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INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

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ANDRÉ BEVILACQUA MENEGHETTI

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ANDRÉ BEVILACQUA MENEGHETTI

Orientadora: Profa. Dra. Christianne Gazzana Salbego

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SUMÁRIO

PARTE I

RESUMO

A doença de Alzheimer é a principal e a mais comum desordem neurodegenerativa relacionada à idade. O número de pessoas afetadas pela doença de Alzheimer vem crescendo bastante nos últimos anos, principalmente devido ao aumento da expectativa de vida da população. As causas para o desenvolvimento da doença de Alzheimer são bastante complexas, envolvendo uma combinação de fatores genéticos, moleculares e ambientais. Os emaranhados neurofibrilares, constituídos pela proteína *tau* hiperfosforilada, e as placas senis, compostas pelo peptídeo β-amiloide, são as duas principais alterações fisiopatológicas encontradas nessa patologia. Existe uma ampla variedade de evidências genéticas, fisiológicas e bioquímicas que suportam a ideia que o peptídeo β-amiloide é ao menos uma das causas que originam a doença de Alzheimer. Na forma monomérica, o peptídeo β-amiloide parece não exercer efeitos tóxicos. Porém, conforme estas formas monoméricas se polimerizam formando intermediários solúveis denominados oligômeros, e por fim protofibrilas e fibrilas, exercem consideráveis efeitos neurotóxicos. Os oligômeros do peptídeo β-amiloide são capazes de se correlacionar de forma mais consistente com os distúrbios cognitivos observados na doença de Alzheimer. Além disso, diversos trabalhos atribuem às alterações sinápticas um dos principais mecanismos de toxicidade dos oligômeros do peptídeo β-amiloide. Para isso, diversas vias de sinalização sofrem alteração no seu funcionamento, como a via das MAPKs, destacando as proteínas JNK1/2 e ERK1/2, e a via Wnt/β-catenina. Neste trabalho nós procuramos avaliar os mecanismos moleculares de toxicidade dos oligômeros do peptídeo β-amiloide em culturas organotípicas de hipocampo de ratos. Para isso, as culturas foram expostas às concentrações de 0,5; 1 e 2 μM por um período de 48h. A morte celular foi avaliada a partir da incorporação do iodeto de propídeo, um marcador de morte celular de células principalmente em processo de necrose. A exposição das culturas a todas as concentrações testadas não foi capaz de causar um aumento significativo na morte celular. Entretanto, observamos um decréscimo nos níveis da proteína sinaptofisina por *Western blotting* em todas as concentrações utilizadas. Essas alterações nos níveis de sinaptofisina foram acompanhadas pela ativação da proteína JNK, ou seja, pelo aumento da sua fosforilação, e pela inibição da proteína ERK, que teve seus níveis de fosforilação diminuídos. Nós também observamos uma diminuição no imunoconteúdo da proteína β-catenina. A avaliação dos níveis de fosforilação da GSK-3β e β-catenina não apresentou resultado significativo. Embora mais estudos sejam necessários para avaliar os mecanismos de toxicidade dos oligômeros do peptídeo β-amiloide, nossos resultados sugerem uma perda sináptica nas culturas organotípicas, uma das primeiras características observadas na doença de Alzheimer. Acreditamos que esse modelo possa ser utilizado no estudo de fatores fisiológicos e compostos farmacológicos relacionados com a doença de Alzheimer.

ABSTRACT

Alzheimer's disease is the principal and the most common neurodegenerative age-related disorder. The number of patients affected by Alzheimer's disease has increased greatly in recent years, mainly due to increase in life expectancy of the population. The causes for the development of Alzheimer's disease are quite complex, involving a combination of genetics, molecular, and environmental factors. Neurofibrillary tangles, formed by hyperphosphorylated *tau* protein, and senile plaques, composed of amyloid-β peptide, are the two major pathological changes found in Alzheimer's disease. A wide variety of genetic, physiological, and biochemical evidences support the idea that amyloid-β peptide is at least one of the main causes of Alzheimer's disease. In monomeric form the amyloid-β peptide appears did not exert toxic effects. However, as these monomers polymerize forming soluble intermediates called oligomers, and after protofibrils and fibrils, exert considerable neurotoxic effects. The amyloid-β oligomers peptide are more consistently correlated to cognitive disturbances observed in Alzheimer's disease. In addition, several works have attributed to synaptic changes a major mechanism of amyloid-β oligomers toxicity. Several signaling pathways become altered in their work due to amyloid-β oligomers, such as MAPK pathway, highlighting JNK1/2 and ERK1/2 proteins, and Wnt/β-catenina. In this work, we evaluated the molecular mechanisms of amyloid-β oligomers toxicity in rat organotypic hippocampal slice cultures. For this purpose, cultures were exposed to concentrations of 0.5, 1, and 2 μM of amyloid-β oligomers for 48h. Cell death was assessed from the incorporation of propidium iodide, a marker of cell that are mainly in the necrosis process death. Exposure of all concentrations tested was not able to induce a significant increase in cell death in cultures. However, a decrease in synaptophysin protein levels by Western blotting occured in all concentrations. These changes in synaptophysin levels were accompanied by JNK activation and ERK inhibition. We also observed a decrease in β-catenin protein immunocontent. The evaluation of GSK-3β and β-catenin phosphorylation showed no significant alterations. Although further studies are necessary for understanding the mechanisms underlying amyloid-β oligomers toxicity, our data suggest synaptic loss in organotypic cultures, one of the earlier characteristics of AD, may be considered a good model to study physiologic factors and pharmacologic compounds AD-related.

LISTA DE ABREVIATURAS

Aβ – beta-amiloide *(Amyloid-beta)*

Aβ1-40 – peptídeo beta-amiloide com aminoácidos 1-40

Aβ1-42 – peptídeo beta-amiloide com aminoácidos 1-42

AβOs – peptídeo beta-amiloide na forma de oligômeros

ADDL – ligantes difundíveis derivados do peptídeo β-amiloide (*Amyloid-beta derived diffusible ligands)*

AICD – domínio APP intracelular *(APP intracelular domain)*

APP – proteína precursora amiloide *(Amyloid precursor protein)*

ApoE – apolipoproteína E

ASPD – agregados esféricos do β-amiloide *(Amylospheroid)*

$sAPP_{\alpha}$ – forma secretada da APP_{α}

$sAPP_{\beta}$ – forma secretada da APP_{β}

BACE 1 – β-secretase *(β-site of beta-amyloid precursor protein cleaving enzyme 1)*

cdk5 – cinase dependente de ciclina-5 (*Cyclin-dependent kinase 5)*

CREB – proteína ligante ao elemento de resposta do cAMP (*cAMP-regulatory element binding)*

CTF83 – fragmento C-terminal da APP associado à membrana contendo 83 aminoácidos

CTF99 – fragmento C-terminal da APP associado a membrana contendo 99 aminoácidos

DA – Doença de Alzheimer

ENFs – emaranhados neurofibrilares

ERK – cinase regulada por sinais extracelulares *(Extracellular Signal-Regulated Kinase)*

FAD – Doença de Alzheimer Familiar *(Familial Alzheimer Disease)*

FDA – *Food and Drug Administration*

GSK-3β – glicogênio sintase cinase-3 beta *(Glycogen Syntase Kinase-3 beta)*

JNK – Proteína cinase c-Jun N-terminal *(Jun N-terminal Kinase)*

LPR1 – receptor de lipoproteína de baixa densidade 1 *(Low-density lipoprotein receptor-related protein 1)*

LTP – potenciação de longa duração *(Long Term-Potentiation)*

LOAD – Doença de Alzheimer de início tardio (*Late-onset Alzheimer's Disease)*

MAPK – proteína cinase ativada por mitógenos *(Mitogen-Activated Protein Kinase)*

MAPs – proteínas associadas aos microtúbulos *(Microtuble Associated Proteins)*

NFTs – emaranhados neurofibrilares (*Neurofibrilary tangles)*

NGF – receptor do fator de crescimento nervoso (*Nerve growth fator)*

NMDA – receptor de glutamato do tipo N-metil-D-aspartato

PHFs – filamentos helicoidais pareados *(Paired Helical Filaments)*

PI – iodeto de propídeo *(propidium iodide)*

PI3K – via da fosfatidilinositol-3-cinase

PKA – proteína cinase A (*Protein kinase A)*

PrP^c – proteína celular príon (*Cellular prion protein)*

PS – presinilina *(Presenilin)*

PSs – placas senis

SAPK – proteína cinase ativada por estresse *(Stress-Activated Protein kinase)*

Wnt – família de proteínas glicosiladas ricas em cisteína derivadas do gene *Wingless* de *Drosophila* e do gene *Int-1* de camundongo.

1. INTRODUÇÃO

1.1. Doença de Alzheimer: prevalência e principais características

A Doença de Alzheimer (DA) é o tipo mais comum de demência entre a população idosa, abrangendo entre 60 a 80% de todos os casos. De acordo com estudos epidemiológicos, entre 7 a 10% dos indivíduos com mais de 65 anos e 50 a 60% acima de 85 anos sofrem com a DA. São estimados que em torno de 35,6 milhões de pessoas apresentaram a DA no mundo em 2010, e que esse número tende a quase duplicar em 20 anos, passando para 65,7 milhões, podendo chegar a 115,4 milhões em 2050 (Mielke et al., 2014; Silva et al., 2014). O principal fator responsável pelo crescente número de pessoas diagnosticadas com DA é o aumento da expectativa de vida. Com os avanços nas áreas da saúde, a humanidade vem passando por uma transição demográfica nas últimas décadas, sendo que o perfil da população está se alterando de um predomínio de jovens e adultos para uma população idosa. De acordo com dados da Organização Mundial da Saúde (OMS), no Brasil, por exemplo, em 1990, a expectativa de vida ao nascer era de 63 anos, em 2012, aumentou para 70 anos. Os números relacionados à DA *per se* já denotam a importância desta patologia, somado a estes, sob o ponto de vista econômico, estima-se um custo de mais de 214 bilhões de dólares em 2014 somente nos Estados Unidos (US Alzheimer's Association, 2014).

Uma das primeiras manifestações clínicas de pacientes acometidos pela DA é a dificuldade em lembrar-se de informações adquiridas recentemente, devido à deterioração de domínios cognitivos seletivos, em especial aqueles relacionados com a memória (Laferla et al., 2007). Com o avanço da doença, os sintomas começam a ficar mais graves, incluindo desorientação, alterações

de humor e comportamento, dificuldade para falar, engolir e andar, que geram um comprometimento significativo da qualidade de vida dos pacientes, levando à dependência absoluta, hospitalização e à morte (Silva et al., 2014). Segundo o *US Food and Drug Administration* (FDA), apenas cinco medicamentos são aprovados para serem utilizados no tratamento da DA, sendo quatro deles inibidores da acetilcolinesterase e a memantina, um antagonista não-competitivo do receptor de glutamato do tipo N-metil-D-aspartato (NMDA - *N-methyl-D-aspartate*). Entretanto, estes se limitam apenas ao tratamento dos sintomas da doença, não impedindo o seu progresso.

As causas para o desenvolvimento da DA são bastante complexas, envolvendo uma combinação de fatores genéticos, moleculares e ambientais. Baseando-se na idade de início de aparecimento dos sintomas, a DA pode ser classificada em DA de início precoce ou familiar (FAD – *Familial Alzheimer's disease*, início < 65 anos) e DA de início tardio ou esporádico (LOAD - *Late-onset Alzheimer's disease,* início > 65 anos). O primeiro tipo é mais raro, acometendo entre 1 a 5% dos casos, e o segundo, mais de 95% do total (Mattson, 2004; Reitz e Mayeux, 2014a). Nos casos de FAD, estudos genéticos demonstram mutações nas presenilinas 1 e 2 (PS1 e PS2) e na proteína precursora amiloide (APP – *Amyloid Precursor Protein*) (Koo e Kopan, 2004; Bettens et al., 2013; Müller et al., 2013). Em relação aos casos de LOAD, apesar de o envelhecimento ser o principal fator de risco para o seu desenvolvimento, outros fatores estão relacionados, tais como: sexo feminino, obesidade, dislipidemia, baixa índice de escolaridade, diabetes, hipertensão e doenças metabólicas (Reitz e Mayeux, 2014a).

