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UNIVERSIDADE DE COIMBRA

Aβ-mediated changes in CREB and ERK activity in cultured cortical neurons: involvement of NMDA receptors

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ACKNOWLEDGMENTS

Vorrei ringraziare alcune persone che sono state fondamentali per la realizzazione di questa tesi e che mi sono state vicine in questo anno di intenso lavoro al CNC di Coimbra:

La Professora Doutora Ana Cristina Rego, per avermi dato l'occasione di far parte del suo gruppo, per la grande generosità dimostratami. Sono molto riconoscente per la disponibilità che mi ha gentilmente concesso e profondamente onorata di aver conosciuta.

La mia tutor Luísa Ferreira, per la grande pazienza e forza nello starmi accanto a insegnarmi tutto ciò che so di laboratorio, per tutto l'aiuto che mi ha dato sottraendolo alla famiglia e alla realizzazione di altri progetti, per tutte le ore passate in compagnia ad analizzare dati e a formattare grafici, per tutte le volte che ha perso la pazienza con me ma rimaneva comunque dietro ad un computer la notte a lavorare, per tutte le correzioni della tesi.

A Gladys, per la grande amicizia e sostegno che mi ha dato sin dall'inizio. La tua conoscenza mi ha fatto apprezzare ancora di più il mondo della ricerca. Sei un bell'esempio di intraprendenza giovanile, correttezza, forza d'animo e solidarietà.

Ad Antonio, per il grande sostegno dato nei momenti di sconforto, per tutti i buoni consigli, per i momenti che abbiamo lavorato insieme, per le chiacchierate, per non avermi abbandonato nei momenti più bui, per essere la persona speciale che sei, per tutte le volte che hai avuto pazienza con me, per tutto: GRAZIE.

A Marcio, Mario, Jorge, Mohammed, Janette, Martina, Denisa, Cristina, Miranda e Rui, Michele, Amelia, Angelo, Agostino e Maria Paola, Diana, Sonia, Ana Placido, Isabel e Dominique del secondo piano, Joana Isabel Real, Joana Guedes, Rachel, Rebecca, per la simpatia dimostrata.

Alle care ragazze che hanno lavorato in laboratorio, Elisabete, Tatiana, Luana, Sandra, Rita e Rui, Ana, Carolina, Jadna, Giorgia, per tutte le volte che mi hanno dato spiegazioni e compagnia.

A Sofia e a Nuno, per l'amicizia che ci lega: Sofi, Grazie per tutte le volte mi hai sostenuta e confortata via Skype.

A Isabel, per la compagnia in sala di coltura, per avermi fatto sempre trovato il materiale per il mio lavoro, per l'amicizia dimostrata, per tutte le volte che mi hai aiutato, persino a trovare un posto dove stampare la tesi.

A Carlo B. Duarte e Emilia Duarte, per tutte le volte che mi hanno chiarito dubbi sull'ERASMUS.

A Dona Isabel e Sara, per i sorrisi e l'aiuto nel mio lavoro.

Alle mie amiche Annalisa, Giusy, Simona, Sara, Rossella, Chiara, per non avermi dimenticata in questo anno di lontananza.

Alla mia famiglia, mamma, papà, Daniela, Clementina, Eleonora, i nonni, le zie, gli zii, che mi seguivano da casa e si preoccupavano per me.

A tutti quelli a cui sono mancata, perché vuol dire che un po' mi hanno pensata…

A tutti voi…. GRAZIE!!!

CONTENTS

ABBREVIATIONS

α7nAChRs	nicotinic acetylcholine α7receptors
α-sAPP	soluble APP fragment
ACE	angiotensin-converting enzyme
ACh	acetylcholine
AChE	acetylcholine-esterase.
AChEI	acetylcholinesterase inhibitor
AD	Alzheimer's disease
ADDLs	Aβ-derived diffusible ligands
AICD	APP containing the intracellular domain
AMPARs	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
APH-1	anterior pharynx-defective 1
APM	Affected Pedigree Member
ApoE ε4	apolipoprotein E ε4
APP	amyloid precursor protein
Aβ	amyloid beta peptide
BACE1	β-site APP-cleaving enzyme 1
BAT	brown adipose tissue
BBB	blood-brain barrier
BDNF	neurotrophin brain-derived neurotrophic factor
BIN1	bridging Integrator 1
CALHM1	calcium homeostasis modulator 1
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CaMKIV	Ca^{2+}/calmodulin-dependent protein kinases IV
CdK-5	Cyclin-dependent Kinase 5
ChAT	ACh Choline Acetyltransferase
CK2	Casein Kinase II
CLU	Clusterin
CR1	Complement component receptor 1
CREB	cAMP response element-binding protein
CREBBP	p300/CBP CREB-binding protein
CRTCs	CREB-regulated transcription coactivators
CT	computerized tomography
CTF	C-terminal fragment

EAATs	excitatory amino acid transporters
ECE	endothelin-converting enzyme
EGF-R	epidermal growth factor receptor
ER	endoplasmic reticulum
EXOC3L2	Exocyst Complex Component 3-like 2
ERK	extracellular-signal-regulated kinase
FAD	familiar AD
FKHR	forkhead in rhabdomyosarcoma
GCN5	General Control Non-Repressed Protein 5
GPCRs	G-Protein Coupled Receptors
HCB	host cell factor binding site
IDE	insulin-degrading enzyme
KPI	Kunitz-type protease inhibitor domain
LRP	Lipoprotein receptor-related protein
LTP	Long Term Potentiation
MAP kinases	Mitogen-Activated Protein Kinases
MAP	microtubule-associated protein
MCI	mild cognitive impairment
MMSE	Mini-Mental State Examination
MRI	magnetic resonance imaging
NCT	nicastrin
NCX	excharger Na^{+}/Ca^{2+}
NEP	neprilysin
NL	neuroligin
NFTs	neurofibrillary tangles
NLSs	Nuclear Localization Signals
NMDARs	*N*-methyl-D-Aspartate Receptors
nNOS	neuronal NOS
NO	nitric oxide
NRFs	nuclear respiratory factors
OX-2	OX-2 antigen domain
PEN-2	presenilin enhancer 2
p-gp	p-glycoprotein
PICALM	phosphatidylinositol-binding clathrin assembly protein
PKA	Protein Kinase A

PKC	Protein Kinase C
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PP2B	protein phosphatase 2B or calcineurin.
PRC	PGC-related coactivator
PSD 95	postsynaptic density 95
PSN1	Presenilin 1
PSN2	Presenilin 2
PTKs	protein tyrosine kinases
PTP	phosphotyrosine phosphatase
RAGE	receptor for advanced glycation end products
RRM	RNA recognition motif
RSK	ribosomal s6 kinase
RTKs	Receptor Tyrosine Kinases
SERCA	sarco-/endoplasmic reticulum calcium ATPase
SOCCs	store-operated calcium channels
SOD	superoxide dismutase sod
SORL1	sortilin-related receptor 1
SPECT	single-photon emission computed tomography
STEP	striatal-enriched tyrosine phosphatase
TFAM	transcription factor A mitochondrial
TGN	trans-Golgi network
TRAP	thyroid receptor TR-associated protein
UCP1	uncoupling protein 1
VGCC	voltage-gated calcium channels
VGLUTs	vesicular glutamate transporters
VLDL	very low-density lipoprotein

ABSTRACT

Alzheimer's disease (AD) is the most common neurodegenerative disorder in elderly. Typical hallmarks of this pathology are the extracellular deposits of amyloid-beta peptide (Aβ) peptide forming plaques and neurofibrillary tangles in the hippocampus and cortex, the main brain areas affected in AD. Many studies consider the accumulation of Aβ as "primum movens" for a cascade of events that ultimately produce massive neuronal death in selective neurons. cAMP response element-binding protein (CREB) is one of the main transcriptional factors involved in gene expression related to cell survival, memory formation and synaptic plasticity. In AD brain, CREB levels and activity were shown to be altered. In additon, extracellular-signal-regulated kinase (ERK) is involved in a cascade of events that can also modulate the activity of CREB through phosphorylation of specific kinases. Both CREB and ERK-associated signaling pathways are sensitive to intracellular Ca^{2+} changes (Ca^{2+}_i), being Ca^{2+}_i dyshomeostasis largely described to occur in AD. *N*-methyl-D-aspartate receptors (NMDARs) have a high Ca^{2+} conductance and are essential for synaptic plasticity, since they are connected to the excitability of post-synaptic membranes; however, overactivation of NMDARs causes excitotoxicity.

In this work we evaluated the changes in CREB and ERK activities in mature cortical neurons exposed to Aβ1-42 oligomers and the involvement of NMDARs activation-mediated Ca^{2+}_i rise in these cells. We further analysed the levels of a CREB target, PGC-1α, involved in mitochondrial biogenesis and in antioxidant response. Our findings show that incubation with Aβ1-42 oligomers produces early changes in the levels of phosphorylated CREB, reflecting CREB activity, in a process mediated by influx of Ca^{2+} occurring through the activation of NMDARs. A similar pattern of activation was observed for ERK, suggesting that both pathways can be connected in the response to Aβ exposure. Interestingly, ERK and CREB activation after Aβ1-42 exposure were largely modulated by GluN2A-composed NMDARs, which are mostly present in synaptic sites. Moreover, protein levels of PGC-1α increased for the same time of exposure and this effect was also mediated by NMDARs activations, suggesting that Aβ-induced early control of PGC-1α levels may help to counteract late neurotoxic effects induced by exposure to Aβ oligomers. Moreover, prolonged Aβ exposure induced a decrease in nuclear CREB, supporting a late decline in pro-survival functions of this transcription factor. Overall, data suggest that early exposure to oligomeric Aβ1-42 exerts beneficial effects in mature cortical neurons through the activation of ERK-CREB signaling pathways, possibly linked to the activation of synaptic NMDARs.

Key words: Aβ, CREB, ERK, PGC-1α, NMDARs

RESUMO

A doença de Alzheimer (DA) é a doença neurodegenerativa mais comum no idoso. De entre os marcadores típicos da doença encontram-se as placas senis, constituídas por depósitos extracelulares do peptídeo beta-amilóide (Aβ) e as tranças neurofibrilares, cuja acumulação ocorre no hipocampo e no córtex, as duas áreas cerebrais mais afetadas na DA. Muitos estudos consideram a acumulação de Aβ como "primo movens" para a cascata de eventos causadores da morte neuronal maciça que ocorre na DA. O CREB é um dos fatores de transcrição mais importantes envolvidos na expressão de genes que conduzem à sobrevivência celular, nos processos de formação da memória, assim como na plasticidade sinática. Na DA, os níveis proteicos e a atividade do CREB têm mostrado estarem alterados. Para além disso, a ERK parece estar envolvida numa cascata de eventos que modulam, por sua vez, a atividade do CREB através da fosforilação de cinases específicas. As vias de sinalização associadas ao CREB e à ERK são sensíveis a alterações dos níveis do cálcio intracelular (Ca2+i) cuja homeostasia está desregulada na DA. Os recetores do NMDA (NMDARs) apresentam uma condutância elevada ao Ca2+, sendo a sua atividade essencial para a plasticidade sinática, uma vez que estes recetores se apresentam acoplados à excitabilidade da membrana pós-sinática; contudo, uma hiperestimulação dos NMDARs causa excitotoxicidade.

Neste trabalho foram avaliadas as alterações na atividade do CREB e da ERK em neurónios corticais expostos a oligómeros de Aβ1-42, bem como o envolvimento dos NMDARs e da dependência do Ca2+ extracelular nestas células. Para além disso, analisaram-se os níveis de PGC-1α, um alvo do CREB envolvido na biogénese mitocondrial e na resposta antioxidante. Os resultados mostram que a incubação com oligómeros de Aβ1-42 produz alterações precoces nos níveis de fosforilação do CREB, refletindo a atividade do CREB, por um processo dependente do influxo de Ca2+ através dos NMDARs. Um padrão de ativação análogo foi observado para a ERK, sugerindo que as duas vias poderão estar interligadas em resposta à exposição a Aβ. Surpreendentemente, a ativação da ERK e do CREB nestas condições parece ser modulada por NMDARs contendo a subunidade GluN2A, presente maioritariamente na sinapse. Para além disso, os níveis proteicos de PGC-1α aumentaram para o mesmo tempo de exposição ao Aβ, sendo este efeito modulado também pelos NMDARs, o que sugere que o Aβ controla precocemente os níveis de PGC-1α no sentido de impedir efeitos neurotóxicos induzidos pela exposição aos oligómeros de Aβ. Também foi observado que a exposição prolongada a Aβ induziu um decréscimo dos níveis nucleares do CREB, o que está de acordo com o decréscimo tardio nas funções de sobrevivência características deste fator de transcrição. No conjunto, os dados apresentados neste trabalho sugerem que uma exposição imediata aos oligómeros de Aβ exerce efeitos benéficos nos neurónios maduros do córtex, através da ativação das vias de sinalização ERK-CREB, possivelmente associadas à ativação dos NMDARs sináticos.

Palavras-chave: Aβ, CREB, ERK, PGC-1α, NMDARs

CHAPTER 1

INTRODUCTION

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Introduction

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is the most common neurodegenerative form of dementia that impairs the quality of life of millions of adult individuals worldwide. The most important clinical hallmarks of this illness, detected in *post-mortem* brain, is the presence of extracellular plaques of amyloid beta peptide (Aβ) (Fig. 1.1) and intracellular neurofibrillary tangles (NFTs) due to hyperphosphorylation of microtubule-associated protein (MAP) Tau that is conjugated with ubiquitin at its microtubule-binding domain (Cripps *et al.*, 2005). Neurofibrillary tangles are made of helical highly soluble Tau that normally provides the stabilization of axonal microtubules (Zhang et al., 2005). Also evident in AD patients is the progressive atrophy in cortical and hippocampal areas involved in processes such as learning and memory. In early stages of illness, patients do not present neuronal loss or any particular neurologic disorder; however with the course of the disease they lose the independence and the relationship with the family, friends and the rest of the world, along with an evident impaired visual and spatial skills, judgment, learning and memory (LaFerla and Oddo 2005).

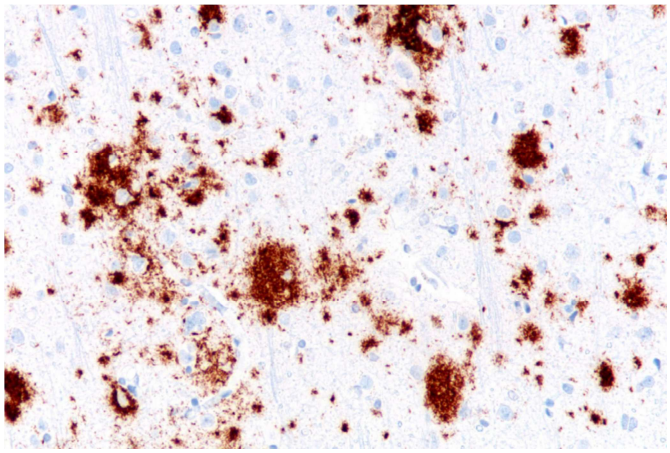

Figure 1.1. Diffuse plaques in the cerebral cortex labeled for Aβ (4G8) by immunohistochemistry (partially modified from Castellani et al., 2010).

The extracellular Aβ plaques, also named neuritic plaques, are small self-aggregating peptides in clumps of spherical shape physiologically produced by brain metabolism. In normal conditions, the extracellular plaques are removed; however, in pathological conditions as in AD, their accumulation occur (Klein et al., 2001). These extracellular deposits are often surrounded by activated microglia and reactive astrocytes, suggesting an involvement of neuroinflammatory components in the pathogenesis of AD (Guo et al., 2002).

The presence of plaques and tangles contribute to the loss of both neurons and synapses, namely in the hippocampus and cortex (Hutton et al., 2001; Lambert and Amouyel, 2011), which has been associated with the manifestation of cognitive symptoms and pathological brain alterations (Carter and Lippa, 2001). Also, both presynaptic vesicle proteins and postsynaptic proteins such as synaptopodin associated to actin microfilaments and post-synaptic density protein 95 (PSD-95) levels are decreased in AD brains (Reddy et al., 2010).

Alterations in number and strength of synapses, measured by following the changes in long term potentiation (LTP) in the brain, have been associated with a decrease in the content of neurotransmitters, such as acetylcholine (ACh) in the basal forebrain, which is related to early cognitive symptoms of AD (Auld et al., 2002). In this respect, acetylcholinesterase inhibitors (AChEI) have been administered in order toprevent the degradation of ACh; however, they are is not effective in all AD patients (Rissner et al., 2004).

In addition to the reduction of cholinergic synapses, increased glutamatergic transmission probably due to deficient glutamate reuptake by astroglial cells near synapses was shown to cause the overactivation of *N*-methyl-D-aspartate receptors (NMDARs). In fact, cholinesterase inhibitors and the NMDAR antagonist memantine help to correct symptomatic neurologic abnormalities associated with AD (Francis 2005).

AD can be classified in early-onset (familial AD) and late-onset (sporadic AD). The early-onset AD are rare hereditary forms (about 5-10% of total cases) of AD that affect people between 30-60 years of age. These familial cases are caused by autosomal dominant mutations in genes encoding for amyloid precursor protein (APP), Presenilin 1 and 2 (PSN1, PSN2) (Bertram and Tanzi, 2012). Patients affected by sporadic forms of AD have more than 65 years old. Apolipoprotein E ε4 (Apo E ε4) polimorphisms seem to be the major risk factors for sporadic AD (Verghese et al., 2011), due to its importance in regulating extracellular and intracellular clearance of Aβ (Castellano et al., 2011).

1.1.1 Clinical stages of Alzheimer's disease

As described before, AD is characterized by cognitive decline. AD patients exhibit deficits in memory and spatial orientation, incapacity to plan skills, poor judgment, changes in mood and personality. They also manifest an altered perception of the world all around, pauperization of speech and difficulty in maintaining a proper gait. The motor functions are progressively impaired and simple actions like the swallowing become very difficult to execute. Patients very often suffer of a lack of independence in their daily life, needing continuous assistance.

The course of all these symptoms can be very variable in different individuals. According to the symptoms showed, AD can be divided in four main stages: mild cognitive impairment (MCI), mild, moderate and severe AD (Braak and Braak, 1997).

MCI is a transitional phase between normal aging and dementia. It refers to a slight mental and mood alterations occurring in daily life; this stage can represent an increased risk to develop an advanced form of AD during the following 10 years (Petersen et al., 2001). Functional impairment is greater than that observed in healthy subjects of the same age (Giovannetti et al., 2008; Masur et al., 1994). Tipical symptoms of MCI are frequent forgetfullness like difficulty in recalling recent events, while childhood/remote memory is inaltered; MCI is also associated to other altered cognitive functions, such as impaired speech, agnosia, lowering of visual-spatial skills, less organizational ability, confusion, changes in mood and increased anxiety.

Moderate AD signs include a worsening of remote memory often linked to increased difficulties to recognize people, even familiars, inarticulate speech progressing to aphasia, agitation and repetitive statements.

In the severe phase of AD the symptoms get worse as the brain undergoes atrophy. The patient lose the ability to recognize family members, to express themselves, to eat, move alone, requiring to be assisted and supervised at all times of the day. Difficulties in swallowing and feeding in this stage are the cause of complications like malnutrition, dehydration or weight loss. Death occurs due to aspiration of food or liquid inside lungs or infectious diseases, especially pneumonia (Selkoe 2001; Kalia, 2003).

1.1.2 Risk Factors

Risk factors for AD are described in two main categories: modifiable and non-modifiable risk factors. Modifiable risk factors refers to the lifestyle, including smoking, alcohol consumption, physical activity, education, social engagement, cognitive stimulation, depression, traumatic brain injury, heavy metals, vascular disease, diabetes mellitus and high fat diet. Non-modifiable risk factors include hereditary gene mutations, age, sex and Down syndrome (Reitz et al., 2011).

Genetic (non-modifiable) risk factors include mutations in APP, presenilin 1 (PSN1), PSN2 and mutations in exon 9 of PSEN1 produces the accumulation of senile plaques morphologically different from those typical in the brain of AD patients (Crook et al., 1998) with no deposited core amyloid fibrils and no inflammatory reactions surrounding the plaque.

