UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: FISIOLOGIA

Efeitos neuroprotetores do 4'-clorodiazepam em modelos experimentais de Doença de Alzheimer in vitro e sobre o desenvolvimento neuronal

BRUNO DUTRA ARBO

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BRUNO DUTRA ARBO

Orientadora: Profa. Dra. Maria Flávia Marques Ribeiro

Tese apresentada ao curso de Pós-Graduação em Ciências Biológicas: Fisiologia da Universidade Federal do Rio Grande do Sul, como requisito à obtenção do grau de Doutor em Fisiologia.

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“El esfuerzo no se negocia”

Diego Pablo Simeone

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LISTA DE ABREVIATURAS

4’-CD – 4’-clorodiazepam

5α-DHP – 5α-dihidroprogesterona

Aβ – Peptídeo beta-amiloide

ACh - Acetilcolina

ACTH – Hormônio adrenocorticotrópico

AD – Doença de Alzheimer

APP – Proteína precursora do amiloide

ATAD3 – Proteína 3 contendo o domínio AAA

ATP – Trifosfato de adenosina

BACE-1 – Enzima clivadora do sítio beta da proteína precursora do amiloide

CCK4 – Tetrapeptídeo colecistocinina

ChE – Colinesterase

CNS – Sistema nervoso central

CRAC – Consenso de aminoácidos reconhecedor do colesterol

CYP11A1 – Citocromo P450 clivadora da cadeia lateral

DA – Doença de Alzheimer

DBI – Inibidor da ligação ao diazepam

DHEA – Dihidroepiandrosterona

DIV – Dias *in vitro*

DMEM F12 – Meio Eagle modificado por Dulbecco/Nutriente F-12 Ham

DMSO – Dimetilsulfóxido

EROs – Espécies reativas de oxigênio

FBS – Soro fetal bovino

FDA – Diacetato de fluoresceína

GAPDH – Gliceraldeído 3-fosfato desidrogenase

JNK – c-Jun N-terminal cinase

LPS - Lipopolissacarídeo

MAPK – Proteínas cinases ativadas por mitógenos

MOMP – Permeabilização da membrana mitocondrial externa

MPTP – Poro de transição de permeabilidade mitocondrial

Ngn3 – Neurogenina 3

NMR – Ressonância magnética nuclear

PBR – Receptor benzodiazepínico periférico

PBS – Solução salina fosfato tamponada

PET – Tomografia por emissão de pósitrons

PDCT – Protocolo Clínico e Diretrizes Terapêuticas

PI – Iodeto de propídeo

PI3K – Fosfatidilinositol-3 cinase

RA – Ácido retinoico

SDS-PAGE – Eletroforese em gel de poliacrilamida e duodecil sulfato de sódio

SNC – Sistema nervoso central

SOD – Superóxido dismutase

StAR – Proteína reguladora aguda da esteroidogênese

SUS – Sistema único de saúde

TBI – Lesão traumática cerebral

TLR – receptor *Toll-like*

TSPO – Proteína translocadora

TTN – *Triakontatetraneuropeptide*

VDAC – Canal aniônico dependente de voltagem

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APRESENTAÇÃO

Esta Tese está organizada em seções dispostas da seguinte maneira: *Introdução*, *Hipóteses, Objetivos, Materiais e Métodos, Capítulos* (I, II, III e IV, referentes a artigos científicos e a outros resultados obtidos ao longo do trabalho), *Discussão*, *Conclusões* e *Referências Bibliográficas*.

A seção *Introdução* apresenta o estado da arte e o embasamento teórico que serviram para a formulação das *Hipóteses* e dos *Objetivos*, que estão descritos na sequência em suas respectivas seções.

A seção *Materiais e Métodos* abrange a descrição de todos os procedimentos e metodologias adotadas para a execução do trabalho.

Na seção *Capítulos*, estão presentes os artigos científicos publicados ou em fase de preparação para serem submetidos. Nesta seção, estão descritos os métodos e as referências bibliográficas específicas de cada artigo. O Capítulo I consiste de um artigo de revisão escrito durante o período do doutorado sanduíche e publicado em colaboração com o Dr. Luis Miguel Garcia-Segura e com o Dr. Fernando Benetti, que contém boa parte do referencial teórico que embasou a realização desse trabalho. Os Capítulos II e III são compostos por experimentos realizados no laboratório do Dr. Luis Miguel Garcia-Segura no Instituto Cajal (Madri/Espanha), enquanto o Capítulo IV engloba experimentos realizados em colaboração com o Grupo de Neuroproteção e Sinalização Celular, coordenado pela Profa. Dra. Christianne Gazzana Salbego do Departamento de Bioquímica (UFRGS).

A seção *Discussão* apresenta a interpretação geral dos resultados obtidos em todos os experimentos apresentados na seção anterior, de forma que as conclusões estão

apresentadas na seção seguinte, intitulada *Conclusões*. As referências citadas ao longo da Tese estão apresentadas na última seção, intitulada *Referências Bibliográficas*.

RESUMO

O aumento da expectativa de vida da população mundial tem se associado com uma maior prevalência de doenças neurodegenerativas. A Doença de Alzheimer (DA) é a doença neurodegenerativa mais comum e a principal causa de demência em indivíduos com mais de 60 anos, sendo caracterizada por um declínio progressivo na memória e função mental dos pacientes. Esses sintomas são acompanhados por alterações histopatológicas no cérebro desses indivíduos, incluindo a presença de uma grande quantidade de placas senis, formadas pela deposição do peptídeo beta-amiloide (Aβ), e de emaranhados neurofibrilares formados pela hiperfosforilação da proteína Tau. Estudos indicam que a deposição do Aβ é uma das principais responsáveis pelo desenvolvimento da DA, causando dano neuronal através da ativação de várias vias pró-apoptóticas e dando origem aos sintomas de demência típicos dessa doença. Até o momento, não existem tratamentos eficazes para o combate à DA, de forma que a maior parte das intervenções farmacológicas é destinada apenas ao tratamento de alguns de seus sintomas. A proteína translocadora (TSPO) se localiza em pontos de contato entre as membranas mitocondriais interna e externa e está relacionada com o transporte de colesterol para o interior da mitocôndria e com a regulação da esteroidogênese e da apoptose. Estudos mostram que ligantes da TSPO apresentam efeitos neuroprotetores em diferentes modelos experimentais de lesão cerebral e doenças neurodegenerativas. Especificamente em relação à DA, um estudo indicou que o 4'-clorodiazepam (4'-CD), um ligante da TSPO, apresenta efeitos neuroprotetores em um modelo animal dessa doença, sendo um possível candidato para o seu tratamento. Dessa forma, o objetivo desse estudo foi verificar o efeito neuroprotetor do 4'-CD em diferentes modelos *in vitro* de toxicidade induzida pelo Aβ, além de seus efeitos sobre o desenvolvimento de neurônios hipocampais. Inicialmente, demonstramos que o 4'-CD reduziu a morte celular de células SH-SY5Y expostas a um modelo de toxicidade induzida pela administração de Aβ. Esses efeitos estiveram associados com a redução da expressão da proteína pró-apoptótica Bax e com um aumento da expressão da survivina, uma proteína anti-apoptótica. A expressão das proteínas Bcl-xl e procaspase-3, por outro lado, não foi alterada pelos tratamentos. Posteriormente, estudamos os efeitos neuroprotetores do 4'-CD contra a toxicidade induzida pela administração do Aβ em culturas organotípicas de hipocampo. Nesses experimentos, foi demonstrado que o 4'-CD reduz a morte celular de culturas organotípicas de hipocampo expostas ao Aβ através de um aumento na expressão da enzima SOD, sem alterar, no entanto, a expressão das proteínas Akt e procaspase-3. Por fim, foi avaliado o efeito do 4'-CD sobre o desenvolvimento de culturas primárias de neurônios hipocampais de camundongos machos e fêmeas. Foi observado que as culturas de neurônios hipocampais das fêmeas apresentaram um desenvolvimento mais rápido do que as dos machos. O 4'-CD acelerou a maturação e aumentou a ramificação neurítica dos neurônios hipocampais dos machos, mas não exerceu qualquer efeito sobre os neurônios das fêmeas. Em suma, foi observado que o 4'-CD apresenta efeitos neuroprotetores contra o Aβ em células SH-SY5Y e em culturas organotípicas do hipocampo, apresentando-se como um fármaco em potencial para o tratamento da DA.

Além disso, foi observado que o 4’-CD exerceu um efeito dependente do sexo sobre o desenvolvimento de culturas primárias de neurônios hipocampais, estimulando o desenvolvimento e a ramificação neurítica de neurônios hipocampais de machos, mas não de fêmeas.

Palavras-chave: proteína translocadora (TSPO); 4’-CD; neuroproteção; beta-amiloide; hipocampo; neuritogênese.

ABSTRACT

The increase in life expectancy of the world population has been associated with a higher prevalence of neurodegenerative diseases. The Alzheimer's Disease (AD) is the most common neurodegenerative disorder and the main cause of dementia among people over 60 years, being characterized by a progressive decline in the memory and mental function of the patients. These symptoms are associated with histopathological changes in the brain of these patients, including the presence of senile plaques, formed by the deposition of amyloid-beta (Aβ), and neurofibrillary tangles, which are related to the hyperphosphorylation of Tau protein. Studies indicate that Aβ deposition is a major contributor to AD progression, promoting neuronal damage through the activation of different pro-apoptotic pathways and giving rise to the typical dementia symptoms of this disease. To date, there are no effective treatments for AD, so that most of the pharmacological intervention is intended for the treatment of some of its symptoms. The translocator protein (TSPO) is located in contact sites between the outer and the inner mitochondrial membranes and is involved in the cholesterol transport into the mitochondria and in the regulation of steroidogenesis and apoptosis. Studies show that TSPO ligands present neuroprotective effects in different experimental models of brain injury and neurodegenerative diseases. Specifically regarding AD, a study indicated that 4'-chlorodiazepam (4'-CD), a TSPO ligand, is neuroprotective in an animal model of this disease, being a possible candidate for its treatment. Therefore, the aim of this study was to evaluate the neuroprotective effect of 4'-CD in different experimental models of Aβ-induced neurotoxicity *in vitro*, as well as its effects on the development of hipocampal neurons. First, it was demonstrated that 4'-CD decreased the cell death of SH-SY5Y cells exposed to the Aβ. This effect was associated with the inhibition of the Aβ-induced upregulation of Bax, a pro-apoptotic protein, and downregulation of survivin, a pro-survival protein. On the other hand, the expression of Bcl-xl and procaspase-3 was not change by the treatments. After, it was studied the neuroprotective effects of 4'-CD against Aβ in organotypic hipocampal cultures. In these experiments, it was shown that 4'-CD decreases the cell death of organotypic hippocampal slices exposed to the Aβ by increasing the protein expression of SOD, but without changing the expression of Akt and procaspase-3. Finally, due to the importance of the processes of neuronal development and maturation in the regeneration of CNS after injury, it was evaluated the effect of 4'-CD on the development of primary hippocampal neurons of male and female mice. It was observed that female primary hippocampal neurons presented an increased rate of development than male neurons. 4'-CD stimulated the development and increased the neuritic branching of male but not from female neurons. In summary, it was observed that 4'-CD presented a neuroprotective effect against Aβ in SH-SY5Y cells and in rat organotypical hippocampal slices, presenting itself as a promising agent for the treatment of AD. Also, it was observed that 4'-CD modulates the development of hippocampal neurons in a sex-dependent manner, stimulating the development of male but not from female cells.

Keywords: **t**ranslocator protein (TSPO); 4’-CD; neuroprotection; amyloid-beta; hippocampus; neuritogenesis.

1 INTRODUÇÃO

1.1 PROTEÍNA TRANSLOCADORA

A proteína translocadora (TSPO) é uma proteína de 18kDa localizada em sítios de contato entre as membranas mitocondriais interna e externa. Inicialmente, essa proteína foi chamada de receptor benzodiazepínico periférico por ser capaz de ligar-se ao diazepam (Papadopoulos et al., 2006). Funcionalmente, a TSPO é conhecida devido a seu papel no transporte do colesterol através do espaço aquoso entre as membranas mitocondriais, e, devido a essa função, essa proteína foi renomeada em 2006 como TSPO (Papadopoulos et al., 2006).