Além disso, mutações no alelo ε4 da apolipoproteína E (ApoE) foram confirmadas como fator que aumenta os riscos de desenvolvimento da forma esporádica da doença e também da forma familiar de herança autossômica dominante. Aproximadamente 50% dos pacientes que desenvolvem a forma esporádica da doença apresentam o alelo ε4 da ApoE. Por outro lado, a presença de uma cópia deste alelo aumenta em três vezes as chances de desenvolver a doença, enquanto que duas cópias em torno de doze vezes (Van Der Flier et al., 2011; Verghese et al., 2011). A ApoE participa da remoção do peptídeo β-amiloide (Aβ), via proteína relacionada ao receptor de lipoproteína de baixa densidade-1 (LPR1), formando um complexo ApoE/Aβ. Entretanto, o alelo ε4 da ApoE liga-se com uma menor afinidade ao peptídeo Aβ, reduzindo a capacidade de remoção do mesmo e promovendo seu acúmulo e formação das placas senis (Bu, 2009).

O cérebro de um paciente com DA apresenta uma redução de tamanho macroscopicamente visível, envolvendo regiões cerebrais relacionadas com o processo de aprendizagem e memória, incluindo os lobos frontal e temporal, resultado da intensa degeneração sináptica e morte neuronal (Mattson, 2004). Em nível molecular, trata-se de uma progressão complexa que envolve uma cascata de interações patológicas sequenciais, incluindo a agregação do peptídeo Aβ através da clivagem proteolítica da APP por β- e ɣ-secretases com o desenvolvimento de placas senis (PSs) extracelulares, e a hiperfosforilação e agregação da proteína *tau* formando os emaranhados neurofibrilares (ENFs) (Laferla et al., 2007; Zhang et al., 2012).

1.2. Proteína *tau*

A *tau* pertence à classe de proteínas associadas aos microtúbulos (MAP – *Microtubule Associated Proteins*) que, em condições fisiológicas, regula a estabilização, a dinâmica e a organização espacial dos microtúbulos. Tem sido demonstrado o envolvimento da *tau* no transporte axonal de organelas, incluindo a mitocôndria. A *tau* é altamente expressa no cérebro, principalmente em axônios de neurônios. Em situações patológicas, como ocorre na DA e em outras doenças neurodegenerativas denominadas taupatias, a *tau* torna-se altamente fosforilada e perde sua estabilidade, e assim, desprende-se dos microtúbulos. Principalmente no corpo celular e dendritos de neurônios, a *tau* hiperfosforilada se acumula e forma os filamentos helicoidais pareados (PHF – *Paired helical filaments*) que, através de um processamento proteolítico, acarreta a formação de oligômeros da *tau* e os ENFs (Figura 1) (Ittner e Götz, 2010; Querfurth e Laferla, 2010; Medina e Avila, 2014).

A hiperfosforilação da *tau* é um evento relacionado diretamente com o aumento da atividade de proteínas cinases e diminuição da atividade de fosfatases. Dentre as principais proteínas que fosforilam a *tau*, destacam-se a glicogênio sintase cinase-3β (GSK-3β), a proteína cinase dependente de ciclina 5 (cdk5), a proteína cinase A (PKA), as proteínas cinase mitógeno-ativadas (MAPK) ERK 1/2, bem como as proteínas cinases ativadas por estresse (SAPks). A perda de atividade da *tau* ocasionada pela hiperfosforilação compromete o transporte axonal e contribui para a disfunção sináptica e morte neuronal observada na DA (Mazanetz e Fischer, 2007; Querfurth e Laferla, 2010; Wang et al., 2013).

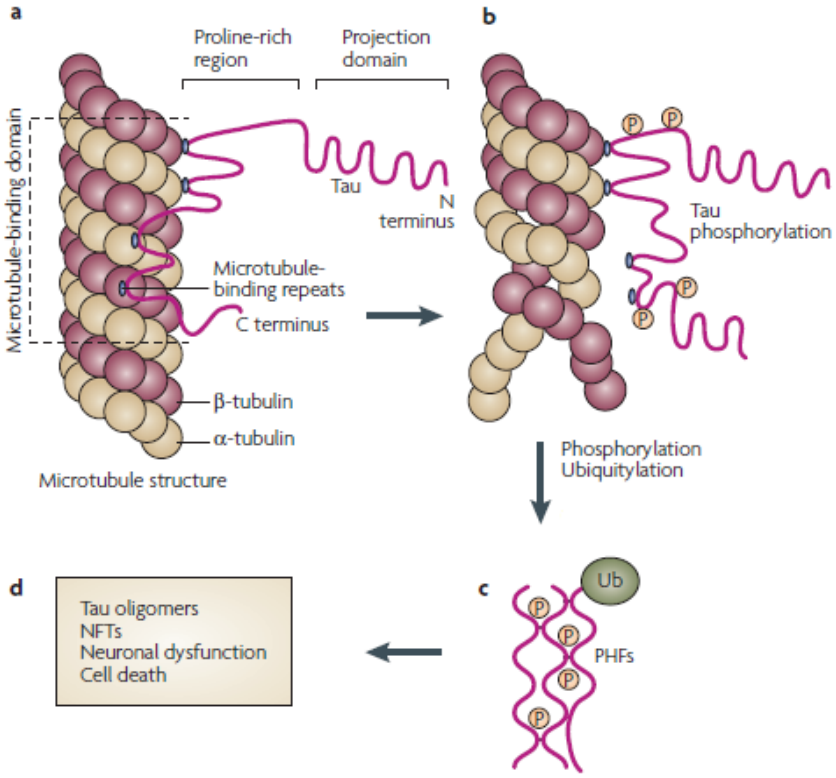

Figura 1 – Mecanismo de formação dos emaranhados neurofibrilares. a) A proteína *tau* ligada aos resíduos de α e β-tubulinas nos microtúbulos, exercendo um papel fundamental para estabilização destes. b) A fosforilação em sítios de serina/treonina altera a afinidade da *tau* com os microtúbulos, desestabilizando-os. c) Agregação da *tau* formando os filamentos helicoidais pareados (PHFs). d) Formação dos emaranhados neurofibrilares (NFTs) contribui para disfunção neuronal e morte celular (adaptada de Mazanetz e Fischer, 2007).

1.3. Peptídeo β-amiloide

O peptídeo Aβ é produzido através da clivagem proteolítica da APP, uma glicoproteína transmembrana amplamente expressa por todos os tipos celulares. A APP é uma proteína integral de membrana tipo I, com um amplo domínio extracelular, uma porção hidrofóbica transmembrana e um pequeno resíduo C-terminal denominado domínio intracelular da APP (AICD – *APP intracelular domain*). O gene da APP está localizado no cromossomo 21 e

contém 18 éxons. Existem 8 isoformas da APP que são geradas por *splicing* alternativo, alcançando um tamanho de 695 a 770 aminoácidos. Dentre estas isoformas, a de 695 aminoácidos (APP695) é a mais expressa no cérebro e é produzida principalmente pelos neurônios (O'brien e Wong, 2011; Zhang et al., 2012).

A APP sofre processamento por diversas secretases e proteases diferentes através de dois caminhos: o processamento amiloidogênico e o não-amiloidogênico. No processamento não amiloidogênico, a APP é sequencialmente clivada pela α-secretase e ɣ-secretase. A α-secretase cliva a APP no meio da sequência de aminoácidos (entre a Lis16-Leu17) no qual geraria o peptídeo Aβ, liberando um grande ectodomínio solúvel chamado sAPPα e um fragmento C-terminal associado a membrana contendo 83 aminoácidos (CTF83). O fragmento CTF83 é posteriormente clivado pela ɣ-secretase para liberar o peptídeo P3 e o AICD, sendo ambos degradados rapidamente. Alternativamente, a geração do peptídeo Aβ ocorre no processamento amiloidogênico através da clivagem da APP inicialmente pela enzima β-secretase ou *β-site APP cleaving enzime 1* (BACE1), formando um domínio extracelular chamado sAPPβ e um fragmento C-terminal associado a membrana contendo 99 aminoácidos (CTF99), que contém a sequência do Aβ e o AICD. Em seguida, a enzima ɣ-secretase cliva o fragmento CTF99 entre os aminoácidos 38 e 43 liberando o peptídeo Aβ (Figura 2) (Laferla et al., 2007; Zhang et al., 2011; Zhou et al., 2011; Zhang et al., 2012).

O peptídeo Aβ produzido a partir do processamento da APP varia no tamanho, sendo a maior produção da sequência contendo 40 aminoácidos (Aβ1-40), e em segundo, com um percentual de aproximadamente 10%, o

peptídeo com 42 aminoácidos (Aβ1-42). Este é a forma mais hidrofóbica e a que apresenta maior facilidade de formar fibrilas, sendo por isso a predominante nas placas senis. Na forma monomérica o peptídeo Aβ parece não exercer efeitos tóxicos. Porém, conforme estas formas monoméricas se polimerizam formando intermediários solúveis denominados oligômeros, e por fim protofibrilas e fibrilas, ele apresenta uma potente atividade bloqueadora da potenciação de longa duração (LTP) e afeta de forma significativa diversas vias de sinalização (Lambert et al., 1998; Laferla et al., 2007; Cavallucci et al., 2012). Quando ocorre um desequilíbrio onde a produção e agregação superam a degradação do peptídeo Aβ, ocorre um acúmulo do mesmo e este excesso pode ser um fator inicial da DA, desencadeando uma série de eventos celulares e moleculares que culminam na disfunção sináptica e na morte neuronal.

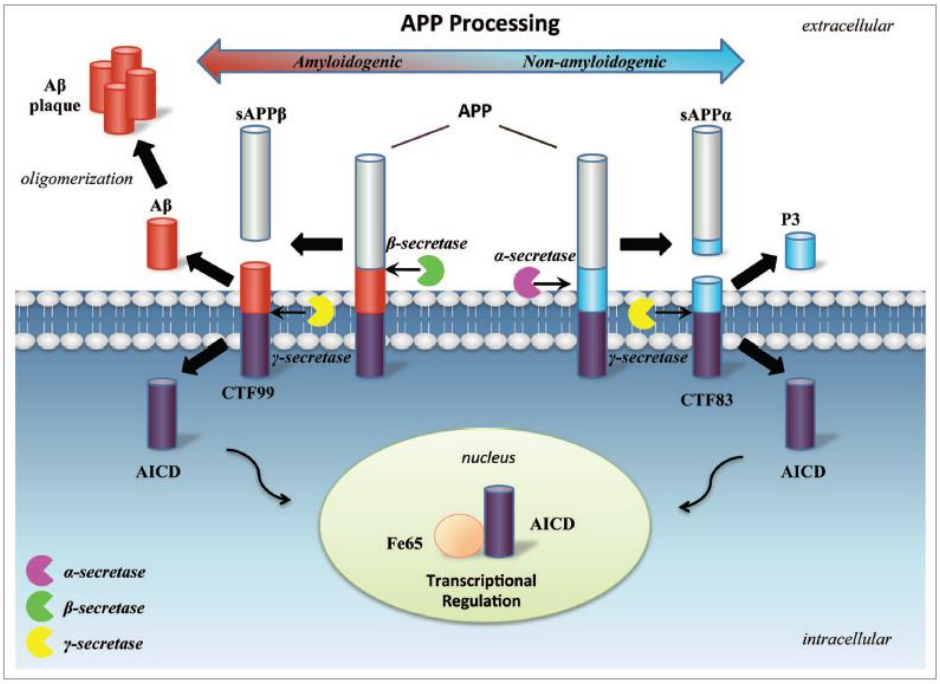

Figura 2 - Processamento proteolítico da APP. Processamento não-amiloidogênico da APP pelas enzimas α- e ɣ-secretases, sem a geração do peptídeo Aβ (direita). Processamento amiloidogênico da APP pelas enzimas β- e ɣ-secretases, formando o peptídeo Aβ de 38 a 43 aminoácidos (esquerda) (adaptada de Zhou et al., 2011).