ApoE is the principal protein component of very low-density lipoproteins (VLDL) that bind to low density lipoprotein receptors and is involved in carrying cholesterol and other lipids in the bloodstream. The three most common human isoforms of ApoE are ApoE2, ApoE3, and ApoE4, encoded by ApoE alleles ε2, ε3, ε4 (Egert et al., 2012) Northern European population presents a frequency of 62.7% for APOE ε4, 42.1% in the middle regions, and 31.5% in the Mediterranean area (Norberg et al., 2011). The presence of APOE ε4 allele is considered a risk factor for sporadic AD (Corder et al., 1993) and the presence of two alleles ε4 predispose the patient to an earlier onset of

AD than those who have only one copy of this allele or a different isoform (Roses, 1996; Cosentino et al., 2008).

In the last years, new genes associated to increased risk to manifest AD were discovered, which function is often connected to the cholesterol metabolism, intracellular transport of Aβ, and autophagy of damaged organelles, such as clusterin (CLU) (Calero et al., 2005; Bell et al., 2007), phosphatidylinositol-binding clathrin assembly protein (PICALM) (Baig et al., 2010), the exocyst complex component 3-like 2 (EXOC3L2) (Munson and Novick 2006), the bridging integrator 1 (BIN1) (Seshadri et al., 2010) and genes related to complement cascade or cytokine production as the complement component receptor 1 (CR1), suggesting a role of inflammatory response in AD pathogenesis (Olgiati et al., 2011).

1.1.3 Diagnosis

The Mini-Mental State Examination (MMSE) is widely used in clinical practice for the evaluation of cognitive functioning in elderly patients, determining the degree of cognitive impairment and in monitoring the progression of dementia with 30 items that evaluate verbal and performance skills, exploring space-time orientation, short-term memory, attention, mental arithmetic, language as comprehension, repetition, naming, reading and writing, and constructional praxis. Also useful are the imaging diagnosis tests such as computerized tomography (CT), able to detect the thickness of the brain, the single-photon emission computed tomography (SPECT)s that measures the blood flow in the brain, which was shown to be reduced in AD patients, or the magnetic resonance imaging (MRI).

However, the definitive AD diagnosis is only obtained after the *post mortem* brain autopsy exhibiting senile plaques and exclusion of other cases of death with laboratory tests (Johnson et al., 2012).

1.1.4 Biomarkers

Currently, there is the no possibility to predict whether a given individual will develop AD or not. The presence of the ApoE4 allelic gene form in the karyotype gives information only about the statistic risk to manifest AD.

Many studies support the diagnostic relevance of the three proteins measured in cerebrospinal fluid (CSF), Aβ1-42, Tau, and phospho-Tau as biomarkers for AD (Sunderland et al., 2003; Blennow et al., 2012). There is evidence that in AD the levels of Tau and Phospho-Tau increase, while Aβ1-42 levels tend to decrease, since Aβ accumulates in the senile plaques in the brain (Andreasen et al., 2001; Blennow, 2004; Fagan et al., 2009).

Tau levels in CSF reflect the extent of neuronal and axonal damage: high levels of Tau have been shown in patients with cerebral stroke, Creutzfeld-Jakob disease and in patients with AD. The

hyperphosphorylated Tau protein is not only a marker of neuronal damage, but because it reflects the state of phosphorylation of Tau in patients with AD provides informations on the progression of neurofibrillary degeneration.

1.2 Aβ and APP processing

Aβ, the major constituent of amyloid plaques, is a polypeptide of 40-42 aminoacids, which was purified and sequenced in 1984 by George Glenner (Glenner and Wong, 1984). Within plaques, Aβ is organized into fibrils of 7-10 nm, mixed with non-fibrillar forms often associated to fragments of degenerated axons and dendrites surrounded by reactive astrocytes and microglial cells, indicating an inflammatory component in the neurodegenerative process (Lemere et al., 1996; Dodart et al., 2002) that complement the well known amyloidogenic hypothesis. According to the neuroinflammatory component of the amyloid cascade hypothesis (Hardy and Selkoe, 2002), Aβ is indirectly responsible for the phosphorylation of Tau and the consequent neurofibrillary degeneration, through the activation of microglia, which may produce and release neurotoxic substances, such as free radicals, proinflammatory cytokines, inflammatory mediators and protein complement, and is ultimately responsible for neuronal death and dementia. Recent findings showed that Aβ1-42 causes degeneration of microtubules through *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) activation in hippocampal matured neurons (Mota et al., 2012).

Aβ results by the proteolysis of a membrane precursor protein named amyloid precursor protein APP (Kang et al., 1987). APP is a type I transmembrane protein synthesized in the endoplasmic reticulum (ER), which is then transported through the Golgi apparatus to the trans-Golgi network (TGN), and then inserted in plasma membrane (Tan and Evin 2012),.

APP full length of 770 aminoacids (APP770) contains the Kunitz-type protease inhibitor (KPI) domain (Tanzi et al., 1988; Kitaguchi et al., 1988) and the OX-2 antigen domain (OX-2) (Weidemann et al., 1989) that is lost in isoforms like APP695 (both domain) and APP751 (without OX-2 domain). APP has the C-terminal end facing the intracellular side and the N-terminal end facing the extracellular side. The Aβ domain is partly included in the plasma membrane with 28 residues protruding outside and other about 12-14 residues inside the plasma membrane. APP protein has three potential cleavage sites called α, β, and γ, according to the sites of cleavage of each one of the secretases (α-, β-, γ-secretase), see Figure 1.2. In the non-amyloidogenic pathway, α-secretase, a disintegrin and metalloproteinase enzyme from ADAM family, such as ADAM9, ADAM10 and ADAM17 (Buxbaum et al., 1998; Lammich et al.,1999), cleaves APP within the Aβ domain to the N-terminal side releasing the large soluble APP fragment (α-sAPP), precluding in this way the formation of Aβ. Then, the C-terminal fragment (CTF), named also α-CTF or C83 because it acts on 83-amino-acid C-terminal fragment, is further cleaved by the γ-secretase complex which components are presenilins 1 and 2

(PSN1, PSN2), nicastrin (NCT), APH-1 (anterior pharynx-defective 1), and PEN-2 (presenilin enhancer 2)(Edbauer et al., 2003), releasing a brief soluble p3 peptide. The C-terminal part of APP containing the intracellular domain (AICD) is released in the cytosol.

In the amiloidogenic pathway, β-secretase (or β-site APP-cleaving enzyme 1, BACE1), a type I integral membrane protein belonging to the pepsin family of aspartyl proteases, cleaves APP protein at the N-terminal side before Aβ domain, releasing soluble β-sAPP. The C-terminal fragment (CTF, β-CTF, or C99) is cleaved by the γ-secretase complex releasing the Aβ peptide as free peptide, which length is variable. The peptide with 40 aminoacids is the most common form, whereas that containing 42 aminoacids is the most toxic. Also in this case, AICD is released in the cytosol, where it can be degradated or take part in a signal transduction within Fe65/Tip60 complex (Marks and Berg, 2010).

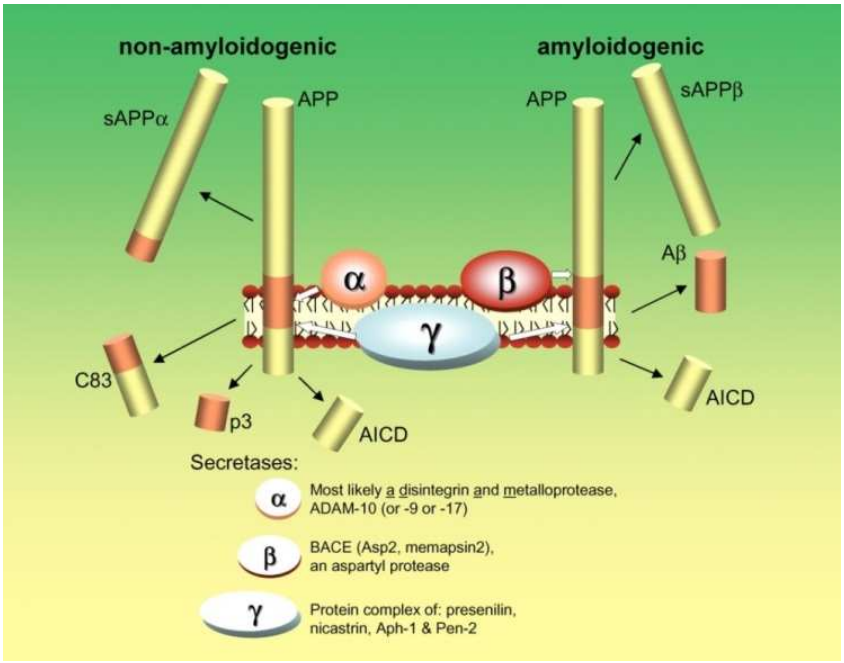

Figure 1.2. Proteolytic processing of amyloid precursor protein (APP) via non-amyloidogenic (left) and amyloidogenic (right) cleavage. Non-amyloidogenic cleavage occurs when α-secretase generate sAPPα and C83, secondary cleaved by γ-secretase form the p3 peptide. Amyloidogenic cleavage by β-secretase generate sAPPβ and the residual peptide is further cleaved producing Aβ. Aβ can be degraded by enzymes including neprilysin, insulin degrading enzyme (IDE) and endothelin cleaving enzyme (not shown in this Figure) (Pearson and Peers, 2006).

The great part of Aβ peptides have a length of 40 residues (Aβ40) and only about 10% is Aβ42. Aβ40 and Aβ42 have a different C-terminal ending (Val40 and Ala42); the presence of the two more hydrophobic amino acids at the C-terminal side of Aβ42 makes this form more hydrophobic and susceptible to form fibrils than Aβ40 (Jarrett et al., 1993). Free Aβ1-40 in preparation rapidally auto-assembles in monomers, dimers, trimers and tetramers, whereas Aβ1-42 peptide preferentially forms pentamers and hexamers that further form oligomers of higher molecular weight, Aβ-derived

diffusible ligands (ADDLs), protofibrils and finally the fibrils and spheroids (Klein et al., 2004; Bitan et al., 2003).

1.3 Amyloid cascade hypothesis

According to the well accepted hypothesis of the amyloid cascade proposed by Hardy in 1992, the tangles formation is a consequence of neurotoxic effect of Aβ. The disrupted balance between the production and degradation of Aβ is the cardinal point that justifies the accumulation of Aβ in the brain and the following toxic effects with synaptic dysfunctions and neuronal death, leading to cognitive and behavioral abnormalities typical of AD (Hardy and Higgins, 1992).

According to the amyloid cascade, in early stages of AD, the imbalance between production and clearance of Aβ cause Aβ to deposit in plaques and synaptic dysfunction, as well as the decrease the LTP in hippocampus of old rats (Gengler et al., 2010). Afterwards, activation of microglia and astrocytes increases the levels of complement factors, cytokines, nitric oxide (NO) and other mediators of inflammation and oxidative stress that lead to ulterior synaptic damage with deficits in neurotransmitters properly firing and onset of first cognitive symptoms. As the impair of synapses progresses, an altered neuronal ionic homeostasis and oxidative injury is shown. At this time, altered activity of kinases and phosphatases lead to tau pathology as tangles appear (Hardy and Higgins, 1992). According with the amyloid hypothesis, Tau pathology, with disassembly of microtubules, loss of transport mechanisms and formation of neurofibrillary tangles in neurons occurs later than the deposit of Aβ. A diffuse synaptic dysfunction and neuronal death, with deficts in neurotransmitters, make the patient seriously affected by dementia (Hardy and Higgins, 1992).

1.3.1 Altered Aβ production and clearance

Evidences of imbalance of Aβ production *versus* clearance come from familial cases of AD (FAD) where mutations in cleavage sites of APP and presenilins are correlated with increased levels of Aβ (Wang et al., 2006;Bates et al., 2009). Patients with Down's syndrome manifest AD earlier in their 4th decade due to a triple chromosome 21, in which the APP gene is located (Rovelet-Lecrux et al., 2006). On the other hand, patients with sporadic or late-onset AD do not present a relevant increase in Aβ production or APP overexpression in the brain, and thus impaired balance mayd probably be due to decreased clearance of Aβ (Wang et al., 2006) and/or to an increase in β- and γ- secretases activities (Yang et al., 2003; Placanica et al., 2009).

The imbalanced clearance can be also due to an altered transport of Aβ from the brain to blood and *vice-versa*. A dysfunction in transcytosis *via* lipoprotein receptor-related protein (LRP) does not allow Aβ to pass the endothelial layer of the blood-brain barrier (BBB), leading to Aβ deposition in the

brain (Kang et al., 2000); on the other hand, the receptor for advanced glycation end products (RAGE) cause increased influx of Aβ in the brain from the bloodstream (Deane et al., 2003).

Aβ increase even further if there is a lack in degradation enzymes such as neprilysin (NEP) and insulin-degrading enzyme (IDE) in the extra- and intracellular space (Iwata et al., 2001; Miller et al., 20031, Farris et al., 2003; Farris et al., 2004), metalloendopeptidases such as endothelin-converting enzyme (ECE) (Eckman et al., 2001; Eckman et al., 2003) and angiotensin-converting enzyme (ACE) (Elkins et al., 2004; Hemming and Selkoe, 2005).

A strategy to improve Aβ clearance is the use of antibodies and substances that bind Aβ and can enter the brain at very low concentrations, preventing Aβ fibril formation (Du et al., 2003).

1.3.2 Oligomeric *versus* fibrillary forms of Aβ

Recent findings suggest that oligomers, but not fibrils of Aβ, are the most responsible of neurotoxic effect of Aβ in AD brain since many evidences like the presence of soluble forms of Aβ in AD brain, in addition to fibrilllary forms, or the loss in synapses is correlated with an increase in soluble oligomers in transgenic animals. Soluble oligomers were shown to modify function and synaptic plasticity, such as as LTP and long-term depression (LTD), and antibodies against oligomers rescue the memory deficit in animal models, without affecting plaque formation. Therefore, the oligomers can be responsible for the impairment of synaptic function that occurs before cellular death, after senile plaque deposits (Gong et al., 2003; Walsh et al., 2002; Wang et al., 2002).

1.3.3 Use of oligomeric form of Aβ in research

In *post-mortem* brain analysis of AD patients, the presence of senile plaques is remarkably evident (LaFerla 2005); however, normal individual brains can also contain senile plaques similar in shape and composition to AD patient's brains, suggesting that eventually other factors are responsible for neurodegeneration in AD (Fukumoto et al.,1996). Moreover, AD-like memory loss and neuronal death appears both in experimental models and in humans, before plaque formation (Price 1999; Aizenstein et al., 2008). The real concentration for neurotoxic effects of Aβ is still unknown and the time of exposure is potentially important. While the disease require years to progress, acute exposures of 16 h in isolated neurons give us phenotypical informations related to its pathological mechanisms, similar to what is observed in transgenic mice overexpressing APP over months (reviewed by Malinow, 2011). Also, attempts to break the plaques lead to neuronal death (Schenk et al., 2012).

Fibrils and soluble oligomeric of Aβ exhibit neurotoxic effects in several mouse cell lines and organotypic slices. Aβ fibrils are mainly toxic in NIH-3T3, SH-SY5Y, HTB186 and M059K cells, while oligomers are toxic in NT-2 cells. Hippocampal formation CA1 is selective vulnerable to soluble oligomeric Aβ, whereas the cerebellum is strongly resistant to soluble oligomers even at highest Aβ concentrations (Kim et al., 2003). Oligomers are shown to be more toxic than fibrils in cortical neurons, since oligomers of Aβ1-42, but not fibrils, promote the release of intracellular Ca^{2+} from the endoplasmic reticulum (ER), contribuiting for intracellular Ca^{2+} dyshomeastasis (Resende et al., 2008). Oligomers can deposit at the level of dendritic spines, triggering the function of membrane receptors such as the NMDAR that leads to alterate communication between neurons. In effect, application of synthetic Aβ decreases cell surface expression of NMDARs, inhibits LTP induction and alters dendritic spine density (Lambert et al., 1998; Snyder et al., 2005; Shrestha et al.,2006).

In hippocampal synapses treated with picomolar concentrations of Aβ oligomers, spine loss is prevented by antibodies binding Aβ or modulators of Aβ aggregation (Shankar et al. 2007), suggesting that low concentrations of Aβ are enough to produce a response in neurons.

Since the preparation of synthetic Aβ could contain an unstable mixture of monomers, dimers, trimers, and higher-order oligomers, it is recommanded a check of the purity of Aβ by Western Blotting, nanoparticle-based detection, conformation-specific antibodies and monoclonal antibody immunoassays. These methods present sometimes differences in sensitivity, specificity, and quantitative reproducibility (Thomas et al., 2013).

1.4 Aβ neurotoxicity

In elderly studies reported that Aβ peptides were neurotoxic *in vitro* as fibrils, but not as monomers (Lorenzo and Yankner, 1994). According to these previous findings, the accumulation of fibrillar Aβ in plaques produce a neuronal damage evoked by two different mechanisms: a direct mechanism and an indirect mechanism. In the direct mechanism, Aβ interacts directly with membrane components, damaging neurons and/or making them more susceptibile to damage such as excitotoxicity, hypoglycemia or oxidative stress (Koh et al., 1990). In the indirect mechanism of Aβ toxicity, there is activation of microglia and astrocytes producing toxic and inflammatory mediators, such as nitric oxid (NO), cytokines and reactive oxygen species (ROS) (Meda et al., 1995; Della Bianca et al., 1999), which cause the death of neurons *via* apoptosis or necrosis.

1.4.1 Extracellular Aβ

Recent studies on solid surface interactions showed that the hydrophobicity, the electrical charge and the surface roughness can influence the fibrillar assembly of amyloid-forming peptides due to a local concentration of Aβ (Linse et al., 2007). Several mechanisms of interactions with membrane were proposed. Aβ can interact electrostatically with phospholipids of the bilayer, exposing the head group charged negatively (Williams et al., 2010). Moreover, the content of cholesterol that regulate the membrane fluidity, permeability and dielectric properties is different in regions of AD and non-demented brains (Mason et al., 1992), suggesting a different Aβ-induced permeation according to the different cholesterol/phospholipid ratio, which cause a change in the distance of the two layers and more accessible sites for enzyme cleavage (Beel et al., 2008; Mason et al., 1993). Futhermore, statins lower the amount of cholesterol and decrease the β and γ-secretase activity, since these two enzymes have cholesterol rich domains (Wolozin, 2004). Using monolayer surface pressure measurements, it was shown that Aβ40 spontaneously inserts into monolayers containing a 30 mol% cholesterol to phospholipid ratio, adopting an α-helical structure (Ji et al., 2002).

Many studies suggest the possibility that Aβ interacts with membrane receptors like glycolipids or glutamate receptors, both ionotropic such as NMDARs or metabotropic receptors, implicated in processes like synaptic plasticity. In fact, Aβ oligomers bind metabotropic mGluR5, triggering an alteration in Ca^{2+} mobilization. In cortical neurons Aβ1-42 alters NMDARs trafficking due to an increase in endocytosis, mediated by the nicotinic α7 acetylcholine receptors (α7nAChRs) (Snyder et al., 2005). Importantly, increased NMDAR endocytosis can be prevented by treatment with γ-secretase inhibitors (Snyder et al., 2005, for review).

Recent findings suggest that the 'solvent' properties of Aβ at the N-terminal region may mediate toxicity by three differents strategies: 1) Aβ could create an asymmetric carpet on the layer of the plasma membrane, removing small molecules; 2) Aβ could induce the formation of pores formed by β-barrels structures, triggering a disruption of Ca^{2+} homeostasis; and 3) at high concentrations, Aβ could produce micelle-like structures that remove lipids from the membrane (Williams and Serpbell 2011). In conclusion, the amphipathic nature of amyloid oligomers has been suggested to contribute to their capacity to penetrate and insert into membranes, coat or lie on the surface of the membranes, or potentially act as cell-penetrating peptides.

An ulterior processing of the N-terminal side of Aβ peptides mediated by amino-peptidases, glutaminyl-cyclases and other modifications may attribute for a neurotoxic property of the amyloidogenic peptides (Iwatsubo et al.,1996; De Strooper 2010) .

