheimer's patients.* J Neurosci Res, 2002. **70**(3): p. 474-83.
358. Grynspan, F., et al., *Active site-directed antibodies identify calpain II as an early-appearing and pervasive component of neurofibrillary pathology in Alzheimer's disease.* Brain Res, 1997. **763**(2): p. 145-58.
359. Adamec, E., et al., *Calpain activation in neurodegenerative diseases: confocal immunofluorescence study with antibodies specifically recognizing the active form of calpain 2.* Acta Neuropathol, 2002. **104**(1): p. 92-104.
360. Noble, W., et al., *Cdk5 is a key factor in tau aggregation and tangle formation in vivo.* Neuron, 2003. **38**(4): p. 555-65.
361. Cruz, J.C. and L.H. Tsai, *Cdk5 deregulation in the pathogenesis of Alzheimer's disease.* Trends Mol Med, 2004. **10**(9): p. 452-8.
362. Granic, I., et al., *Calpain inhibition prevents amyloid-beta-induced neurodegeneration and associated behavioral dysfunction in rats.* Neuropharmacology, 2010. **59**(4-5): p. 334-42.
363. Bonfoco, E., et al., *Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures.* Proc Natl Acad Sci U S A, 1995. **92**(16): p. 7162-6.
364. Slee, E.A., et al., *Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD.FMK) inhibits apoptosis by blocking the processing of CPP32.* Biochem J, 1996. **315 (Pt 1)**: p. 21-4.
365. Kaplan, D.R. and F.D. Miller, *Neurotrophin signal transduction in the nervous system.* Curr Opin Neurobiol, 2000. **10**(3): p. 381-91.
366. Tong, L., et al., *Beta-amyloid peptide at sublethal concentrations downregulates brain-derived neurotrophic factor functions in cultured cortical neurons.* J Neurosci, 2004. **24**(30): p. 6799-809.