1.4. A hipótese da cascata amiloide e a toxicidade dos oligômeros

O peptídeo Aβ está naturalmente presente no cérebro e fluido cefalorraquidiano de seres humanos saudáveis durante toda a vida. A hipótese da cascata amiloide surgiu em 1992, quando Hardy e Higgins (Hardy e Higgins, 1992) colocaram as fibrilas insolúveis do peptídeo Aβ como as primeiras espécies tóxicas na DA, e a formação dos emaranhados neurofibrilares, perda sináptica e morte das células neuronais como um evento secundário. A origem desta hipótese veio da descoberta de que uma das variantes da DA possui herança autossômica dominante, a partir de uma mutação no gene que codifica a APP e nas presinilinas, o que aumentaria a produção do peptídeo Aβ, evento suficiente para desenvolver a doença. Entretanto, esta hipótese não considerava a interação do Aβ com a proteína *tau.* Descobertas de que mutações no gene que codifica a proteína *tau* podem levar a um tipo de demência autossômica dominante no lobo frontotemporal, sem o aparecimento de placas senis, mostram que os emaranhados neurofibrilares podem causar perda neuronal *per se.* Essas observações colocaram as alterações patológicas na *tau* como um evento *downstream* da formação das placas senis (Hardy e Selkoe, 2002; Karran et al., 2011; Carrillo-Mora et al., 2014).

Embora a hipótese amiloide forneça um panorama geral para explicar a patogênese da DA, ela carece de detalhes, e algumas observações não se encaixam com a versão mais simples da hipótese. Primeiro, a quantidade de placas e sua distribuição se correlacionam fracamente com a severidade dos sintomas em pacientes com a DA (Perrin et al., 2009). Segundo, em vários modelos animais transgênicos para APP a perda neuronal e anormalidades

comportamentais têm surgido antes da formação das placas senis (Mucke et al., 2000). Em terceiro, a concentração de peptídeo Aβ necessária para fibrilação e neurotoxicidade são maiores que as concentrações fisiológicas (Gilbert, 2013). Com base nessas evidências e questionando a hipótese da cascata amiloide, uma nova versão foi elaborada, a hipótese dos oligômeros tóxicos de Aβ.

No final da década de 90, dois trabalhos mostraram que os oligômeros do peptídeo Aβ (AβOs – *Aβ oligomers*) são capazes de se correlacionar de forma mais consistente com os distúrbios cognitivos observados na DA, sendo capazes de mediar sua toxicidade distante das placas senis, pois ao contrário destas, são solúveis (Lue et al., 1999; Mclean et al., 1999; Larson e Lesné, 2012). O mecanismo de formação dos AβOs ainda não é totalmente elucidado. Diferentes tamanhos e formatos já foram descritos com vias de formação diferentes, indicando a complexidade do mecanismo de formação, como pode ser observado na Figura 3. Além disso, a formação parece também diferir para oligômeros intracelulares e extracelulares (Sakono e Zako, 2010; Benilova et al., 2012).

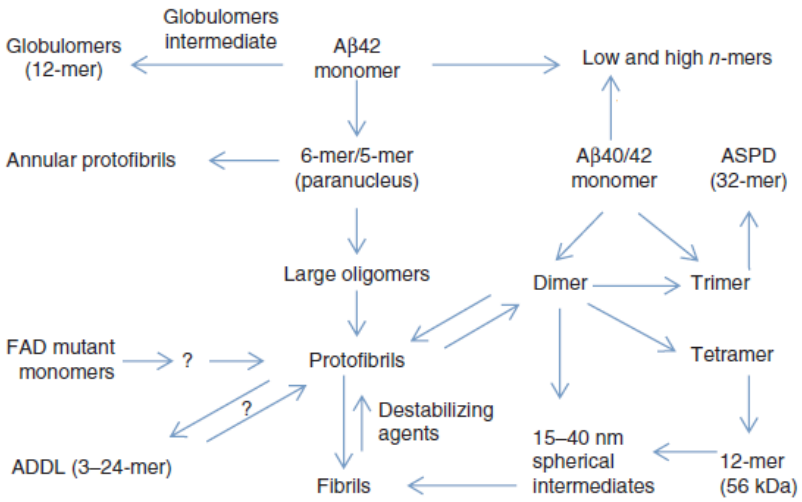

Figura 3 – Esquema de interconversão entre as diferentes espécies naturais e sintéticas do peptídeo Aβ. Monômeros, oligômeros e fibrilas de Aβ existem em um complexo equilíbrio. A coexistência de populações diferentes de oligômeros que podem ou não converter-se em fibrilas é possível. Apesar das diferenças estruturais, estabilidade e concentração, todos os oligômeros podem contribuir para toxicidade do peptídeo Aβ (adaptada de Benilova et al., 2012).

A toxicidade dos AβOs está bem documentada. Por exemplo, baixas concentrações de dímeros e trímeros solúveis do peptídeo Aβ são capazes de reduzir a densidade dos espinhos dendríticos e o número de sinapses eletrofisiologicamente ativas em neurônios piramidais de culturas de hipocampos de ratos. Além disso, são capazes de ativar receptores de glutamato NMDA, induzindo a depressão de longa duração (Mclean et al., 1999; Benilova et al., 2012; Cavallucci et al., 2012). Diversos outros receptores proteicos têm sido apontados como capazes de se ligarem as várias formas dos AβOs, incluindo os receptores de insulina, o receptor do fator de crescimento nervoso (NGF – *Nerve growth factor*), a proteína celular príon (PrP^c – *Cellular prion protein*) e os receptores *Frizzled*, contribuindo para as alterações fisiológicas que levam a morte celular (Figura 4) (Sakono e Zako, 2010; Benilova et al., 2012; Giuffrida et al., 2012; Patel e Jhamandas, 2012).

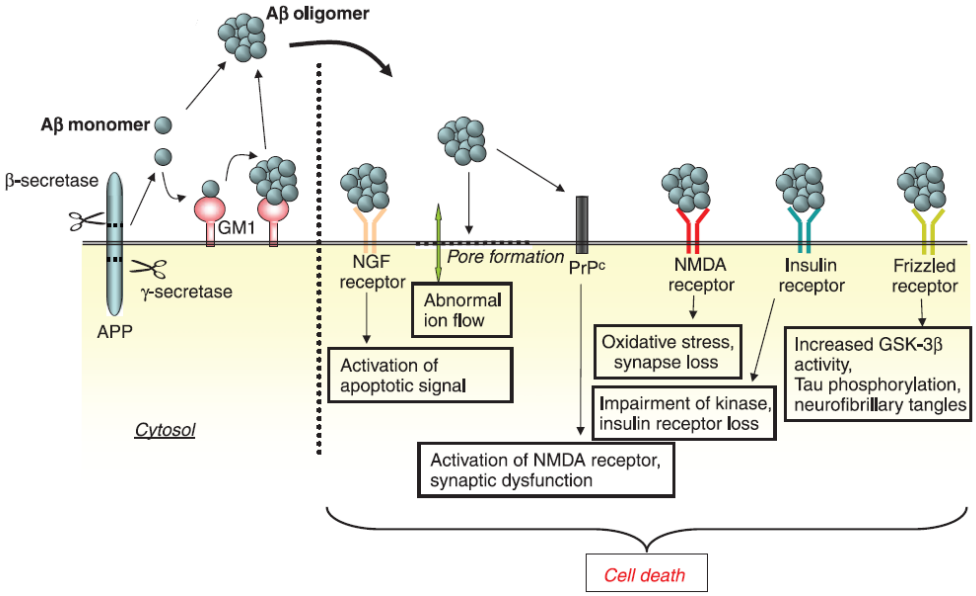

Figura 4 – Mecanismos de toxicidade de oligômeros extracelulares. Após a clivagem da APP, o peptídeo Aβ é liberado para o meio extracelular. Os oligômeros podem ser formados na presença do gangliosídeo GM1 na membrana celular e causar morte celular mediada pelo receptor do fator de crescimento nervoso (NGF). A proteína celular príon (PrP^c) pode atuar como um receptor dos oligômeros mediando a disfunção sináptica. O poro na membrana pode ser formado pelos oligômeros, causando um influxo de Ca^{2+}, provocando disfunção celular. Além disso, a toxicidade dos oligômeros pode ser mediada através da ligação aos receptores NMDA, de insulina e Frizzled (adaptada de Sakono e Zako, 2010).

1.5. Sinalização celular e oligômeros do peptídeo Aβ

Boa parte do mecanismo de homeostase das células passa pelo bom funcionamento das vias de sinalização intracelulares, ou seja, o controle inadequado dessas vias pode ser responsável pelo desencadeamento ou agravo de diversas patologias, entre elas as doenças neurodegenerativas, incluindo a DA. As vias de sinalização envolvidas na DA foram resumidas em um mapa denominado *AlzPathway*, onde pode ser observada a complexidade da sinalização intra, inter e extracelular envolvida na doença. O mapa é composto por 1347 espécies (proteínas, complexos, moléculas simples, RNAs,

genes) e 1070 reações (Ogishima et al., 2013), e pode ser acessado em alzpathway.org.

As alterações cognitivas associadas aos primeiros estágios da DA são atribuídas às falhas sinápticas (Selkoe, 2002; Coleman e Yao, 2003; Bate e Williams, 2010). Além disso, diversos trabalhos atribuem às alterações sinápticas um dos principais mecanismos de toxicidade dos AβOs (Lacor et al., 2007; Overk e Masliah, 2014). Diversas vias de sinalização ao sofrem alteração no seu funcionamento, podem levar à perda sináptica, tais como a via das MAPKs, destacando as proteínas JNK (c-*Jun N-terminal kinase*) e ERK1/2 (Kim e Choi, 2010; Sclip et al., 2011; Sclip et al., 2014). Além desta, a via da fosfatidilinositol-3-cinase (PI3K/Akt) apresenta um grande papel na sobrevivência celular e também na plasticidade sináptica (Kim e Chung, 2002; Horwood et al., 2006; Chiang et al., 2010), sendo diretamente influenciada pela exposição ao peptídeo Aβ (Figura 5). Dentro desta via, a GSK-3β é a principal cinase envolvida na fosforilação da proteína *tau*, é uma proteína altamente expressa no cérebro, está envolvida em importantes processos celulares e em uma variedade de vias intracelulares, incluindo diferenciação celular e apoptose. A GSK-3β é considerada uma proteína pró-apoptótica por inibir uma série de fatores de transcrição importantes para sobrevivência celular (Li et al., 2002; Reddy, 2013).

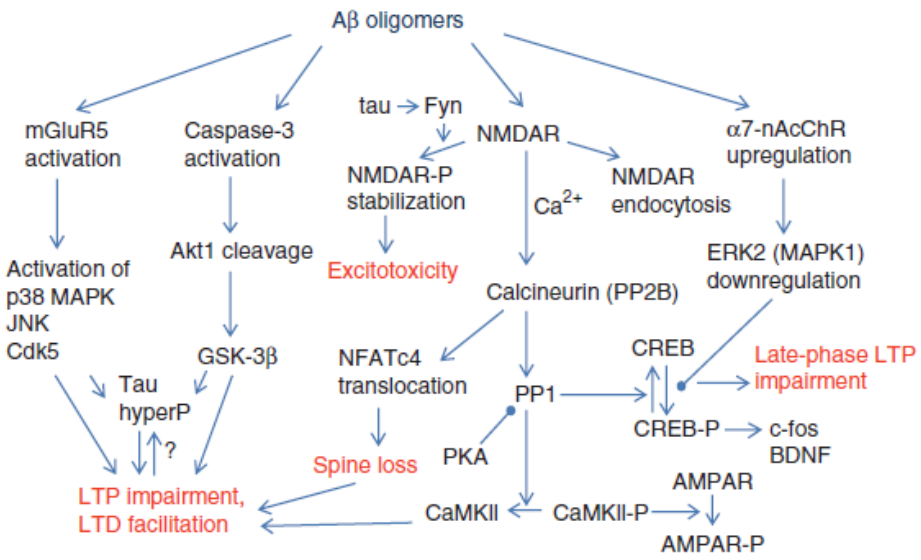

Figura 5 – Mecanismos moleculares propostos na literatura para a sinaptotoxicidade do peptídeo Aβ. O diagrama ilustra algumas vias de sinalização que são requeridas em diferentes paradigmas experimentais para explicar a sinaptotoxicidade. Ainda não existe um consenso sobre qual mecanismo seria mais relevante na DA (adaptada de Benilova et al., 2012). Abreviaturas: mGluR5, receptor metabotrópico de glutamato 5; AMPAR, receptor α-amino-3-hidroxil-5-metil-4-isoxazolepropionato; CaMKII, proteína cinase dependente de cálcio/calmodulina II; PKA, proteína cinase A; PP1, proteína fosfatase 1; NFATc4, fator nuclear de células T ativadas; α7-nAcChR, receptor nicotínico de acetilcolina α7; BDNF, fator neurotrófico derivado do cérebro.