A TSPO é uma proteína integral de membrana de 169 aminoácidos, bastante conservada durante a evolução (Batarseh e Papadopoulos, 2010). Essa proteína está arranjada em cinco domínios alfa-hélice transmembrana, e apresenta alta afinidade pelo colesterol devido à presença de um domínio CRAC (*cholesterol recognition amino acid consensus*) na sua região C-terminal (Li e Papadopoulos, 1998; Jaremko et al., 2014). Inicialmente, acreditava-se que a TSPO poderia funcionar como um canal, recebendo o colesterol através da proteína reguladora aguda da esteroidogênese (StAR), todavia, estudos mais recentes sugerem que o transporte do colesterol estaria relacionado com um mecanismo de deslizamento envolvendo a associação da TSPO com outras proteínas (Li et al., 2015). Dentro desse contexto, sabe-se que a TSPO faz parte de um complexo multiproteico chamado de transduceossomo, composto pela TSPO e por outras proteínas envolvidas no transporte do colesterol para o interior da mitocôndria, incluindo o canal aniônico dependente de voltagem (VDAC), a proteína 3 contendo o domínio AAA (ATAD3) e a proteína StAR (Midzak et al., 2011; Rone et al., 2012) (Figura 1). O transporte de colesterol para o interior da mitocôndria é a etapa limitante no processo de esteroidogênese. Na mitocôndria, a síntese dos hormônios esteroides inicia com a

clivagem enzimática da cadeia lateral do colesterol, efetuada pela enzima citocromo P450 clivadora da cadeia lateral (CYP11A1), que dá origem à pregnenolona, o primeiro esteroide formado na rota da esteroidogênese (Miller e Auchus, 2011). Posteriormente, a pregnenolona deixa a mitocôndria e vai para o retículo endoplasmático, onde sofre diversas transformações enzimáticas que darão origem aos demais hormônios esteroides (Lacapère e Papadopoulos, 2003).

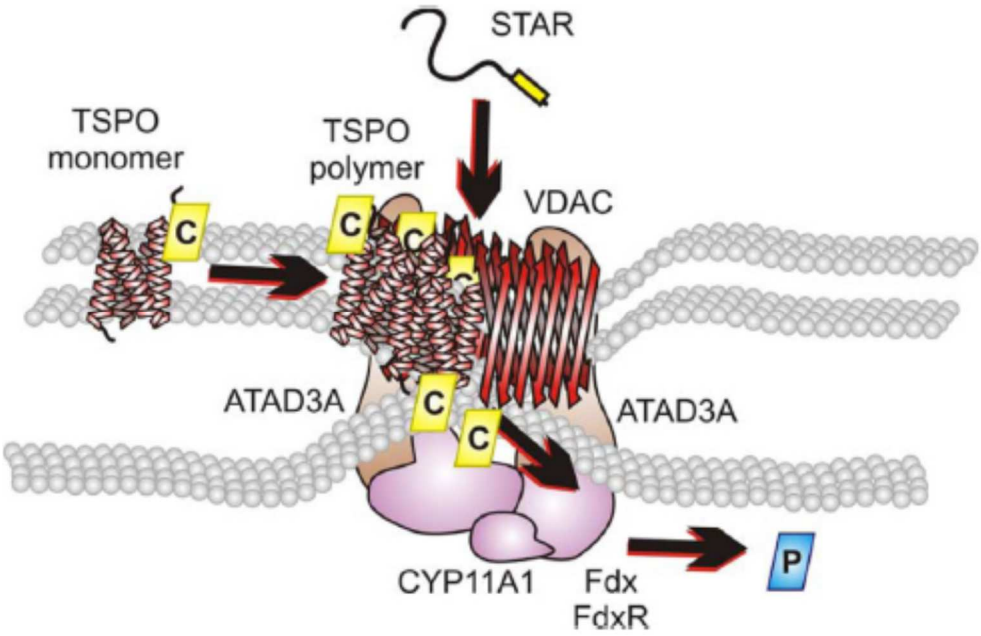

Figura 1 - Transporte do colesterol para o interior da mitocôndria.

Legenda: O transporte do colesterol (em amarelo) para o interior da mitocôndria é mediado por uma estrutura chamada de transduceossomo, formado por diversas proteínas incluindo a TSPO, a StAR, o VDAC e a ATAD3. Após sua chegada no interior da mitocôndria, o colesterol é convertido em pregnenolona (em azul) pela ação da CYP11A1

Fonte: Rone et al., 2012.

Além do seu papel na regulação do transporte do colesterol e da esteroidogênese, a TSPO tem sido relacionada com o controle de outras funções mitocondriais, como a respiração mitocondrial, a abertura do poro de transição de permeabilidade mitocondrial (MPTP), a apoptose e a proliferação celular, embora ainda exista alguma controvérsia

sobre a participação da TSPO no controle de algumas dessas funções (Hirsch et al., 1989; Azarashvili et al., 2007; Veenman et al., 2007; Corsi et al., 2008, Sileikyte et al., 2011, 2014).

A expressão da TSPO no encéfalo no estado saudável é relativamente baixa, sendo encontrada principalmente em células gliais e em níveis mais baixos nos neurônios (Chen e Guilarte, 2008; Papadopoulos e Lecanu, 2009). Em condições patológicas, por outro lado, como na doença de Alzheimer (DA), na doença de Parkinson, na doença de Huntington e na esclerose múltipla, a expressão da TSPO no sistema nervoso central (SNC) aumenta em locais de dano e inflamação, especialmente nos locais mais afetados pelos mecanismos neurodegenerativos dessas doenças (Arbo et al., 2015). Dentro desse contexto, estudos têm utilizado radioligantes da TSPO para a realização de exames de imagem pela técnica de tomografia por emissão de pósitrons (PET), de forma que a ligação desses compostos às células cuja expressão da TSPO é maior permite a análise das regiões cerebrais mais afetadas nessas doenças (Ouchi et al., 2005; Pavese et al., 2006; Edison et al., 2008; Batarseh e Papadopoulos, 2010; Colasanti et al., 2014; Rissanen et al., 2014).

Além do seu uso na área do diagnóstico, alguns estudos têm mostrado que os ligantes da TSPO poderiam exercer efeitos neuroprotetores em diferentes modelos experimentais. Levando em consideração que diversos estudos já mostraram que esteroides neuroativos, como a progesterona, o estradiol, a dihidroepiandrosterona (DHEA) e a testosterona exercem efeitos protetores em diferentes modelos experimentais (De Nicola et al., 2013; Persky et al., 2013; Grimm et al., 2014; Arevalo et al., 2015; Arbo et al., 2016; Grimm et al., 2016), e tendo em mente o papel da TSPO na regulação da esteroidogênese e da apoptose, o estudo das ações neuroprotetoras dos ligantes da TSPO

em diferentes modelos experimentais ganhou força na última década (Papadopoulos e Lecanu, 2009; Rupprecht et al., 2010; Arbo et al., 2015). Dentro desse contexto, já foi mostrado que ligantes da TSPO poderiam exercer efeitos neuroprotetores em modelos experimentais de lesão traumática cerebral (Soustiel et al., 2008, 2011), excitotoxicidade (Veiga et al., 2005) e doenças neurodegenerativas, incluindo a DA (Barron et al., 2013), a esclerose múltipla (Daugherty et al., 2013) e a neuropatia diabética (Giatti et al., 2009), além de efeitos benéficos em modelos de transtornos psiquiátricos (Kita et al., 2004; Verleye et al., 2005; Rupprecht et al., 2009; Zhang et al., 2014).

1.2 DOENÇA DE ALZHEIMER

O aumento da expectativa de vida da população mundial vem sendo acompanhado de um crescimento exponencial do número de pessoas afetadas por doenças neurodegenerativas associadas à senilidade. A Doença de Alzheimer (DA) é a doença neurodegenerativa mais comum e a principal causa de demência em indivíduos com mais de 60 anos, acometendo cerca de 8 a 15% da população acima de 65 anos de idade, o que equivale a cerca de 35 milhões de pessoas em todo o mundo (Querfurth e LaFerla, 2010). Essa doença foi descrita no início do século XX pelo médico alemão Alois Alzheimer, após receber uma paciente do sexo feminino no Hospital de Frankfurt em 1901 que apresentava perda progressiva de memória, delírios e alucinações, que a definiu como uma patologia neurológica desconhecida, associada com demência, sintomas de déficit de memória, alterações comportamentais e dificuldade para a realização de atividades de rotina (Alzheimer, 1907). Posteriormente, a análise do cérebro da paciente após sua morte revelaria a presença de placas senis e de emaranhados neurofibrilares, as duas principais características histopatológicas dessa doença, que permitiram que Alzheimer a

classificasse como uma doença distinta das patologias conhecidas até então (Alzheimer, 1911).

O principal fator de risco para a DA é a idade. A incidência da doença dobra a cada 5 anos após os 65 anos de idade, de forma que após os 85 anos, a chance de os indivíduos serem diagnosticados com Alzheimer é maior do que 1:3 (Hirtz et al., 2007). Estudos mostram que existem atualmente cerca de 5,3 milhões de indivíduos com DA nos EUA, e, que com o aumento progressivo da expectativa de vida das pessoas, a prevalência da DA nos EUA poderá alcançar até 16 milhões de casos até 2050 (Hebert et al., 2013, Alzheimer's Association, 2015). Dados do ano de 2013 mostram que, ao passo que a taxa de mortalidade relacionada com doenças cardiovasculares e acidentes vasculares cerebrais diminuiu em 14% e 23%, respectivamente, a taxa de mortalidade relacionada com a DA aumentou em 71%, tornando-a a sexta causa de morte nos EUA e a quinta causa de morte em indivíduos acima de 65 anos (Alzheimer's Association, 2015). Estima-se que o custo relacionado ao tratamento e ao cuidado de pacientes com DA nos EUA tenha alcançado 226 bilhões de dólares no ano de 2015 (Alzheimer's Association, 2015), enquanto que os gastos com demência na Europa no ano de 2010 alcançaram a marca de 105 bilhões de euros (Gustavsson et al., 2011). No Brasil, estudos da década passada estimavam que a prevalência de demência na população acima de 65 anos era de 7,1%, com a DA sendo responsável por mais da metade dos casos (Herrera Jr et al., 2002). Levando em consideração o aumento da expectativa de vida no país (dados do Instituto Brasileiro de Geografia e Estatística de 2014 indicam que a expectativa de vida no país subiu para 75,2 anos, contra 74,9 anos segundo dados de 2013), a prevalência da DA no país é provavelmente maior do que a prevista por essas estimativas anteriores. No país, em 2002, foi criado no âmbito do Sistema Único de Saúde (SUS) o Programa de

Assistência aos Portadores da Doença de Alzheimer e, desde então, o tratamento farmacológico da DA possui um Protocolo Clínico e Diretrizes Terapêuticas (PDCT) específico, de forma que os medicamentos recomendados no PDCT são financiados pelo Ministério da Saúde. Dentro desse contexto, estima-se que entre 2008 e 2013, mais de 47 milhões de unidades de medicamentos para o tratamento da DA foram adquiridos pelo governo federal, ao custo de R$90,1 milhões (Costa et al., 2015).

A DA é uma doença complexa, envolvendo a combinação de fatores genéticos, moleculares e ambientais. Normalmente, a DA se manifesta de maneira mais precoce e apresenta uma evolução mais rápida em pacientes com história familiar dessa doença (Casserly e Topol, 2004; Blennow et al., 2006). Os primeiros sintomas da DA normalmente se manifestam na forma de leves déficits de memória, afetando principalmente a memória de curto-prazo, com os pacientes podendo apresentar dificuldade em lembrar de pequenos eventos do cotidiano, por exemplo. Posteriormente, em estágios mais avançados, ocorre o desenvolvimento progressivo de uma demência severa, que leva progressivamente os pacientes à incapacitação. Em média, os indivíduos afetados pela DA podem conviver com esse distúrbio por cerca de oito anos, podendo chegar a até 20 anos em alguns casos (Alzheimer's Association, 2015).

O diagnóstico definitivo da DA é obtido apenas através da análise *post mortem* do cérebro dos pacientes, com a detecção das alterações histopatológicas descritas por Alzheimer. Essas alterações incluem a presença de placas senis, formadas pelo acúmulo e agregação do peptídeo beta-amiloide (Aβ), formando filamentos cercados por células gliais reativas e neuritos distróficos, e também a presença de emaranhados neurofibrilares formados pelo acúmulo da proteína Tau, que se encontra hiperfosforilada na DA e se acumula dentro das células na forma de filamentos emaranhados helicoidais pareados

(LaFerla et al., 2007; Ballard et al., 2011; Ittner e Gotz, 2011). Em geral, o cérebro desses pacientes apresenta atrofia cortical bastante acentuada no lobo temporal, especialmente no córtex e na formação hipocampal, de forma que o volume cerebral reduzido é consequência da neurodegeneração progressiva e da morte neuronal nessas estruturas (Mattson et al., 2004; Heneka et al., 2010).