367. Ancot, F., et al., *Proteolytic cleavages give receptor tyrosine kinases the gift of ubiquity.* Oncogene, 2009. **28**(22): p. 2185-95.
368. Tauszig-Delamasure, S., et al., *The TrkC receptor induces apoptosis when the dependence receptor notion meets the neurotrophin paradigm.* Proc Natl Acad Sci U S A, 2007. **104**(33): p. 13361-6.
369. Canas, P.M., et al., *Predominant loss of glutamatergic terminal markers in a beta-amyloid peptide model of Alzheimer's disease.* Neuropharmacology, 2014. **76 Pt A**: p. 51-6.
370. Bell, K.F., et al., *Structural involvement of the glutamatergic presynaptic boutons in a transgenic mouse model expressing early onset amyloid pathology.* Neurosci Lett, 2003. **353**(2): p. 143-7.
371. Lambert, M.P., et al., *Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins.* Proc Natl Acad Sci U S A, 1998. **95**(11): p. 6448-53.
372. Smith, J.P., et al., *Stimulus pattern dependence of the Alzheimer's disease amyloid-beta 42 peptide's inhibition of long term potentiation in mouse hippocampal slices.* Brain Res, 2009. **1269**: p. 176-84.
373. Gomes, J.R., et al., *Excitotoxicity downregulates TrkB.FL signaling and upregulates the neuroprotective truncated TrkB receptors in cultured hippocampal and striatal neurons.* J Neurosci, 2012. **32**(13): p. 4610-22.
374. Vidaurre, O.G., et al., *Imbalance of neurotrophin receptor isoforms TrkB-FL/TrkB-T1 induces neuronal death in excitotoxicity.* Cell Death Dis, 2012. **3**: p. e256.
375. Spires-Jones, T. and S. Knafo, *Spines, plasticity, and cognition in Alzheimer's model mice.* Neural Plast, 2012. **2012**: p. 319836.
376. Marques, S., et al., *Modulating Alzheimer's disease through caffeine: a putative link to epigenetics.* J Alzheimers Dis, 2011. **24 Suppl 2**: p. 161-71.
377. Arendash, G.W., et al., *Caffeine protects Alzheimer's mice against cognitive impairment and reduces brain beta-amyloid production.* Neuroscience, 2006. **142**(4): p. 941-52.
378. Espinosa, J., et al., *Caffeine consumption prevents memory impairment, neuronal damage, and adenosine A2A receptors upregulation in the hippocampus of a rat model of sporadic dementia.* J Alzheimers Dis, 2013. **34**(2): p. 509-18.
379. Jarvis, M.F., et al., *[3H]CGS 21680, a selective A2 adenosine receptor agonist directly labels A2 receptors in rat brain.* J Pharmacol Exp Ther, 1989. **251**(3): p. 888-93.
380. Cunha, R.A., M.D. Constantino, and J.A. Ribeiro, *ZM241385 is an antagonist of the facilitatory responses produced by the A2A adenosine receptor agonists CGS21680 and HENECA in the rat hippocampus.* Br J Pharmacol, 1997. **122**(7): p. 1279-84.
381. Rahman, A., *The role of adenosine in Alzheimer's disease.* Curr Neuropharmacol, 2009. **7**(3): p. 207-16.
382. Dall'Igna, O.P., et al., *Caffeine and adenosine A(2a) receptor antagonists prevent beta-amyloid (25-35)-induced cognitive deficits in mice.* Exp Neurol, 2007. **203**(1): p. 241-5.
383. Canas, P.M., et al., *Adenosine A2A receptor blockade prevents synaptotoxicity and memory dysfunction caused by beta-amyloid peptides via p38 mitogen-activated protein kinase pathway.* J Neurosci, 2009. **29**(47): p. 14741-51.
384. Cunha, R.A., et al., *Adenosine A2A receptors stimulate acetylcholine release from nerve terminals of the rat hippocampus.* Neurosci Lett, 1995. **196**(1-2): p. 41-4.
385. Jin, S. and B.B. Fredholm, *Adenosine A2A receptor stimulation increases release of acetylcholine from rat hippocampus but not striatum, and does not affect catecholamine release.* Naunyn Schmiedebergs Arch Pharmacol, 1997. **355**(1): p. 48-56.
386. Mayne, M., et al., *Adenosine A2A receptor activation reduces proinflammatory events and decreases cell death following intracerebral hemorrhage.* Ann Neurol, 2001. **49**(6): p. 727-35.
387. Dijkhuizen, P.A. and A. Ghosh, *BDNF regulates primary dendrite formation in cortical neurons via the PI3-kinase and MAP kinase signaling pathways.* J Neurobiol, 2005. **62**(2): p. 278-88.
388. Ji, Y., et al., *Cyclic AMP controls BDNF-induced TrkB phosphorylation and dendritic spine formation in mature hippocampal neurons.* Nat Neurosci, 2005. **8**(2): p. 164-72.
389. Cheng, H.C., H.M. Shih, and Y. Chern, *Essential role of cAMP-response element-binding protein activation by A2A adenosine receptors in rescuing the nerve growth factor-induced neurite outgrowth impaired by blockage of the MAPK cascade.* J Biol Chem, 2002. **277**(37): p. 33930-42.