Além disso, a GSK-3β está envolvida na regulação dos níveis da proteína β-catenina, que faz parte da via canônica da Wnt (Inestrosa et al., 2012). Na sua forma ativada, a GSK-3β atua fosforilando a β-catenina, desencadeando sua ubiquitinação e encaminhando-a para degradação no proteassoma, diminuindo desta forma sua atividade. Na forma desfosforilada, a β-catenina apresenta estabilidade e é capaz de se translocar para o núcleo

ativando uma série de fatores de transcrição, levando a efeitos neuroprotetores frente à toxicidade do peptídeo Aβ (Inestrosa et al., 2012; Purro et al., 2014; Vargas et al., 2014).

1.6. Modelos experimentais de toxicidade do peptídeo Aβ

Com finalidade de se obter uma melhor compreensão sobre os mecanismos envolvidos na fisiopatologia da DA, a busca por modelos *in vivo* e *in vitro* de toxicidade do peptídeo Aβ, que mimetizem ao máximo as alterações encontradas na doença, segue como um desafio para os pesquisadores. Devido ao alto custo desses modelos, peptídeos sintéticos do Aβ já foram desenvolvidos e surgem como importante ferramenta para o estudo desta patologia.

Dentre os modelos utilizados para compreensão dos mecanismos envolvidos na neurotoxicidade do peptídeo Aβ, modelos em animais com injeção intracerebral do peptídeo Aβ têm sido utilizados com a finalidade de observar as alterações comportamentais e fisiológicas, além de ser um modelo utilizado para o teste de substâncias possivelmente terapêuticas (Bernardi et al., 2012; Frozza et al., 2013; Hoppe, Juliana B et al., 2013).

Nos modelos *in vitro*, o cultivo de tecido é uma importante alternativa aos modelos com animais, incluindo-se neste a cultura organotípica de hipocampo (Frozza et al., 2009; Hoppe et al., 2010). Ao contrário do cultivo celular que permite a análise em um conjunto de células isoladas, a cultura organotípica combina a acessibilidade com a preservação da multiplicidade celular original do tecido cerebral e das conexões interneurais (Noraberg et al.,

2005). Dessa forma, a utilização desse modelo permite o estudo molecular das vias de sinalização envolvidas com a morte neuronal e danos na plasticidade sináptica induzida pela toxicidade do peptídeo Aβ (Holopainen, 2005).

2. OBJETIVOS

2.1. Objetivo Geral

Avaliar os mecanismos moleculares de toxicidade dos AβOs em culturas organotípicas de hipocampo de ratos.

2.2. Objetivos específicos

a) Avaliar a morte celular induzida pelos AβOs nas culturas organotípicas através da incorporação do iodeto de propídeo.

b) Avaliar algumas das vias de sinalização que poderiam estar envolvidas na toxicidade dos AβOs, tais como PI3K, Wnt/β-catenina, MAPK, bem como o imunoconteúdo da proteína sinaptofisina.

PARTE II

3. CAPÍTULO I

Artigo científico a ser submetido ao periódico *Neurobiology of aging*

Evaluation of the molecular mechanisms of Amyloid-β oligomers toxicity in organotypic hippocampal slice cultures

André Meneghetti, Juliana Bender Hoppe, Leon de Morais Lisboa, Bruna de Melo Menezes, and Christianne Gazzana Salbego*.

Department of Biochemistry, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul – UFRGS, Porto Alegre, Brazil.

* Correspondig author:

Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS.

Av. Ramiro Barcelos, 2600 – anexo, Porto Alegre, RS, CEP 90035-003, Brasil.

Tel.: +55 51 3308 5547

E-mail address: salbego@terra.com.br

Abstract

Senile plaques, composed of the Aβ peptide, are a hallmark of Alzheimer's disease (AD). A wide variety of evidences support the idea that Aβ peptide is at least one of the main causes of AD. Aβ oligomers peptides (AβOs) are able to correlate more consistently than fibrils with cognitive disturbances and synaptic changes observed in AD. In this work, we evaluated the molecular mechanisms of AβOs toxicity in organotypic hippocampal slice cultures. Cell death was assessed by propidium iodide incorporation. Exposure to AβOs for 48h was not able to induce a significant cell death in cultures. However, a decrease in synaptophysin levels was detected by Western blotting. These changes were accompanied by JNK activation and ERK inhibition, as well as a decrease in β-catenin immunocontent. The evaluation of GSK-3β and β-catenin phosphorylation showed no significant alterations. Although further studies are necessary for understanding the mechanisms underlying AβOs toxicity, our data suggest synaptic loss, one of the earlier characteristics of AD, and may be considered relevant to study physiologic factors and pharmacologic compounds AD-related.

Keywords: Alzheimer's disease; amyloid-β oligomers; organotypic culture; MAPK.

1. Introduction

Alzheimer's disease is the most common neurodegenerative disorder in the elderly. It is clinically characterized by memory loss and progressive deterioration in cognition, function, and behavior (Reitz e Mayeux, 2014b; Silva et al., 2014). The clinical symptoms result from deterioration of selective cognitive domains, particularly those related to memory, such as the hippocampus and cortex (Laferla et al., 2007). These regions are reduced in size in AD patients as the result of synaptic degeneration and neurons death (Mattson, 2004). At molecular level, there is a complex cascade involving several pathologic sequential interactions that include the formation of intracellular neurofibrillary tangles (NFTs), which resulted from the paired helical filaments of hyperphosphorylated *tau* protein, and extracellular deposits of fibrillar amyloid-β (Aβ) peptide into dense senile plaques (Zhang et al., 2011).

In accord to amyloid cascade hypothesis, neurodegeneration in AD begins from the abnormal processing of amyloid precursor protein (APP) resulting in the production, aggregation, and deposition of Aβ peptide into senile plaques. These deposits of Aβ peptide were considered the first toxic species in AD, and the formation of NFT, synaptic loss and neuronal cell death as a secondary event (Hardy e Selkoe, 2002; Karran et al., 2011). Although the amyloid cascade hypothesis provides an overview to explain the pathogenesis of AD, it lack details, and some observations do not correspond to the simplest version of the hypothesis. The most important is that the amount and distribution of plaques weakly correlated with the severity of symptoms in patients with AD (Perrin et al., 2009). Moreover, in several APP transgenic

animal models, neuronal loss and behavioral abnormalities were shown to occur before plaque formation (Gilbert, 2013). Finally, Aβ fibrils derived from AD brain did not cause consistent *in vitro* toxic effects in rat and human neurons (Gschwind e Huber, 1995). Based on these evidences questioning the amyloid hypothesis, a new evidence was developed, the toxic Aβ oligomers (AβOs) hypothesis began to emerge.

According to this new hypothesis, between the monomeric Aβ and the Aβ fibrils in the plaques, intermediary soluble oligomers must exist and they might explain the neurotoxicity related to soluble fractions in many experiments (Benilova et al., 2012). In the late 90s, two studies showed that AβOs were able to correlate more consistently with cognitive disturbances and synaptic losses observed in AD, being able to mediate its toxicity away from senile plaques, since unlike them, AβOs are soluble (Lue et al., 1999; Mclean et al., 1999). The mechanism formation of AβOs *in vivo* is still not fully understood. Many types of natural and synthetic AβOs of different sizes and shapes have been reported, which accounts for their biologic and structural diversity and for the complexity of AD pathology (Glabe, 2008; Sakono e Zako, 2010). Cognitive changes associated with early stages of AD are attributed to synaptic loss. In addition, several reports consider that synaptic alterations may be the major mechanism of AβOs toxicity (Selkoe, 2002; Bate e Williams, 2010). Synthetic or AβOs extracted from cerebral cortex of humans with AD can disrupt molecular processes involving long-term potentiation (LTP) in hippocampal slices (Shankar et al., 2008; Pozueta et al., 2013).

Access to central nervous system is a limiting factor to study neurodegenerative diseases. Organotypic slice cultures, widely used in our

group to investigate neuronal death associated with oxygen and glucose deprivation (Simão et al., 2012; Simões Pires et al., 2014) and Aβ fibrils toxicity (Hoppe, Juliana Bender et al., 2013a; Hoppe, Juliana Bender et al., 2013b), are a valuable alternative to animal experiments, because they combine accessibility and maintenance of *in vitro* culture systems while preserving intact hippocampal synaptic circuitry and anatomy (Stoppini et al., 1991; Bruce et al., 1996).

Although many studies have reported the toxicity mechanisms of AβOs, there are few works showing their effect on organotypic hippocampal slice cultures. In the present study, we investigated some signaling pathways in hippocampal slices exposed to AβOs. Therefore, we decided to focus on signaling pathways that could be involved in the synaptic loss, as the Extracellular signal-Regulated Kinases (ERK1/2) and c-Jun *N*-terminal kinase (JNK), members of the mitogen-activated protein kinases (MAPK) family. We also investigated the immunocontent and phosphorylation of β-catenin and Glycogen synthase kinase-3β (GSK-3β), members of phosphatidylinositide 3-kinases pathway (PI3K), as well as synaptophysin levels.

2. Materials and methods

2.1. Organotypic hippocampal culture

All procedures used in the present study followed the 'Principles of Laboratory Animal Care' from NIH publication No. 85-23 and were approved by the Ethics Committee on the Use of Animals (CEUA-UFRGS, protocol number

23521). Organotypic hippocampal cultures were prepared from 6- to 8-day-old male Wistar rats, according to the method of Stoppini et al. (1991), with some modifications. Briefly, the animals were euthanized, the brains were removed, the hippocampi were isolated, and transverse hippocampal slices (400 µM thickness) were obtained using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Guildford, UK). Slices were separated in a Hank's balanced salt solution (HBSS) (Gibco) supplemented with 25 mM HEPES, Fungizone® 1% and 0.100 mg/mL Gentamicin, pH 7.2. The slices were placed on membrane inserts (Millicell- CM culture plate inserts, 0.4 µm) and transferred to a six-well culture plates (Cell Culture Cluster, TPP).Each well contained 1mL of culture medium consisting of 50% minimum essential medium (MEM) (Gibco), 25% heat inactivated horse serum (Gibco) and 25% HBSS (Gibco), supplemented with glucose 36 mM, glutamine 2 mM, HEPES 25 mM, and $NaHCO_3$ 4 mM (final concentrations). Fungizone® 1% and Gentamicin 0.100 mg/mL were added to the medium, and the pH was adjusted to 7.3. Organotypic cultures were maintained in a humidified incubator gasified with $5\%CO_2/95\%O_2$ atmosphere at 37°C. Culture medium was changed twice a week and experiments were carried out after 21 days *in vitro*.

2.2. Preparation and characterization of AβOs

The AβOs were prepared according to Lambert et al. (2001) with some modifications. Aβ1-42 peptide (American Peptide, Sunnyvale, CA, USA) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to a final concentration of 1 mM to establish monomerization and randomization of structure. After that,

solution was aliquotted to microcentifuge tubes and aged 24h in room temperature to evaporate the HFIP and the residual was removed by evaporation under vacuum in a SpeedVac; the tubes were stored at -40 ºC. Prior to use, an aliquot of Aβ1-42 was dissolved in anhydrous, sterile dimethyl sulfoxide (DMSO) to make a 5 mM solution, which was diluted to 100 µM with phosphate-buffered saline (PBS), pH 7.4. This solution was incubated at 4ºC for 24h and then centrifuged at 14.000 *g* for 10min to remove any insoluble aggregates. The supernatant containing soluble AβOs was transferred to clean tubes and stored at 4 ºC. Oligomer solutions were used within 24h of preparation. Protein concentration was determined using BCA assay kit (Pierce® BCA Protein Assay kit, Rockford, IL, USA).