1.4.2 Intracellular role of Aβ

Aβ can also enter in the neurons and impair their normal function. The highly Ca^{2+}-permeable α7nAChRs often co-localize with Aβ1-42 within neurons of AD brains. The rate of Aβ internalization is dependent on α7nAChRs content and effectively blocked by α-bungarotoxin, an α7nAChR receptor antagonist, and by phenylarsine oxide, an inhibitor of endocytosis, suggesting that intraneuronal accumulation of Aβ1-42 occurs predominantly in neurons expressing α7nAChRs and is mediated by endocytosis (Nagele et al., 2002).

In previous studies, Aβ oligomers were often found in neurons associated with NFT, suggesting a link between the two pathologies; interestingly, the most frequent form of Aβ found in these conditions was Aβ1-42 (LaFerla et al., 2007).

Despite the finding that Aβ may accumulate intracellularly, La Ferla et al. (2007) also suggested that since APP localizes not only at the plasma membranes, but also to the trans-Golgi network, ER, endosomal, lysosomal and mitochondrial membranes, and that β- and γ-secretases are present in several cellular compartments, intracellular Aβ could be generated intracellulary and not secreted; in alternative, secreted Aβ could be taken back up by the cell to form Aβ intracellular pools. The first evidence that Aβ could be generated intracellularly as well as at the plasma membrane was provided in 1993 when human NT2N cell line differentiated into neurons with retinoic acid was able to produce intracellular Aβ in a constitutive manner (Wertkin et al., 1993.)

Interestingly, a new genetic variant of the sortilin-related receptor 1 (SORL1) gene that is linked to late-onset AD appears to regulate the trafficking of APP from the plasma membrane into retromer (a complex of proteins important in recycling transmembrane receptors from endosomes to the *trans*-Golgi network) recycling endosomes, allowing the recovering of APP holoprotein. APP holoprotein that is not cleaved at the plasma membrane is transferred to early/late endosomes, which are also sites for Aβ generation due to their acidic nature, since BACE1 shows an optimal activity. Mutations in SORL1 increase APP in these endosomes and this corresponds to increased risk for late-onset AD. Blocking the endocytosis of APP by removing its cytoplasmic domains significantly reduced Aβ levels (LaFerla et al., 2007).

Moreover, internalization of extracellular Aβ seems to be mediated by membrane receptors. Recent studies demonstrated that apolipoproteinE (APOE) receptor members of the low-density lipoprotein receptor (LDLR) family can modulate the production and cellular uptake of Aβ, as Aβ internalization was decreased in ApoE KO PDAPP transgenice mice (Zerbinatti et al., 2006).

In addition to nicotinic receptors and LRP, Aβ internalization has been reported to occur through the RAGE, since both co-localize in lysosomes of AD patient's brains (Sasaki et al., 2001). The binding of Aβ to RAGE also produces a cascade of events that result in oxidative stress and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation (Deane et al., 2003).

Aβ neuronal uptake has been also shown to be mediated through NMDARs and this effect was prevented by memantine (Snyder et al.,2005).

Several evidences suggest that intracellular Aβ may contribute to AD pathology by facilitating tau hyperphosphorylation, disrupting proteasome and mitochondrial function, and triggering Ca^{2+} and synaptic dysfunction, and generation of reactive oxygen species (ROS) (LaFerla et al., 2007).

In conclusion, these findings show the importance of Aβ intracellular pool for cognitive impairment in AD.

1.4.3 Synaptic dysfunction

The amyloid hypothesis suggests that Aβ oligomers are the main responsible for synaptic failure both in human and AD animal models. In literature, it is widely demonstrated that Aβ are neurotoxic both in mice overexpressing human mutant APP and in slices from wild-type mice exposed to Aβ. Neurotoxic effects can appear before plaque deposition in specific subdomains of neurons like cholinergic and glutamatergic neurons in cortex and hippocampus. Plaques are probably "reservoirs" of Aβ since the great part of dysmorfic neuronal features like spine loss or synapse loss are concentrated nearby the senile plaques (Sheng et al., 2012). Synapse impairment is characterized by decreased levels of synaptic proteins like presynaptic vesicle proteins and postsynaptic density, previously described in the section 1.1 of the Introduction (Reddy *et al.*, 2010). The disruption of cholinergic synapses may be due to an impairment in the activity of the ACh synthesizing enzyme, choline acetyltransferase (ChAT), and ACh degradating enzyme, acetylcholinesterase (AChE). The vesiclular transport of ACh or cholinergic receptors is also impaired in AD. ACh muscarinic are essentially downregulated by Aβ (Pavía et al., 2000), whereas the different subtypes of nicotinic receptors show a different pattern of upregulation/downregulation when exposed to Aβ (Yakel, 2013). Within nicotinic receptors, the Ca^{2+}-permeable α7nAChR, previously described in this Chapter, seems to have a major importance in the context of AD (Snyder et al., 2005; Nagele et al., 2002).

Aβ oligomers impair the activity and the surface expression of both muscarinic and nicotinic receptors, leading to a failure in synaptic plasticity. Extracellular Aβ cause an increase in glutamate and the NMDAR co-agonist D-serine, leading to the overactivation of glutamate receptors, which is accompanied by a massive influx of Ca^{2+} that has excitotoxic effects in neurons (Paula-Lima et al., 2013). This activates many cytosolic Ca^{2+}-dependent enzymes, leading to an impairment in energy metabolism, ROS production and neuronal death (Paula-Lima et al., 2013). Vesicular glutamate transporters (VGLUTs) and excitatory amino acid transporters (EAATs) are altered in AD patient's prefrontal cortex (Chen et al., 2011), accounting for desregulated extracellular glutamate levels. In cortex and hippocampi, astrocytes mechanisms of reuptake (through the EAATs) (Jacob et al., 2007) and convertion of glutamate into glutamine by glutamine synthase are also compromised, leading to

an accumulation of glutamate at synapses (Robinson 2001). In transgenic mice with human mutant APP and PS1, the overload of Ca^{2+} is higher in the proximity of Aβ plaques, suggesting a role of Ca^{2+} dyshomeostasis in early stages of AD (Kuchibhotla et al., 2008).

A possible neural hyperpolarization through GABA has been proposed as a therapy in AD. GABA is physiologically released as consequence of glutamate excitotoxicity, acting as a compensatory mechanism to glutamatergic overactivation.

Recent studies focused on excitatory post-synaptic receptors as possible receptors for Aβ on central synapses, due to the decrease in PSD-95, present in glutamatergic synapses, associated to (NMDARs and neuroligin (NL) (Dinamarca et al., 2012). Since the NMDARs play a central role in cellular models of learning, as well as neurotoxicity, abnormal function of this receptor caused by Aβ could be a potential mechanism in the pathophysiology of AD.

1.4.4 NMDARs: composition, localization and function

NMDARs are ionotropic glutamate receptors (Fig. 1.3) permeable to cations, specially high permeable to Ca^{2+}, which are mostly present at post-synaptic density of dendritic spines and are involved in physiological and pathological mechanisms such as glutamatergic transmission and synaptic plasticity regulating processes like learning and memory or involved in neuronal death after excitotoxic injury. These different effects were reported to be evoked by a different localization of the receptor, synaptic or extrasynaptic (Hardingam and Bading, 2010).

Structurally, these receptors are heterotetramers, composed by GluN subunits, which expression is different in the developmental stages, and composition may change among synapses. NMDARs assembly require two GluN1 and two GluN2 (GluN2A-D) or GluN3 (GluN3A-B) subunits; a tri-heteromer may be also present, formed by GluN1/GluN2B/GluN3A or GluN1/GluN2B/GluN2D complexes, in early stages of development, and GluN1/GluN2A/GluN2B or GluN1/GluN2A/GluN2C in adulthood (Sanz-Clemente et al., 2013). GluN1 is encoded as splicing variants of one gene, whereas GluN2 and GluN3 are encoded by six genes. A specific cassette to the C-terminal side of GluN1 modulate NMDAR trafficking (Horak et al., 2009).

NMDARs are widely expressed in the brain with a specific distribution and composition in subunits. GluN2A and GluN2B subunits are mostly expressed in cortex and hippocampus, with a preference in expression in mature neurons of GluN2A at synapses and GluN2B at extrasynaptic sites (Sanz-Clemente et al., 2013). The shift between GluN2B-containing NMDARs to GluN2A at synapses in neurons is made possible by experience and activity (Sanz-Clemente et al., 2013).

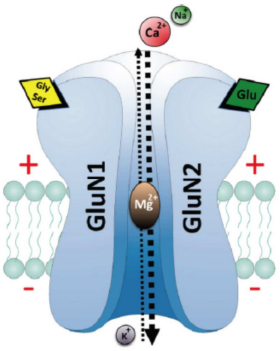

Figure 1.3. NMDA receptor. Coagonists for NMDARs binds exposed sites of the receptor while Mg^{2+} takes place in the inner channel that allows flu of Na^{+} and Ca^{2+} when the block of the Mg^{2+} is removed by depolarization (Sanz-Clemente et al., 2013).

The rapid and massive influx of Ca^{2+} through NMDARs is responsible of a phenomenon called synaptic plasticity, confirmed by strengthening of synapses with LTP at hippocampus. In LTP is associated with an increase in post-synaptic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors and in the number of dendritic spines. On the other hand, a low Ca^{2+} influx produces LTD linked to a decrease in the number of AMPA receptors and decreased number of dendritic spines (Holtmaat and Svoboda, 2009).

NMDARs activation is more complicated than a simple ligand-binding receptor, because at resting membrane potential Mg^{2+} blocks NMDARs channel pore and only a post-synaptic depolarization as a consequence of activation of AMPA receptors, which have a higher affinity for glutamate compared to NMDARs, can induce the release of Mg^{2+}. Glutamate binds to GluN2 subunit, whereas the co-agonists (either glycine or D-serine) bind to the GluN1 subunit. D-serine was recently shown to bind to synaptic NMDARs, whereas glycine preferentially binds to extrasynaptic localization NMDARs (Papouin et al., 2012).

Several kinases and phosphatases can modulate the gating and the activation of NMDARs, acting on serine/threonine or tyrosine residues. NMDARs are connected to anchoring proteins that approach these enzymes to NMDAR subunits, enhancing their efficiency and specificity of the signaling pathways. Intracellular serine/threonine residues of NMDARs are regulated by protein kinase A (PKA), protein kinase C (PKC), casein kinase II (CK2), cyclin-dependent kinase 5 (CdK-5) and Ca^{2+}/CaM dependent protein kinase II (CaMKII). NMDARs subunits are dephosphorylated and inactivated by other serine/threonine protein phosphatase 1 (PP1), 2A (PP2A), and 2B (PP2B or calcineurin). GluN2B is phosphorylated at Ser1303 by PKC and is dephosphorylated by protein PP1, but not PP2A in isolated postsynaptic density. Other enzymers such as members of the Src family of protein tyrosine kinases upregulate NMDAR function in equilibrium with the activity of members of phosphotyrosine phosphatase like the striatal-enriched tyrosine phosphatases (STEP)s. In AD impaired NMDAR phosphorylation was shown to result from the activation of α7nAChRs by Aβ, leading toCa^{2+} influx and activation of calcineurin (or PP2B), which dephosphorylates and activates STEP, and in return

dephosphorylates GluN2B subunit at Tyr1472, promoting the internalization of NMDAR. Synaptic and extrasynaptic NMDARs (GluN2 but not GluN1) are also regulated by cleavage mediated by calpains at the C-terminal side of the receptor (Gladding and Raymond, 2011). In this respect, NMDAR overactivation produces a modulation of the receptor by negative feedback, since calpains are activated through a rise in intracellular Ca^{2+}.

A physiological activation of NMDAR may promote neuronal survival through the activation of PI3K/Akt signaling pathway (Hetman and Kharebava, 2006) that phosphorylates and inactivates both glycogen synthase kinase-3β (GSK-3β) and the pro-apoptotic bcl-2 family member BAD (Brunet et al., 2001).

On the other hand, excessive activation of NMDARs leads to intracellular Ca^{2+} dyshomeostasis, causing acute excitotoxic effects in ischemic stroke through calpain-mediated cleavage of plasma membrane Na^{+}/Ca^{2+} exchanger (NCX), mitochondrial Ca^{2+} uptake through the mitochondrial Ca^{2+} uniporter and overactivation of neuronal NOS (nNOS), leading to mitochondrial dysfunction and TRPM (transient receptor potential ion channels), also permeable to Mg^{2+} and Ca^{2+}. This two-faced neuroprotective and neurotoxic role of NMDARs can be regulated by different amounts of Ca^{2+} influx or by the different localization of the receptor, which seems to activate distinct intracellular signaling pathways (Hardingham, 2006).

Stimulation of extrasynaptic NMDARs containing GluN2B subunits seems to have neurotoxic effects, whereas synaptic NMDARs preferentially composed by GluN2A subunits are neuroprotective through activation of cAMP/PKA/CREB pathway (Vitolo et al., 2002), namely by the phosphorylation of CREB at Ser133 by PKA (Snyder et al., 2005). In effect, in hippocampus, decreased activity of PKA induced by Aβ1-42 was reverted by rolipram and forskolin, which increase the intracellular levels of cAMP, resulting in recovery of its activity (Vitolo et al., 2002).

Moreover, stimulation of NMDAR containing GluN2B subunits was involved in reducing dendritic spines after intracellular Ca^{2+} rise (Shankar et al., 2007) andimpairing ERK pathway activation, leading to the downregulation of CREB and synaptic dysfunction, which was reverted by NMDAR antagonists (Li et al., 2001). Conversely, synaptic activation of NMDARs promote the activation of Ras-ERK1/2, having pro-survival effects such as the activation of CREB, BAD inactivation and antagonizing apoptosis induced by GSK-3β. (Hardingham, 2006).

NMDARs are also neuroprotective in the hippocampus by recruiting α-secretase (ADAM10), involved in the non-amyloidogenic pathway, in association with synapse associated protein 97 (SAP97) (Marcello et al. 2007), thus reducing Aβ production and its release in cortical neurons (Hoey et al., 2009).

1.4.5 Link between NMDARs and Aβ

As reviewed by Malinow (2011), NMDARs can be potential receptors for Aβ since they can directly or indirectly mediate the effects of Aβ on neurons. As shown in Figure 1.4 NMDARs may: 1) directly bind Aβ or through an X molecule; 2) mediate the actions of Aβ on signaling pathways linked to synaptic transmission and plasticity; 3) influence Aβ formation; and 4) have their activity controlled by Aβ.

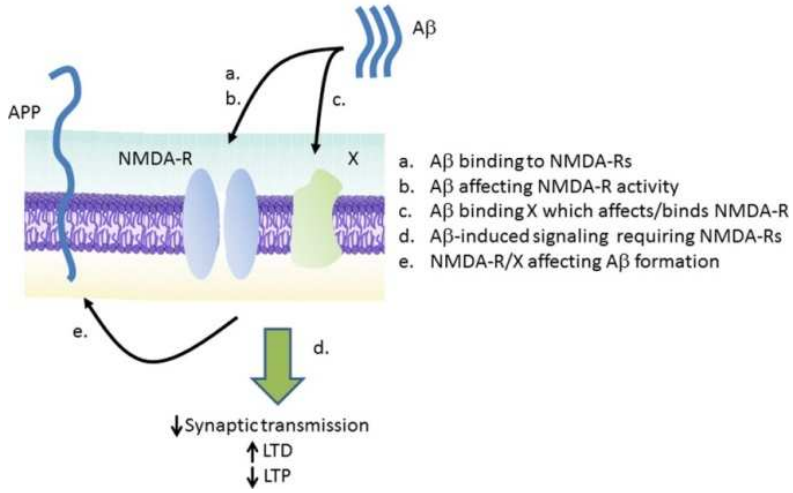

Figure 1.4. Several potential roles for NMDARs in the effects of Aβ (Malinow 2011).

1.5 Ca^{2+} dyshomeostasis in AD

Many studies focused on the role of altered Ca^{2+} homeostasis in AD, since Ca^{2+} is a central regulator of many signaling pathways, contributing to cellular functions such as membrane excitability, neurotransmitter release, synaptic plasticity, gene expression, free radical species formation and neuronal death. Ca^{2+} levels increase both in human and experimental models during the early phases of the illness, before appearance of symptoms. AD transgenic mice show alterations in Ca^{2+} homeostasis before extracellular Aβ deposition (LaFerla, 2002).

Cytosolic Ca^{2+} levels are normally about 100 nM; however after electrical, synaptic or receptor mediated mechanisms intracellular Ca^{2+} levels can increase to concentrations that lead to toxic effects related with the activation of calcium-dependent enzymes and alteration of intracellular pattern of kinase/phosphatases, among other pathological events. Indeed, under physiological conditions, Ca^{2+} is tighty regulated by many mechanisms, as shown in Figure 1.5. These include Ca^{2+} binding by intracellular calcium buffering proteins like calbindin, the extrusion of cytosolic calcium across the plasma membrane through Ca^{2+}-ATPase and NCX or by sequestration into intracellular

stores such as the ER, through the sarco-/endoplasmic reticulum calcium ATPase (SERCA) pump, or the mitochondria, through the mitochondrial Ca^{2+} uniporter.

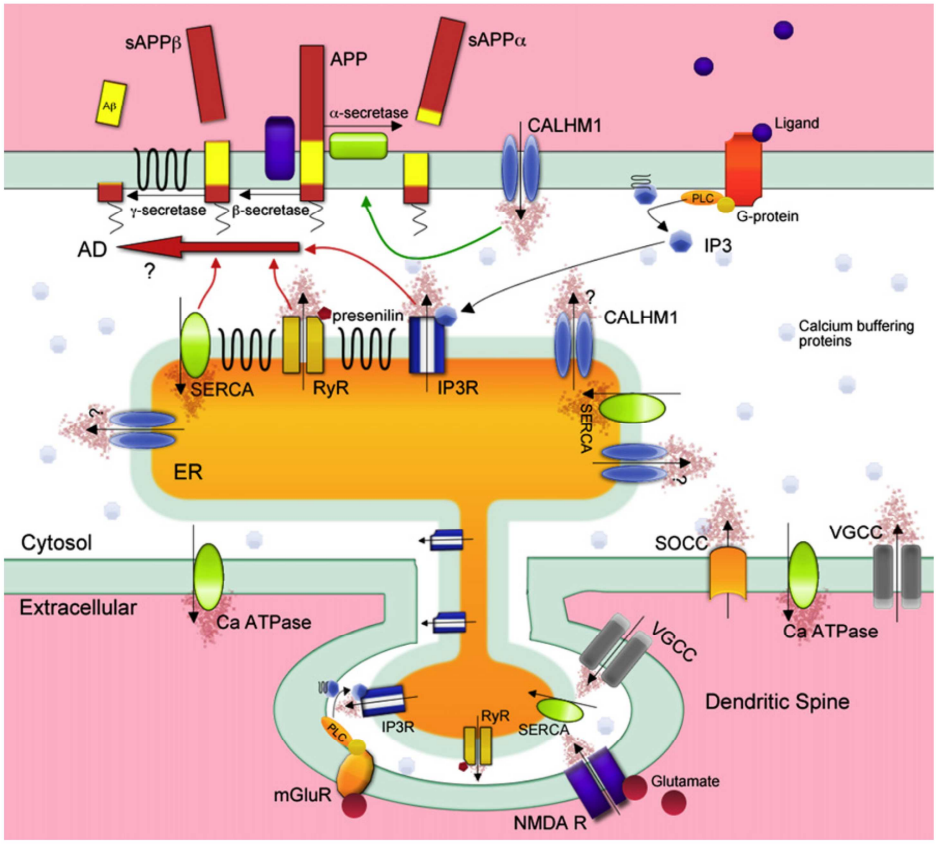

Figure 1.5. Ca^{2+} signaling pathway in a neuronal model (Green and LaFerla 2008).

In pathological conditions, like AD, several pathways can be perturbed by dyshomeostasis of Ca^{2+}. Aβ can trigger an intracellular Ca^{2+} overload. Aβ oligomers can form pores in plasma membrane allowing a passive flux of Ca^{2+}, making the neurons more vulnerable to excitotoxic damage, includingapoptosis (Bezprozvanny and Mattson, 2008). In combination with Fe^{2+} or Cu^{+}, Aβ oligomers produce ROS (through the Fenton reaction), leading to lipid peroxidation; the formation of resulting aldehydes may impair the function of of plasma-membrane Ca^{2+}-ATPase (PMCA). The consequent plasma membrane depolarization opens channels such as NMDARs and VGCCs, producing an entry of Ca^{2+} in the cytosol (Berridge, 2010). Aβ also acts on mitochondria causing increased ROS production, decreased ATP production and depolarization of the mitochondrial membrane (e.g. Eckert et al., 2011). Moreover, in AD the mitochondrial NCX (NCX_{mito}) is impaired(Castaldo et al., 2009). In addition, mutated presenilins are reported to cause an early accumulation of Ca^{2+} through SERCA in ER, followed by extrusion through RyR and InsP3R channels (Bezprozvanny and Mattson 2008).