390. Batalha, V.L., et al., *Adenosine A2A receptor activation-trigger to aging-like modifications on adenosine modulation in the hippocampus.* J. Neurochem, 2013b. **125**(125): p. 153-153.
391. Orru, M., et al., *Striatal pre- and postsynaptic profile of adenosine A(2A) receptor antagonists.* PLoS One, 2011. **6**(1): p. e16088.
392. Orru, M., et al., *Pharmacological evidence for different populations of postsynaptic adenosine A2A receptors in the rat striatum.* Neuropharmacology, 2011. **61**(5-6): p. 967-74.
393. Deogracias, R., et al., *Expression of the neurotrophin receptor trkB is regulated by the cAMP/CREB pathway in neurons.* Mol Cell Neurosci, 2004. **26**(3): p. 470-80.
394. Kingsbury, T.J., et al., *Ca(2+)-dependent regulation of TrkB expression in neurons.* J Biol Chem, 2003. **278**(42): p. 40744-8.
395. Fredholm, B.B., et al., *International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors.* Pharmacol Rev, 2001. **53**(4): p. 527-52.
396. Martin, S.J., P.D. Grimwood, and R.G. Morris, *Synaptic plasticity and memory: an evaluation of the hypothesis.* Annu Rev Neurosci, 2000. **23**: p. 649-711.
397. Liu, I.Y., et al., *Brain-derived neurotrophic factor plays a critical role in contextual fear conditioning.* J Neurosci, 2004. **24**(36): p. 7958-63.
398. Takei, S., et al., *Enhanced hippocampal BDNF/TrkB signaling in response to fear conditioning in an animal model of posttraumatic stress disorder.* J Psychiatr Res, 2011. **45**(4): p. 460-8.
399. Wang, K.K., *Calpain and caspase: can you tell the difference?* Trends Neurosci, 2000. **23**(1): p. 20-6.
400. Smith, M.A. and R.G. Schnellmann, *Calpains, mitochondria, and apoptosis.* Cardiovasc Res, 2012. **96**(1): p. 32-7.
401. Momeni, H.R., *Role of calpain in apoptosis.* Cell J, 2011. **13**(2): p. 65-72.
402. Rodrigues, T.M., et al., *Challenges and Promises in the Development of Neurotrophic Factor-Based Therapies for Parkinson's Disease.* Drugs Aging, 2014.
403. Fifre, A., et al., *Microtubule-associated protein MAP1A, MAP1B, and MAP2 proteolysis during soluble amyloid beta-peptide-induced neuronal apoptosis. Synergistic involvement of calpain and caspase-3.* J Biol Chem, 2006. **281**(1): p. 229-40.
404. Raynaud, F. and A. Marcilhac, *Implication of calpain in neuronal apoptosis. A possible regulation of Alzheimer's disease.* FEBS J, 2006. **273**(15): p. 3437-43.
405. Kim, D.H. and X. Zhao, *BDNF protects neurons following injury by modulation of caspase activity.* Neurocrit Care, 2005. **3**(1): p. 71-6.
406. Han, B.H., et al., *BDNF blocks caspase-3 activation in neonatal hypoxia-ischemia.* Neurobiol Dis, 2000. **7**(1): p. 38-53.
407. Zhou, H., et al., *Akt regulates cell survival and apoptosis at a postmitochondrial level.* J Cell Biol, 2000. **151**(3): p. 483-94.
408. Lesne, S., et al., *Akt-dependent expression of NAIP-1 protects neurons against amyloid-{beta} toxicity.* J Biol Chem, 2005. **280**(26): p. 24941-7.
409. Rebola, N., et al., *Different roles of adenosine A1, A2A and A3 receptors in controlling kainate-induced toxicity in cortical cultured neurons.* Neurochem Int, 2005. **47**(5): p. 317-25.
410. Valadas, J.S., et al., *Neuroprotection afforded by adenosine A2A receptor blockade is modulated by corticotrophin-releasing factor (CRF) in glutamate injured cortical neurons.* J Neurochem, 2012. **123**(6): p. 1030-40.
411. Dall'Igna, O.P., et al., *Neuroprotection by caffeine and adenosine A2A receptor blockade of beta-amyloid neurotoxicity.* Br J Pharmacol, 2003. **138**(7): p. 1207-9.
412. Cunha, G.M., et al., *Adenosine A2A receptor blockade prevents memory dysfunction caused by beta-amyloid peptides but not by scopolamine or MK-801.* Exp Neurol, 2008. **210**(2): p. 776-81.
413. Kemppainen, S., et al., *Impaired TrkB receptor signaling contributes to memory impairment in APP/PS1 mice.* Neurobiol Aging, 2012. **33**(6): p. 1122 e23-39.
414. Hossain, M.I., et al., *A truncated fragment of Src protein kinase generated by calpain-mediated cleavage is a mediator of neuronal death in excitotoxicity.* J Biol Chem, 2013. **288**(14): p. 9696-709.
415. Sola, S., et al., *p53 interaction with JMJD3 results in its nuclear distribution during mouse neural stem cell differentiation.* PLoS One, 2011. **6**(3): p. e18421.