Atualmente, as estratégias de tratamento da DA são bastante limitadas, sendo baseadas na modulação da ação de neurotransmissores, em especial da acetilcolina (ACh). Os inibidores das colinesterases (ChE) são os principais fármacos utilizados dentro desse contexto, atenuando o déficit colinérgico ocasionado pela degeneração de neurônios colinérgicos no prosencéfalo basal que ocorre com a progressão da doença e melhorando sintomas cognitivos e comportamentais associados à doença (Lane et al., 2006; Hroudová et al., 2016). A Tacrina foi o primeiro inibidor reversível da ChE a ser sintetizado e o primeiro fármaco a ser utilizado para o tratamento da DA, todavia, devido à sua baixa seletividade e à ocorrência de efeitos colaterais colinérgicos, como náuseas, cólicas abdominais e hepatotoxicidade, acabou sendo posteriormente retirado do mercado (Korabecny et al., 2010), sendo substituída por outros fármacos inibidores da ChE como a rivastigmina, a galantamina e o donepezil (Hroudová et al., 2016). Além dos inibidores da ChE, a memantina, um inibidor não-competitivo dos receptores NMDA, também tem sido utilizada para o tratamento da DA em casos moderados ou graves, bloqueando a ativação excessiva dos receptores NMDA sem bloquear, no entanto, sua ativação fisiológica e sem interferir na transmissão sináptica normal (Lipton, 2005). Devido à limitação das estratégias terapêuticas disponíveis e à ausência de cura da DA, muitos estudos têm sido realizados visando a descoberta de novas estratégias terapêuticas para essa doença. Estudos mostraram resultados promissores com o uso de inibidores da γ e

da β-secretase, enzimas envolvidas na síntese do Aβ, reduzindo o acúmulo de Aβ no SNC e melhorando o déficit de memória de camundongos transgênicos utilizados como modelos experimentais da DA (Chang et al., 2004; Lahiri et al., 2007; Imbimbo et al., 2007). Esses resultados foram corroborados por ensaios clínicos de fase I e II, nos quais se observou que o uso desses fármacos causou uma melhora moderada nos déficits cognitivos de pacientes com DA branda ou moderada (Siemers et al., 2006; Fleisher et al., 2008). Posteriormente, porém, ensaios clínicos de fase III verificaram que a administração desses fármacos não ocasionou qualquer melhora cognitiva em pacientes com DA leve ou moderada, e ainda aumentou o risco de eventos adversos graves nesses pacientes (Green et al., 2009; Carlson et al., 2011). Dessa forma, apesar do grande impacto econômico e dos efeitos ocasionados pela DA sobre a saúde e a qualidade de vida dos pacientes, os tratamentos para essa doença ainda são limitados, de forma que existe a necessidade do desenvolvimento de estratégias terapêuticas que sejam capazes de atuar sobre os mecanismos patogênicos e impedir a progressão da doença (Sugino et al., 2015).

1.3 PEPTÍDEO BETA-AMILOIDE (Aβ)

Conforme mencionado anteriormente, a presença de uma grande quantidade de placas-senis formadas pelo acúmulo do Aβ é uma das principais alterações histopatológicas encontradas no cérebro de pacientes acometidos pela DA. Os peptídeos Aβ são produtos naturais do metabolismo, sendo formados por cerca de 36 até 43 aminoácidos, e originam-se da proteólise da proteína precursora do amiloide (APP) (Querfurth e LaFerla, 2010). Essa proteína pode ser processada através de duas vias, uma via não-amiloidogênica, que não forma peptídeos Aβ, envolvendo a ação de uma enzima

α-secretase, seguida da ação de uma enzima γ-secretase, ou através de uma via amiloidogênica, envolvendo a ação sequencial de uma enzima β-secretase, chamada enzima clivadora do sítio beta da proteína precursora do amiloide (BACE-1), e de uma enzima γ-secretase, formando peptídeos Aβ (Figura 2) (Haass e Selkoe, 2007; Querfurth e LaFerla, 2010). A atividade da BACE-1 parece ser o passo limitante na produção dos peptídeos Aβ, sendo responsável pelo processamento de cerca de 10% da APP, enquanto os outros 90% normalmente são processados através da via não-amiloidogênica (Murphy e LeVine, 2010). A clivagem realizada pela γ-secretase, por sua vez, é um pouco variável, podendo formar peptídeos com diferenças na sua porção C-terminal. Dessa forma, existem diferentes tipos de peptídeos Aβ, incluindo aqueles que terminam na posição 40 (Aβ40), que correspondem a cerca de 80 a 90% dos peptídeos formados, além de alguns outros peptídeos como o Aβ42, que corresponde a cerca de 5 a 10% dos peptídeos sintetizados (Selkoe et al., 2001; Qiu et al., 2015). Um desequilíbrio entre a produção e a eliminação dos peptídeos faz com que o Aβ se acumule, e esse fenômeno poderia ser um dos desencadeadores da DA, segundo a hipótese amiloidogênica (McGeer e McGeer, 2013).

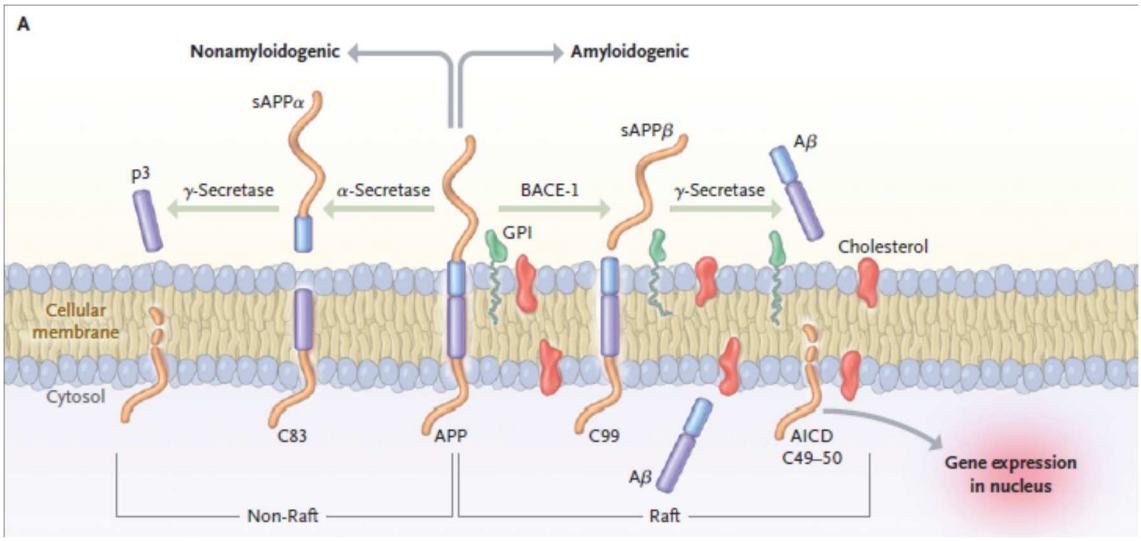

Figura 2 – Processamento da APP e síntese do Aβ.

Legenda: A APP pode ser processada pela via não-amiloidogênica (lado esquerdo da figura), envolvendo a ação de uma α-secretase ou pela via amiloidogênica (lado direito da figura), envolvendo a ação da BACE-1. O processamento da APP pela via amiloidogênica é responsável pela formação dos peptídeos Aβ.

Fonte: Querfurth e LaFerla, 2010.

Os peptídeos Aβ normalmente são sintetizados em uma forma solúvel e posteriormente se agregam espontaneamente em múltiplas formas coexistentes. Inicialmente são formados pequenos dímeros e trímeros, que posteriormente dão origem aos oligômeros. Os oligômeros então podem se agregar formando as fibrilas, que se arranjam em folhas β pregueadas para formar as placas senis características dos estágios avançados da DA (Stine et al., 2003; Querfurth e LaFerla, 2010). Ao passo que o Aβ não parece exercer efeitos tóxicos no estado monomérico, os seus oligômeros solúveis e os amiloides intermediários são bastante neurotóxicos, com seus níveis estando frequentemente associados com os déficits cognitivos na DA (Lue et al., 1999). As fibrilas insolúveis, por outro lado, são menos tóxicas, todavia, as placas senis formadas pela deposição dessas fibrilas acabam servindo como uma espécie de reservatório de peptídeos Aβ, apresentando dímeros e oligômeros difusíveis em suas adjacências, que podem se deslocar para locais mais distantes das placas, causando danos aos neurônios e às sinapses (Shankar et al., 2008; Selkoe, 2011).

O acúmulo de peptídeos Aβ na DA causa dano neuronal por meio de diversos mecanismos distintos. O Aβ promove a ativação de várias vias pró-apoptóticas que contribuem para a formação dos emaranhados fibrilares intracelulares, que aceleram a morte neuronal e causam os sintomas de demência típicos da DA (Takata e Kitamura, 2012). Estudos mostram que o Aβ reduz a expressão da Bcl-2, uma proteína anti-apoptótica, e aumenta a expressão da Bax, uma proteína pró-apoptótica, regulando a

sobrevivência neuronal através da modulação do balanço entre as proteínas pró e anti-apoptóticas (Paradis et al., 1996; Selznick et al., 2000; Clementi et al., 2006). Também, sabe-se que a elevação dos níveis de Aβ desencadeia uma resposta crônica do sistema imune, envolvendo a ativação de *Toll-like receptors* 2 (TLR 2), TLR4 e TLR6, o que resulta em inflamação e na liberação de citocinas inflamatórias e outras moléculas que podem afetar a depuração do Aβ e de restos neuronais e aumentar a morte neuronal mediada por células microgliais (Neniskyte et al., 2011; Liu et al., 2012; Heneka et al., 2015; Minter et al., 2016). Além disso, o Aβ está relacionado com a regulação do estado redox no encéfalo, causando dano oxidativo através da reação de radicais livres com diferentes classes de lipídios e proteínas, alterando suas estruturas e/ou funções e provocando danos na membrana e no DNA celular (Varadarajan et al., 2000; Butterfield et al., 2013; Sun et al., 2015). O acúmulo de Aβ tem sido associado com o aumento dos níveis de espécies reativas de oxigênio (EROS) e com a redução da atividade e/ou expressão de enzimas antioxidantes, incluindo a superóxido dismutase (SOD), catalase e glutationa peroxidase (Turunc Bayrakdar et al. 2014; Liu et al. 2015; Zhang et al. 2015). Por fim, estudos têm associado o acúmulo de Aβ com alterações na função mitocondrial, incluindo a inibição de enzimas mitocondriais como a citocromo c oxidase e alterações no transporte de elétrons, na produção de trifosfato de adenosina (ATP), no consumo de oxigênio e no potencial de membrana mitocondrial, além de estimular a liberação de radicais livres (Smith et al., 1996; Caspersen et al., 2005; Hauptmann et al., 2006; Reddy e Beal, 2008).

1.4 POTENCIAL NEUROTERAPÊUTICO DOS LIGANTES DA TSPO

Conforme mencionado anteriormente, nos últimos anos, diversos estudos têm mostrado que os ligantes da TSPO apresentam efeitos neuroprotetores em diferentes modelos experimentais, incluindo modelos de doenças neurodegenerativas, lesões traumáticas cerebrais e distúrbios psiquiátricos (Papadopoulos e Lecanu, 2009; Rupprecht et al., 2010; Arbo et al., 2015). Especificamente em relação à DA, estudos mapeando a expressão da TSPO em células gliais em diferentes modelos animais de DA mostraram que as células microgliais com alta expressão da TSPO exercem um papel neurotóxico, enquanto astrócitos com alta expressão da TSPO exercem um papel neuroprotetor na DA. Ainda, foi observado que durante a progressão da doença, a ação neurotóxica exercida pela microglia se sobrepõe ao efeito neuroprotetor dos astrócitos (Ji et al., 2008). Posteriormente, visando estudar os possíveis efeitos neuroprotetores de ligantes da TSPO em um modelo animal de DA, Barron et al., (2013) mostraram que a administração de 4'-clorodiazepam (4'-CD, também conhecido como Ro5-4864), um ligante da TSPO, exerce efeitos neuroprotetores em camundongos 3xTgAD, reduzindo o acúmulo de Aβ-40 e a gliose reativa, e melhorando o desfecho funcional dos animais. Além disso, outro estudo mostrou que a administração de PK11195, outro ligante da TSPO, é capaz de inibir a elevação dos níveis de Aβ-42 ocasionada pela administração de lipopolissacarídeo (LPS) em camundongos (Ma et al., 2016). Esses resultados levaram à formulação de hipóteses a respeito do uso de ligantes da TSPO como fármacos em potencial para o tratamento da DA (Chua et al., 2014; Repalli, 2014), todavia, mais estudos são necessários visando a identificação dos mecanismos de ação possivelmente envolvidos nos efeitos neuroprotetores dos ligantes da TSPO em modelos experimentais de DA para a confirmação e o refinamento dessas hipóteses.