Routine characterization of oligomer preparations was performed by Western blotting using a monoclonal antibody 6E10 (Covance, Emeryville, California), that recognize Aβ residues 1-16 (De Felice et al., 2008). Samples were mixed with Tricine sample buffer and resolved on a 4-20% Tris-Glycine gel (Novex®, Cartsbad, CA, USA) at 120V for 80 min at room temperature. The gel (10 µg of Aβ/lane) was electroblotted onto Hybond ECL nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany) using a semi-dry transfer apparatus (Bio-Rad, Trans-Blot SD, Hercules, CA, USA) at 15 V for 50 min. The blots were blocked with 5% nonfat milk in Tris-buffered saline Tween 20 (TBS-T; 0.1% Tween 20) for 1h at 4 ºC. Anti-Aβ monoclonal antibody 6E10 (1:2000) was diluted in 5% milk/TBS and incubated with the blots overnight at 4 ºC. Following three 5-min washes with TBS-T, the blots were incubated with horseradish peroxidase-linked anti-mouse IgG antibody (1:1000) for 2h. The

blots were washed five times for 5 min with TBS-T and the chemiluminescence was detected using X-ray films.

2.3. Culture treatment

Organotypic cultures treatment occurred after 21 days *in vitro*. At the 21st day, the cultures were treated with 0.5, 1, and 2 μM of AβOs for 48h. Vehicle group received PBS with 2% of DMSO in the same volume of the higher concentration of AβOs.

2.4. Quantification of Cellular Death

Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI, Calbiochem, San Diego, CA, USA) uptake (Noraberg et al., 2005). PI is a polar compound that is impermeable to an intact cell membrane, but it penetrates damaged cell membranes of dying cells and binds to nuclear DNA to generate a bright red fluorescence. Organotypic cultures were incubated with 5 μM PI 1h before the end of the treatment with AβOs and then were observed with an inverted microscope (Nikon Eclipse TE 300) using a standard rhodamine filter. Images were captured and analyzed using Scion Image software (www.scioncorp.com). The area where PI fluorescence was detectable above background levels was determined using the ‘‘density slice’’ option of Scion Image software and compared to the total slice area to obtain the percentage of damage. PI intensity, meaning cell death, was expressed as a percentage of cell damage: cell death (%) = *Fd/Fo x* 100, where *Fd* is the PI

uptake fluorescence of dead area oh hippocampal slices ad *Fo* is the total area of each hippocampal slice (Hoppe et al., 2010).

2.5. Western blotting analysis

After obtaining fluorescent images, slices were homogenized on lyses buffer (4% sodium dodecylsulfate [SDS], 2 mM EDTA, 50 mMTris), aliquots were taken for protein determination and β-mercaptoethanol was added to a final concentration of 5%. Equal amounts of proteins were resolved (30µg per lane) on 12% SDS-PAGE. After electrophoresis, proteins were electro transferred to nitrocellulose membranes using a semi-dry transfer apparatus. Membranes were incubated for 60 min at 4ºC in blocking solution (Tris-buffered saline containing 5% powdered milk and 0.1% Tween-20, pH 7.4) and further incubated with the appropriate primary antibody dissolved in the blocking solution overnight at 4ºC. Primary antibodies anti-phospho-GSK3β Ser^{9} (1:1000; Cell Signaling Technology Beverly, MA, USA), anti-GSK3β (1:1000; Cell Signaling Technology), anti-phospho-JNK (1:1000; Cell Signaling Technology), anti-JNK (1:1000; Cell Signaling Technology), anti-phospho-ERK (1:1000; Cell Signaling Technology), anti-ERK (1:1000; Cell Signaling Technology), anti-phospho-β-catenin Ser45 (1:1000; Sigma Aldrich), anti-β-catenin (1:1000; Sigma-Aldrich), anti-synaptophysin (1:4000; Millipore®, Bradford, MA, USA) and anti-β-actin (1:4000; Sigma-Aldrich) were used. The membranes were then incubated with appropriate secondary antibody horseradish peroxidase-conjugated (1:1000) for 2h. The chemiluminescence was accessed using ECL™ Prime Western Blotting Detection Reagent (GE

Healthcare UK) and detected using ImageQuant LAS 4000 (GE Healthcare Life Sciences). Data are expressed as percentage of control cultures.

2.6. Data analysis

The results were presented as the mean ± SEM. The statistical comparisons of the data were performed by One-way analysis of variance (ANOVA) followed by Tukey post-hoc test using Graph Pad Prism software version 5.01 (Graph Pad Software Inc. La Jolla, CA, USA). *P*-values lower than 0.05 ($P<0.05$) were considered significant.

3. Results

3.1. Characterization and toxicity of amyloid-β oligomers

Each preparation was evaluated by SDS-PAGE to confirm the presence of soluble oligomers; gels were processed for Western blotting using 6E10 monoclonal antibody, a non-selective antibody that binds all forms of Aβ (Lambert et al., 2007). As can be observed (Fig. 1A), gels routinely showed Aβ monomers and SDS-resistant oligomers, including low molecular weight trimers and tetramers, as well as high molecular weight oligomers (~50 a 150KDa), but not fibrils (Fig. 1A). This result is in according with other studies (De Felice et al., 2008; Sebollela et al., 2012).

The analyses of cell death showed that the exposure of cultures to different concentrations of AβOs (0.5, 1, and 2 μM) for 48h did not cause a

significant increase of PI fluorescence in hippocampal slices (Fig. 1B and C). Quantification of PI fluorescence showed that concentrations of 0.5, 1, and 2μM of AβOs caused around 1.9, 2.3 and 2.2% of damage, respectively, which was not a significant increase when compared to controls cultures (about 0,8% of cellular damage). The vehicle group showed similar values of controls (data not shown).

3.2. AβOs cause synaptic dysfunction

Despite the fact that AβOs did not cause significantly cell death, to further evaluate the neuronal integrity, we performed Western blotting analysis for the presynaptic protein synaptophysin (a specific pre-synaptic marker). A significant reduction in the synaptophysin levels was found in all concentrations of AβOs-used, after 48h of culture exposure. The treatment with 0.5 and 1 μM of AβOs significantly decreased the immunocontent of synaptophysin to approximately 74 and 77%, respectively (P<0.05), when compared to controls. Cultures treated with 2 μM of AβOs presented a reduction about 70% in the levels of synaptophysin (P<0.01), compared to control (Fig. 2A and B).

3.3. ERK1/2 inhibition and activation of JNK1/2 pathway plays a role in AβOs toxicity

ERK1/2 are ubiquitously expressed hydrophilic non-receptor proteins that participates in the Ras-Raf-MEK-ERK signal transduction cascade, denoted as the MAPK cascade (Wortzel e Seger, 2011). This cascade participates in the

regulation of a variety of process including cell survival, differentiation, metabolism, proliferation and transcription (Roskoski Jr, 2012). JNK also belongs to MAPK family, and it is activated in response to a wide range of cellular stresses, as well as, in response to inflammatory mediators (Mehan et al., 2011). Cultures exposure to 0.5 μM of AβOs significantly decreased phospho-ERK1/2 (42-44 kDa) levels when compared to controls (P<0.05). Treatment with 1 and 2 μM of AβOs presented a stronger decrease in phospho-ERK1/2 levels (approximately 50%; P<0.01) (Fig. 3A and B). As shown in Fig 3C and D, the exposure to 1 and 2 μM significantly induced an increase on JNK1/2 (46-54 KDa) phosphorylation when compared to control cultures (P<0,05).

3.4. AβOs had no effect on GSK-3β phosphorylation

Considering that GSK-3β is one of the most important kinases that phosphorylate *tau* protein, we decided to investigate the effect of AβOs on its phosphorylation/inativation state. Western blotting was performed with antibodies against the inactive form of GSK-3β, phosphorylated at Ser9, as well as against its total immunocontent (Fig. 4A). As presented in Fig. 4B, cultures exposure to all three concentrations of AβOs for 48h did not cause any significant difference in phospho-GSK-3β intensity, although a tendency could be observed at the concentration of 1 μM.

3.5. Effect of AβOs on β-catenin phosphorylation and total immunocontent

We next ascertained the effect of AβO on intracellular signaling proteins, which are critical for cell survival. Wnt pathway disruption represents a pivotal event in synaptic plasticity impairment in the adult brain, and it is well establish that regulation of β-catenin stability is a crucial control mechanism in Wnt signaling (Inestrosa et al., 2012). We analyzed, by Western blotting, the effect of AβOs on β-catenin levels and phospho-β-catenin levels. AβOs treated slices did not show significantly alteration on phosphorylated β-catenin levels, although a tendency could be observed at the concentration of 1 μM (Fig. 5A and B). However, the culture exposure to 2 μM of AβOs caused a significant decrease in the total immunocontent of β-catenin (P<0.05; Fig. 5C and D).

4. Discussion

Neurodegenerative diseases are one of the most enigmatic problems in medicine, among them AD is distinguished by its high prevalence in the elderly population. Although basic and clinical research advances over the past decades have provided progress about understanding on course of AD, a full understanding of the mechanisms involved in this disease are still far from being elucidated. The amyloid cascade theory is the most accepted by AD researchers, it postulates that gradual changes in Aβ peptide aggregation initiate the cascade of neuronal and inflammatory injuries that culminate into extensive neuronal dysfunction and cellular loss and may lead to dementia (Selkoe, 1991; Laferla et al., 2007). Synaptic loss strongly correlates with AD-associated cognitive deficits (Selkoe, 2002; Nisticò et al., 2012), however the mechanism responsible for connection losses is not established. First, synaptic

loss was thought to occur because insoluble amyloid fibrils, but recently, it has been hypothesized that synapse dysfunction and degeneration are the early consequences of soluble Aβ oligomers (AβOs) (Klein, 2006; Benilova et al., 2012).

In this study, we evaluated the molecular mechanisms of AβO-induced neurotoxicity using an organotypic hippocampal slice culture model. We verified the toxicity of AβOs by propidium iodide incorporation and by Western blotting to proteins that could be involved in synaptotoxicity. Although the incorporation of propidium iodide has increased when organotypic cultures were exposed to 0.5 to 2 μM of AβOs, this was not significant in our experimental conditions. These results are similar with those found by Chong et al. (2006), despite differences in technique. Despite the fact that we have not observed cellular death, we decided to analyze the immunocontent of synaptophysin, the major synaptic vesicle protein, and the results showed a decrease in this synaptic marker. This effect appears to be mediated via modulation of cellular signaling ERK1/2 and JNK, as well as β-catenin.

The mammalian family of MAPKs includes ERK1/2, p38, and JNK. Members of MAPK family play crucial roles in regulating responses to various stresses, in neuronal development, inflammation and apoptosis (Kim e Choi, 2010). The JNK signaling pathway is activated by pro-inflammatory cytokines or in response to cellular stresses such as genotoxic, osmotic or oxidative stress. The activation of JNK has been described in cultured neurons after Aβ exposure, and its inhibition attenuates Aβ toxicity (Bozyczko-Coyne et al., 2001; Morishima et al., 2001). Furthermore, JNK regulates various processes such as brain development, repair and memory formation. Additionally, JNK is closely

associated with neuronal dysfunction in AD (Mehan et al., 2011). Sclip et al. (2014) showed that JNK is activated at the spine prior to the onset of cognitive impairment and synaptopathy in a transgenic *in vivo* AD model. Our results have shown that when organotypic cultures were exposed to AβOs, JNK activity becomes increased. These results suggest that JNK pathway could be involved in the synaptic loss observed in organotypic cultures after AβOs exposure, however we may further investigate this mechanism.

ERK pathway, one of the three best-characterized MAPKs pathway, is emerging as an important regulator of neuronal function, since ERK1/2 are abundant in adult brain and its pathway can play multiple roles in the activity-dependent regulation of neuronal function (Zhu et al., 2003). At cellular level, ERK1/2 regulate a diverse array of functions including cell growth and proliferation, differentiation and cell survival or apoptosis, being considered to act as an anti-apoptotic factor (Rosen et al., 1994; Ghasemi et al., 2014). Additionally, in the brain, strong evidences suggest that ERK1/2 are important components of activity-dependent signaling cascades within neurons and that modulation of their activity may be required for both synaptic plasticity and learning and memory (Sweatt, 2001). The activity and expression of β-secretase (BACE 1), the endoprotease essential for Aβ peptide production, seems to be negatively modulated by activated ERK1/2 pathway (Tamagno et al., 2009). However, the Aβ effects in ERK1/2 activity are still controversial. Some studies have shown a decrease in ERK activity after Aβ exposure (Bell et al., 2004; Townsend et al., 2007; Li et al., 2011), while other demonstrated the opposite effect (Chong et al., 2006; Ghasemi et al., 2014), which could be explained by different levels of aggregation, concentration, brain area and/or

time exposure to Aβ. In our study, we observed that organotypic cultures exposure to AβOs leads to a decrease in ERK1/2 activity. Considering the fact that, among other functions, ERK plays a role in regulating neuronal gene expression, its inactivation may decrease the transcription of several factors, such as cAMP-regulatory element binding (CREB), by decreasing its phosphorylation (Adams et al., 2000; Benilova et al., 2012). Thus, CREB-mediated transcription of genes responsible for synaptic plasticity is altered, resulting in damage to cognitive functions (Zhang et al., 2013).