10. Appendix

10.1. TrkB-ICD: putative conformation and localization.

Preliminary data obtained in this thesis, indicate that the TrkB-ICD fragment, which is produced upon calpain-mediated cleavage of TrkB-FL receptor, adopts a different conformation after the cleavage. Indeed, it was observed that the c-terminal TrkB antibody is only able to immunoprecipitate TrkB-FL, but not TrkB-ICD (Figure 10.1A). Given that both TrkB-FL and TrkB-ICD share the same c-terminal domain (see Figure 10.1B) and are both recognized by the c-terminal TrkB antibody in western-blot (denaturing conditions), our hypothesis would be that after the cleavage of TrkB-FL, the TrkB-ICD fragment would adopt a different structure in solution, masking the c-terminal epitope and preventing the immunoprecipitation with the c-terminal TrkB antibody in native conditions.

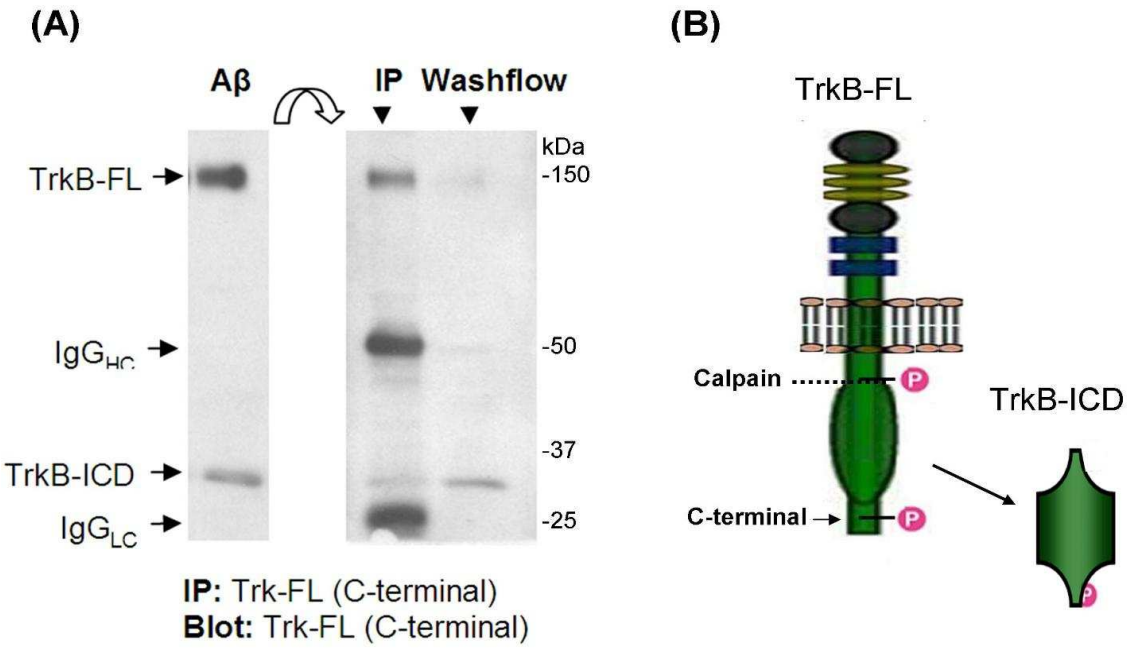


Figure 10.1 – TrkB-ICD conformation

(A) Left: Western-blotting, probed with TrkB c-terminal antibody, showing the presence of TrkB-FL and the TrkB-ICD fragment on Aβ-treated neuronal cultures. Right: Immunoprecipitation (IP) of Aβ-treated neuronal cultures lysate with the TrkB c-terminal antibody. This antibody fully immunoprecipitates TrkB-FL from the Aβ-treated neurons, but it failed to immunoprecipitate the 32kDa TrkB-ICD. Thus, the supernatant (washflow) does not contain TrkB-FL, since it was fully immunoprecipitated, and it contains almost all original amount of TrkB-ICD. **(B)** Schematic representation of TrkB-FL, showing calpain cleavage site, c-terminal, Y515 and Y816 (indicated with P), and TrkB-ICD with a different conformation.

To identify the subcellular localization of TrkB-ICD, it was performed a cytosolic and nuclear protein extraction, as described in [415], from Aβ-treated cortical neurons. By analysing the levels of GAPDH (a cytosolic protein), we observed that TrkB-ICD is not enriched in cytosolic (GAPDH) fraction. However, we