1.5 EFEITOS DOS LIGANTES DA TSPO SOBRE O DESENVOLVIMENTO NEURONAL

O desenvolvimento e a maturação neuronal são processos essenciais dentro da fisiopatologia de doenças neurodegenerativas e da regeneração do SNC após as injúrias (Royo et al., 2003; Liu et al., 2011; Winner et al., 2011; Doron-Mandel et al., 2015). Sabe-se que progenitores neurais localizados no giro denteado são capazes de gerar novos neurônios e células gliais no cérebro de mamíferos durante a vida adulta, de forma que essas células são integradas estruturalmente e funcionalmente aos circuitos hipocampais, contribuindo de maneira importante para os processos de aprendizado e memória (Kee et al., 2007; Aimone et al., 2011; Gu et al., 2012). Dentro desse contexto, estudos em modelos animais e análises *post morten* no cérebro de pacientes portadores da DA têm mostrado que a inibição da neurogênese poderia anteceder as lesões características observadas no cérebro de pacientes portadores da DA, exercendo um papel importante no desencadeamento e na progressão da doença (Lazarov e Marr, 2010; Demars et al., 2010; Perry et al., 2012; Gomez-Nicola et al., 2014; Ekonomou et al., 2015). Devido a isso, uma possibilidade de abordagem terapêutica para o combate à DA seriam tratamentos ou outras intervenções capazes de aumentar a neurogênese no hipocampo e melhorar os déficits de memória associados à DA (Schaeffer et al., 2009; Valero et al., 2011; Richetin et al., 2015).

Além de exercerem efeitos neuroprotetores em diversos modelos experimentais, sabe-se que os hormônios esteroides regulam o desenvolvimento neuronal e o processo de neuritogênese através de diferentes mecanismos, incluindo a modulação das vias das proteínas cinases ativadas por mitógenos (MAPK), da fosfatidilinositol-3 cinase (PI3K)

e da Notch/Neurogenina 3 (Arevalo et al., 2012). Entretanto, embora alguns estudos tenham avaliado os efeitos de hormônios como o estradiol sobre a neuritogênese, o desenvolvimento axonal e a sinaptogênese no hipocampo (von Schassen et al., 2006; Ruiz-Palmero et al., 2011; Fester et al., 2012), até o momento não existem estudos que tenham abordado os efeitos de ligantes da TSPO sobre o desenvolvimento neuronal. Além disso, recentemente foi demonstrado que existem diferenças sexuais na diferenciação neuronal no hipotálamo (Scerbo et al., 2014), todavia, ainda necessita ser esclarecido se essas mesmas diferenças também estão presentes na diferenciação e desenvolvimento de neurônios hipocampais.

2 HIPÓTESES

H_0 – O 4’-CD não apresenta efeitos neuroprotetores contra a toxicidade induzida pelo Aβ e não altera o desenvolvimento de cultivos primários de neurônios hipocampais de camundongos machos e fêmeas.

H_1 – O 4’-CD apresenta efeitos neuroprotetores contra a toxicidade induzida pelo Aβ e estimula o desenvolvimento de cultivos primários de neurônios hipocampais de camundongos machos e fêmeas.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Verificar o efeito neuroprotetor do 4’-CD, um ligante da TSPO, em diferentes modelos *in vitro* de toxicidade induzida pelo Aβ, além de seus efeitos sobre o desenvolvimento de neurônios hipocampais.

3.2 OBJETIVOS ESPECÍFICOS

- Verificar o efeito neuroprotetor do 4’-CD sobre a viabilidade de células SH-SY5Y expostas ao Aβ e avaliar o possível envolvimento de proteínas relacionadas com a regulação da sobrevivência e da morte celular nesse efeito.

- Avaliar o efeito do 4’-CD sobre a morte celular em um modelo de cultura organotípica de hipocampo de ratos e avaliar o possível envolvimento de proteínas relacionadas com a regulação da sobrevivência e da morte celular nesse efeito.

- Investigar os efeitos do 4’-CD sobre o desenvolvimento de cultivos primários de neurônios hipocampais de camundongos machos e fêmeas, avaliando os efeitos do 4’-CD sobre a neuritogênese e o desenvolvimento axonal.

- Verificar a existência de diferenças sexuais no desenvolvimento de cultivos primários de neurônios hipocampais de camundongos machos e fêmeas, e se existem diferenças sexuais nos efeitos do 4’-CD sobre o desenvolvimento desses cultivos.

4 MATERIAIS E MÉTODOS

4.1 REAGENTES

O dimetilsulfóxido (DMSO), o 4'-CD, o ácido retinóico (RA), a DNAse I, a poli-L-lisina, o diacetato de fluorosceína (FDA) e o iodeto de propídeo (PI) foram adquiridos da Sigma-Aldrich (St Louis, MO, EUA). Os meios de cultura, o B27 e o GlutaMAX I foram adquiridos da Invitrogen (Invitrogen, Crewe, Reino Unido). A tripsina foi fornecida pela Worthington Biochemicals (Worthington Biochemicals, Freehold, NJ, EUA). O $A\beta_{1-40}$ foi fornecido pela Polypeptide (Estrasburgo, França), enquanto o $A\beta_{1-42}$ foi fornecido pela Bachem (Bubendorf, Suiça).

4.2 CULTURAS DAS CÉLULAS DE NEUROBLASTOMA SH-SY5Y

Células de neuroblastoma SH-SY5Y (American Type Culture Collection, Manassas, VA, USA) foram cultivadas em meio Eagle modificado de Dulbecco/F-12 Ham (DMEM-F12) suplementado com 10% de soro fetal bovino (FBS) inativado por calor e 1% de antibióticos. As células foram mantidas à 37ºC em uma atmosfera contendo 95% de ar e 5% de CO_2. Elas foram passadas duas vezes por semana e não foram usadas após 10 passagens.

4.3 AVALIAÇÃO DOS EFEITOS NEUROPROTETORES DO 4'-CD CONTRA A TOXICIDADE INDUZIDA PELO Aβ EM CÉLULAS SH-SY5Y

Após alcançarem 80% de confluência, as células foram semeadas em placas de 48-poços (para os ensaios de viabilidade) ou em placas de 6 poços (para as análises por

Western Blot). Para a obtenção de células diferenciadas, elas foram cultivadas na presença de ácido retinoico (RA) (10 μM) por seis dias conforme descrito por outros estudos (Guarneri et al., 2000; Jantas et al., 2014), com o meio de cultura sendo trocado a cada dois dias durante este período. Cinco horas antes do início dos tratamentos, as células foram lavadas com solução salina fosfato tamponada (PBS) e incubadas com meio novo sem a adição de soro. As células foram pré-tratadas por 1h com DMSO ou 4'-CD (1, 10, 100 e 1000 nM) e então expostas ao veículo (PBS) ou ao $A\beta_{1-40}$ (12 μM, solubilizado em PBS a 37ºC por 24h para a agregação do Aβ) mais DMSO ou 4'-CD nas doses previamente descritas por 24h.

4.4 AVALIAÇÃO DA VIABILIDADE DAS CÉLULAS SH-SY5Y

A viabilidade celular foi avaliada pelo ensaio do diacetato de fluoresceína (FDA)/iodeto de propídeo (PI) (Astiz et al., 2014). O FDA é um composto que é captado pelas células vivas, onde é convertida em fluoresceína, um composto fluorescente verde. Por outro lado, o PI é um composto que penetra apenas nas células mortas ou em processo de morte celular, onde se intercala ao DNA e emite uma fluorescência vermelha. Após os tratamentos, as células foram incubadas por 50 minutos a 37ºC com FDA (100 μM) e PI (15 μM). Posteriormente, as células foram lavadas com meio de cultivo e as absorbâncias posteriormente lidas em um fluorímetro. A viabilidade celular é expressada pela relação entre as absorbâncias para o FDA e o PI e é dada como percentual do grupo controle. Pelo menos quatro experimentos independentes foram realizados.

4.5 CULTURAS ORGANOTÍPICAS DE HIPOCAMPO

Fatias hipocampais foram obtidas de ratos Wistar de 8 dias de idade conforme descrito por Hoppe et al. (2013). Em resumo, os animais foram mortos, seus encéfalos removidos e seus hippocampos dissecados, e posteriormente foram obtidas fatias (400 μm) usando um chopper de tecidos McIlwain (Mickle Laboratory Engineering Co., Guilford, UK). As fatias foram colocadas sobre insertos de membrana inserts (Millicell®-CM 0.4μm, Millipore) em placas de 6-poços. Cada poço foi preenchido com 1 mL de meio de cultura contendo 50% de meio essencial mínimo (MEM), 25% de solução de sais balanceada de Hank (HBSS) e 25% de soro de cavalo suplementado com glicose (36 mM), HEPES (25 mM), $NaHCO_3$ (4 mM), fungizona (1%) e gentamicina 0,100 $mg.ml^{-1}$. As culturas foram mantidas a 37ºC em uma atmosfera contendo 5% de CO_2 por 25 dias *in vitro* antes do seu uso. Durante esse período, o meio de cultura foi trocado duas vezes por semana.

4.6 AVALIAÇÃO DOS EFEITOS NEUROPROTETORES DO 4'-CD CONTRA A TOXICIDADE INDUZIDA PELO AB EM CULTURAS ORGANOTÍPICAS DE HIPOCAMPO

Após 25 DIV, as culturas foram tratadas com $A\beta_{1-42}$ (5 μM) por 72h em combinação com o veículo (DMSO) ou 4'-CD nas doses de 10 nM, 100 nM e 1 μM. Visando a agregação do peptídeo, o $A\beta_{1-42}$ foi dissolvido em H_2O Milli-Q e incubado a 37ºC por 72h antes do seu uso.

4.7 AVALIAÇÃO DA MORTE CELULAR NAS CULTURAS ORGANOTÍPICAS DE HIPOCAMPO

O dano celular nas culturas organotípicas foi avaliado pela análise da incorporação do PI nas fatias. Setenta e uma horas após os tratamentos, o PI foi adicionado ao meio de cultura na concentração de 5 mM por 1 hora e a fluorescência emitida pelo PI foi observada em um microscópio de fluorescência invertido (Nikon Eclipse TE 300). As imagens foram capturadas através de uma câmera acoplada ao microscópio (Visitron Systems, Puchheim, Alemanha), armazenadas e subsequentemente analisadas no software Scion Image. A quantidade da fluorescência foi determinada por análise densitométrica, após a transformação das imagens em vermelho para o cinza. Para a quantificação do dano neural, o percentual da área contendo fluorescência emitida pelo PI acima dos valores do background foi calculada em relação à área total de cada fatia. A intensidade da marcação com o PI foi expressada como percentual do dano celular (Hoppe et al., 2013). Os dados são apresentados como % da incorporação do PI.

4.8 WESTERN BLOT

Após os ensaios de viabilidade, as células SH-SY5Y foram homogeneizadas em tampão de lise (pH= 7,4) contendo inibidores de protease e detergentes (150 mM NaCl, 20 mM Tris–HCl, 5 mM EDTA, 10% glicerol, 0,5% Nonidet P40) e um coquetel com inibidores de proteases. Os homogeneizados foram centrifugados a 14000xg por 5 minutos a 4ºC para a eliminação de restos celulares, e o sobrenadante foi usado para a análise por Western Blot. Por outro lado, as fatias hipocampais foram homogeneizadas em um tampão de lise contendo 4% de duodecil sulfato de sódio (SDS), 2 mM de EDTA, 50 mM de Tris e 1% de inibidores de proteases. Após a extração de proteínas, as amostras

foram fervidas por 5 minutos. Os níveis de proteínas foram quantificados através do método de Bradford (1979). Posteriormente, quantidades semelhantes de proteínas (25 μg no caso das células SH-SY5Y e 50 μg no caso das fatias hipocampais) foram separadas em géis de poliacrilamida e transferidas para membranas de nitrocelulose conforme previamente descrito (Arbo et al., 2014). Nos experimentos envolvendo as células SH-SY5Y, as membranas foram processadas para imunodetecção através do uso de anticorpos policlonais para survivina (16 kDa) (diluição 1:1000) (Cell Signaling, Danvers, MA, EUA), Bax (23 kDa) (diluição 1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, EUA), Bcl-xl (30 kDa) (diluição 1:50) (Santa Cruz) e procaspase-3 (32 kDa) (diluição 1:1000) (BD Biosciences, San Jose, CA, EUA). Por outro lado, nos experimentos envolvendo as culturas organotípicas de hipocampo, as membranas foram processadas para imunodetecção através do uso de anticorpos policlonais SOD1 (16 kDa) (diluição 1:500), p-Akt (60 kDa) (diluição 1:500), Akt (60 kDa) (diluição 1:500) e procaspase-3 (32 kDa) (diluição 1:500). Após as incubações *overnight* com os anticorpos primários, as membranas foram lavadas com TTBS e incubadas por 2h com os anticorpos secundários conjugados à peroxidase cabra anti-coelho ou cabra anti-camundongo (diluição 1:10000) (Millipore, Billerica, MA, EUA). Posteriormente, após serem lavadas com TBS (20 mM Tris-HCl, 140 mM NaCl, pH= 7,4), as membranas foram reveladas por quimiluminescência seguidas da exposição das membranas a filmes autorradiográficos (Hyperfilm ECL, Amersham). Os filmes foram digitalizados e analisados através do software ImageJ (NIH, Bethesda, MD, EUA). Os resultados obtidos para cada membrana foram normalizados através da relação com a expressão da gliceraldeído 3-fosfato desidrogenase (GAPDH) ou da β-actina conforme indicado. Para minimizar a variação entre os ensaios, as amostras de todos os grupos experimentais foram processadas em paralelo. Os valores para a expressão proteica foram calculados

como unidades densitométricas arbitrárias, e foram expressos como percentual do grupo controle.