1.6 Transcription factors

Since several stimuli such as ligands for GPCRs, neuronal growth factors, stress and excitatory neurotransmitters or eventually Aβ, through stimulation of NMDARs (Ferreira et al., 2012), produce influx of Ca^{2+} (Fig. 1.6), in this work the investigation focused on CREB and ERK pathway. CREB is activated at Ser133 by phosphorylation from different kinases such as PKA, CaMKIV, MAPKAP K2, ERK, RSK and MSK leading to an activation/inactivation of this transcription factor, which is known to regulate the downstream transcription of targets gene, such as PGC1α, involved in mitochondrial biogenesis.

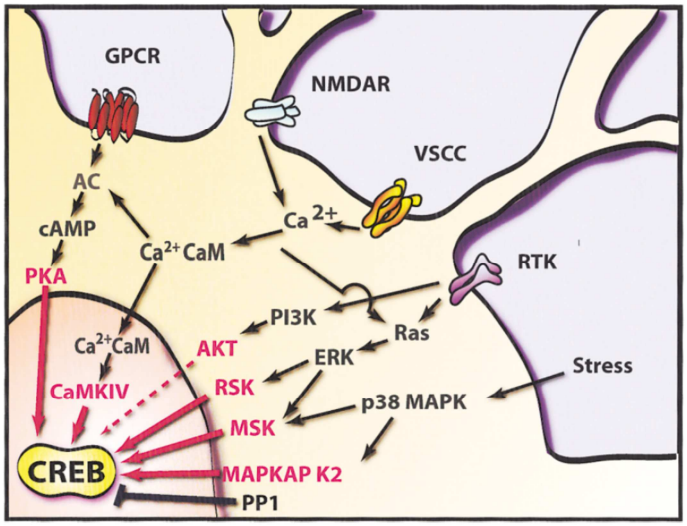

Figure 1.6. Signaling pathways that converge on CREB in a complex Ca^{2+}-mediated cross-talk (Lonze and Ginty 2002).

1.6.1 P(Ser133)CREB - CREB

The cAMP response element-binding protein (CREB) is a 43 kDa nuclear widespread transcription factor discovered in 1987 as regulator of somatostatin gene expression (Montminy and Bilezikjian, 1987).

CREB seems to be one of the main transcription factors that specifically modulates the downstream expression of genes related to memory formation and synaptic plasticity (Alberini, 2009), involved in the conversion of short-term to long-term memory in *Drosophila*, *Aplysia* and mice (Yin and Tully, 1996) or the regulation of GluN1 and GluN2B subunits of NMDARs (Lee and Silva, 2009). Increased CREB activity increases both NMDAR-mediated synaptic currents and surface level of NMDARs, while inhibition of NMDARs abolishes the effect of CREB on upstate duration (Huang et al., 2008).

CREB belongs to the dimerizing bZIP transcription factor superfamily with a C-terminal side binding the DNA on 5'-TGACGTCA-3' consensus sequences upstream CRE (cAMP responsive element). The N-terminal side contains two glutamine rich regions (Q1 and Q2), a region with serine residues named KID (kinase inducible domain) susceptible to be phosphorylated and leucine-zipper domain for homo- or heterodimerization with other members of ATF family, such as ATF1 and CREM. Heterodimerization decreases its stability and CRE binding affinity (Shaywitz and Greenberg, 1999).

CREB is located in cytosol as an inactive form. Stimuli that increase the levels of second messengers such as Ca^{2+} and cAMP, which mediate the specific pathway activation of kinases such as CaMKII/IV and PKA, respectively, or the MAPK/ERK pathway and the MAPK-activated kinase RSK. Phosphorylation of CREB on Ser133 allows the recruitment of co-activators like the p300/CBP (CREB-binding protein or CREBBP) *via* its KIX domain. CBP and p300 are highly conserved co-activators homologues of CREB that further recruit transcriptional factors such as p53, ATFs, c-jun, c-myc. HAT (histone acetyl transferase) activity of CBP further modifies chromatin through histone acetylation, making the site accessible to RNA polymerase II transcription (Goodman and Smolik, 2000).

Efficiency of gene expression is dependent on the time of activation of CREB. Inactivation of phosphatases such as calcineurin or the nuclear phosphatase PP1 increases the levels of phosphorylated CREB (Bito et al.,1996).

Phosphorylated CREB can increase after influx of Ca^{2+}, which stimulates CaM translocation to the nucleus and the sequential activation of CaMKII and CaMKIV that act on CREB (Saura and Valero 2011). In AD patients a dyshomeostasis of Ca^{2+} can interfer with the balance between kinases and phosphatases that control the activation of CREB (LaFerla, 2002). Aβ may thus interfer with CREB-regulated pathways, including NMDARs, L-type VGCCs, PKA and calcineurin (Saura and Valero, 2011).

CREB is well expressed in cortex and hippocampus, two regions associated with learning and memory (Lonze and Ginty, 2002). In AD patients a decrease in CREB phosphorylation and gene transcription

(e.g. BDNF) was previously demonstrated (Yamamoto-Sasaki et al., 1999; Pugazhenthi et al., 2011; España et al., 2010; Tapia-Arancibia et al., 2008).

1.6.2 P(Thr202/Tyr204)ERK - ERK

ERK1 and 2, of 44 and 42 kDa, respectively, also known as MAPKs (Mitogen-Activated Protein Kinases) are serine/threonine-specific protein kinases involved in physiological processes such as proliferation, differentiation, survival, apoptosis and stress response ERK was initially considered important in synaptic plasticity and memory (e.g. Sweatt, 2004), but in last years it has emerged as having a role in pro-death processes in neurons (Subramaniam et al., 2010). ERK1 ⁄ 2 may be transiently induced by growth factors, resulting in promotion of neuronal survival whereas oxidative stress may result in a sustained induction of ERK1/2, which may promote neuronal death (Subramaniam et al., 2010). Oxidants can activate ERK1/2 either through acting on receptors, Ca^{2+} channels, or directly on Src-tyrosine kinase. When ERK is activated can interact with cytoplasmatic target or if the stimulus is sustained, ERK migrated to the nucleus activating pro-death transcription of genes that evoke neuronal death, independently by caspase activity (Subramaniam et al., 2010). ERK dysregulation is associated with many neurodegenerative disorders, such as AD and Parkinson's disease (Colucci-D'Amato et al., 2003).

The MAPK/ERK signaling cascade is activated by a wide range of receptors involved in growth and differentiation, including GPCRs (G-Protein Coupled Receptors), RTKs (Receptor Tyrosine Kinases), integrins, ionic channels, CaMII, Src, or EGF-R (epidermal growth factor receptor). Several components make part of this cascade, including adaptors like Grb2, SHC, Crk and exchange factors like SOS that induce the activation of Ras protein. Ras-GTP activated transduces the signal to little kinases named Rafs (Raf-1, A-Raf, B-Raf) (MAPKKK). Rafs activate the dual specific kinase MEK1/2 (MAPKK), which in return activates ERK1/2 by phosphorylation on Thr202 and Tyr204 residues in humans.

Activated ERK dimerizes and may regulate hundreds of targets in the cytosol or translocate to the nucleus where it phosphorylates a huge variety of transcription factors, thus regulating gene expression.

1.6.3 PGC-1α

PGC-1α is a 90 kDA protein belonging to the PGC-1 family in conjunction with PGC-1β and PGC-related coactivator (PRC) which are strong promoters of mitochondrial biogenesis and antioxidant regulation (Andersson and Scarpulla, 2001; Lin et al., 2002). With the exception of PRC that is ubiquitously expressed, PGC-1α and PGC-1β are well expressed in oxidative tissues such as the brain,

heart, kidney, muscle, liver, brown adipose tissue (BAT) and pancreas with specific functions (Uldry et al.2006).

PCG-1α has a length of 798 aminoacids and from the N-terminal contains three LxxLL nuclear receptor binding motifs, three p38 MAPK phosphorylation sites, an host cell factor binding site (HCB) where binds MEF2C, a DEAD box, two RS protein interaction domains, a high conserved nuclear localisation signal (NLS) and a RNA recognition motif (RRM). The N-terminal contains residues for the binding of TRAP220 and splicing factor U1-70K (Soyal 2006).

PGC-1α activity is regulated by phosphorylation by AMPK in Thr178 and Ser539, promoting PGC-1α co-transcriptional activity (Jager et al., 2007), whereas Akt phosphorylation at Ser571 downregulate this activity (Li et al., 2007). This latter effect is achieved by an initial Ser571 phosphorylation, followed by acetylation by general control non-repressed protein 5 (GCN5, an ubiquitous histone acetyltransferase) that promotes PGC-1α dissociation from target gene promoters. PGC-1α can be deacetylated by SIRT1 in low nutrients/high NAD^+ conditions, leading to the activation of PGC-1α.

Activated PGC-1α migrates to the nucleus and binds co-activators or co-repressors to the N-terminal side undergoing conformational changes that allow the interaction with transcription factors such as CBP/p300 and steroid receptor coactivator (SRC-1) having HAT activity (Puigserver et al. 1999).

PGC-1α levels are also controlled by CREB, in conjunction with Ca^{2+} and cAMP-sensitive co-activators (TORC), since cytosolic TORC1 and TORC2 move to the nucleus as a consequence of an increase in Ca^{2+} and cAMP levels (Herzig et al., 2001; Wu et al., 2006), and by the forkhead in rhabdomyosarcoma (FKHR or FoxO1)

Impaired in PGC-1α transcription occurs when the CREB binding sites are mutated, suggesting a central role of CREB in regulating PGC-1α promoter activity (St Pierre et al., 2006).

PGC-1α increases the expression and acts as a co-activator for nuclear respiratory factors NRFs (1 and 2) which activation coordinates the expression of genes encoding mitochondrial proteins (Scarpulla et al., 2002) or transcription factor A mitochondrial (TFAM), involved in transcription and replication of mtDNA (Wu et al., 1999), among other relevant mitochondrial proteins and antioxidants. PGC-1α increase the expression of ROS-detoxifying enzymes such as GPx1 and SOD2. PGC-1α protein levels are negatively associated with both AD-type neuritic plaques and Aβ content in human AD brains and in the Tg2576 model mouse (Qin et al., 2009; Sheng et al., 2012). In human hippocampi and in M17 cells overexpressing FAD-causing APP mutant (APPswe) a lower number of mitochondria were associated to reduced expression of PGC-1α, NRF 1, NRF 2, and TFAM. APPswe M17 cells showed a reduction in mitochondrial/nuclear DNA ratio, correlated with reduced ATP content and decreased cytochrome C oxidase activity. In PGC-1α KO animals the mitochondrial biogenenesis was strongly impaired, whereas this effect was rescued after an overexpression of this transcription factor (Sheng et al., 2012).

PGC-1α transcription levels increase two weeks postnatally in regions like cortex, hippocampus and cerebellum in GABAergic areas undergoing high remodelling and mitochondrial changes (Cowell et al., 2007). Interestingly, PGC-1α responds to changes in neuronal activity, since KCl depolarizing stimulus increased PGC-1α levels in the nucleus and cytoplasm at 0.5h of treatment and this level was sustained up to 3h (Meng et al., 2007).

In addition, PGC-1α is a regulator of adaptive thermogenesis in brown adipose tissue (BAT), mediating the expression of mitochondrial uncoupling protein 1 (UCP1) (Puigserver et al., 1998) and increasing the expression of transcription factors, such as NRF-1, NRF-2, PPARα and PPARγ that result in the increase in expression of genes involved in fatty acid oxidation and in mitochondrial respiratory chain (Vega et al., 2000; Wu et al., 1999).

OBJECTIVES

Previous studies have shown that extracellular Aβ oligomers might be responsible for synaptotoxic effects on synapses (Klein, 2013). Moreover, our group showed that NMDARs can be directly activated by Aβ1-42 oligomers, allowing intracellular Ca^{2+} influx through the receptor (Ferreira et al., 2012; Costa et al., 2012). Additionally, some transcription factors, namely CREB and PGC-1α, were shown to be altered in AD (Saura and Valero, 2011; Qin et al., 2009; Sheng et al., 2012). Thus, in the present study we focused on intracellular pathways functionally linked to processes like learning and memory, such as CREB and ERK, and the role played by NMDARs in mature cortical neurons (maintained *in vitro* for 15 days) following exposure to Aβ1-42 oligomers (0.5 μM). Furthermore, we verified another transcription factor that is linked to CREB, PGC-1α, which is known to regulate mitochondrial biogenesis and antioxidant response.

Our main investigation was directed to:

1) Determine if CREB and ERK activities were influenced by extracellular Aβ oligomeric stimulus;
2) Check if these changes were regulated by the presence of extracellular Ca^{2+};
3) Verify the role played by NMDARs composed by GluN2A or GluN2B subunits, by using selective antagonists, on Aβ-induced changes in CREB and ERK pathways;
4) Evaluate the changes in the transcription factors, CREB and PGC-1α, following exposure to Aβ oligomers.

CHAPTER 2

MATERIAL AND METHODS

2.1 Materials

Neurobasal medium and B27 supplement were purchased from GIBCO (Paisley, UK); BSA, trypsin, trypsin inhibitor, ifenprodil, FCCP, oligomycin, ionomycin is from Sigma Chemical Co. (St. Louis, MO, USA). (+)-5-Methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate (MK-801) was obtained from Calbiochem (Darmstadt, Germany); memantine was a kindly gift from Lundbeck Portugal; [(R)-[(S)-1-(4-bromophenyl)-ethylamino]-(2,3-dioxo-1,2,3,4 tetrahydroquinoxalin-5-yl)-me thyl]-phosphonic acid (NVP-AAM077) was a generous gift from Novartis Pharma AG, (Basel, Switzerland). Synthetic amyloid-beta 1-42 peptide was purchased from American Peptide (Sunnyvale, California, USA). PVDF membrane was from BioRad Laboratories, Inc.. Fura-2AM was purchased from Molecular Probes and antibodies origin are described in table 2.1 and 2.2.. All other reagents were from analytical grade.

2.2 Primary neuronal cultures

Primary cultured cells were obtained from Wistar fetal rats at embryonic 16 day as described previously (Agostinho and Oliveira, 2003) with minor modifications by Ferreira et al., 2012. The pregnant female was anesthetized with 2-bromo-2-chloro-1,1,1-trifluoroethane and then sacrificed by cervical dislocation. Embryos were separated from placenta and washed in Ca^{2+} and Mg2+-free Krebs medium (120 mM NaCl, 4.83 mM KCl, 1.22 mM KH2PO4, 25.5 mM NaHCO3, 13 mM glucose, pH 7.2). Frontal cortices, free from meninges, were stored in 0.3% (w/v) BSA-containing-Krebs medium and then treated with 0.035% trypsin in BSA-containing Krebs medium to perform the enzymatic digestion for 5 min at 37 °C followed by addition of 0.075% trypsin inhibitor to block the enzymatic reaction. Cells were then mechanically digested in Krebs medium and centrifuged at 180 x g for 5 min. The final pellet was resuspended in Neurobasal Medium supplemented with 2% B27 supplement, 0.5 mM glutamine and 50 µg/ml gentamicin. Cell counts and viability were performed by trypan blue dye exclusion test by using a hemocytometer (see appendix for the details). Cortical cells were then plated at a density of 0.16 × 106 cells/cm2 in both poly-D-lysine coated multiwells (MW6) plates for Western Blotting analysis or MW96 for fluorimetric analysis.

Primary cortical cells were cultured for 15 days in a humidified incubator chamber with 95% air and 5% CO2 at 37 °C. Half medium was changed with fresh medium at day 8 and 12 in culture.

All experiments using animals were carried out following the Guide for laboratory animal practice of the Center for Neuroscience and Cell Biology, University of Coimbra, and experiments planned in order to minimize the number of animals used and their suffering.

2.3 Cell viability

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells have intact cell membranes that exclude certain dyes, such as trypan blue (Strober, 2001). An aliquot of cell suspension was diluted 1:2 in 0.1 % Trypan blue and then counted in an hemocytometer (see appendix for details about hemocytometer use).

2.4. Preparation of amyloid-peptide

The preparation of Aβ1–42 peptides required the resuspention of Aβ1–42 peptides powder in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to a final concentration of 1 mM. HFIP was removed by liophilization in a Speed Vac (Ilshin Lab. Co. Ltd., Ede, The Netherlands) and dried HFIP film was stored at −20 °C. The peptide film was resuspended in anhydrous dimethyl sulfoxide (DMSO) to make a final solution of 5 mM. Peptides were supplemented with a phenol red-free Ham's F-12 medium without glutamine to a final concentration of 100 µM and incubated overnight at 4 °C. The solution was centrifuged at 15.000 x g for 10 min at 4 ◦C to separate the pellet with insoluble aggregates from the supernatant containing soluble oligomers and monomers. Supernatant was transferred to clean Costar tubes and stored at 4 °C. Protein concentrations of Aβ1–42 peptides were determined using the BioRad protein dye assay reagent. Samples containing 10 µg of protein were diluted 1:2 with sample buffer containing 40% glycerol, 2% SDS, 0.2 M Tris-HCl, pH 6.8 and 0.005% Comassie G-250). The presence of different assembly peptide forms (monomers, oligomers and/or fibrils) in the preparation was evaluated by 4-16% Tris-Tricine SDS-PAGE gel electrophoresis and further stained with Coomassie blue.

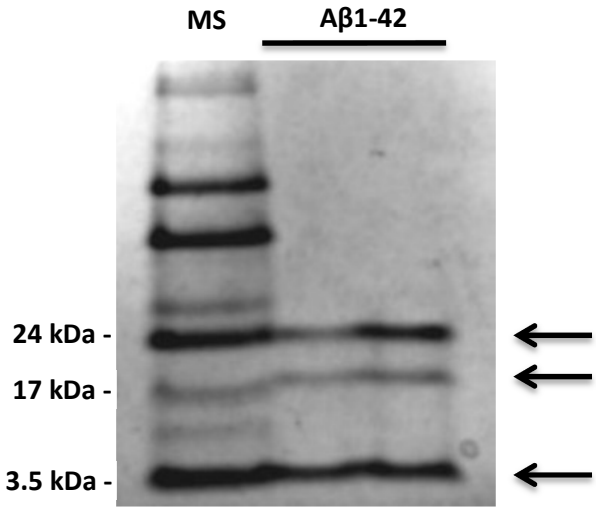

Figure 2.1. Representative gel of two independent Aβ samples prepared from synthetic Aβ 1–42 as described in Materials and methods (MS, molecular weight standard).

2.5. Incubation of cortical cells with Aβ

Cortical neurons cultured for 15 days were treated with Aβ (0.5 µM) at 37°C for 5 min, 30 min, 2h or 24h) in the contioned culture medium (medium in which cells were grown). Alternatively, cells were incubated with Aβ in a Na^+ medium (containing: 140mM NaCl, 5 mM KCl, 1mM CaCl2, 1 mM MgCl2, 10 mM Glucose, 10 mM HEPES, pH 7.4 for 5 min. When the effect of the NMDARs antagonists (MK-801, Ifenprodil, mamantine, and NVP-AAM077) was tested, a preincubation of 5 min was performed.