cannot conclude that TrkB-ICD is enriched in nuclear (Lamin) fraction, since this fraction was contaminated with other fractions and it was also detected the presence of TrkB-FL (transmembrane protein located in plasma membrane and intracellular membranes). Thus, our preliminary data suggests that TrkB-ICD is not enriched in the cytosolic fraction.

The indication that TrkB-ICD has a different conformation and that TrkB-ICD seems to be enriched in nucleus or inside other organelles, suggest that this fragment may have a distinct function.

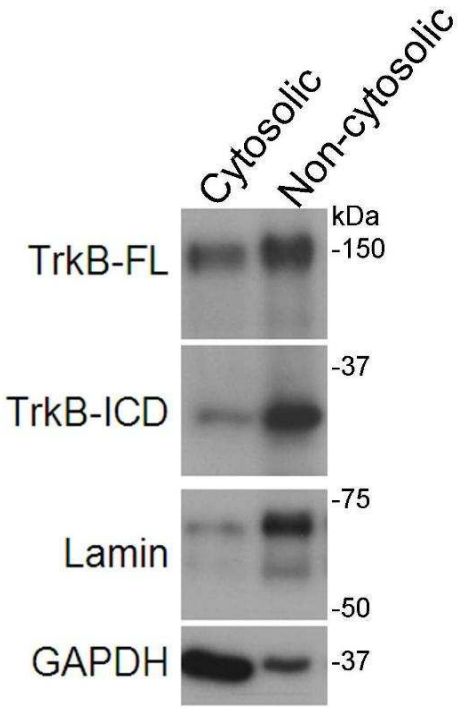


Figure 10.2 – TrkB-ICD subcellular localization

Western-blot of cytosolic and non-cytosolic fractions from Aβ-treated cultured neurons, showing the levels of TrkB-ICD and TrkB-FL (transmembrane protein), Lamin A/C (nuclear protein) and GAPDH (cytosolic protein).

10.2. TrkB-ICD: cloning and transfection

In order to study the putative biological role of TrkB-ICD fragment, and also to evaluate its subcellular localization by microscopy, we already cloned the fragment sequence into a mammalian expression vector (by using a PCR cloning kit – pcDNA Gateway Directional TOPO expression by *Invitrogen*).

Given that we identified the TrkB cleavage site by N-sequencing, we were able to design a pair of PCR primers with the proper sequences to amplify the TrkB-ICD sequence. In addition, to be able to amplify the TrkB-ICD sequence, the primers must also have the required sequence to facilitate directional cloning and the required Kozak sequence to promote the initiation of translation. Moreover, the reverse primer should not contain the termination codon in order to fuse the TrkB-ICD product in frame with the c-terminal V5 tag. Thus, the forward primer was: 5'-CACCATGAGCCAGCTCAAGC-3' (the first 4 nucleotides CACCA will promote directional cloning, and the ATG is the initiation codon); and the reverse primer was 5'-GCCTAGGATGTCCAGGTAGAC-3'.