4.9 CULTURAS DE NEURÔNIOS HIPOCAMPAIS DE CAMUNDONGOS

Camundongos CD1 foram criados no Instituto Cajal e usados para a geração de embriões para esse estudo. O dia do tampão vaginal foi definido como o dia embrionário 0 (E0). Todos os protocolos usados foram aprovados pelo comitê de ética institucional e estão de acordo com as diretrizes do Conselho das Comunidades Europeias (86/609/CEE).

Embriões de camundongos (E17) foram sexados através da identificação dos testículos dos embriões machos com o uso de um microscópio de dissecção, e os seus hipocampos foram dissecados e dissociados em células isoladas após digestão enzimática com 0,5% de tripsina e DNAse I a 37ºC por 15 minutos (Goslin e Banker, 1989). As células foram lavadas com HBSS livre de Ca^{2+} e Mg^{2+} e os neurônios foram contados e semeados em lamínulas de vidro cobertas por poli-L-lisina em uma densidade de 200 neurônios/mm^2. As células foram cultivadas em meio Neurobasal sem vermelho-fenol suplementado com GlutaMAX I e B27. Sob essas condições, o nível de contaminação por astrócitos deve ser menor do que 5% após 1 dia *in vitro* (DIV) (Ruiz Palmero et al., 2011). Após 1 DIV, neurônios hipocampais de machos e fêmeas foram tratados com DMSO ou 4'-CD (10 nM, 100 nM e 1000 nM) por 24h.

4.10 IMUNOCITOQUÍMICA E ANÁLISE MORFOLÓGICA

Após o tratamento, as células foram fixadas em paraformaldeído 4% por 20 min à temperatura ambiente e permeabilizadas com 0,12% de triton-X mais 0,12% de gelatina diluídos em PBS por 4 minutos. A seguir, as células foram lavadas com PBS/gelatina e incubadas por 1h com anticorpos policlonais de coelho anti-Tau (diluição 1:500) e de galinha anti-MAP-2 (diluição 1:4000) (Abcam, Cambridge, UK) para a marcação dos axônios e dendritos, respectivamente. Em seguida, as células foram novamente lavadas com PBS/gelatina e incubadas por 1h em temperatura ambiente com os anticorpos secundários Alexa Fluor® 568 cabra anti-coelho (diluição 1:1000) (Abcam) para a detecção da Tau e Fluoroscein (FITC) AffiniPure burro anti-galinha (diluição 1:1000) (Jackson ImmunoResearch, West Grove, PA, EUA) para a detecção da MAP-2. Os núcleos celulares foram corados com DAPI. As imagens foram obtidas em um microscópio de fluorescência equipado com uma câmera digital (Leica, Heidelberg, Alemanha) em um aumento de 40x.

Para a avaliação dos efeitos do 4'-CD sobre o desenvolvimento neuronal, as células foram classificadas em três estágios de desenvolvimento (I, II e III) de acordo com sua morfologia (Scerbo et al., 2014). Em resumo, no estágio I, as células ainda não apresentam neuritos bem definidos; no estágio II, os neurônios apresentam neuritos curtos ou prolongamentos relativamente pequenos, enquanto no estágio III, os neurônios apresentam um neurito longo Tau-positivo, relativamente uniforme em diâmetro, que corresponde ao axônio (Scerbo et al., 2014). Para cada grupo, pelo menos 100 células foram avaliadas em cada experimento, e pelo menos três experimentos independentes foram realizados. Além disso, para a avaliação dos efeitos do 4'-CD sobre o desenvolvimento neuronal, foi contado o número de neuritos primários nas células nos

estágios II e III, enquanto o comprimento axonal das células no estágio III foi medido utilizando o software ImageJ (NIH, Bethesda, MD, USA) e expressado como pixel/μm. Ainda, a ramificação neurítica foi avaliada pelo método de Sholl, com o uso do software CellTarget (Garcia-Segura e Perez-Marquez, 2014). Basicamente, uma grade contendo sete círculos concêntricos com raios aumentando progressivamente em 20 μm foi superposta às imagens, com o círculo mais interno sendo fixado em torno do corpo neuronal, e o número de instersecções dos neuritos em cada um dos círculos foi avaliado (Sholl, 1953).

4.11 ANÁLISE ESTATÍSTICA

A análise estatística foi realizada usando o software GraphPad Prism 5.0 (La Jolla, CA, EUA). A análise de variância (ANOVA) de uma via foi realizada para a comparação entre a viabilidade celular e a expressão proteica entre os diferentes grupos nos experimentos envolvendo o estudo dos efeitos neuroprotetores do 4'-CD contra o efeito neurotóxico do Aβ. Quando apropriado, a ANOVA foi seguida do pós-teste de Student-Newman-Keuls (SNK). Para a análise das diferenças morfológicas entre as culturas primárias de neurônios hipocampais, foi realizada uma ANOVA de duas vias, usando-se como fatores independentes sexo e tratamento. Quando apropriado, a ANOVA de duas vias foi seguida do pós-teste de Bonferroni. Todos os resultados foram expressados como média ± erro padrão da média. O nível de significância estatística foi definido como $P<0,05$.

5 CAPÍTULO I

Artigo: Therapeutic actions of translocator protein (18kDa) ligands in experimental models of psychiatric disorders and neurodegenerative diseases

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Therapeutic actions of translocator protein (18kDa) ligands in experimental models of psychiatric disorders and neurodegenerative diseases

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Abstract

Translocator protein (TSPO) is an 18 kDa protein located at contact sites between the outer and the inner mitochondrial membrane. Numerous studies have associated TSPO with the translocation of cholesterol across the aqueous mitochondrial intermembrane space and the regulation of steroidogenesis, as well as with the control of some other mitochondrial functions, such as mitochondrial respiration, mitochondrial permeability transition pore opening, apoptosis and cell proliferation. In the brain, changes in TSPO expression occur in several neuropathological conditions including neurodegenerative diseases and psychiatric disorders. Furthermore, TSPO ligands have been shown to promote neuroprotection in animal models of brain pathology. At least in some cases, the mechanisms of neuroprotection are associated with modifications in brain steroidogenesis. In addition, regulation of neuroinflammation seems to be a common mechanism in the neuroprotective actions of TSPO ligands in different animal models of brain pathology.

Keywords: PBR; steroidogenesis; neuroprotection; traumatic brain injury; anxiety; Alzheimer's disease.

1. Introduction

Translocator protein (TSPO) is an 18 kDa protein located at contact sites between the outer and the inner mitochondrial membranes. It was previously known as peripheral benzodiazepine receptor (PBR), due to its property to bind to diazepam [1]. Functionally, TSPO is well known for its role in the translocation of cholesterol across the aqueous mitochondrial intermembrane space. Due to this specific function, the protein was renamed in 2006 as TSPO [1].

TSPO is an integral membrane protein of 169 amino acids, well conserved throughout evolution [2]. It is arranged in five transmembrane alpha helices, an extra-mitochondrial C-terminal, an intramitochondrial N-terminal and two extramitochondrial and two intramitochondrial loops [3]. It presents high-affinity for cholesterol, having a cholesterol recognition amino acid consensus (CRAC) domain at its C-terminal region [4]. While first studies suggested that TSPO could function as a channel, receiving cholesterol from the steroidogenic acute regulatory protein (StAR) and mediating its transport to the inner mitochondrial membrane, recently, Li et al. [5] studied the crystal structure of TSPO and suggested a possible sliding mechanism of transport for cholesterol on the external protein surface, that would require the association of TSPO with other proteins. Indeed, TSPO forms part of a multiprotein complex, termed the transduceosome, composed by other molecules involved in the transport of cholesterol into the mitochondria such as the voltage-dependent anion channel (VDAC), the 67-kDa long isoform of the adenosine triphosphatase (ATPase) family, AAA domain-containing protein 3 (ATAD3) and StAR [6-11]. The transport of cholesterol into the mitochondria is the rate-limiting step in steroidogenesis. In the mitochondria, the biosynthesis of steroid hormones is initiated with the enzymatic cleavage of the side chain of cholesterol by the

cytochrome P450 side chain cleavage enzyme (CYP11A1), which forms the first steroid, pregnenolone [12]. Pregnenolone, then, leaves the mitochondria towards the endoplasmic reticulum, where it undergoes further enzymatic transformations that will form the final steroid products [13].

Numerous studies have addressed the possible role of TSPO in cholesterol transport and have associated this protein with the regulation of steroidogenesis. The first evidence came from studies showing that TSPO ligands stimulated the biosynthesis of steroids in different cell types in vitro [14-18]. In vivo observations also helped to uphold the role of TSPO in steroidogenesis, such as the increased plasma levels of testosterone in men treated with diazepam [19], the increased corticosterone levels in rats treated with diazepam [20, 21], and the increased synthesis of pregnenolone in the forebrain of rats treated with high-affinity TSPO ligands [22]. In agreement with its proposed function on steroidogenesis, the expression of TSPO is higher in steroid-synthesizing tissues, such as the gonads and adrenals [1]. It should be mentioned, however, that the results of some recent studies using a conditional knockout mice with TSPO deletion in testicular Leydig cells [23] or global TSPO knockouts [24, 25] have challenged the functional implication of TSPO on gonadal steroidogenesis. In addition, another recent study has shown that TSPO deletion had little effect on gonadal steroidogenesis, but affected adrenocorticotropic hormone (ACTH)-mediated steroidogenesis in the adrenals, increasing the number of lipid droplets and the levels of neutral lipids, suggesting an impairment of mitochondrial cholesterol transport and steroidogenesis [26]. These mice presented an up-regulation of *Scarb1* in the adrenals, a gene that is involved in the main pathway of cholesterol uptake from the periphery, facilitating the uptake of cholesteryl esters from high-density lipoproteins, indicating an adaptive response to the lack of

substrate availability for steroidogenesis [26]. Another interesting observation of this study was that the viability of two different lines of Cre-mediated TSPO conditional knockout mice was different [26], indicating that the methodology used to produce TSPO deletion plays a key role in the results obtained. Therefore, a better understanding of these knockout models is necessary to elucidate the function of TSPO on the regulation of steroidogenesis. Findings arising from studies involving transgenic animal models should be analyzed with caution, as complex interactions of the thousands of genes in a multicellular organism may obscure the functions of any individual gene [27]. On the other hand, even if the protein in its quiescent state is not involved in gonadal steroidogenesis, drug activation of the protein affects cholesterol transport and steroidogenesis in every tissue tested so far. This effect is highly specific as shown using drugs of different chemical structure, affinities, stereoisomers, agonists/antagonists, recombinant protein reconstituted alone in liposomes, and based on nuclear magnetic resonance (NMR) and crystal structure information [28]. Moreover, blocking the CRAC domain also blocks steroidogenesis [29]. Thus, the presence of TSPO allows for such control of cholesterol transport and steroid production by the drug ligands.

In addition to the possible relationship of TSPO with the regulation of cholesterol transport and steroidogenesis, TSPO is thought to mediate other mitochondrial functions, such as mitochondrial respiration, apoptosis and cell proliferation [30-34]. Moreover, TSPO has been linked with the control of the mitochondrial permeability transition pore (MPTP) opening, therefore regulating cytochrome C release, caspase activation and apoptosis [35-38]. However, this role is still under debate [39].

2. TSPO in the brain

Brain expression of TSPO in physiological conditions is low. In the CNS, TSPO is mainly found in glia and at very low levels in neurons [32, 37, 40]. TSPO ligands are used for brain imaging of neuroinflammation, since TSPO is upregulated at sites of injury and inflammation, as well as in several neuropathological conditions including stroke and neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, multiple sclerosis and amyotrophic lateral sclerosis [2, 41-43]. Under these conditions, the expression of TSPO is highly enhanced in reactive microglia and astrocytes [7, 44-46]. Steroid hormones may also regulate the expression and activity of TSPO in neural cells. Rats submitted to a model of neuropathic pain and treated with progesterone present a higher spinal expression of TSPO than vehicle-treated animals [47]. Furthermore, estradiol has been recently shown to activate TSPO in hypothalamic astrocytes, promoting the synthesis of progesterone [48]. Interestingly, TSPO levels are also affected by stress. Rats exposed to forced swimming test, a stress-inducing condition, showed higher density of TSPO ligand binding in the cerebral cortex [49], while inescapable tailshocks decreased TSPO ligand binding in the same brain region [50].

Numerous studies have shown that neuroactive steroids, either from peripheral or central origin, exert neuroprotective and anxiolytic actions [51-54]. Given the role of TSPO on steroidogenesis and apoptosis, this molecule has been explored as a therapeutic target for psychiatric disorders (Table 1), neurodegenerative diseases (Table 2) and neurotrauma (Table 3). In the next sections we will examine the studies conducted with TSPO ligands in animal models of these pathological conditions.