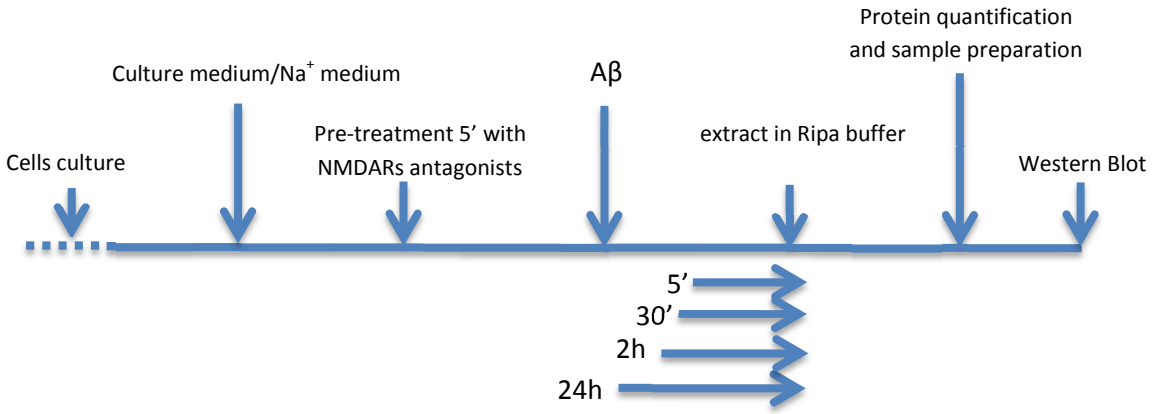

Figure 2.2. Schematic representation of the treatment protocol of cells with Aβ with or without NMDARs antagonists.

2.6 Preparation of total extracts

Cells subjected to the desired stimulation, as shown in the figure legends, were washed twice in ice cold PBS (containing: 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM $Na_2HPO_4.2H_2O$, pH 7.4) and then scrapped with Ripa buffer (containing: 150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS) freshly supplemented with 1 mM DTT, 1 mM PMSF, 100 nM okacaid acid, 25 mM NaF, 1mM Na_3VO_4 and 1 µg/mL protease inhibitors (chymostatin, pepstatin A, leupeptin and antipain). Then the cell extracts were further collected in chilled clean tubes and centrifuged at 20,800 x *g* for 10 minutes at 4°C.

The supernatant (total extract) of proteins were then submitted to protein quantification by BioRad method and stored at -80°C until use.

2.7 Preparation of nuclear extracts

Nuclear extracts were performed by using BioVision Nuclear/Cytosolic Fractionation Kit (BioVision, CA, USA), following the manufacturer instructions with minor modifications, freshly supplemented with protease inhibitors and DTT provided by the kit. After two washes in ice-cold PBS, cells were extracted with 100 µl of Cytosol Extraction Buffer A Mix (CEB-A Mix), vortexed at highest settings for 15 seconds and further incubated on ice for 10 minutes. Briefly, 5,5 µl of Cytosol Extraction Buffer B (CEB-B) was added to each sample, vortexed 5 seconds at highest settings and incubated on ice for 1 minute. Following steps included 5 seconds vortexing followed by 5 minutes centrifugation at maximal speed 20,800 x *g* at 4°C in order to separate the pellet (nuclear extract) form the supernatant (cytosolic extract). Each tube was resuspended in 35 µl of Nuclear Extraction Buffer Mix (NEB-Mix), vortexed at highest settings 15 seconds and stored on ice for 10 minutes. This step was replicated 5 times followed by centrifugation ad 20,800 x *g* for 10 minutes 4°C. The supernatant was stored in clean pre-chilled tubes, immediately submitted for protein quantification by BioRad method and stored at -80°C until use.

2.8 Western Blot analysis

Total and nuclear extracts obtained as described above were used for Western Blot analysis. After protein concentration quantification by BioRad method (see appendix for details), samples were denaturated with sample buffer containing: Tris-HCl 300mM pH 6,8; SDS 12%, glycerol 30%, bromofenol blue 0,06%, DTT 600mM at 95 ◦C, for 5 min.

Equal amounts of the desired protein (20-30 µg for CREB, P(SER133)-CREB, ERK, P(Thr202/Tyr204)-ERK and PGC-1α or 100 µg for GluN2A, GluN2B, P(Ser1232)-GluN2A and P(Tyr1472)-GluN2B) were applied in a 10% or 8%, respectively, SDS-PAGE gel electrophoresis and further transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% fat-free milk or 5% BSA when phosphorylated proteins were evaluated in order to prevent following unspecific binding of antibody. Then, membranes were incubated with the respective primary antibody (Table 2.1) with gentle agitation, overnight at 4°C. After a washing step (three times for 10 minutes) in 0,1% TBS-T (containing: 25 mM Tris, 150 mM NaCl and 0,1% Tween-20), to remove antibody solution, membranes were incubated with an alkaline phosphatase-conjugated secondary antibody (anti-rabbit, anti-mouse or anti-goat, Table 2.2) with gentle agitation for 2 hours, at room temperature and then washed three times for 10 minutes in 0,1% TBS-T. To control for loading of the gels, anti-tubulin, laminB1 and anti-actin antibodies were used. Immunoreactive bands were detected after incubation of membranes with ECF reagent and visualized in a BioRad Versa Doc 3000 Imaging System. Densitometric analysis was performed by using Quantity One software (BioRad)

Table 2.1. Primary antibodies used for Western Blotting.

Primary Antibody	Diluition	Host	Manufacturer
CREB (#9192)	1:1000	Rabbit	Cell Signaling
P(SER133)-CREB (#9196)	1:500	Mouse	Cell signaling
p44/42 MAPK (#9102)	1:1000	Rabbit	Cell Signaling
P-p44/42 MAPK (#4377)	1:1000 or 1:500	Rabbit	Cell Signaling
PGC-1alpha K-15 (sc5816)	1:500	Goat	Santa Cruz
GluN2A (#07-632)	1:1000	rabbit	Millipore
GluN2B (MAB 5778)	1:500	mouse	Millipore
P-GluN2A (2056)	1:500	rabbit	Tocris
P-GluN2B (XPS-1019)	1:1000	rabbit	ProSci incorporated
Actin (5316)	1:20000	Mouse	Sigma Aldrich
LaminB1 (ab16048)	1:1000	rabbit	Abcam
α-Tubulin (T-6199)	1:20000	Mouse	Sigma

Table 2.2. Secondary antibodies used for Western Blotting.

Secondary antibody	Diluition	Manufacturer
Anti-Rabbit (Alkaline Phosphatase)	1:20000	GE Healtcare, UK
Anti-Mouse (Alkaline Phosphatase)	1:20000	GE Healtcare, UK
Anti-Goat IgG-AP (sc2022)	1:3000	Santa Cruz Biotecnology

2.9 Intracellular free Ca^{2+} determination

Cells were washed in Na^+ medium containing 140mM NaCl, 5 mM KCl, 1mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Glucose, 10 mM HEPES, pH 7.4 and then incubated with 10 µM FURA-2AM in Na^+ medium for 30 minutes at 37°C. After a washing step in Mg^{2+}-free Na^+ medium, intracellular Ca^{2+} determinations were performed in cells exposed to Aβ in the absence or in the presence of NMDARs antagonists in glycine (20 µM)-containing Mg^{2+}-free Na^+ medium in order to maximize the effect at the NMDAR. Intracellular levels of Ca^{2+} were measured in a microplate reader spectrofluorometer Gemini EM (Molecular Devices, USA) with excitation wavelengths of 340 and 380 nm and 510 nm emission. The basal recording was obtained each 30 seconds for 1 min 30 seconds, followed by the addition of Aβ;

fluorescence levels were measured each 15 seconds for 3 minutes. Fluorescence values (ratio 340/380) were normalized to the baseline. See appendix for details about this method.

2.10 Data and statistical analysis

Data were analyzed by using Excel (Microsoft, Seattle, WA, USA) and GraphPad Prism (GraphPad Software, San Diego, CA, USA) softwares. Data were expressed as the mean ± S.E.M. of the number of experiments indicated in legends. Comparisons among multiple groups were performed by one-way ANOVA, followed by Dunnett's Multiple Comparison post hoc test. Student's *t*-test was also performed for comparison between two Gaussian populations. Significance was accepted at $p < 0.05$. Data from fluorimeter were normalized to the baseline and plotted in GraphPad Prism (GraphPad Software, San Diego, CA, USA).

CHAPTER 3

RESULTS

3.1 Characterization of subcellular nuclear fractions

Because part of the work performed in the present thesis used nuclear fractions obtained from primary cortical neurons, we initiated by evaluating the purity of nuclear extracts by western blotting, after labeling with antibodies against Lamin B1, a marker of nuclear protein extracts, Hsp60, a mitochondrial marker, and α-tubulin, a cytosolic marker, as shown in Figure 3.1. Our results demonstrate that the extracts are enriched in nuclear fraction, as depicted by increased labeling of Lamin B1.

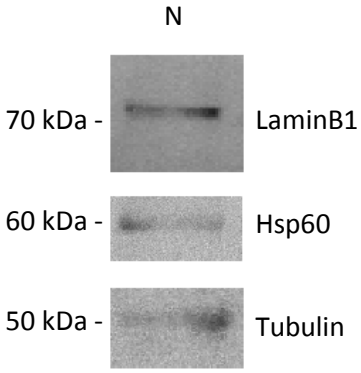

Figure 3.1. Characterization the nuclear fraction (**N**). The purity of the fractions was evaluated by Western Blotting.

3.2 Effect of Aβ1-42 oligomers on the levels of phosphorylation of GluN2A and GluN2B subunits of the NMDA receptor

Cortical neurons cultured for 15 days were exposed to 0.5 μM Aβ1-42 oligomers (prepared as described by Ferreira et al., 2012) for 2 and 5 min and total extracts were subjected to Western Blotting for analysis of P(Ser1232)-GluN2A and P(Tyr1472)-GluN2B, two NMDARs subunits highly expressed in the cortex (Cull-Candy et al., 2001). As presented in Figure 3.2 a tendency for an immediate increase in phosphorylation levels of both subunits, namely P(Ser1232)-GluN2A and P(Tyr1472)-GluN2B, occurs in response to Aβ exposure.

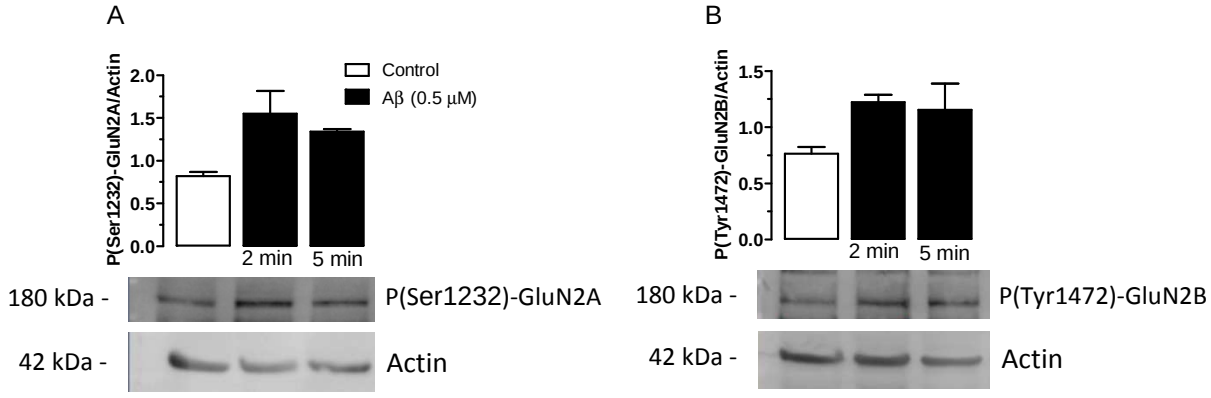

Figure 3.2. Levels of phosphorylation of (**A**)GluN2A and (**B**)GluN2B subunits in cortical neurons (15DIV) exposed to Aβ1-42 oligomers for 2 and 5 min. Data are the mean±SEM of 3 independent experiments.

3.3 Changes in phosphorylation of CREB transcription factor following exposure to Aβ oligomers

Changes in the activity of CREB, an important transcription factor known to regulate pro-survival signals in neurons (Bok et al., 2007) was investigated by western blotting with an antibody against P(Ser 133)CREB in total extracts of cultured cortical neurons with 15DIV exposed to Aβ1-42 oligomers for 5 min, 30 min, 2 h and 24 h in culture medium (Fig. 3.3A). Our results show that 5 min incubation with Aβ oligomers induced a significant increase in P(Ser133)-CREB levels; however, 30 min, 2 h and 24 h treatments did not produced any increase in P(Ser133)-CREB levels when compared to control conditions (Fig. 3.3A). The same stimulation protocol did not significantly affect the levels of total CREB (Fig. 3.3B).

The effect of antagonists of NMDARs, namely MK-801 (10 μM), ifenprodil (10 μM), memantine (10 μM) and NVP-AAM077 (1 μM), were then evaluated during 5 min stimulation with Aβ. Results depicted in Fig 3.3C demonstrate that the immediate increase in P(Ser133)-CREB that occurred upon 5 min incubation with Aβ was completly prevented by NMDARs antagonists MK-801 (non-competitive antagonist), ifenprodil (non-competitive, selective for GluN2B subunit), memantine (uncompetitive, lower affinity antagonist) and NVP-AAM077 (selective for GluN2A). In fact, all the antagonists reduced P(Ser133)-CREB levels to those achieved in control conditions; moreover, both memantine and NVP-AAM077 showed a significant decrease in P(Ser133)-CREB when compared with 5 min exposure to Aβ in the absence of the antagonists.

These findings suggest that Aβ induced an immediate increase in P(Ser133)-CREB levels through NMDARs activation since this effect is prevented by NMDAR antagonists.

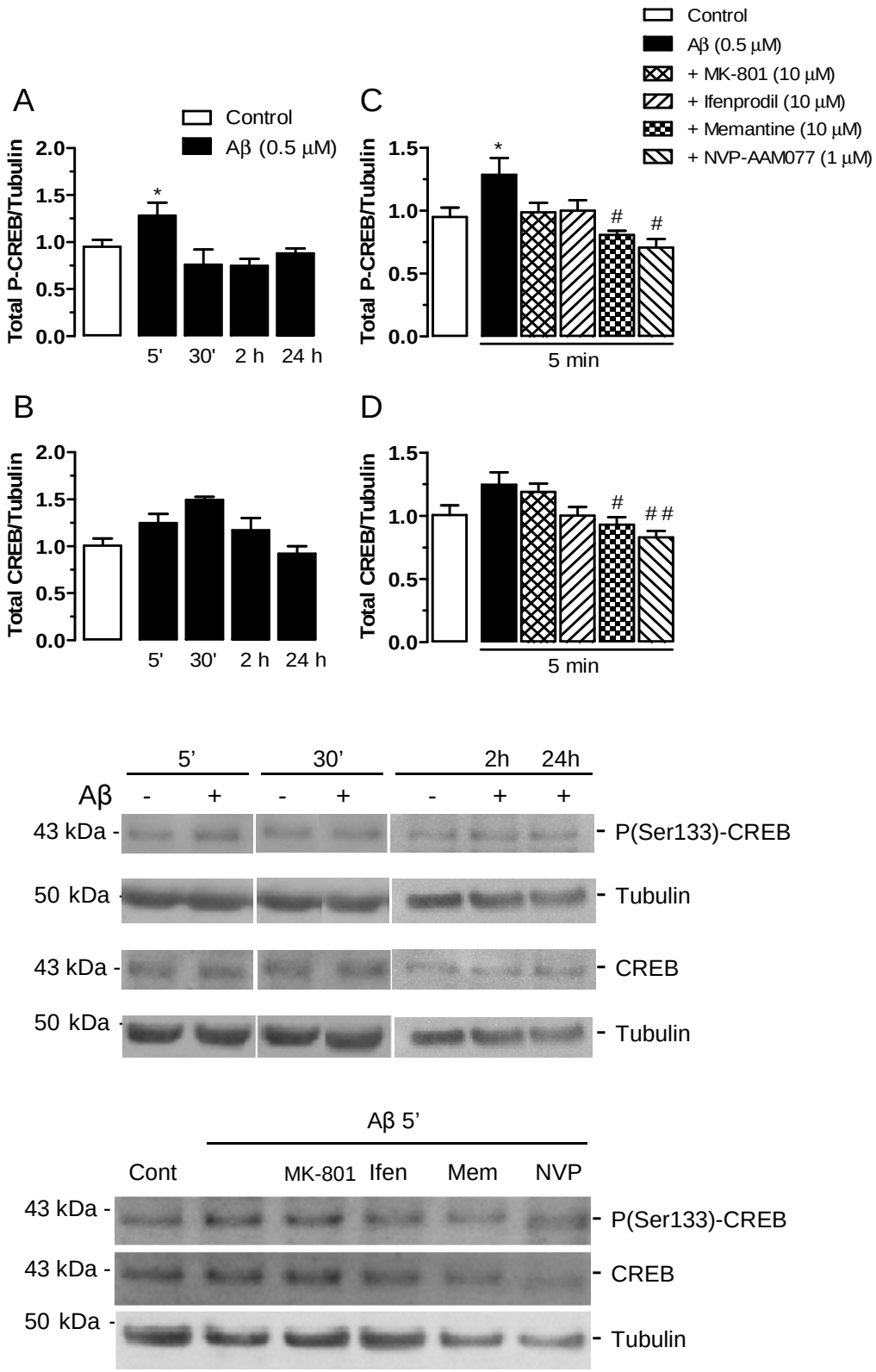

Figure 3.3. Effect of Aβ oligomers on P(Ser133)-CREB levels - the involvement of NMDARs. Total extracts were obtained from cultured rat cortical neurons with DIV 15 exposed to 0.5 μM Aβ1-42 for 5 min, 30 min, 2 or 24 hours in the absence (Control) or in the presence of NMDARs antagonists. The levels of (**A**) P(Ser133)-CREB/tubulin, (**B**) CREB/tubulin, and the effect of NMDARs antagonists (MK-801 10 μM, ifenprodil 10 μM, memantine 10 μM, NVP-AAM077 1 μM) on (**C**) P(Ser133)-CREB and (**D**) CREB levels, as determined by Western Blotting. α-Tubulin levels were used as loading control. Data are the mean±SEM of 15 independent experiments. Statistical analysis: Dunnett's multiple comparison test. *$p < 0.05$ when compaired to control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, when compaired to Aβ 5 min.

Previous results demonstrated that exposure of cortical neurons to Aβ1-42 oligomers evoked an immediate increase in Ca^{2+}_i (Ferreira et al., 2012). In order to evaluate if the increase in P(Ser133)-CREB depends on Ca^{2+} influx induced by Aβ1-42 oligomers, cells were incubated for 5 min with 0.5 μM Aβ in Na^+ medium in the presence (1 mM Ca^{2+}) or in the absence of Ca^{2+} (0 mM Ca^{2+}) (Fig. 3.4). Our results demonstrate that Aβ exposure promoted a significant increase in P(Ser133)-CREB levels when compared to control conditions; moreover, this effect was shown to be completely prevented by the NMDAR antagonist memantine (Fig. 3.4A). Conversely, the absence of external Ca^{2+} (0 mM Ca^{2+}) completely abolished the increase in P(Ser133)-CREB in cortical cells in response to Aβ1-42 oligomers (Fig. 3.4B). These results suggest that Ca^{2+} influx through NMDARs seems to mediate P(Ser133)-CREB increase in response to Aβ exposure. Our results also demonstrate that total CREB levels are not altered in the presence or absence of extracellular Ca^{2+}, following incubation of cells with Aβ1-42 oligomers for 5 min (Fig. 3.4C and D).