By performing a RT-PCR with the selected primers, we were able to amplify a ~910bp product, which as the expected size of TrkB-ICD product (Figure 10.3A). The TrkB-ICD band obtained with 64ºC of annealing temperature was cut and gel-purified using a DNA extraction kit. One fifth of total purified TrkB-ICD DNA was applied in the agarose gel, and its purity was confirmed by the appearance of a single band (Figure 10.3B).

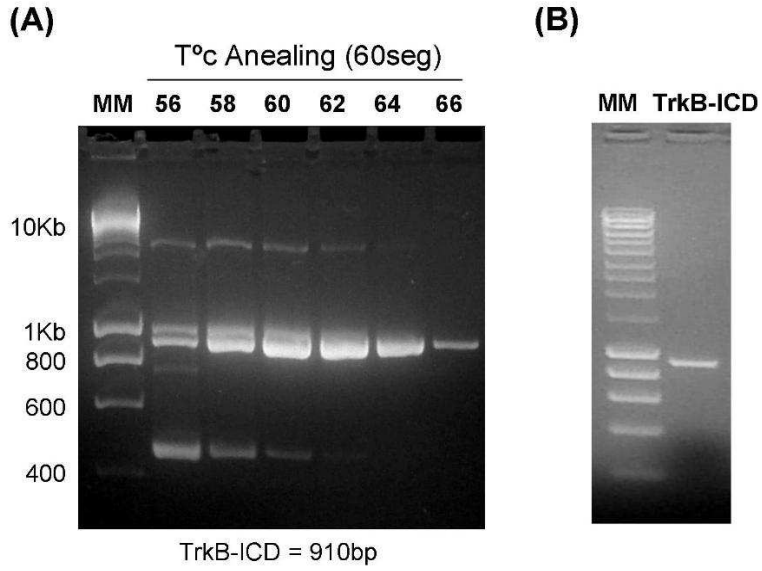


Figure 10.3 – Amplification of TrkB-ICD by RT-PCR

(A) Agarose electrophoresis showing the amplification of TrkB-ICD fragment by RT-PCR in different annealing temperatures, from the total mRNA extracted from a rat hippocampus. The expected size of TrkB-ICD product is 910bp. (B) Agarose electrophoresis showing the gel-purified TrkB-ICD product.

After the purification process, 3 ng of purified TrkB-ICD amplification product were cloned into 3 ng of linearized pcDNA 3.2 TOPO vector (*Invitrogen*). After the cloning reaction, the chemically competent E.coli cells (TOP10, *Invitrogen*) were transformed and plated in selective media overnight at 37ºC. Then, six colonies were picked and analysed by colony PCR and three of them contained the plasmid of interest with the TrkB-ICD inserted in the correct direction (Figure 10.4). The results were further confirmed by restriction analyses (not shown). One of the plasmids was selected and its sequence was ultimately confirmed by DNA sequencing.

Finally, after the successful cloning of TrkB-ICD into pcDNA 3.2, a neuroblastoma cell line (SH-SY5Y) was lipo-transfected with the expression vector (Figure 10.5). The results obtained indicate the presence of a ~35kDa protein containing the V5-tag, which matches with the expected weight of TrkB-ICD plus the linker and V5-Tag (4 kDa). This TrkB-ICD-V5 was not detected by the c-terminal antibody of TrkB (not shown), possibly due to the epitope alteration on c-terminus. The results obtained also indicate a slight increase in the levels of SBDP120 (caspase-3 specific SBDP) in the presence of the TrkB-ICD-V5 (Figure 10.5).