In an attempt to better explain the synaptotoxicity observed in organotypic cultures after AβOs exposure, we evaluated two important signaling pathways implicated in synaptic loss: PI3K, represented by GSK-3β, and Wnt/β-catenin. GSK-3β is an abundant protein in the central nervous system and has been shown to be a key component in signaling pathways that underlie neurodegeneration (Balaraman et al., 2006). Recent studies revealed that elevated GSK-3β activity is directly linked to increase levels of Aβ production and Aβ deposits, *tau* hyperphosphorylation and synaptic damage in AD patients and AD animal models (Kremer et al., 2011; Darocha-Souto et al., 2012; Hurtado et al., 2012; Reddy, 2013). Beyond the fact that GSK-3β is the major contributor to *tau* phosphorylation (Ryder et al., 2004), it can also phosphorylate β-catenin, linking PI3K and Wnt/β-catenin pathways. This reaction targets β-catenin to the proteasome for ubiquitin-mediated degradation, promoting neuronal degeneration in AD. Disrupt Wnt/β-catenin signaling may be a direct association between Aβ-toxicity and *tau* hyperphosphorylation, ultimately leading to impaired synaptic plasticity and/or neuronal degeneration (Inestrosa et al., 2012). Based on that, our results showed that 48h of cultures exposure to

2 μM of AβOs was able to decrease significantly the total immunocontent of β-cateninin of organotypic slices. However, we did observe neither an increase in β-catenin phosphorylation nor GSK-3β dephosphorylation. More studies are necessary to elucidate this question. Lack of differences on phosphorylation of GSK-3β and β-catenin in organotypic hippocampal slices treated with AβOs for 48h can be due to the fact these changes may be happening earlier than 48h. Although alterations of these pathways seem to be transient in our *in vitro* model, it may be sufficient to transmit signals that culminate in the decrease of synaptophysin levels.

5. Conclusions

In conclusion, the results presented here provide evidence supporting that this *in vitro* model of AβOs toxicity was able to generate synaptic loss in organotypic hippocampal slice cultures, one of the earlier characteristics of AD, using oligomers concentrations similar to those found in AD patients. Although further studies are necessary to understanding the precise mechanism of AβOs toxicity, this is a great model to study physiologic factors and pharmacologic compounds AD-related.

Acknowledgements

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Legends of figures

Figure 1. Representative Western blotting of AβOs and their toxicity in organotypic hippocampal slices cultures. (A) Representative Western blotting of AβOs probed with anti-Aβ antibody (6E10). Note the presence of both low-n (trimers and tetramers) and higher molecular weight oligomers (approx. 50-150 KDa). **(B)** Representative photomicrographs of PI uptake in hippocampal slices 48h after exposure to 0.5, 1, and 2 μM of AβOs. **(C)** Quantification of PI uptake in response to AβOs demonstrated no differences compared to controls. Values are expressed as % of cell death in hippocampus. Bars represent the mean ± SEM, n=6 (One-way ANOVA followed by Tukey's test).

Figure 2. AβOs exposure causes synaptotoxicity. (A) Representative Western blotting showing synaptophysin levels 48h after AβOs exposure. **(B)** Bands quantification showed a decrease in synaptophysin levels 48h after cultures exposure to 0.5, 1, and 2 μM of AβOs. The results were normalized to the total amount of β-actin. Bars represent the mean ± SEM, n=10. * Significantly different from control cultures, $p<0.05$, ** Significantly different from control cultures, $p<0.01$ (One-way ANOVA followed by Tukey's test).

Figure 3. Effects of AβOs-treatment on phosphorylation of JNK and ERK in organotypic hippocampal slice cultures. (A) Representative Western blotting of phosphoERK1/2, ERK1/2, and β-actin loading control. **(B)** Quantification showed a decrease in ERK1/2 phosphorylation 48h after cultures

exposure to 0.5, 1 and 2 μM of AβOs. **(C)** Representative Western blotting of phosphoJNK1/2, JNK1/2, and β-actin loading control. **(D)** Graphic showed an increase in JNK phosphorylation 48h after cultures exposure to 1 and 2 μM of AβOs. Data are expressed as a ratio of phosphorylated and total protein immunocontent and normalized to their respective controls nonexposed to AβOs toxicity (control bar; 100%). Uniformity of gel loading was confirmed with β-actin as the standard. Bars represent the mean ± SEM, n=10. * Significantly different from control culture, $p<0.05$, ** Significantly different from control culture $p<0.01$ (One-way ANOVA followed by Tukey's test).

Figure 4. AβOs had no effect on GSK-3β activity in hippocampal slices cultures. (A) Representative Western blotting showing phospho-GSK-3β, GSK-3β, and β-actin. **(B)** Quantification of GSK-3β phosphorylation levels 48h after exposure to AβOs (0.5, 1, and 2 μM). Data are expressed as a ratio of phosphorylated and total protein immunocontent and normalized to their respective controls nonexposed to AβOs (control bar; 100%). Uniformity of gel loading was confirmed with β-actin as the standard. Bars represent the mean ± SEM, n=6 (One-way ANOVA followed by Tukey's test).

Figure 5. AβOs decrease the immunocontent of free β-catenin while not altering their phosphorylation levels. (A and C) Representative Western blotting showing phospho-β-catenin and β-catenin, respectively. **(B)** Quantification of β-catenin phosphorylation levels 48h after AβOs exposure presented no significant differences when compared to controls. **(D)** Graphics showed a decrease in β-catenin immunocontent 48h after cultures exposure to

2 μM of AβOs. The results were normalized to the total amount of β-actin. Bars represent the mean ± SEM, n=6. * Significantly different from control cultures, $p<0.05$ (One-way ANOVA followed by Tukey's test).

Figure 1

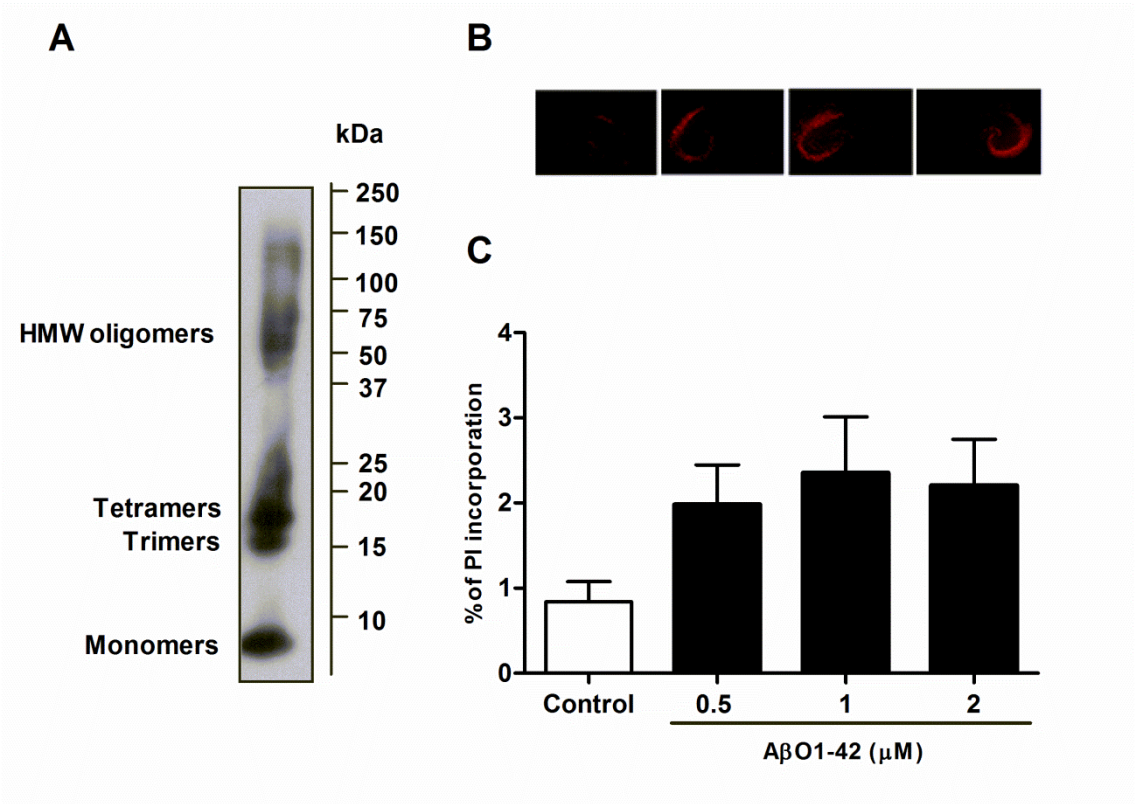

A
kDa
250
150
100
75
50
37
25
20
15
10
HMW oligomers
Tetramers
Trimers
Monomers
B
C
% of PI incorporation
4
3
2
1
0
Control
0.5
1
2
AβO1-42 (μM)

Figure 2

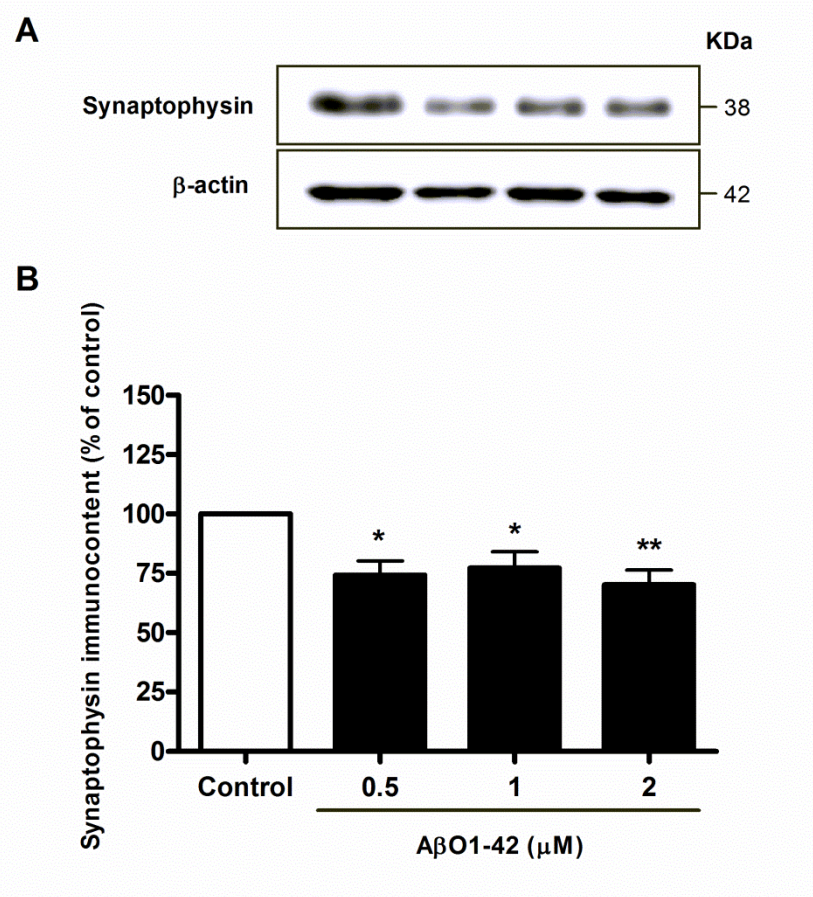

A
KDa
Synaptophysin
38
β-actin
42
B
Synaptophysin immunocontent (% of control)
150
125
100
75
50
25
0
*
*
**
Control
0.5
1
2
AβO1-42 (μM)