3. TSPO ligands as neurotherapeutics

3.1 TSPO ligands and psychiatric disorders

Several studies have associated psychiatric disorders with a downregulation of TSPO expression in peripheral cells. Decreased TSPO expression has been found in the platelets and lymphocytes of patients with anxiety disorders [55-57], in the platelets of patients suffering from schizophrenia [58] and post-traumatic stress disorder [59] and in a suicidal adolescent population [60]. However, increased TSPO density, measured by distribution volume by positron emission tomography, has been detected in the prefrontal cortex, the anterior cingulate cortex and insula of patients with a major depressive episode [61]. In these patients, greater TSPO density in the anterior cingulate cortex correlated with greater depression severity [61].

Different TSPO ligands presented anxiolytic effects in rodents and humans. Etifoxine was the first TSPO ligand to be used in clinics due to its anxiolytic effects, showing an efficacy similar to the benzodiazepine lorazepam [62]. XBD173, a new selective and high affinity TSPO ligand, presents anxiolytic and antidepressant effects in different experimental models, without causing the side effects normally associated with conventional benzodiazepines, such as myorelaxant effects, tolerance and withdrawal symptoms [63, 64]. Also, XBD173 exerted anti-panic effects in rodents and humans, in the absence of sedation, tolerance development and withdrawal symptoms [65]. More recently, this compound has been shown to improve the behavioral deficits involving freezing and anxiety-like behaviors in a mouse model of post-traumatic stress disorder [66]. However, Owen et al. [67] reported high TSPO binding variability of XBD173 across human subjects, and XBD173 did not show superiority over placebo in a phase II trial with patients with generalized anxiety disorder [68]. Other TSPO ligands, such as

ZBD-2 and YL-IPA08, have been shown to exert anxiolytic effects in rats and mice [69-71]. Furthermore, YL-IPA08 reduced contextual fear in rodent models of post-traumatic stress disorder [69, 70] (Table 1).

3.2 TSPO ligands and neurodegenerative diseases

Neurodegenerative diseases include a wide range of acute and chronic conditions characterized by the loss of neuronal and glial cells, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and amyotrophic lateral sclerosis [72]. As mentioned before, increased TSPO expression has been detected in the brain of patients with these pathologies, especially at the sites most affected by degenerative changes [73-81]. However, few studies have investigated the role of TSPO in these diseases (Table 2).

Barron et al [82] have shown that the TSPO ligand 4'-chlorodiazepam (4'-CD, also known as Ro5-4864), exerts neuroprotective actions in a mouse model of Alzheimer's disease, reducing hippocampal amyloid-beta (Aβ) accumulation and gliosis in 3xTgAD mice. These findings correlated with marked improvements in functional outcomes, including increased working memory performance and reduced anxiety behaviors in 3xTgAD mice. Another study examined the neuroprotective effects of other TSPO ligand, etifoxine, in a mouse model of multiple sclerosis. In this study, etifoxine attenuated the severity of the disease when administered before the development of clinical signs and improved symptomatic recovery when administered at the peak of the disease. Etifoxine decreased peripheral immune cell infiltration in the spinal cord and increased oligodendrial regeneration after inflammatory degeneration [83].

The protective effect of TSPO ligands have also been assessed in a model of peripheral diabetic neuropathy caused by the injection of streptozotocin to male rats. Treatment of these animals with 4'-CD resulted in an improvement of nerve conduction velocity, thermal threshold, and skin innervation and improved expression of myelin proteins and Na+,K+-ATPase activity in the sciatic nerve [84]. In addition, the TSPO ligand etifoxine has been shown to promote axonal regeneration and functional recovery after peripheral nerve freeze injury [85] and to enhance peripheral nerve regeneration through large acellular nerve grafts [86].

3.3 TSPO ligands and traumatic brain injury

Traumatic brain injury (TBI) is one of the leading causes of morbidity and mortality in young adults, being caused by direct neural tissue damage and secondary sequelae [87]. Reactive gliosis is one of the main responses of the brain to injuries, and TSPO upregulation in microglia and astrocytes in response to lesions is directly related with the degree of damage. Several studies suggest that TSPO ligands could be used as markers for the state and progression of TBI [40, 88]. In addition, some studies have addressed the neuroprotective effects of TSPO ligands in experimental models of brain injury (Table 3). Soustiel et al [89] have shown that 4'-CD is neuroprotective in a rat model of cortical injury, increasing the number of surviving neurons and the density of the neurofilament network in the perilesional cortex. These effects were correlated with a decreased activity of caspase-9 and caspase-3, indicating that 4'-CD could be acting through the inhibition of the caspase-dependent apoptosis pathway, which is triggered by the cytochrome c release from the mitochondria [89]. The same group described that 4'-CD decreases intracranial pressure after a cortical contusion, reducing water content and

improving cerebral metabolism and neurological recovery, probably through a mechanism involving a protective effect of 4'-CD in mitochondria as evidenced by electron microscopy analysis [90].

4. TSPO and the control of neuroactive steroid levels

As previously mentioned, neuroactive steroids exert neuroprotective actions in a variety of experimental models of neurodegenerative diseases and also have positive outcomes in models of psychiatric disorders [51-54]. Therefore, since TSPO is known to be involved in steroidogenesis, it has been proposed that TSPO ligands may exert neurotherapeutic actions by regulating the synthesis of neuroactive steroids. Indeed, changes in the levels of neuroactive steroids in the brain have been detected after the administration of different TSPO ligands, including etifoxine [91-93], XBD173 [65], 4'-CD [82, 94] and YL-IPA08 [69], among others.

There is evidence that alterations in the regulation of steroidogenesis by TSPO are associated with psychiatric disorders. For instance, a polymorphism (rs6971) in the TSPO gene has been associated with the diagnosis of bipolar disorder [95]. This polymorphism causes an amino acid substitution within the transmembrane domain, which is in the same location of the cholesterol binding pocket [4] and therefore it may affect cholesterol transport. Interestingly, this polymorphism is also associated with an altered peripheral production of pregnenolone [96].

The effects of TSPO ligands on animal models of psychiatric disorders and neurodegeneration are often associated with increased local levels of neuroactive steroids. The anxiolytic effects of etifoxine and imidazopyridine acetamides are associated with an

increase in the brain levels of several neuroactive steroids, such as pregnenolone, progesterone, 5α-dihydroprogesterone (5α-DHP), allopregnanolone and THDOC [91, 92]. Moreover, Verleye et al [92] reported that when etifoxine was administered in combination with finasteride, an inhibitor of the 5α-reductase, the enzyme responsible for the conversion of 5α-DHP in allopregnanolone, its anxiolytic effects were attenuated, indicating that the anxiolytic effects of etifoxine could be mediated by an increased synthesis of allopregnanolone. The neuroprotective effects of etifoxine in a mouse model of multiple sclerosis were associated with an increase in the mRNA expression of 3α-hydroxysteroid dehydrogenase, an enzyme responsible for the production of allopregnanolone [83]. Concerning the TSPO ligand XBD173, which exerts anxiolytic and antidepressant effects, Rupprecht et al. [65] have shown that it increased the production of neurosteroids in brain slices and enhanced GABAergic neurotransmission, possibly through the action of neuroactive steroids. In addition, the anxiolytic effects of YL-IPA08 were associated with an increase in the levels of allopregnanolone in the prefrontal cortex [69]. Furthermore, the protective effects of 4'-CD in the sciatic nerve of diabetic rats were associated with an increase in the local levels of pregnenolone, progesterone and dihydrotestosterone [84].

All these findings suggest that the therapeutic actions of TSPO ligands in the nervous system may be mediated by an increase in the levels of neuroactive steroids. However, there is still a lack of direct evidence on the link between the increase in neuroactive steroids and the actions of TSPO ligands in neural cells. Furthermore, in some cases, as reported for etifoxine on frog hypothalamic explants [93], the increase in steroidogenesis produced by TSPO ligand may be mediated by TSPO independent mechanisms [97].

5. TSPO and the control of neuroinflammation

Neuroinflammation is a common component of neurodegenerative diseases and traumatic brain injury and may also play a role in psychiatric disorders [98]. Both microglia and astrocytes participate in the neuroinflammatory response of the central nervous system. TSPO is highly expressed in astrocytes and microglia under neurodegenerative conditions and TSPO seems to participate in the control of reactive gliosis and neuroinflammation. Administration of 4'-CD to male rats reduced neuronal loss, reactive astrogliosis and reactive microgliosis in the hippocampus caused by the systemic administration of the excitotoxin kainic acid [99]. Another TSPO ligand, PK11195 was shown to reduce microgliosis and microglia proliferation in the hippocampus of male rats that received an intracerebroventricular infusion of bacterial lipopolysaccharide (LPS) [100]. In contrast to the systemic administration of kainic acid, the treatment with LPS does not induce significant neuronal loss in the hippocampus. Therefore, it can be concluded that the reduction of microgliosis by PK11195 is not a secondary effect due to decreased neuronal loss, suggesting that microglia are direct targets of TSPO ligands. This is also suggested by the fact that the TSPO ligand XBD173 decreased the expression of proinflammatory markers in the microglia cell line BV-2 exposed to LPS [45]. Furthermore, the over-expression of TSPO on microglia decreased its production of proinflammatory cytokines induced by LPS. In contrast, TSPO knock-down in microglia increased the effects of LPS on the expression of inflammatory markers [101, 102]. This effect of TSPO was at least in part mediated by the modulation of NF-κB activity [101].

TSPO may also participate in the communication of astrocytes and microglia in the regulation of neuroinflammation. In the retina TSPO is upregulated in microglia under conditions of inflammation. At the same time the TSPO endogenous ligand diazepam-binding inhibitor (DBI) is upregulated in astrocytes and Müller cells, a specialized macroglia cell type of the retina. Triakontatetraneuropeptide (TTN), a DBI derived peptide and a TSPO ligand, reduced microglia activation in vitro and reduced retinal inflammatory responses in vivo, suggesting that TSPO ligands mediate astroglia-microglia communication to regulate the neuroinflammatory response [102].

6. Conclusion and future directions

The evidence reviewed here indicates that TSPO is a potential therapeutic target for psychiatric disorders and neurodegenerative diseases. Thus, TSPO ligands have been documented to be effective in different experimental animal models of neurodegenerative diseases, TBI and psychiatric disorders. Increased neuroactive steroid synthesis and decreased neuroinflammation may be involved in the neural actions of TSPO ligands. However, a direct proof for these possible mechanisms is still lacking. Further investigations focused on the study of the effects of conditional TSPO knockout in brain cells are necessary. Recently, a NMR structure of the mouse TSPO [3] and high resolution crystal structures for TSPO from two distinct bacteria were obtained [5, 103], allowing a correlation of TSPO structure and function at the molecular level for the first time. Although these studies showed a similar overall topology of the monomer, differences between the crystallographic and NMR structures were found, including the ligand binding residues and oligomeric states. Therefore, further studies should be focused on the elucidation of the functional attributes that may arise from these structures, trying to

address at which extent TSPO acts alone or in combination with other proteins in both health and disease states, and how it interacts with different drugs.

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Table 1 - The use of TSPO ligands as neurotherapeutic agents in different experimental models of psychiatric disorders.

Experimental models	TSPO ligands	Effect
Water-lick conflict test	Etifoxine and imidazopyridine acetamides	Anxiolytic effects [91, 92].
Different anxiety and depression animal models	XBD173	Anxiolytic and antidepressant-like effects [63, 64].
	YL-IPA08	Anxiolytic and antidepressant-like effects [70].
Elevated plus-maze and social exploration test	XBD173	Anxiolytic effects [65].
Lactate- or CCK4-induced panic in rodents	XBD173	Anti-panic effects [65].
Mouse model of post-traumatic stress disorder	XBD173	Decreased freezing and anxiolytic-like behaviors [66].
	YL-IPA08	Decreased anxiolytic-like behavior and contextual fear [69].
CCK4-induced panic in healthy male volunteers	XBD173	Anti-panic effects [65].
Patients with generalized anxiety disorder	XBD173	Did not show superiority over placebo [68].
Animal model of chronic pain	ZBD-2	Anxiolytic effect [71].

Abbreviations: 4'-CD: 4'-chlorodiazepam; CCK4: cholecystokinin tetrapeptide; TSPO: translocator protein (18 kDa).

Tabela 2 - The use of TSPO ligands as neurotherapeutic agents in different experimental models of neurodegenerative diseases.