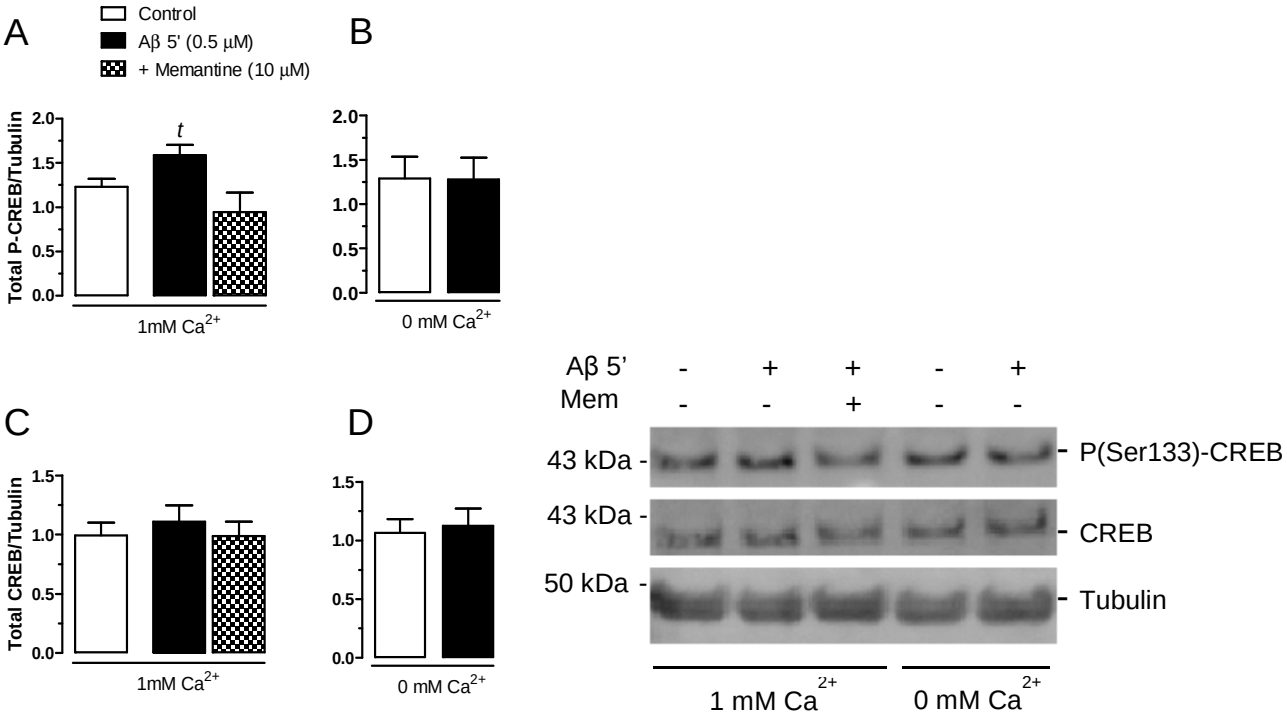

Figure 3.4. Dependence of extracellular Ca^{2+} on P(Ser133)-CREB in cortical neurons exposed to Aβ1-42 oligomers. Total extracts were obtained from cultured rat cortical neurons at DIV 15 exposed to 0.5 μM Aβ1-42 oligomers for 5 minutes in Na^+ medium in the presence (1 mM) or absence (0 mM) of Ca^{2+}. Aβ1-42 was added to the culture medium 5 minutes after NMDARs antagonist memantine (10 μM) treatment, which remained during the 5 minutes of Aβ1-42 exposure. Levels of (**A,B**) P(Ser133)-CREB/tubulin and (**C,D**) CREB/tubulin were determined by Western Blotting. Data are the mean±SEM of 7 independent experiments. Statistical analysis: $^t p<0.05$ *vs* control (Student's *t* test).

We next followed the changes in P(Ser133)-CREB in nuclear extracts obtained from primary cortical cells. We found a significant decrease in CREB levels after 24 hours of incubation with Aβ1-42 oligomers (Fig. 3.5A); this effect was slightly, but not significantly, prevented by memantine and, to a lesser extent, by ifenprodil (Fig. 3.5B). No significant changes were observed in nuclear P(Ser133)-CREB levels (Fig. 3.5C,D) in response to Aβ treatment for 5 min, 2h or 24 h.

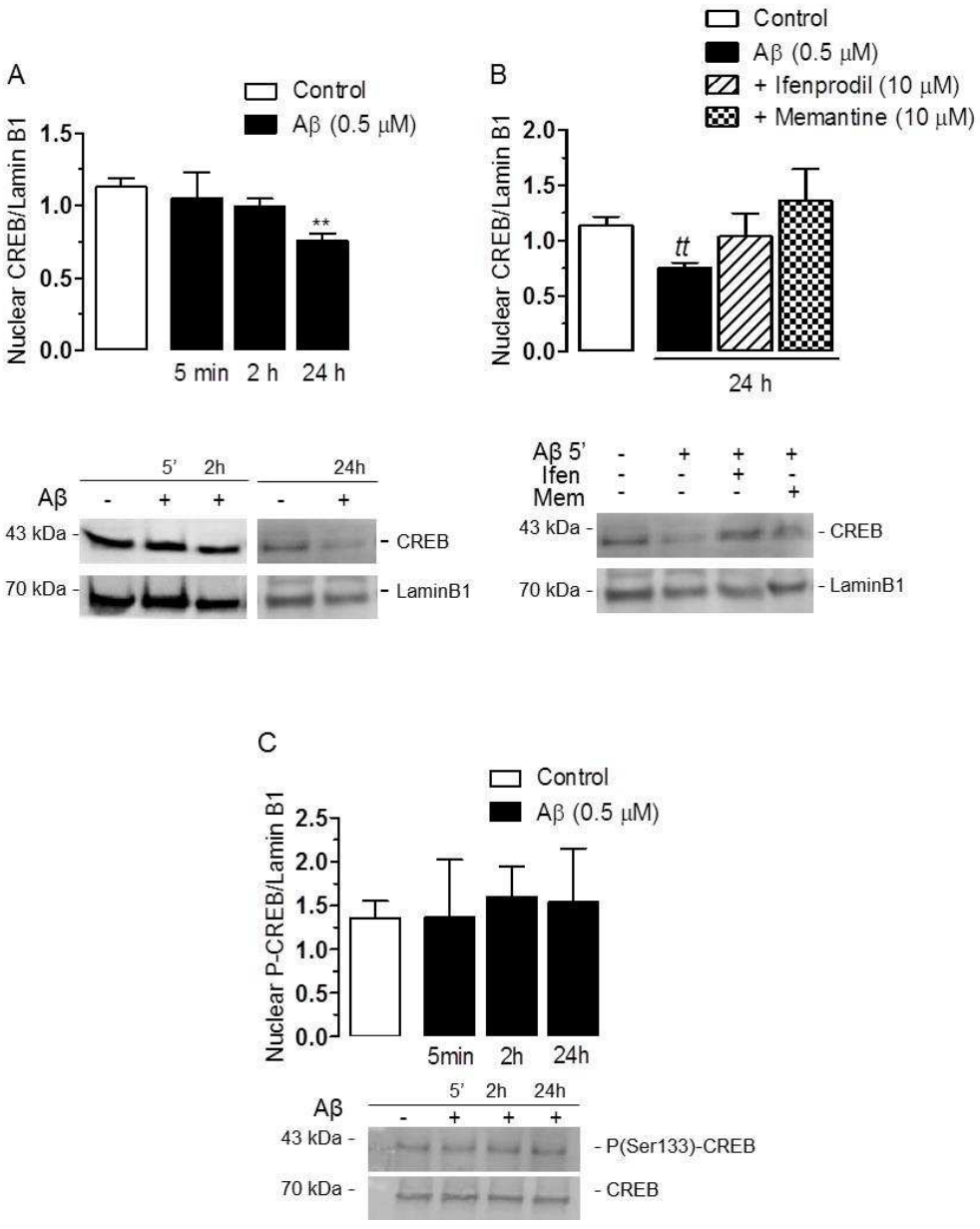

Figure 3.5. Time-dependent changes in nuclear transcription factor CREB. Primary cortical neurons at DIV 15 were exposed to 0.5 μM Aβ1-42 oligomers in culture conditioned medium for 5 min, 2 or 24 h and expression levels of (**A,B**) CREB and (**C,D**) P(Ser133)-CREB were analysed by Western Blotting using LaminB1 as a loading control. In **B, D** we tested the effect of NMDAR antagonists, memantine (10 μM) and ifenprodil (10 μM), by cells treated with antagonists exposed for 5 min prior to Aβ1-42 treatment. Data are the mean±SEM of 8-9 independent experiments. Statistical analysis (Dunnett's multiple comparison test or Student's t-test): **p < 0.01 or ^{tt}p < 0.01 when compared to the control.

3.4 Effect of Aβ1-42 oligomers on PGC-1α levels

Since Aβ1-42 oligomers affect CREB activity and CREB has been involved in the regulation of transcription factors related to mitochondrial biogenesis, such as PGC-1α well known to have elements responsive to CREB (CRE) in its promoter (Fernandez-Marcos and Auwerx, 2011), and signaling pathways through NMDARs (Sala et al., 2000), we tested in primary cultures of cortical cells the effect of Aβ1-42 oligomers exposure for 5 minutes, 2 or 24 hours on nuclear and total protein levels of PGC-1α.

Nuclear PGC-1α levels did not change upon addition of Aβ to cortical neurons (Fig. 3.4A). However, 5 minutes, but not 2 or 24 hours of incubation to Aβ1-42 oligomers produced a significant increase in total PGC-1α levels (Fig. 3.4B). Moreover, this increase was abolished in the presence of NMDARs antagonists MK-801 (10 μM), ifenprodil (10 μM), memantine (10 μM) and NVP-AAM077 (1 μM), suggesting an involvement of NMDARs on Aβ1-42-induced increase in total PGC-1α levels (Fig. 3.4C).

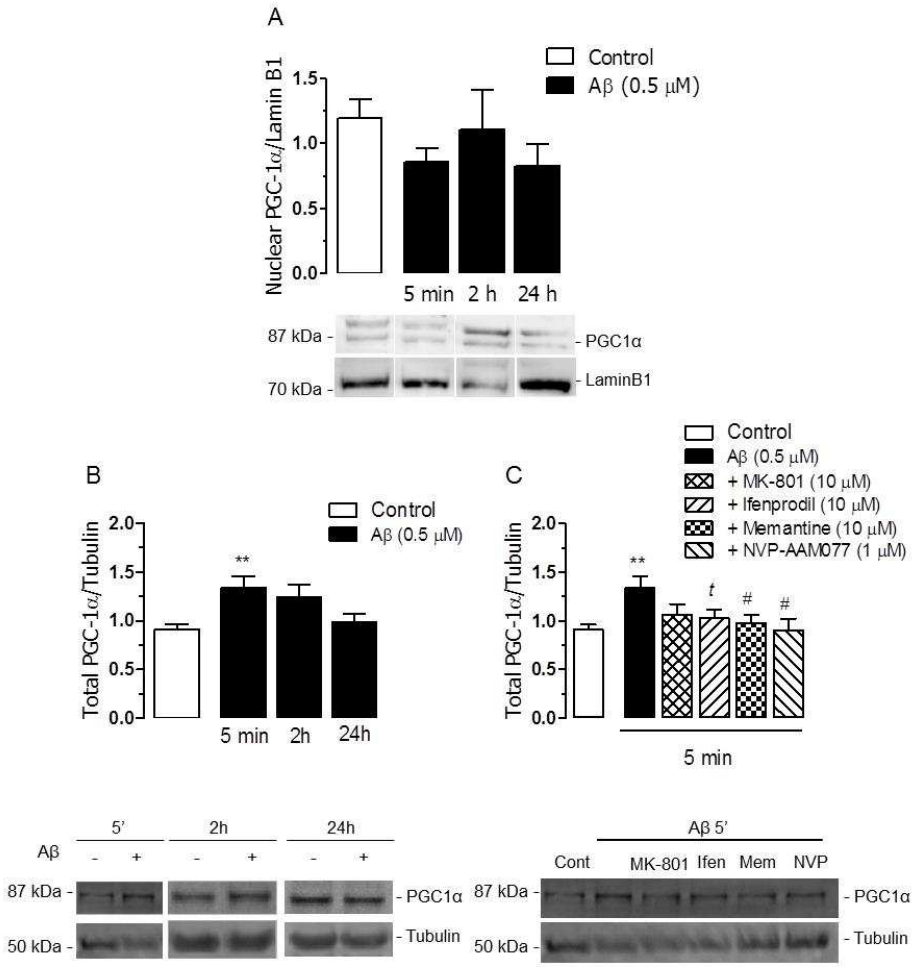

Figure 3.6. Time-dependent changes in transcription factor PGC-1α levels in nuclear and total fractions of rat primary cortical neurons exposed to Aβ1-42 oligomers. Cortical neurons at 15DIV were exposed to Aβ1-42 oligomers for 5 min, 2 and 24 hours. Cells treated with antagonists (MK-801 10 μM, ifenprodil 10 μM, memantine 10 μM, NVP-AAM077 1 μM) were pre-exposed for 5 minutes before adding Aβ1-42 in culture medium for 5 minutes, 2 or 24 hours. The levels of PGC-1α in nuclear and total extracts were analyzed by Western Blotting. Data represents the mean ± SEM of 10-15 independent experiments. Statistical analysis: **p < 0.01 when compared to control; $^{\#}$p < 0.05 when compared to Aβ 5 min (Dunnett's multiple comparison test); ^{t}p<0.05 *vs* control (Student's *t* test).

3.5 Effect of Aβ1-42 oligomers on phosphorylation levels of ERK

Since ERK and CREB pathway can crosstalk, we also investigated the changes in P(Thr202/Tyr204)-ERK in total extracts from cortical neurons exposed to 0.5 µM Aβ1-42 oligomers for 5 min, 30 min, 2 h and 24 h in culture medium. Both subunits 42 and 44 kDa of ERK P(Thr202/Tyr204)-ERK (42) and P(Thr202/Tyr204)-ERK (44)) showed relevant significant increase in phosphorylation after 5 minutes of exposure to 0.5 µM Aβ1-42, which was coincident with the increase observed in P(Ser133)-CREB; however, no effect of Aβ1-42 was detectable for all incubation times, namely 30 minutes, 2 h and 24 h (Fig. 3.7A and E). The increase in P(Thr202/Tyr204)-ERK (42) and P(Thr202/Tyr204)-ERK (44) at 5 minutes was shown to be completely prevented by the NMDAR antagonists MK-801 (10 µM), memantine (10 µM), NVP-AAM077 (1 µM), but not by Ifenprodil (10 µM) (Fig. 3.7B and F), suggesting that NMDARs are involved in the mechanisms that allows the phosphorylation of ERK for short times of exposure to Aβ1-42, but not those composed by GluN2B subunits. Within the antagonists, memantine statistically prevented the increase in P(Thr202/Tyr204)-ERK (44) when compared with the condition of exposure to Aβ1-42 (Fig. 3.7F).

Total ERK 42 levels did not change following incubation Aβ1-42 for the different times of exposure (Fig. 3.7C); however, 2h incubation to Aβ1-42 oligomers significantly increased the ERK 44 levels, suggesting a difference between ERK 44 and ERK 42 levels achieved after a prolonged exposure to Aβ. Nevertheless, the increase in total levels of ERK 44 were not inhibited by any of the NMDAR antagonists tested, suggesting that increased total ERK 44 levels occur independently of NMDARs activation.

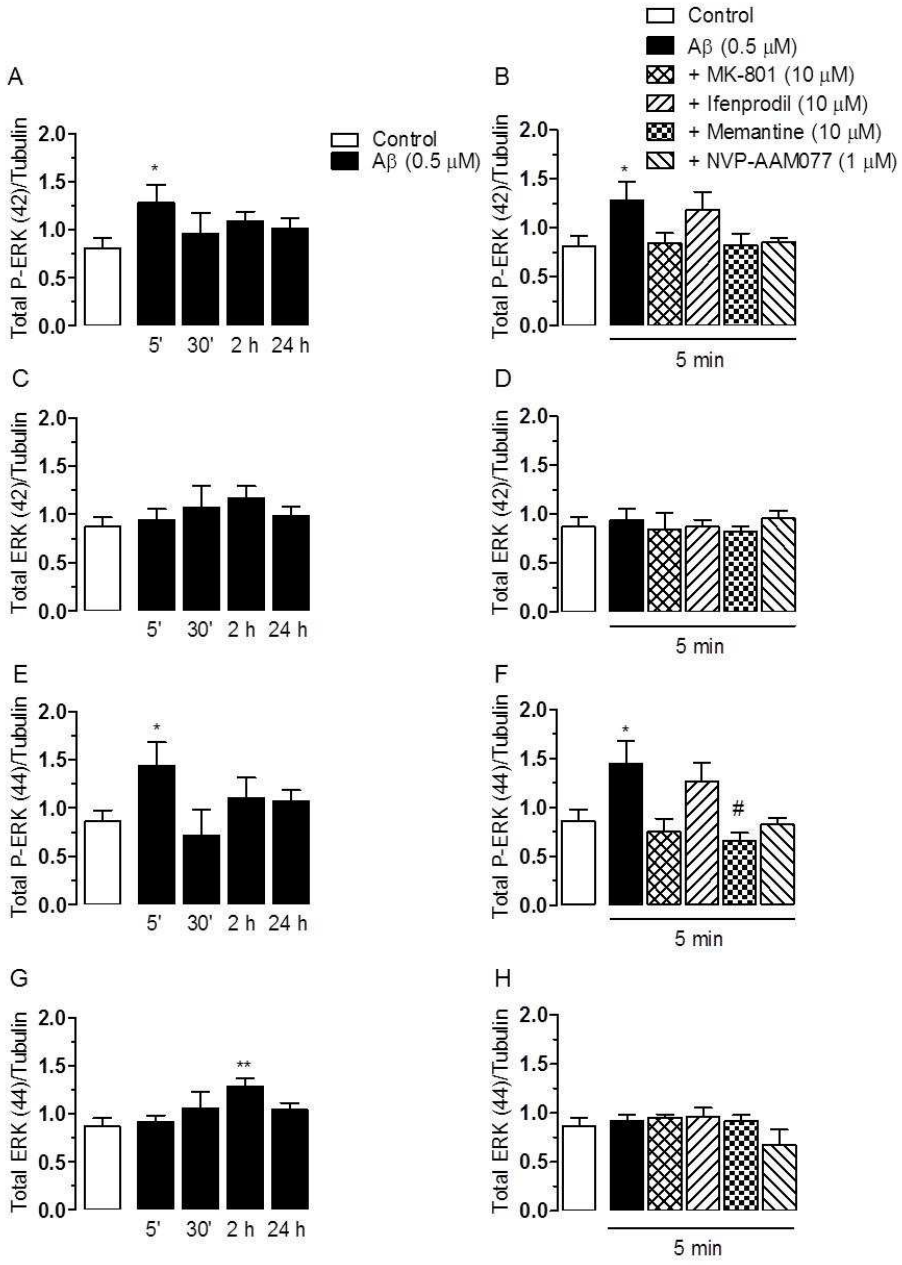

Figure 3.7. Effect of Aβ oligomers on ERK phosphorylation and total ERK levels: effect of NMDAR antagonists. Total extracts were obtained from cultured rat cortical neurons (DIV 15) exposed to 0.5 μM Aβ1-42 oligomers for 5 min, 30 min, 2 h or 24 h. Time-dependent changes in the levels of (**A,E**) P(Thr202/Tyr204)-ERK/Tubulin and (**C,G**) ERK/Tubulin determined for both 42 (**A,C**) and 44 (**E,G**) kDa subunits by Western Blotting, using tubulin as a control loading; the effect of NMDAR antagonists (MK-801 10 μM, ifenprodil 10 μM, memantine 10 μM, NVP-AAM077 1 μM) is depicted in (**B,F**) P(Thr202/Tyr204)-ERK/Tubulin and (**D,H**) ERK/Tubulin levels for both 42 (**B,D**) and 44 (**F,H**) kDa subunits. Representative blots are shown in Figure 3.8. Data are the mean±SEM of 11 independent experiments. Statistical analysis (Dunnett's multiple comparison test): *$p < 0.05$ when compared to the control; $^{\#}p < 0.05$ when compared to Aβ 5 min.

According to the findings obtained when ERK (42) was plotted independently of ERK (44), the same pattern was observed when the results were plotted considering P(Thr202/Tyr204)-ERK (42+44) and ERK (42+44) levels (Fig 3.8).