Figure 3

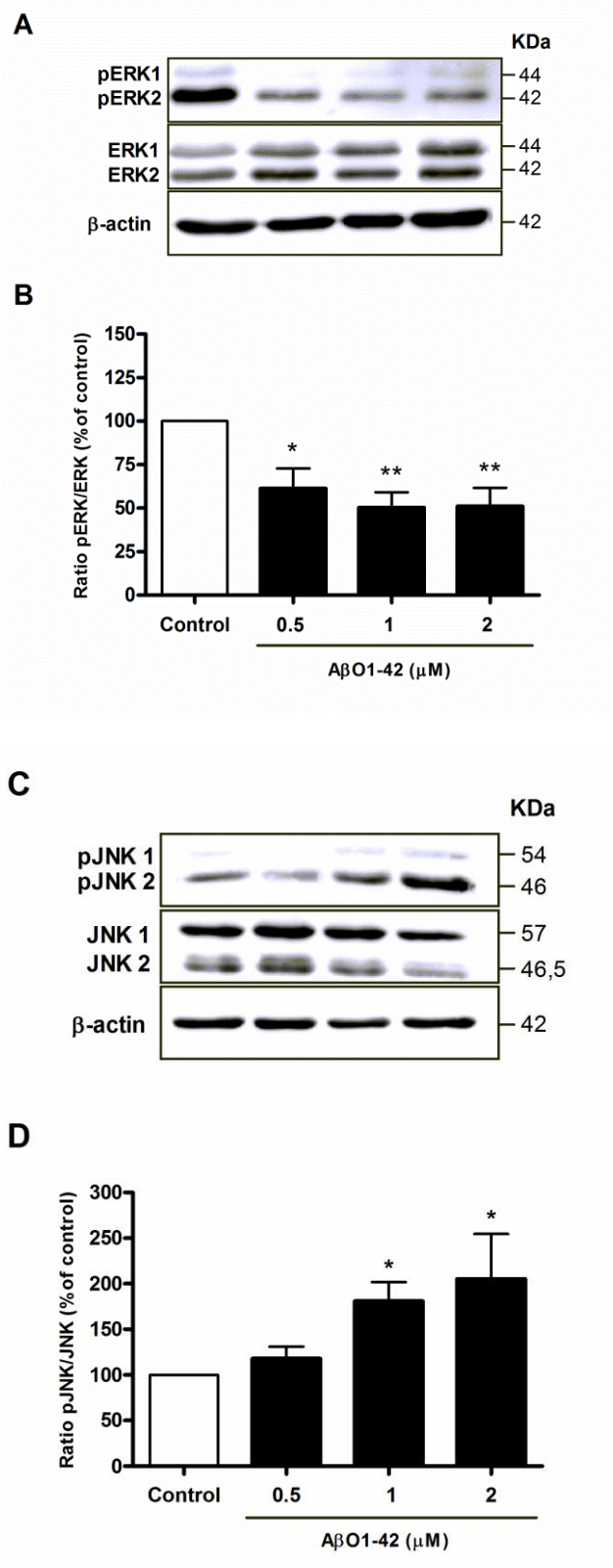

A
KDa
pERK1
pERK2
44
42
ERK1
ERK2
44
42
β-actin
42
B
Ratio pERK/ERK (% of control)
150
125
100
75
50
25
0
*
**
**
Control
0.5
1
2
AβO1-42 (μM)
C
KDa
pJNK 1
pJNK 2
54
46
JNK 1
JNK 2
57
46,5
β-actin
42
D
Ratio pJNK/JNK (% of control)
300
250
200
150
100
50
0
*
*
Control
0.5
1
2
AβO1-42 (μM)

Figure 4

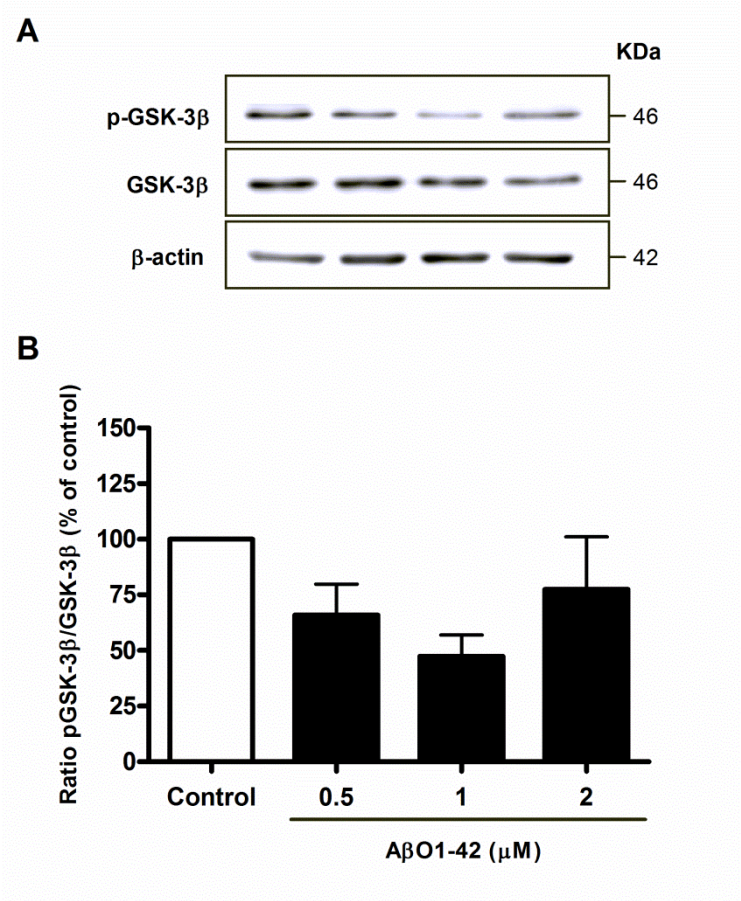
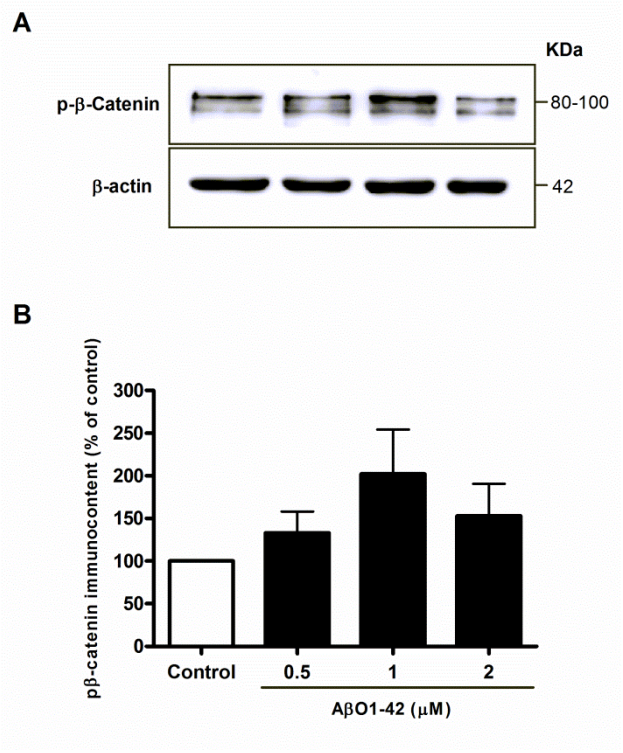
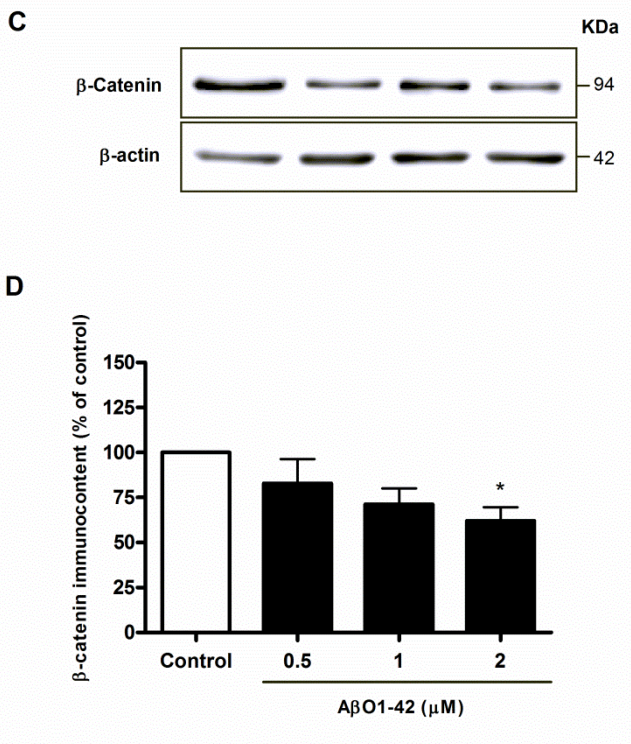

A
KDa
p-GSK-3β
GSK-3β
β-actin
46
46
42
B
Ratio pGSK-3β/GSK-3β (% of control)
150
125
100
75
50
25
0
Control
0.5
1
2
AβO1-42 (μM)

Figure 5


A
KDa
p-β-Catenin
80-100
β-actin
42
B
pβ-catenin immunocontent (% of control)
300
250
200
150
100
50
0
Control
0.5
1
2
AβO1-42 (μM)


C
KDa
β-Catenin
94
β-actin
42
D
β-catenin immunocontent (% of control)
150
125
100
75
50
25
0
*
Control
0.5
1
2
AβO1-42 (μM)

PARTE III

4. DISCUSSÃO

A Doença de Alzheimer, uma desordem neurodegenerativa multifatorial, é a principal forma de demência relacionada à idade, responsável por mais de 60% dos casos, com tendência a se tornar um problema de saúde pública em razão do aumento da expectativa de vida da população mundial. Por isso, a DA tem atraído cada vez mais a atenção da comunidade médica e científica. Doenças neurodegenerativas estão entre os problemas mais desafiadores da medicina. Embora os avanços nas pesquisas básicas e clínicas tenham mostrado progresso em relação ao entendimento do curso da DA, ela ainda é considerada uma das doenças mais complexas, uma vez que o completo entendimento dos seus mecanismos ainda está longe de ser elucidado. A teoria da cascata amiloide é a mais aceita pelos pesquisadores da DA. Essa teoria propõe que mudanças graduais na produção e agregação do peptídeo Aβ iniciam uma cascata de eventos moleculares que progridem para uma extensiva disfunção neuronal e morte celular, causando uma diminuição nos níveis de neurotransmissores e consequentemente ao desenvolvimento de um quadro de demência (Selkoe, 1991; Laferla et al., 2007). A perda sináptica está associada com os déficits cognitivos observados na DA (Selkoe, 2002; Nisticò et al., 2012), entretanto o mecanismo responsável pela perda das conexões ainda não está estabelecido. Primeiramente, acreditava-se que a perda sináptica ocorria devido às fibrilas amiloides insolúveis, porém, recentemente, vem sendo demonstrado que a disfunção sináptica e a neurodegeneração são as primeiras consequências da toxicidade dos AβOs (Klein, 2006; Benilova et al., 2012).

Mais de 20 anos atrás, foi demonstrado que o peptídeo Aβ monomérico não tóxico foi convertido para espécies tóxicas após incubação por alguns dias

em solução tamponada (Pike et al., 1991). A toxicidade foi associada ao aparecimento de espécies de alto peso molecular, confirmado por eletroforese em gel de poliacrilamida, mostrando a propriedade do peptídeo Aβ de se auto-agregar. A polimerização do peptídeo Aβ é um processo complexo e ocorre via intermediários metaestáveis (Lee et al., 2011). As placas amiloides podem existir à parte ou em equilíbrio com os AβOs, resultando em um misto de espécies neurotóxicas ao redor das placas que podem causar danos neuronais (Benilova et al., 2012). A multiplicidade de espécies de Aβ é derivada não somente de variações biológicas nativas, mas também de diferentes técnicas de preparação *in vitro* ou do isolamento *post mortem* de cérebro de pacientes com DA. Trabalhos anteriores do nosso grupo de pesquisa já demonstraram o efeito do peptídeo Aβ fibrilado em modelos *in vitro* e *in vivo* (Bernardi et al., 2012; Frozza et al., 2013; Hoppe, Juliana Bender et al., 2013a; Hoppe, Juliana Bender et al., 2013b). Este é o primeiro trabalho em nosso grupo que utiliza a metodologia de preparação dos AβOs, visto que, atualmente, este é o modelo de preparação mais utilizado na literatura. A caracterização dos oligômeros por *Western blotting* utilizando o anticorpo 6E10 mostrou a presença de monômeros, trímeros, tetrâmeros, além de oligômeros de alto peso molecular, e confirmou também a ausência de fibrilas, de acordo com trabalhos anteriores (De Felice et al., 2008; Sebollela et al., 2012).