Experimental models	TSPO ligands	Effect
Neurodegenerative diseases		
Mouse model of Alzheimer's disease	4'-CD	Decreased hippocampal Aβ accumulation and gliosis, increasing working memory performance and reducing anxiety behaviors [82].
Mouse model of multiple sclerosis	Etifoxine	Decreased inflammation and peripheral immune cell infiltration of the spinal cord, and increased oligodendroglial regeneration after inflammatory demyelination [83].
Rat model of peripheral diabetic neuropathy	4'-CD	Improved nerve conduction velocity, thermal threshold, skin innervation and the expression of myelin proteins and Na+,K+-ATPase activity in the sciatic nerve [84].
Peripheral nerve freeze injury	Etifoxine	Promoted axonal regeneration and functional recovery [85].

Abbreviations: 4'-CD: 4'-chlorodiazepam; Aβ: amyloid-beta; TSPO: translocator protein (18 kDa).

Tabela 3 - The use of TSPO ligands as neurotherapeutic agents in different experimental models of traumatic brain injury and neuroinflammation.

Experimental models	TSPO ligands	Effect
Traumatic brain injury		
Rat model of cortical injury	4'-CD	Increased the neuronal survival and the density of the neurofilament network in the perilesional cortex, decreasing the activity of caspase-9 and caspase-3 [89].
	4'-CD	Decreased intracranial pressure, reducing the water content and improving the cerebral metabolism and the neurological recovery [90].
Neuroinflammation		
BV-2 cells exposed to LPS	XBD173	Decreased the expression of proinflammatory markers [45].
Kainic acid excitotoxicity	4'-CD	Reduced neuronal loss, reactive astrogliosis and reactive microgliosis in the hippocampus [99].
Intracerebroventricular infusion of LPS	PK11195	Reduced microgliosis and microglia proliferation in the hippocampus [100].
LPS-induced retinal inflammation	TTN	Reduced microglia activation in vitro and reduced retinal inflammatory responses [102].

Abbreviations: 4'-CD: 4'-chlorodiazepam; TSPO: translocator protein (18 kDa); TTN: Triakontatetraneuropeptide.

6 CAPÍTULO II

Artigo: 4'-Chlorodiazepam is neuroprotective against amyloid-beta through the modulation of survivin and bax protein expression in vitro

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4'-Chlorodiazepam is neuroprotective against amyloid-beta through the modulation of survivin and bax protein expression in vitro

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Abstract

The translocator protein of 18 kDa (TSPO) is located in the outer mitochondrial membrane and is involved in the cholesterol transport into the mitochondria and in the regulation of steroidogenesis, mitochondrial permeability transition pore opening and apoptosis. TSPO ligands have been investigated as therapeutic agents that promote neuroprotective effects in experimental models of brain injury and neurodegenerative diseases. The aim of this study was to identify the neuroprotective effects of 4'-chlorodiazepam (4'-CD), a ligand of TSPO, against amyloid-beta (Aβ) in SH-SY5Y neuroblastoma cells and its mechanisms of action. Aβ decreased the viability of SH-SY5Y neuroblastoma cells, while 4'-CD had a neuroprotective effect at the doses of 1 nM and 10 nM. The neuroprotective effects of 4'-CD against Aβ were associated with the inhibition of Aβ-induced upregulation of Bax and downregulation of survivin. In summary, our findings indicate that 4'-CD is neuroprotective against Aβ-induced neurotoxicity by a mechanism that may involve the regulation of Bax and survivin expression.

Keywords: translocator protein (TSPO); steroids; steroidogenesis; apoptosis; Alzheimer's disease.

1. Introduction

Translocator protein (TSPO) is an 18 kDa protein located at contact sites between the outer and the inner mitochondrial membranes, which was previously known as peripheral benzodiazepine receptor (PBR), due to its property to bind diazepam [1, 2]. This protein is arranged in five transmembrane alpha helices and presents a cholesterol recognition amino acid consensus (CRAC) domain at its C-terminal region, presenting high-affinity for cholesterol [3, 4].

Functionally, TSPO is thought to mediate the translocation of cholesterol across the aqueous mitochondrial intermembrane space, and due to this function, this protein was renamed in 2006 as TSPO [1]. TSPO forms part of a multiprotein complex, termed the transduceosome, composed by other molecules involved in the transport of cholesterol into the mitochondria such as: (i), the voltage-dependent anion channel (VDAC); (ii), the 67-kDa long isoform of the adenosine triphosphatase (ATPase) family, AAA domain-containing protein 3 (ATAD3) and (iii), the steroidogenic acute regulatory protein (StAR) [5-7]. The transport of cholesterol into the mitochondria is the rate-limiting step in steroidogenesis. In the mitochondria, the biosynthesis of steroids is initiated with the enzymatic cleavage of the side chain of cholesterol by the cytochrome P450 side chain cleavage enzyme (CYP11A1), which forms the first steroid, pregnenolone [8]. Pregnenolone, then, leaves the mitochondria towards the endoplasmic reticulum, where it undergoes further enzymatic transformations that will form the final steroid products, including progesterone, testosterone and estradiol [9].

In addition to its relationship with the regulation of cholesterol transport and steroidogenesis, TSPO is thought to mediate other mitochondrial functions, such as mitochondrial respiration and cell proliferation and differentiation [10, 11]. Moreover,

TSPO has been associated with the control of the mitochondrial permeability transition pore (MPTP) opening, therefore regulating cytochrome C release, caspase activation and apoptosis [12-14].

TSPO is widely distributed throughout the body, being found in most tissues, including the adrenal, pineal and salivary glands, the olfactory epithelium, ependyma, gonads, heart, kidney, liver, lung, bone, marrow, brain, spinal cord and peripheral nerves [2, 15]. In the nervous system, the expression of TSPO in physiological conditions is low, however, this protein is upregulated at sites of injury and inflammation, as well as in several neuropathological conditions including stroke and neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, multiple sclerosis and amyotrophic lateral sclerosis [16-21]. Some studies have shown that TSPO ligands may be neuroprotective in experimental models of different neurodegenerative diseases, as well as in experimental models of brain injuries and psychiatric disorders (see Arbo et al. [22] for review). In addition, Girard et al. [23] have shown that the TSPO ligand etifoxine is able to improve nerve regeneration and functional recovery after nerve transection in rats. Furthermore, Giatti et al [24] demonstrated that the TSPO ligand 4'-chlorodiazepam (4'-CD, also known as Ro5-4864) is able to increase the levels of pregnenolone, progesterone and dihydrotestosterone in the sciatic nerves of diabetic rats and to decerase the severity of diabetic peripheral neuropathy. In relation to AD, it was observed that 4'-CD exerts neuroprotective actions in a mouse model of AD, reducing hippocampal amyloid-beta (Aβ)-40 accumulation and gliosis, and improving the functional outcomes of 3xTgAD mice [25].

AD pathological hallmarks include the presence of extracellular senile plaques mainly composed of Aβ peptide and intracellular neurofibrillary tangles formed by hyper-

phosphorilated aggregates of tau, which is a microtubule-associated protein [26]. Aβ accumulation causes neuronal damage in AD through the activation of pro-apoptotic pathways [27-29] and contributes to the formation of intracellular neurofibrillary tangles, which further accelerates neuronal loss and causes the symptoms of dementia [30]. It is known that Aβ downregulates the expression of Bcl-2, an anti-apoptotic protein, and upregulates the expression of Bax, a pro-apoptotic protein, regulating neuronal survival through the modulation of the balance between pro-apoptotic and anti-apoptotic proteins [31-33]. Therefore, the aim of this study was to identify the neuroprotective effects of 4'-CD against amyloid-beta (Aβ) in SH-SY5Y neuroblastoma cells and its mechanisms of action.

2. Material and Methods

2.1 Chemicals

Dimethyl sulfoxide (DMSO), 4'-CD, retinoic acid (RA), fluorescein diacetate (FDA) and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Culture mediums were purchased from Invitrogen (Invitrogen, Crewe, UK). $A\beta_{1-40}$ was supplied by Polypeptide (Strasbourg, France).

2.2 Cell Cultures

Female human SH-SY5Y neuroblastoma cells (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle Medium/Nutrient F-12 Ham (DMEM F12) supplemented with 10% heat-inactivated fetal

bovine serum (FBS) and 1% of antibiotics. Cells were maintained at 37º C in a saturated humidity atmosphere containing 95% air and 5% CO_2. They were passaged twice per week and were not used after 10 passages.

2.3 Evaluation of the neuroprotective effects of 4'-CD against Aβ induced neurotoxicity

After reaching 80% confluence, cells were seeded into 48-well plates (for viability assays) or into 6-well plates (for Western Blot analysis). In order to obtain differentiated cells, they were cultured with RA (10μM) for six days as described elsewhere [34, 35] and the culture medium was changed every two days during this period. Five hours before treatments, cells were washed with phosphate buffered solution (PBS) and incubated with fresh serum-free medium. Cells were pre-treated for 1h with DMSO or 4'-CD (1nM, 10nM, 100nM and 1000nM), and then exposed to vehicle (PBS) or $A\beta_{1-40}$ (12μM, solubilized in PBS at 37ºC for 24h in order to obtain aggregated Aβ) plus DMSO or 4'-CD in the doses previously described for 24h. The dose of Aβ used in this study was based in a pilot study (data not shown).

2.4 Cell Viability Assays

To assess cell viability we performed the fluorescein diacetate (FDA)/propidium iodide (PI) assay [36]. FDA is a cell-permeant esterase substrate that stain live cells. Living cells actively convert the non-fluorescent FDA into the green compound fluorescein. FDA assay allows the evaluation of cell-membrane integrity, which is required for intracellular retention of its fluorescent product. In contrast, PI is a membrane

impermeable compound commonly used for the identification of dead cells. After treatments, cells were incubated for 50 min at 37ºC with FDA (100µM) and PI (15µM). After that, cells were washed with culture medium and plates were read in a fluorimeter. The viability ratio is expressed by the ratio of the FDA/PI absorbance and is given as percentage of control, and at least four independent experiments were performed.

2.5 Western Blotting

After viability assay, cells were homogenized in lysis buffer (pH 7.4) containing protease inhibitors and detergents (150mM NaCl, 20mM Tris–HCl, 5mM EDTA, 10% glycerol, 0.5% Nonidet P40 and protease inhibitor cocktail). The homogenates were centrifuged at 14000×g for 5 min at 4ºC to discard cell debris, and the supernatant fraction obtained was used for Western blot assay. After protein isolation, the samples were boiled for 5 min. The protein levels were measured by the method of Bradford [37]. After protein measurement, sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) on 12 or 15% (w/v) was carried out using a mini-protean system (Bio-Rad, Hercules, CA, USA) with broad range molecular weight standards (Precision Plus Protein Dual Color Standards, Bio-Rad). Protein (25µg) was loaded in each lane with loading buffer containing 65mM Tris (pH= 6.8), 50% glycerol, 10% SDS, 0.5M mercaptoethanol, 0.002% bromophenol blue. Samples were heated at 94ºC for 2 min prior to gel loading. After electrophoresis, proteins were transferred to 0.2 µm nitrocellulose membranes (Trans-Blot, Bio-Rad) by a semi-dry system 25V, 2.5A, 5 min (Trans-Blot Turbo Transfer System, Bio-Rad). The membranes were blocked with 5% (w/v) BSA in TTBS (138 mM NaCl, 25 mM Tris, pH 8.0, and 0.1% (w/v) Tween-20) at room temperature for 2h, and then incubated overnight at 4°C with the primary antibody diluted in this same blocking

solution. The membranes were processed for immunodetection using rabbit polyclonal antibodies for survivin (16kDa) (1:1000 dilution) (Cell Signaling, Danvers, MA, USA), Bax (23kDa) (1:500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bcl-xl (30kDa) (1:50 dilution) (Santa Cruz) and procaspase-3 (32kDa) (1:1000 dilution) (BD Biosciences, San Jose, CA, USA). After washing with TTBS, the membranes were incubated for 2h at room temperature with goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA, USA) or goat anti-mouse (Bio-Rad) peroxidase-conjugate secondary antibodies (1:10000 dilution) and washed with TBS (20mM Tris-HCl, 140mM NaCl, pH= 7.4). The blots were revealed for chemiluminescence followed by apposition of the membranes to autoradiographic films (Hyperfilm ECL, Amersham). The densitometric analysis of the autoradiographies was performed with the image ImageJ software (NIH, Bethesda, MD, USA). The results from each membrane were normalized to β-actin (42kDa) (1:4000 dilution) (Sigma-Aldrich) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (36kDa) (1:1000 dilution) (Millipore, Billerica, MA, USA) where indicated. To minimize interassay variations, samples from all experimental groups were processed in parallel. Protein expression values were calculated as arbitrary densitometric units.

2.6 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 5.0 software (La Jolla, CA, USA). A one-way analysis of variance (one-way ANOVA) was performed to evaluate cell viability and protein expression between different groups. When appropriate, ANOVA was followed by the Student-Newman-Keuls (SNK) *post hoc* test. All results were

expressed as mean ± standard error (SEM). The level of statistical significance was set at $P< 0.05$.