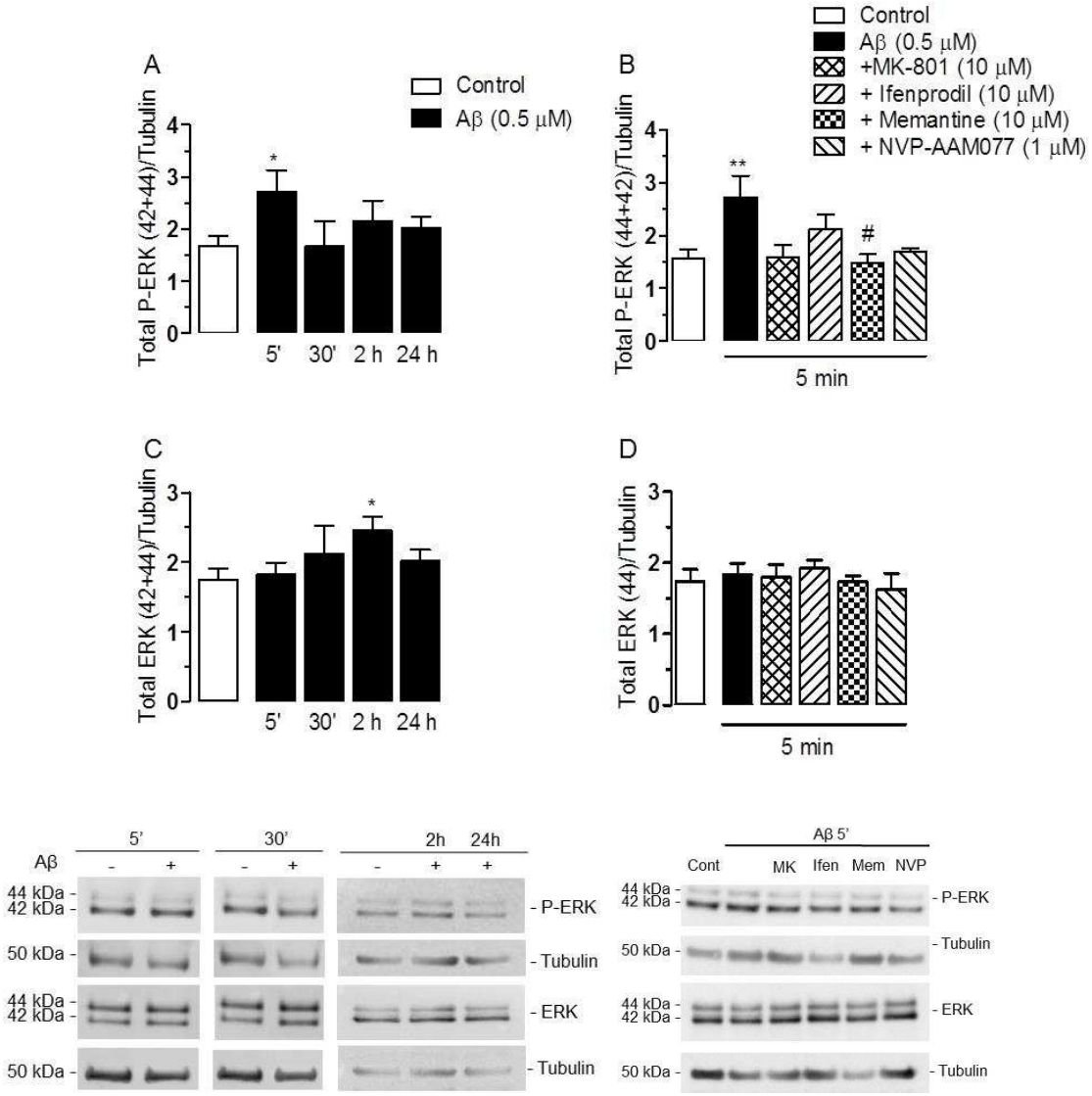

Figure 3.8. Effect of Aβ oligomers on ERK (42+44) phosphorylation and total ERK (42+44) levels: effect of NMDAR antagonists. Total extracts were obtained from rat cortical neurons (15DIV) exposed to 0.5 μM Aβ1-42 oligomers for 5 min, 30 min, 2 h or 24 h. Time-dependent changes in (**A**) P(Thr202/Tyr204)--ERK(42+44)/Tubulin, (**C**) ERK(42+44)/tubulin, and the effect of NMDAR antagonists (MK-801 10 μM, ifenprodil 10 μM, memantine 10 μM, NVP-AAM077 1 μM) in (**B**) P(Thr202/Tyr204)-ERK (42+44)/tubulin and (**D**) ERK(42,44)/tubulin levels were determined by Western Blotting. Data are the mean±SEM of 11 independent experiments. Statistical analysis (Dunnett's multiple comparison test): *$p < 0.05$ when compared to the control; **$p < 0.01$ when compared to the control; $^{\#}p < 0.05$ when compared to Aβ 5 min.

In order to elucidate if the increase in phosphorylation levels of ERK depends on extracellular Ca^{2+} present in culture medium (Fig. 3.7 and 3.8), we performed experiments by using cortical neurons exposed to Aβ1-42 oligomers for 5 minutes in Na^{+} medium in presence (1 mM) or absence of Ca^{2+} (0 mM). As previously shown for CREB in Figure 3.4A, 15DIV cortical neurons showed a significant increase in both P(Thr202/Tyr204)-ERK (42) and P(Thr202/Tyr204)-ERK (44) in response to Aβ stimulation in a Ca^{2+}-dependent manner (Fig. 3.9A and C). In the absence of external Ca^{2+} (Fig. 3.9A and C) no effect on phosphorylation levels was observed for both subunits when the cells were exposed to Aβ. Moreover, no significant changes in total ERK were observed upon exposure to Aβ for 5 minutes in the presence or absence of 1 mM Ca^{2+} (Fig. 3.9B and D). When the results were plotted considering the sum of both subunits (42+44) the same pattern was observed (Fig 3.9 E and F).

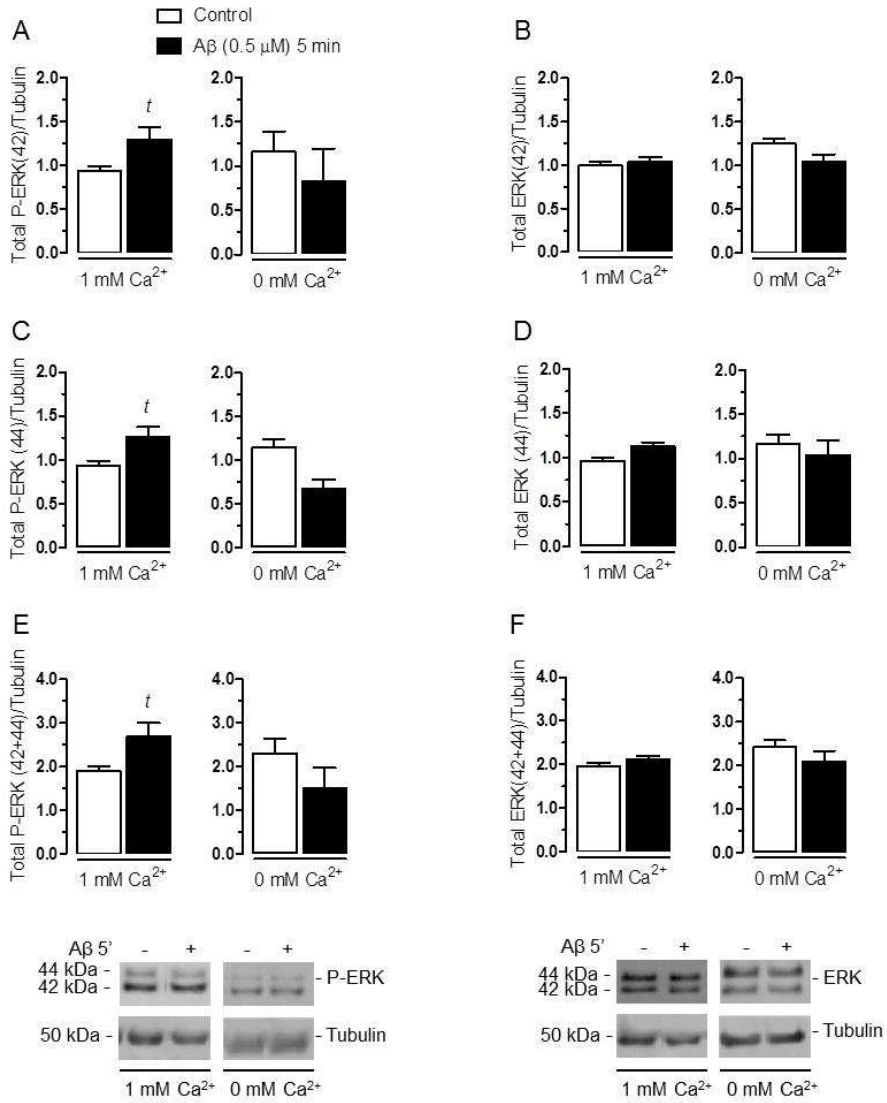

Figure 3.9. Dependence of extracellular Ca^{2+} on P(Thr202/Tyr204)-ERK in cortical neurons exposed to Aβ1-42 oligomers. Total extracts were obtained from cultured rat cortical neurons (15 DIV) exposed to 0.5 μM Aβ1-42 oligomers for 5 minutes in Na^{+} medium in the presence (1 mM) or absence (0 mM) of Ca^{2+}. Protein levels of (**A,C,E**) P(Thr202/Tyr204)-ERK and (**B,D,F**) ERK were determined by Western Blotting. Data are the mean±SEM of 8 independent experiments. Statistical analysis: $^{t}p<0.05$ *vs* control (Student's *t* test).

3.6 Intracellular Ca^{2+} recording

Since we observed a dependence on external Ca^{2+} in P(Ser133)-CREB and P(Thr202/Tyr204) (42+44) induced by Aβ, changes in intracellular free Ca^{2+} (Ca^{2+}_i) were evaluated in cells immediately exposed to Aβ in the presence or absence of NMDAR antagonists, namely MK-801 (10 μM), ifenprofil (10 μM), memantine (10 μM) and NVP-AA0M77 (50 nM and 10 μM). Results presented in Figure 3.10 show that Aβ induced an immediate increase in Ca^{2+}_i mostly mediated by NMDARs. Surprisingly, ifenprodil did not significantly inhibit the Ca^{2+} rise induced by Aβ.

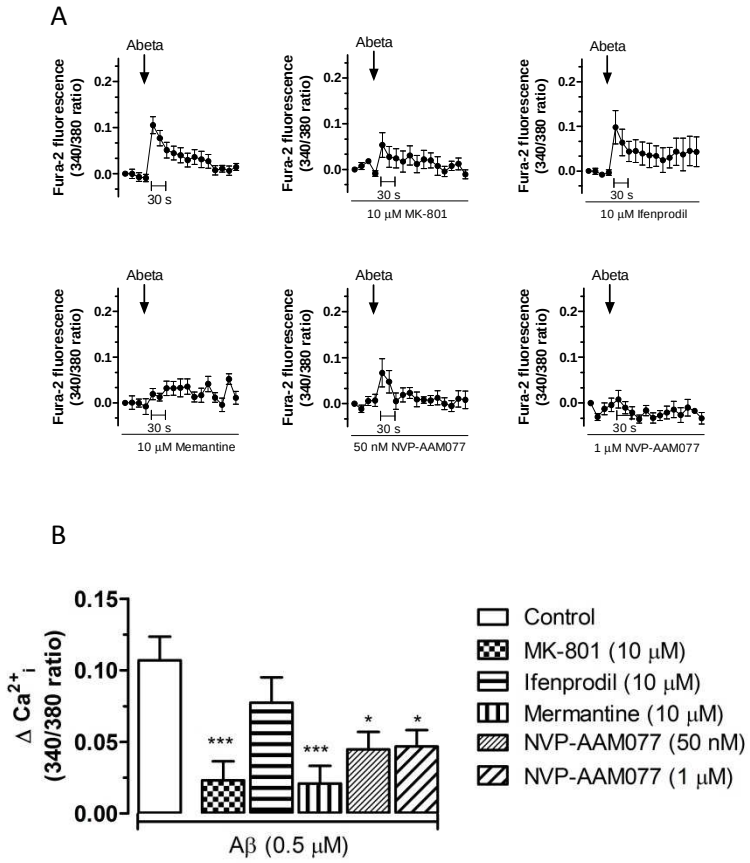

Figure 3.10. Changes in the intracellular Ca^{2+} levels induced by Aβ: effects of NMDAR antagonists. Cortical cells were stimulated with 0.5 μM Aβ1-42 oligomers in the absence or presence of NMDARs antagonists (10 μM MK-801, 10 μM ifenprofil, 10 μM memantine and 0.05 and 1 μM NVP-AA0M77). (**A**) Representative tracings showing the basal Fura-2 fluorescence recording (340/380 ratio) and the effect of Aβ. (**B**) Results were plotted as the difference between the maximun value achieved and the basal value before Aβ addition. Data are the mean±SEM of 7 independent experiments. Statistical analysis (Dunnett's multiple comparison test): *p < 0.05 and ***p < 0.001 when compared to the control.

CHAPTER 4

DISCUSSION

DISCUSSION

In this present work we evaluated changes in activity of CREB, ERK and PGC-1α in cultured cortical neurons exposed to Aβ1-42 oligomers in a AD cell model. Our data show that the presence of extracellular Aβ induces rapid changes in phosphorylation levels in ERK and CREB, which was prevented by NMDARs antagonists and that this effect depends on extracellular Ca^{2+}. Previous reports have shown that NMDAR activation induces long-lasting changes through phosphorylation of CREB at Ser133 in immature neurons, but only transiently in mature neurons (Sala et al., 2000), like in our case (using neurons with 15DIV).

Since CREB and PGC-1α are transcription factors and ERK can modulate CREB response, and the effects were prevented by NMDAR antagonists, data uggest a possible changes in transcriptional activity involving the NMDARs as neurodegenerative mechanisms in AD mature cortical neurons.

Until now there is no definitive cure for AD. In fact, strategies to reduce the processing of Aβ (such as β- and γ-secretase inhibitors) or its aggregation (chelating agents for metals Cu^{2+} and Zn^{2+}) are not sufficient to block the progession of the illness. Even a new immunotherapy strategy was succefull in mice, the same was not efficient in humans due to disruption of plaques and release of Aβ as oligomers still showing toxic effects, activation of microglia with inflammatory response and encephalitic response in part of patients (Schenk et al., 2012). The few treatments currently used in the clinic take into account NMDARs overactivation, by using memantine in moderate-severe AD stages, and modifications of acetylcholine levels by using AChEI, both as palliative drugs of symptoms. The latter is used considering that the main cognitive deficits of AD patients are caused by impairment of the Meynert Nucleus that spreads cholinergic projections to all the brain mainly to the hippocampus, amigdala and cortex. Therefore, there is a need to continue to search for new targets for therapeutic intervention based on the early pathological effects of Aβ and tau.

Vitolo et al. (2002) has previously proposed that there might be a membrane receptor, not well identifed, that could mediate the effects of Aβ1-42 oligomers, given in a sublethal dosage, in hippocampal cultured neurons through rapid and protract decrease of PKA that causes a decrease in CREB phosphorylation. This effect was prevented by drugs such as rolipram and forskolin that increase the intracellular levels of cAMP, decreasing its degradation by the phosphodiesterase PDE4 and increasing its synthesis by adenylate cyclase, respectively. Increased levels of cAMP make the catalytic subunit of PKA free to migrate to the nucleus and activate CREB, as detected by increased phosphorylation at Ser133. In this situation, reversal of PKA inhibition was sufficient to restore CREB phosphorylation and LTP, which is implicated in memory formation.

Taking in account the typical features of AD, namely a decrease in postsynaptic excitatory proteins, alterations in the number and morphology of the dendritic spines and a decrease in LTP in central synapses, recently Dinamarca et al. (2012) reviewed the post-synaptic receptors as target for Aβ oligomers. These receptors are supposed to be mediators of neuronal damage in AD. In this scenario, glutamate receptors (ionotropic NMDARs and metabotropic) and cholinergic receptor α7-nAChR were considered as mediators of Aβ toxicity. In Ferreira et al. (2012), the GluN2B subunit of NMDAR is namely responsible for intracellular rising in Ca^{2+}, whereas GluN2A-NMDARs antagonism potentiates this increase at high concentrations of Aβ (1 μM), suggesting that GluN2A and GluN2B subunits play an opposite roles in regulating intracellular Ca^{2+} content. Aβ oligomers appear to affect the trafficking of NMDARs, inducing endocytosis in a α7-nAChR-dependent manner (Snyder et al., 2005). From the literature, it is evident that NMDARs are important for LTP and synaptic transmission and that Aβ interferes in this mechanism at the plasma membrane (Snyder et al., 2005)(Shankar et al., 2007). Interestingly, GluN1 and GluN2B subunits of NMDARs in hippocampus can be bound by Aβ oligomers (Lacor et al., 2007), although the exact binding domain is still unknown. GluN2B subunits were previously proposed as the main NMDAR subunit implicated in regulating the intracellular influx of Ca^{2+} in dendritic spines, causing a decrease in dendritic spines and synaptic density, and leading to synaptic dysfunction (Shankar et al., 2007). The binding of GluN2B subunits with Aβ oligomers leads to ER stress in a mechanism that induces NADPH oxidase (NOX)-mediated superoxide production and prevented by ifenprodil and slightly by NVP-AAM077 (Costa et al., 2012). Moreover, Li et al. (2011) suggested that the stimulation of this same subunit triggered the activation of MAPK with down-regulation of CREB, and that low doses of NMDAR antagonists were sufficient to revert synaptic dysfunction. Importantly, Hardingham and Bading (2010) reviewed that different NMDAR signaling might after the activation of extrasynaptic or synaptic NMDAR receptors, mainly composed of GluN2B or GluN2A subunits, respectively (Hardingham and Bading, 2010), which could lead to the activation of pro-death or pro-survival neuronal responses. Synaptic responses are pro-survival including induction of survival genes like CREB or suppression of death genes related to the intrinsic apoptotic cascade like Puma (upstream of cytochrom c release), Apaf-1 and pro-caspase 9 (downstream), and also suppression of pro-death FOXO. Extrasynaptic responses are pro-death with shut-off of CREB pathway, inactivation of ERK 1/2 , activation of FOXO, activation of calpains with following cleavage of Ca^{2+}excharger 3 (NCX3)(leading to delayed Ca^{2+} deregulation) and STEP.

Increasing evidence suggests a connection between Aβ and alterations in transcription factors such as CREB (Saura et Valero, 2011), PGC-1α (Qin et al., 2009; Sheng et al., 2012)., NF-kB and STAT1 (Kitamura et al., 1997).

In this work we found that exposure of cultured cortical neurons to Aβ1-42 (0.5 μM) oligomers for 5 min was sufficient to produce changes in CREB phosphorylation levels and that this effect was prevented by all antagonists of NMDARs tested, namely by memantine (10 μM) and NVP-AAM077 (1 μM). A similar patten was observed in Na^+ medium containing Ca^{2+}, but not in the absence of Ca^{2+}, indicating that the increase in CREB phosphorylation largely depend on extracellular Ca^{2+}. Indeed, mature cortical neurons exhibited increased intracellular Ca^{2+} levels following exposure to Aβ1-42 oligomers, that was prevented by NMDAR antagonists, with the exception of ifenprodil. Since NMDARs antagonists prevent this increase in phosphorylation, we may affirm that CREB phosphorylation needs the entry of Ca^{2+} through NMDARs. These data corroborate the entry of Ca^{2+} occurring after activation of NMDARs evoked by Aβ1-42 oligomers, as demonstrated previously by our group to occur in cultured cortical (Ferreira et al., 2012) and hippocampal (Costa et al., 2012) neurons.

When we checked for nuclear levels of P(Ser133)-CREB for the same time of exposure to Aβ1-42 oligomers (5 min), we did not find any tendency for an increase, suggesting that short times of exposure are not sufficient to produce changes in CREB-mediated transcription. However, after 24 hours of exposure to the amiloidogenic peptide, the levels of CREB decreased and this was prevented by ifenprodil and memantine, while P(Ser133)-CREB was not significantly changed up to 24h. In literature there are evidence for decreased levels of CREB phosphorylation and transcription in AD patients (Yamamoto-Sasaki et al., 1999; Pugazhenthi et al., 2011; España et al., 2010). Caldeira (2012) previously described that CREB levels decrease in 15 month-old (aged) 3xTg-AD mice; however, neither P(Ser133)-CREB nor CBP levels were altered in young or aged 3xTg-AD mice. CREB is a transcription factor acting in nucleus in many promoters for genes encoding for survival, synaptic plasticity and memory. For istance, the neurotrophin brain-derived neurotrophic factor BDNF, as target of CREB, is involved in the long term memory process. For the formation of a lasting memory, it is essential that changes occur in the long term in neuronal networks, a process known as synaptic remodeling (Saura and Valero, 2011).