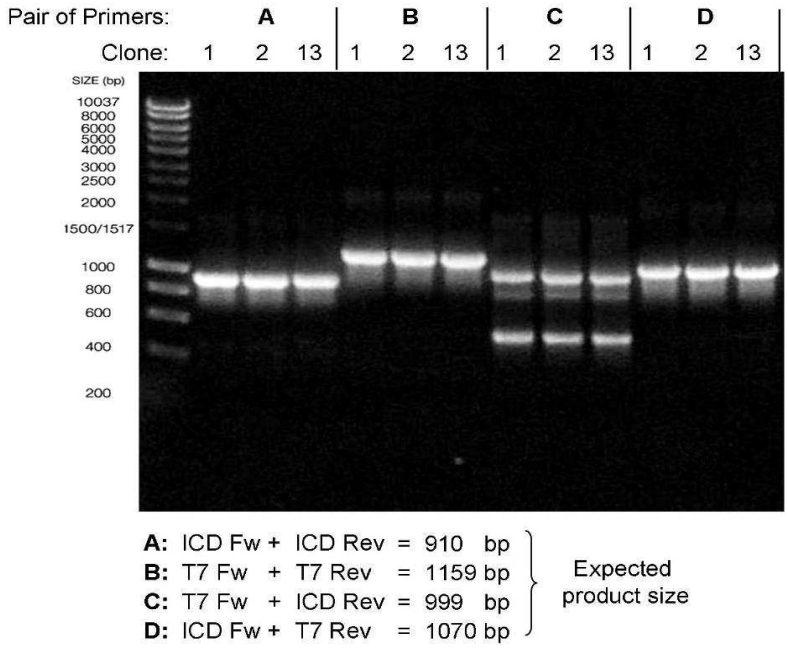


Figure 10.4 – Analysis of selected E.coli colonies by RT-PCR.

Agarose electrophoresis showing the RT-PCR amplification of distinct products based on the mentioned primers combination. T7 sequences are located on the plasmid and flank the TrkB-ICD insertion product. All products sizes observed matched with the expected sizes for a plasmid with the TrkB-ICD inserted in the correct direction.

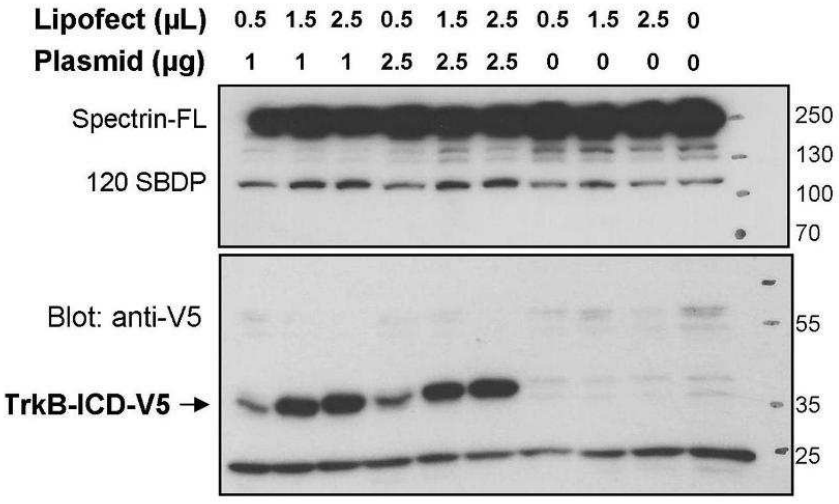


Figure 10.5 – Transfection of SH-SY5Y cell line with pcDNA-TrkB-ICD-V5.

Western-blot probed with anti-αII-spectrin antibody (upper panel) and anti-V5-tag antibody (lower panel) for SH-SY5Y cells 72hours after transfection with different amounts of lipofectamine2000 and plasmid in 24-well plates ($0.25x10^6$ cells/ well).