Neste estudo, nós avaliamos os mecanismos moleculares pelos quais os AβOs induzem neurotoxicidade utilizando um modelo de cultura organotípica de fatias de hipocampo de ratos. Nossos resultados demonstraram a toxicidade dos AβOs pela incorporação do iodeto de propídeo e por *Western blotting* para algumas proteínas que poderiam estar envolvidas na sinaptotoxicidade. No

presente trabalho, culturas organotípicas de fatias de hipocampo foram expostas a concentrações de 0,5 a 2 μM de AβOs por um período de 48h. Nestas condições não foi observada morte celular necrótica, uma vez que não houve um aumento significativo na incorporação do iodeto de propídeo. Esse resultado foi similar aos encontrados por Chong et al. (2006), apesar dos modelos apresentarem diferenças. Uma possível explicação pode ser o fato de que o tempo de exposição utilizado no experimento não foi suficiente para que a morte celular por necrose possa ser detectada nas culturas. Ainda, deve-se considerar que outros mecanismos de morte celular podem estar envolvidos, os quais não são detectados pela metodologia utilizada.

Apesar do fato de não ter sido observada morte celular neste tempo de exposição, os AβOs podem estar interferindo em outros processos celulares que levam à disfunção sináptica. Para verificar esta possibilidade, optou-se por analisar os níveis de sinaptofisina, a principal proteína sináptica vesicular, e os resultados mostraram uma diminuição nesse marcador sináptico (Figura 2 do artigo). Esse efeito parece ser mediado através da modulação das vias de sinalização celular das proteínas ERK1/2 e JNK (Figuras 3 e 4 do artigo), bem como da proteína β-catenina (Figura 5 do artigo). A família das MAPKs inclui a ERK1/2, p38 e JNK. Membros da família das MAPKs desempenham um papel crucial na regulação de respostas a vários estresses no desenvolvimento neuronal, resposta inflamatória e apoptose (Kim e Choi, 2010). A via de ativação da JNK pode ocorrer pela liberação de citocinas pró-inflamatórias ou em resposta a estresses celulares genotóxicos, osmóticos ou oxidativos (Shen e Liu, 2006). A ativação da JNK tem sido descrita em cultura de neurônios após exposição ao peptídeo Aβ, e sua inibição apresenta efeitos que diminuem a

toxicidade do Aβ (Bozyczko-Coyne et al., 2001; Morishima et al., 2001). Além disso, a JNK regula vários processos como o desenvolvimento cerebral, formação e reparo da memória. Ainda, a JNK está estritamente relacionada com a disfunção neuronal na DA (Mehan et al., 2011). Sclip et al. (2014) mostraram que a ativação da JNK ocorre precocemente antes dos processos que levam a sinaptopatia e aos danos cognitivos em um modelo transgênico *in vivo* da DA. Aqui, mostramos que quando as culturas organotípicas foram expostas aos AβOs, a atividade da JNK se mostrou aumentada. Esses resultados sugerem que a via da JNK pode estar envolvida na perda sináptica observada nas culturas organotípicas após a adição dos AβOs. Entretanto, futuras investigações precisam ser feitas para melhor elucidação desse mecanismo.

A via da ERK, umas das mais bem caracterizadas entre as três MAPKs, tem sido apontada como uma via importante na regulação da função neuronal, uma vez que a ERK é uma proteína abundante no cérebro de adultos e sua sinalização pode desempenhar múltiplos papeis na regulação atividade-dependente da função neuronal (Zhu et al., 2003). A nível celular, ERK1/2 regula uma diversidade de funções incluindo crescimento, proliferação, diferenciação e sobrevivência celular ou apoptose, sendo considerada como um fator antiapoptose (Rosen et al., 1994; Ghasemi et al., 2014). Além disso, no cérebro, fortes evidências sugerem que a ERK1/2 é um importante componente das cascatas de sinalização nos neurônios e que a modulação da sua atividade pode ser necessária tanto para plasticidade sináptica quanto para os processos de aprendizado e memória (Sweatt, 2001). Por outro lado, a atividade e expressão da β-secretase (BACE 1), a endoprotease essencial para

produção do Aβ, parece ser negativamente modulada pela ativação da via da ERK1/2 (Tamagno et al., 2009). Entretanto, os efeitos do peptídeo Aβ na atividade da ERK1/2 ainda são controversos. Alguns estudos têm mostrado uma diminuição da atividade da ERK após a exposição ao peptídeo Aβ (Bell et al., 2004; Townsend et al., 2007; Li et al., 2011), enquanto outros mostraram o efeito oposto (Chong et al., 2006; Ghasemi et al., 2014), o que poderia ser explicado pelos diferentes níveis de agregação, concentração, área cerebral e/ou tempo de exposição ao peptídeo Aβ. Em nosso estudo, observou-se que a exposição das culturas organotípicas aos AβOs por um período de 48h levou a uma diminuição da atividade da ERK1/2. Considerando o fato que, entre outras funções, a ERK desempenha um papel na regulação da expressão gênica neuronal, sua inativação pode levar à diminuição da transcrição de diversos fatores, como por exemplo, da proteína ligante ao elemento de resposta do cAMP (CREB), através da diminuição da sua fosforilação (Adams et al., 2000; Benilova et al., 2012). Desta forma, a transcrição mediada pelo CREB de genes responsáveis pela plasticidade sináptica fica alterada, implicando em danos nas funções cognitivas (Zhang et al., 2013).

Com a finalidade de tentar explicar melhor a sinaptotoxicidade observada nas culturas organotípicas após a exposição aos AβOs, foram avaliadas outras duas importantes vias de sinalização implicadas na perda sináptica: PI3K, representada pela GSK-3β, e a Wnt/β-catenina. GSK-3β é uma proteína abundante no sistema nervoso central e tem se mostrado ser um componente chave nas vias de sinalização que levam à neurodegeneração (Balaraman et al., 2006). Estudos recentes revelaram que a elevação da atividade da GSK-3β está diretamente relacionada com o aumento dos níveis

de produção e depósitos do peptídeo Aβ, hiperfosforilação da *tau* e danos sinápticos em pacientes com a DA e em modelos animais da mesma (Kremer et al., 2011; Darocha-Souto et al., 2012; Hurtado et al., 2012; Reddy, 2013). Além do fato de a GSK-3β ser o principal mediador da fosforilação da t*au* (Ryder et al., 2004), ela também pode fosforilar a β-catenina, gerando um elo entre as vias da PI3K e Wnt/β-catenina. Essa reação encaminha a β-catenina para o sistema proteassomo para degradação mediada por ubiquitinação, promovendo a degeneração neuronal observada na DA. Um distúrbio na sinalização da Wnt/β-catenina pode ser uma ligação direta entre a toxicidade do peptídeo Aβ e a hiperfosforilação da *tau,* levando a uma diminuição da plasticidade sináptica e/ou degeneração neuronal (Inestrosa et al., 2012). Sendo assim, nossos resultados demonstraram que a exposição das culturas por um período de 48h à concentração de 2 μM dos AβOs foi capaz de diminuir significativamente o imunoconteúdo total da β-catenina no citoplasma das fatias de tecido. Entretanto, não foram observadas alterações significativas nos níveis de fosforilação da β-catenina, nem da GSK-3β. Uma razão que poderia explicar o motivo de não terem sido observadas alterações nas fosforilações da β-catenina e da GSK-3β nas culturas organotípicas expostas aos AβOs por 48h pode ser o fato de que essas mudanças podem ocorrer mais precocemente (6-24h de exposição), o que não foi realizado neste estudo. Embora as alterações nos níveis de fosforilação dessas proteínas possam ser transientes em nosso modelo *in vitro* de toxicidade dos AβOs, mostraram ser suficientes para transmitir sinais que culminaram na diminuição dos níveis de sinaptofisina.

5. CONCLUSÕES

Os resultados obtidos nesta dissertação nos permitem concluir que:

a) A cultura organotípica mantida por um longo período de maturação é um bom modelo para estudar a toxicidade e as cascatas de sinalização desencadeadas pelos AβOs.
b) Este modelo *in vitro* de toxicidade dos AβOs foi capaz de causar uma diminuição no conteúdo sináptico nas culturas organotípicas, uma das primeiras características neuropatológicas evidenciadas na DA.
c) O dano sináptico pôde ser observado inclusive em concentrações nanomolares dos AβOs, similares às encontradas em pacientes com a DA.
d) Este efeito parece ser mediado pela alteração nos níveis de fosforilação das proteínas JNK e ERK, bem como no imunoconteúdo da proteína β-catenina.
e) Embora mais estudos sejam necessários para compreender o preciso mecanismo de toxicidade dos AβOs nas culturas organotípicas, este é um bom modelo para estudar fatores bioquímicos e compostos farmacológicos com potencial para o tratamento da DA.

6. PERSPECTIVAS

- Avaliar o conteúdo de sinaptofisina nas fatias hipocampais expostas aos oligômeros do peptídeo Aβ (AβOs) por imunohistoquímica.
- Estudar o envolvimento da morte celular por apoptose nas culturas expostas aos AβOs através da marcação com anexina V, bem como utilizando um ensaio de caspases.
- Avaliar o efeito dos AβOs nas culturas organotípicas de hipocampo de ratos utilizando moduladores seletivos da via das MAPKs.
- Avaliar o efeito do resveratrol em uma formulação nanoencapsulada na possível prevenção da toxicidade dos AβOs nas culturas organotípicas.
- Estabelecer um modelo *in vivo* através da injeção intracerebroventricular dos AβOs, comparando as alterações morfológicas desencadeadas com o modelo *in vitro.*
- Avaliar os efeitos oxidativos e sobre o metabolismo energético mitocondrial dos AβOs em culturas organotípicas hipocampais e em modelo *in vivo* por infusão intracerebroventricular em ratos.

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LISTA DE FIGURAS

INTRODUÇÃO

8. ANEXOS

7.1. Carta de aprovação da CEUA

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

PROPESQ

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 23521
Título: Investigação do efeito neuroprotetor de nanocápsulas contendo resveratrol sobre um modelo in vitro de toxicidade induzida por oligômeros do peptídeo β-amilóide

Pesquisadores:

Equipe UFRGS:

CHRISTIANNE GAZZANA SALBEGO - coordenador desde 01/08/2012
BRUNA DE MELO MENEZES - pesquisador desde 01/08/2012
Juliana Bender Hoppe - Aluno de Doutorado desde 01/08/2012
Leon de Moraes Lisbôa - Aluno de Mestrado desde 01/08/2012
André Bevilacqua Meneghetti - Aluno de Mestrado desde 01/08/2012

Comissão De Ética No Uso De Animais aprovou o mesmo em seus aspectos éticos e metolodológicos, para a utilização de 42 ratos, Wistar, de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Terça-Feira, 13 de Novembro de 2012

STELA MARIS KUZE RATES
Coordenador da comissão de ética

7.2. Normas do periódico *Neurobiology of aging*, no qual o artigo científico será submetido.

Preparation

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It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier:http://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

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Negative results will be published as one journal page (3 double-spaced typed manuscript pages),with supplementary material to be posted at the journal website. The 3 double-spaced pages should include a brief abstract, a brief introduction, a few sentences of methods,core data and discussion of the data. If room allows, an abbreviated reference list with the most critical references should also be included in these 3 double-spaced pages.

Supplementary material for the website should include amore detailed introduction, more details of the methods, the complete reference list and any additional data. The supplementary material should be sufficient to convince the interested reader of the validity and reliability of the results. It should be made clear which material is for printing in thejournal and which is supplementary material for the website. Since the net effect of a Negative Result is to discourage repetition, the standards for acceptance as a Negative Result will be highly demanding (see Announcement "Negative results can be valuable", Neurobiol. Aging 25(10):iii;2004).

Length of paper
The Editors insist upon clear, concise statements of facts and conclusions. Regular manuscripts should be no longer that 10 printed journal pages (30 doubled-spaced pages, including references, figures and tables) and should include only the most essential figures and tables. Brief Communications should be restricted to eight double-spaced pages (including references, figures and tables) and should not present more than one figure and one table, or two figures, or two tables. Fragmentation of material into numerous short reports is discouraged.

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Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

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Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation
A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

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Results should be clear and concise.

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A concise and factual abstract is required. The abstract, not to exceed 170 words, should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

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