The observation that CREB levels are affected only after a prolonged exposure to Aβ1-42 oligomers and in old 3xTg-AD mice suggest that, in initial stages of AD, Aβ1-42 does not affect transcription events mediated by CREB, and that deregulation of long last functions, such as memory and synaptic plasticity, might occur later, along the progression of AD. Nevertheless, no changes were observed in P(Ser133)-CREB, despite the fact that many stimuli may converge to the nucleus activating CREB through several kinases like PKA, CaMKII and CaMKIV, ERK and RSK, being some of them directly regulated by intracellular Ca^{2+} levels (e.g. CaMK). CaM binds Ca^{2+} and activates CaMKII that phosphorylates CREB in the nucleus. Calcineurin also affects CREB pathway by dephoshorylating the transcriptional coactivator TORC (Transducers of Regulated CREB activity) and allowing its migration

to the nucleus, where it binds CREB, increasing gene transcription (Saura and Valero, 2011, for review). Conversely, calpains are cytosolic cysteine proteinases that cleave protein targets essential for neuronal survival (Wu et al., 2007) and the use of calpains inhibitors was demonstrated to improve spatial-working memory and associative fear memory in APP/PS1 mice (Trinchese et al., 2008).

Since CREB phosphorylation is perturbated by changes in intracellular Ca^{2+}, in our work we have also taken in account the changes in its direct target, PGC-1α, sice it contains CRE elements in its promoter and the activation of CREB can bring to an increase of transcription of PGC-1α. As described before in this thesis, PGC1-α is activated by phosphorylation (Puigserver *et al.*, 2003) and controlled by CREB, in conjunction with TORCs in the nucleus (Herzig et al., 2001; Wu et al., 2006). We found that nuclear levels of PGC1-α had a slight tendency to increase at 2 hours, suggesting a possible increase in mitochondrial biogenesis, probably as a compensatory response to Aβ1-42 exposure and resulting mitochondrial dysfunction. Indeed, Aβ was found to impair the activity of complex IV and V of electron transport chain (ATP synthase) leading to increase in ROS production and decrease in ATP levels (Parks et al., 2001; Eckert et al., 2011). PGC-1α promoter activity is increased following oxidative strees and regulate components of the ROS defense system (St-Pierre et al., 2006). In our group increased nuclear levels of PGC-1α were previously observed in both in young and aged 3xTg-AD mice (Caldeira et al., unpublished data). Surprisingly, we found increased PGC-1α levels in total extracts from cultured neurons after 5 minutes of exposure to Aβ, which was prevented by NMDARs antagonists, suggesting that the protein levels of PGC-1α can be regulated by an increased protein synthesis by local translation of mRNA in response to NMDAR activation. Since all NMDAR antagonists prevented the increase in PGC-1α levels, data suggest that both GluN2A and GluN2B-containing NMDARs are involved in this process. This part of the work suggests that NMDAR-dependent increases intracellular Ca^{2+} for short time of exposure to Aβ1-42 (5 min) are sufficient to promote the protein levels of PGC-1α either by decreasing its degradation and/or stimulating protein synthesis by translation of local mRNA. Later (after 2 h of Aβ1-42 exposure), PGC-1α may in return migrate into the nucleus, where it may potentially activate genes for mitochondrial biogenesis and ROS defence such as mitochondrial transcription factor A (TFAM) and superoxide dismutase (SOD). In future studies, analysis of mRNA levels of PGC-1α could give us more about the role of PGC-1α following exposure to Aβ1-42 oligomers. Despite increased levels of PGC-1α in 3 and 15 month-old 3xTg-AD mice, no changes in TFAM (a mitochondrial transcription factor, which levels are regulated by PGC-1α) have been found in this animal model (Caldeira et al., unpublished data). Published studies reported that PGC-1α protein levels are negatively associated with both AD-type neuritic plaques and Aβ content in human AD brains and in the Tg2576 model mouse (Qin *et al.*, 2009; Sheng

et al., 2012), also showing reduced expression of NRF-1, NRF-2 and TFAM, a reduction in mitochondrial/nuclear DNA ratio, which correlated with reduced ATP content, and decreased cytochrome C oxidase activity.

In addition to CREB pathway, our investigation focused on changes in ERK activation in mature cultured cortical neurons maintained *in vitro* for 15 days. Interestingly, similarly to CREB, ERK was activated following a short exposure to Aβ1-42 oligomers, in a NMDAR- and Ca^{2+}-dependent process. Indeed, increased P(Thr202/Tyr204)-ERK was recapitulated in Na^{+} medium containing Ca^{2+}, but in its absence. Moreover, with the exception of ifenprofil, all the other NMDAR antagonists tested (MK-801, memantine and NVP-AAM077) prevented ERK phosphorylation. This apparently suggests that ERK is activated by NMDARs enriched in GluN2A subunits, which are mainly expressed at synapses, possibly having pro-survival effects in neurons, as previously suggested (Hardingam and Bading 2010). Nevertheless, a role for ERK as a death promoter in nervous system was also proposed (Subramaniam and Unsicker, 2010), for istance with a sustained activation of ERK after oxidative stress, through activation of receptors, Ca^{2+} channels or directly by Src-tyrosine kinase. We analysed both subunits of ERK as singular subunits and as sum. Memantine highly prevented the phosphorylation of ERK subunit with 44 kDa, when compared with Aβ exposure for 5 min. The same pattern was verified when we considered the sum of both proteins (42 and 44 kDa). Moreover, increased levels of ERK44 were observed after 2 h of exposure to Aβ1-42 oligomers, which may underlie decreased protein degradation induced by the oligomeric peptide.
Our data show that the levels of P-ERK increased for short time of exposure to Aβ, suggesting that it is ready to interact with cytosolic targets, like p90RSK, which in turn activate CREB (Boglári G. and Szeberényi J. 2002). In order to verify if the changes on P(Ser133)-CREB mediated by Aβ1-42 oligomers are dependent on ERK pathway activation, in near future experiments we will test an inhibitor of ERK (e.g. U0126) and test the levels of P(Ser133)-CREB.

In summary, both CREB and ERK pathways seem to be affected by external stimulus of Aβ and this occurs through a mechanism linked to increased intracellular Ca^{2+} levels triggered by NMDARs activation. Since memantine was efficient in blocking the activation of these two pathways, data suggest that NMDARs channel pore is open upon incubation with Aβ1-42. Using mature cortical neurons, was further assessed NMDAR activation in the presence of Aβ1-42 oligomers, by following GluN2B and GluN2A phosphorylation. Indeed, data indicate a tendency for increased activity of both receptor subunits after a very short incubation with Aβ1-42 (2-5 min).

Sustained influx of Ca^{2+} occurring through NMDARs in response to Aβ stimulus may produce long-term excitotoxic effects on neurons, as previously demonstrated (Kho et al., 1990). However, short-

term exposure to Aβ to mature cortical neurons may favor the activation of pro-survival signaling pathways. Indeed, Aβ seems to preferentially activate GluN2A-composed NMDAR subunits, which exist mostly in synaptic sites, previously linked to survival pathways (Hardingam and Bading, 2010). In fact, the selective antagonist of GluN2B-containing NMDARs, ifenprodil, did not prevent the effects of Aβ on ERK or CREB phosphorylation. Protective effects of Aβ have been previously reported (Castellani et al., 2009), but the intricate cascade of events may be rather complex. The localization (synaptic *versus* extrasynaptic) and/or the type of the NMDARs activated (containing GluN2A or GluN2B subunits) can present different outcomes upon stimulation with extracellular Aβ oligomers. In this perspective, it will be important to determine whether activation of extrasynaptic or synaptic NMDARs have differential effects on CREB and ERK pathways, by using Na^+ medium containing glycine or D-serine as NMDAR co-agonists (Papouin et al., 2012). Moreover, extrasynaptic and synaptic NMDAR can be preferentially activated using bicuculline (competitive $GABA_A$ receptor antagonist) , 4-aminopyridine (4-AP, a blocker of voltage-activated K^+ channels) and MK-801 to block synaptic receptors and activate preferentially the extrasynaptic, or using only bicuculline plus 4-AP to preferentialy activate the synaptic receptors (Hardingham et al., 2001).

CONCLUSION

This work shows changes in transcription factors, CREB and PGC-1α, in mature (15DIV) cortical neurons exposed to Aβ1-42 oligomers as a model of initial stages of AD. Moreover, we observed Aβ1-42-evoked early concomitant ERK and CREB activation, along with increased levels of PGC-1α, which have been related to pro-survival processes as well as learning and memory formation (in the case of ERK and CREB) and mitochondrial biogenesis (in the case of PGC-1α), which have been described to be impaired in AD advanced stages. Indeed, the initial pathogenic mechanisms of this neurodegenerative disease still remain unclear. In this work we show NMDAR-dependent perturbations in intracellular Ca^{2+} that influence CREB and ERK activation. This might represent an initial mechanism that may modify relevant neurophysiological circuits, leading to changes in intracellular signaling pathways and expression of proteins that, if sustained, may favor the development of neuronal death. Thus, the understanding of these processes may be useful for the formulation of more selective pharmacotherapies, able to slowdown AD progression.

APPENDIX

A.1 Hemocytometer

The hemocytometer is a device used to count cells. The number of cells in the chamber of the hemocytometer is used to calculate the concentration or density of the cells in the mixture the sample comes from. The hemocytometer is a glass platform engraved with a laser-etched grid of perpendicular lines forming squares with a surface area of $1mm^2$ covered by a thin coverglass producing a depth between of 0.1mm (Fig. A.1). Each square of the hemocytometer represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 = 1 ml, the subsequent cell concentration per ml can be determined as media of number of cells contained in the two squares multiplied for the dilution factor and multiplied for 10^4. A count less than 50 or superior to 200 cells needs to adjust the initial dilution. The grid lines are well focused using the 10X objective of the microscope.

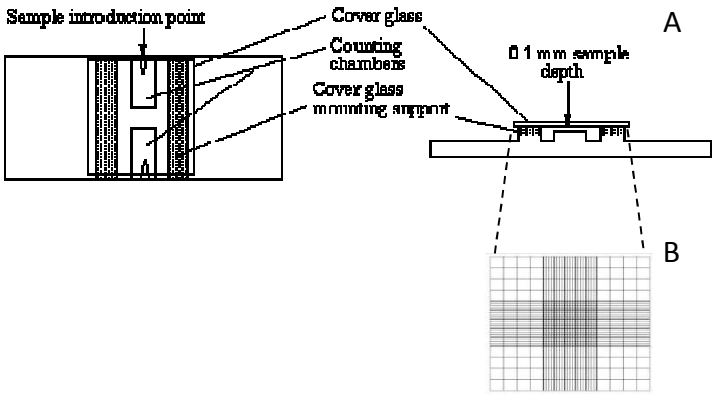

Figure A.1. Schematic representation of an hemocytometer (**A**) showing laser-etched grid of perpendicular lines forming squares in which the cells are counted (**B**).

A.2 Protein quantification by the Bio-Rad method

The Bio-Rad protein assay is a simple colorimetric assay for measuring total protein concentration and is based on the Bradford dye-binding method. It involves the addition of an acidic dye to protein solution, and subsequent measurement at 595 nm with a spectrophotometer or microplate reader. Comparison to a standard curve provides a relative measurement of protein concentration. The reagent Coomassie Brilliant Blue turns blue when it binds to arginine and aromatic amino acids present in proteins, thus increasing the absorbance of the sample. The absorbance is measured using a spectrophotometer, at the maximum absorbance frequency (A_{max}) of the blue dye (which is 595 nm). In this case, greater is the absorbance, higher is the protein concentration.

A.3 Standard Curve

Standard curves are most commonly used to determine the concentration of a substance such as protein or DNA. For example, a standard curve for protein concentration is often created using known concentrations of bovine serum albumin. Protein solutions are normally assayed in duplicate and the absorbance read is the arithmetic media of the two values obtained. Known concentrations of BSA are used to make the standard curve (Table A.1), plotting concentration on the X axis, and the assay measurement on the Y axis. The same assay is then performed with samples of unknown concentration (Table A.1). To analyze the data, one locates the measurement on the Y-axis that corresponds to the assay measurement of the unknown substance and follows a line to intersect the standard curve. The corresponding value on the X-axis is the concentration of substance in the unknown sample.

Table A.1. Scheme for standard curve and samples preparation used in protein quantification by the BioRad method

Standard	H_20 (µl)	Buffer (µl)	BSA 0.1% (µl)	BioRad reagent (µl)
1	79	1	0	120
2	78	1	1	120
3	77	1	2	120
4	76	1	3	120
5	75	1	4	120
6	74	1	5	120
Samples	79	1	-	120

Note: Incubation at room temperature for 5-10 minutes in dark

A.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis and protein transfer

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), is the most widely used technique to separate proteins from a mixture. Being present electricity, proteins migrate inside the poly-acrylamide gel under denaturing conditions achieved by using denaturing conditions (achieved by SDS present in the sample buffer described in Material and Methods section). SDS and a heating step determine that the electrophoretic mobility of a single kind of protein is only affected by its molecular weight in the running step.

Inserted between two glasses spacers, the gel require the co-polymerization of acrylamide and N,N-methylenebisacrylamide (Bis-acrylamyde) in a vinyl addiction reaction initiated by free radical-generating system from ammonium persulfate (APS) and catalyzed by N,N,N',N'-tetramethylethylenediamine (TEMED). The APS free radicals convert acrylamide monomers to free radicals which start a chain reaction with inactivated monomers (Fig. A.2).

The separation of molecules within this gel is determined by the relative size of the pores that depends on condition of polymerization and monomer concentration. The percentage of acrylamide in the gel is chosen according the molecular weight of the protein. In general, an higher percentage of acrylamide makes smaller pores that block the migration of high molecular weight proteins. Samples are then applied in the gel and exposed to an electric field in a chamber apparatus. Negative electrical charged proteins can pass through the gel subjected to an electrical field onto a support (nitrocellulose or PVDF) membrane. After blocking in BSA 5% or milk 5%, the membrane containing proteins is incubated with first and secondary antibodies before the revelation with ECF solution.

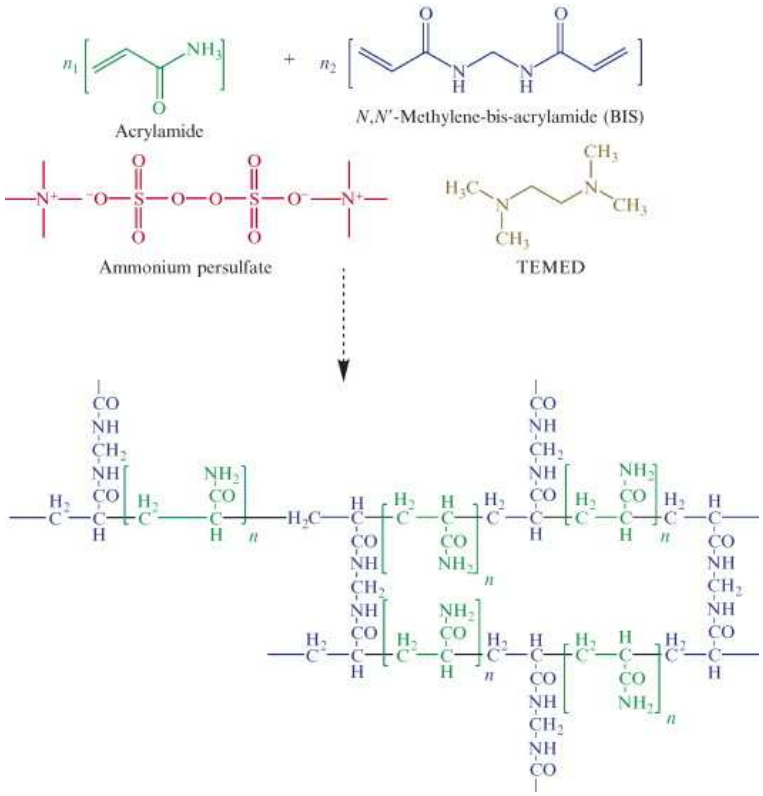

Figure A.2. - Polymerization reaction of acrylamide in SDS-PAGE gel (from Kandow et al., 2007).

Contaminations by buffer reagents (Tris, borate, acetate, glycine etc.), gel additives, detergents (SDS) and the water of the laboratory can produce changes in the polymerization that usually give an higher porosity to the gel and the molecules rapidly migrates, impairing the quality of experiments. The external temperature can impair the polymerization of the gel. However, a rapid polymerization produces an exothermic reaction producing heat that make in turn the reaction of polymerization more rapid.

A.5 Chemifluorescent detection by ECF

ECF™ Western is a highly sensitive system for chemifluorescent detection of Western Blots analyzed by VersaDoc System. ECF Western Blotting permits immunodetection of proteins electroblotted onto the membrane, by using a primary antibody directed towards the target protein, followed by incubation with alkaline phosphatase-linked antibody (secondary antibody) as shown in Figure A.3. The alkaline phosphatase of the secondary antibody catalyzes the conversion of ECF to a new product that fluoresces strongly at 550–570 nm when the blots are illuminated with UV light with a peak of excitation at 430 nm. ECF revelation shows a linear proportionality between the amount protein and the relative fluorescent output. This method makes a safe handling and reliable, highly sensitive detection of target proteins.

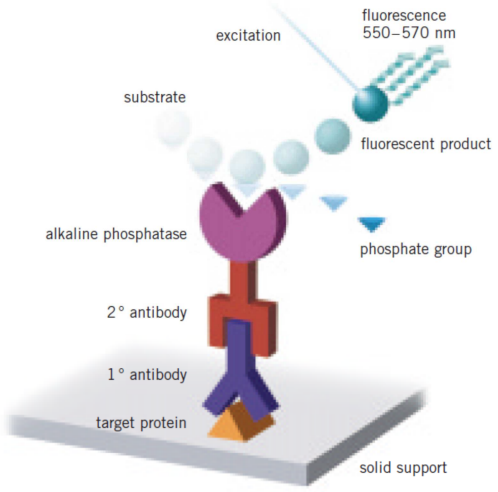

Figure A.3. Schematic diagram of the ECF Western Blotting detection. Proteins are detected by chemifluorescence using alkaline phosphatase-labeled secondary antibody. Alkaline phosphatase cleaves a phosphate group from the ECF substrate to yield a highly fluorescent product. (from www.gelifesciences.com).

A.6 Fura-2 Ratiometric Ca^{2+} Indicator

Fura-2-acetoxymethyl ester (or Fura-2AM) is one of the most popular fluorescent probes used to register dynamic changes in cytosolic free Ca^{2+} in intact living cells. Fura-2AM is lipophilic and, once inside the cell, is target of esterases that remove the acetoxymethyl groups, regenerating Fura-2 form, the pentacarboxylate Ca^{2+} indicator, no more membrane permeable. The cytosolic concentration of Ca^{2+} can be continuously monitored because the complex Ca^{2+}-Fura 2 emits fluorescence directly proportional to Ca^{2+}content. Measurement of Ca^{2+} are indicated as ratio of fluorescence registered at 340 nm and 380 nm (Fig. A.4).

The FURA-2 manifests a shift of the absorption spectrum as result of Ca^{2+} binding. In the absence of Ca^{2+}, the FURA-2 excitation spectrum has a fairly broad, with a peak at about 380 nm. When binded to the Ca^{2+}, the excitation spectrum shifts even more in the UV side with a peak at

340 nm and, the intensity of the fluorescence emitted by the FURA-2 (measured at 510 nm) increases if one excites at 340 nm (F_{340}) and decreases if it excites at 380 nm (F_{380}). In conclusion, exciting at the two wavelengths of 340 nm and 380 nm allows to collect a pair of signals at the wavelength of emission of 510 nm for each experimental point. Making the ratio of this two values is possible, therefore, to obtain a measure that is independent of the concentration of the indicator in the sample.

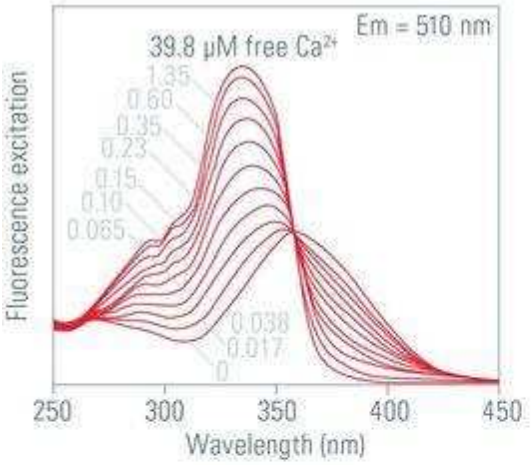

Figure A.4. Spectrum of Ca^{2+}-sensitive dyes FURA-2 AM with emission at 510 nm for different excitation wavelengths 340 nm and 380 nm.

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