3. Results

3.1 4'-CD is neuroprotective against Aβ induced neurotoxicity

To assess its neuroprotective effects, four different doses of 4'-CD were tested against Aβ-induced neurotoxicity. It was observed that 4'-CD was neuroprotective against Aβ when administered at 1nM or 10nM ($F_{(6,30)}= 8.093$, $P<0.0001$) (Figure 1). However, when cells were treated with higher doses of 4'-CD (100nM or 1μM), its neuroprotective effect disappeared. Based on these findings, we selected the dose of 10nM to be used in the subsequent experiments.

3.2 The neuroprotective effect of 4'-CD against Aβ is related with the modulation of the protein expression of Bax and survivin

After the identification of the neuroprotective effect elicited by 4'-CD administration against Aβ-induced neurotoxicity, we studied the expression of some proteins implicated in the regulation of cell survival and apoptosis, namely Bax, survivin, Bcl-xl and procaspase-3, which could mediate the neuroprotective effects of 4'-CD. We observed that Aβ increased the protein expression of Bax ($F_{(3,20)}= 5.916$, $P=0.0046$) (Figure 2) and decreased the protein expression of survivin ($F_{(3,18)}= 3.454$, $P=0.0385$) (Figure 3), while the concomitant administration of 4'-CD inhibited these effects. No differences were

found in the expression of Bcl-xl (Figure 4) and procaspase-3 (Figure 5) between the experimental groups.

4. Discussion

In the last decade, several studies have assessed the neuroprotective actions of TSPO ligands in different experimental models. These compounds, which are currently used for brain imaging of neuroinflammation due to the upregulation of TSPO in reactive microglia and astrocytes, have been shown to be neuroprotective in different experimental models of neurodegenerative diseases, brain injuries and psychiatric disorders (as reviewed by Arbo et al. [22]). In this study, we observed that the TSPO ligand 4'-CD was neuroprotective against Aβ treatment in female human neuroblastoma cells. These data corroborate previous data by Barron et al. [25], which found that 4'-CD was neuroprotective in a mouse model of AD, reducing hippocampal Aβ-40 accumulation and gliosis, and improving the functional outcomes of 3xTgAD mice.

As previously mentioned, it is known that Aβ may affect neuronal survival through the modulation of the balance between pro-apoptotic and anti-apoptotic proteins [31]. We observed here that Aβ neurotoxicity was associated with a decreased expression of survivin, which was reversed by the co-administration of 4'-CD. Survivin is a downstream protein of the β-catenin pathway and a member of the inhibitor of apoptosis protein family, which exerts anti-apoptotic effects by inhibiting caspase activation and activated caspases, including caspase-3 and -7 [38, 39]. Although we did not observe changes in the levels of procaspase-3, survivin could be acting modulating its cleavage into the active caspase-3, whose levels were not detected in the Western-blots. Indeed,

data suggest that although survivin tightly binds to active caspases such as caspase-3 and -7, it may not bind to its inactive proforms, acting mainly by inhibiting the activated caspases and regulating caspase activation by preventing the cleavage of its proforms [38]. Therefore, this could explain the fact that the changes in the levels of survivin were not correlated with significant differences between the levels of procaspase-3. In addition, it should be mentioned that the role of caspase-3 in the Aβ-induced neuronal death is still controversial, as studies have suggested that Aβ neurotoxicity may be also caspase-independent [32, 40-42].

In addition to the modulation of survivin protein expression, the neuroprotective effects of 4'-CD were also associated with a decreased protein expression of Bax. As previously mentioned, several studies have shown that Aβ-induced neuronal damage is associated with the modulation of the balance between pro-apoptotic and anti-apoptotic proteins, including the upregulation of Bax and the downregulation of anti-apoptotic proteins from the Bcl-2 family [31-33]. Bax is a pro-apoptotic protein from the Bcl-2 protein family that is found mainly in the cytosol as soluble monomers, and to a lesser extent, loosely associated to mitochondria [43]. Bax activity is under the control of prosurvival members of the Bcl-2 family, including Bcl-2 and Bcl-xl, which heterodimerize with Bax and other pro-apoptotic proteins, inhibiting their activation [44]. After apoptotic stimulation, Bax undergoes conformational changes and is translocated from the cytosol to the mitochondria, where it regulates the formation of pores and leads to the mitochondrial outer membrane permeabilization (MOMP), resulting in the release of cytochrome c and other pro-apoptotic factors from the mitochondria, which lead to caspase activation and apoptosis [45]. Therefore, the neuroprotective actions of 4'-CD against Aβ could be related with a downregulation of Bax expression, which could be

associated with decreased cytochrome c release and increased cell viability. Although the changes in the Bax expression were not directly related to significant changes in the expression of Bcl-xl, other proteins of the Bcl-2 family such as Bcl-2 could be involved in the regulation of Bax expression after 4'-CD treatment.

As previously mentioned, functionally, TSPO is thought to regulate cholesterol transport and steroidogenesis [5, 6, 22]. Therefore, one of the hypothesis regarding the neuroprotective effects of TSPO ligands against Aβ is that the activation of TSPO could be related with the stimulation of the biosynthesis of neurosteroids, which could mediate the neuroprotective effects of TSPO ligands. Regarding this aspect, it is known that estradiol is able to regulate the expression of proteins from the Bcl-2 family, decrease mitochondrial cytochrome c release and protect cortical and hippocampal neurons against Aβ [46, 47]. These data were corroborated by another study, which found that estradiol protects cerebellar granule cells against Aβ through a reduction in the Aβ-induced upregulation of Bax and downregulation of Bcl-xl, inhibiting mitochondrial cytochrome c release and apoptosis [48]. Moreover, Qin et al. [49] demonstrated that progesterone is neuroprotective against Aβ-induced neurotoxicity in primary cultured rat cortical neurons through the inhibition of JNK signaling and the inhibition of the mitochondrial apoptotic pathway. More recently, Grimm et al. [50] (2015) showed that different neurosteroids, including progesterone, estradiol and testosterone, are able to rescue the bioenergetics deficits induced by the overexpression of Aβ in SH-SY5Y cells. In addition, other study showed that both estradiol and progesterone are able to regulate the expression of Aβ clearance factors in primary neuron cultures and female rat brain [51], corroborating a possible role of neurosteroids in the regulation of Aβ actions in the brain. On the other hand, it is also known that neurosteroidogenesis is affected by Aβ in SH-SY5Y cells [52]

and that 3xTg-AD mice show age-related changes in neuroactive steroid levels [53], corroborating the relationship between Aβ and these molecules. Therefore, as 4'-CD elicit neuroprotection against Aβ through similar mechanisms than neurosteroids, including the modulation of the balance between pro-apoptotic and anti-apoptotic proteins, it is possible that these effects could be mediated by increased neurosteroidogenesis, and this hypothesis should be tested by further studies.

In summary, we showed that 4'-CD is neuroprotective against Aβ-induced neurotoxicity and that this effect may be related with the inhibition of Aβ-induced downregulation of survivin and upregulation of Bax. Further studies may investigate if the neuroprotective effects of 4'-CD against Aβ are mediated by increased neurosteroidogenesis related to the stimulation of TSPO activity.

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Figure Legends

Figure 1. Effect of different doses of 4'-CD in the viability of SH-SY5Y cells exposed to Aβ. Cells were pre-treated with vehicle or different doses of 4'-CD for 1h, and then treated with vehicle or 4'-CD and exposed to Aβ (1-40) (12μM) for 24h. At least four independent experiments were performed. Data represent the mean ± SEM and are expressed as percentage of control values. #Different from DMSO; *Different from Aβ (ANOVA/SNK, $P<0.05$).

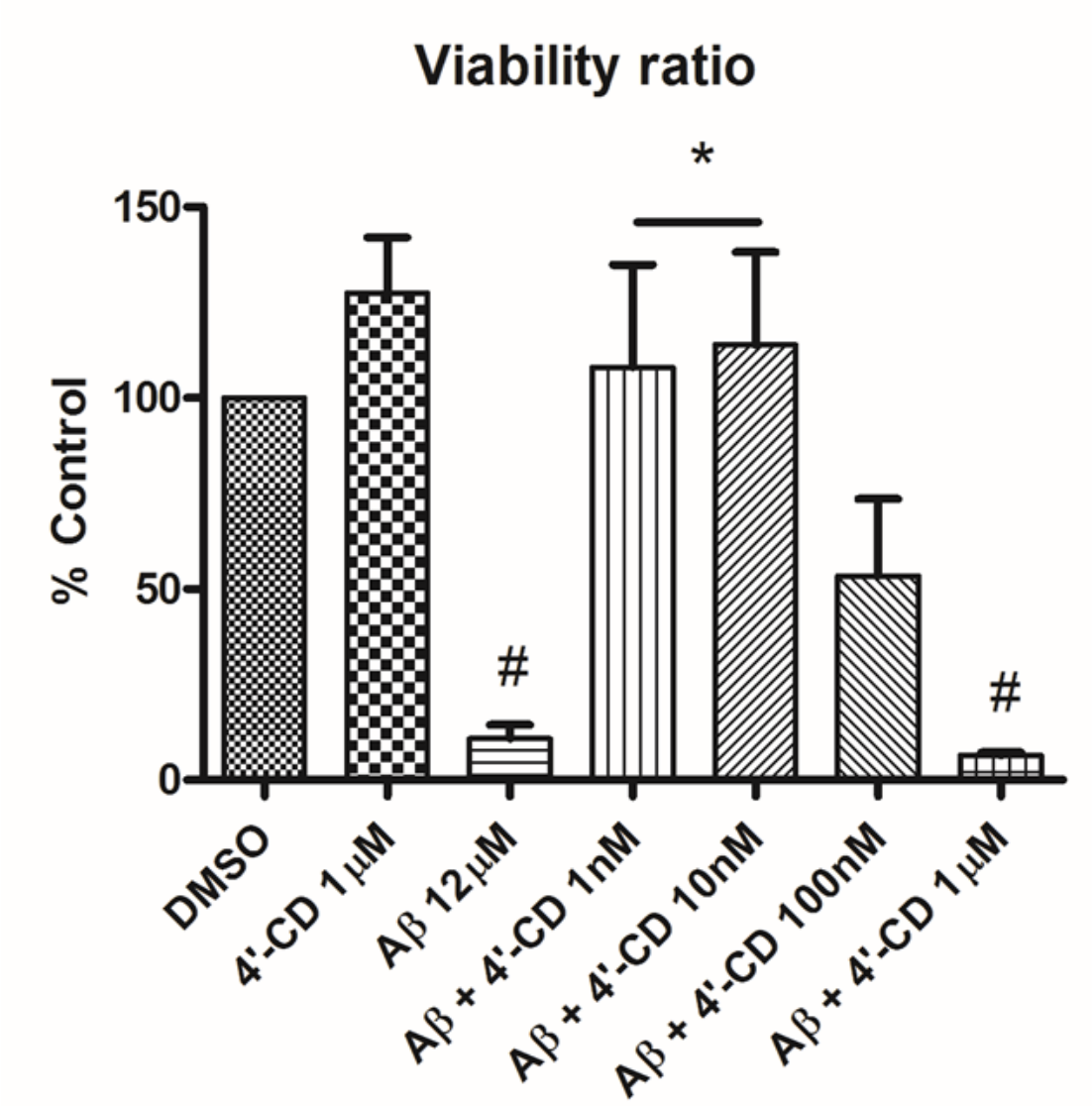
Figure 2. Effect of 4'-CD in the protein expression of Bax in SH-SY5Y cells exposed to Aβ. Cells were pre-treated with vehicle or 4'-CD (10 nM) for 1h, and then treated with vehicle or 4'-CD (10 nM) and exposed to Aβ (1-40) (12μM) for 24h. Data represent the mean ± SEM. *Different from other groups (n= 5-6/group; ANOVA/SNK, $P=0.0046$).

Figure 3. Effect of 4'-CD in the protein expression of survivin in SH-SY5Y cells exposed to Aβ. Cells were pre-treated with vehicle or 4'-CD (10 nM) for 1h, and then treated with vehicle or 4'-CD (10 nM) and exposed to Aβ (1-40) (12μM) for 24h. Data represent the mean ± SEM. *Different from other groups (n= 6/group; ANOVA/SNK, $P=0.0385$).

Figure 4. Effect of 4'-CD in the protein expression of Bcl-xl in SH-SY5Y cells exposed to Aβ. Cells were pre-treated with vehicle or 4'-CD (10 nM) for 1h, and then treated with vehicle or 4'-CD (10 nM) and exposed to Aβ (1-40) (12μM) for 24h. Data represent the mean ± SEM (n= 5-6/group).

Figure 5. Effect of 4'-CD in the protein expression of procaspase-3 in SH-SY5Y cells exposed to Aβ. Cells were pre-treated with vehicle or 4'-CD (10 nM) for 1h, and then treated with vehicle or 4'-CD (10 nM) and exposed to Aβ (1-40) (12μM) for 24h. Data represent the mean ± SEM (n= 6/group).

Figure 1


Viability ratio
% Control
150
100
50
0
*
#
#
DMSO
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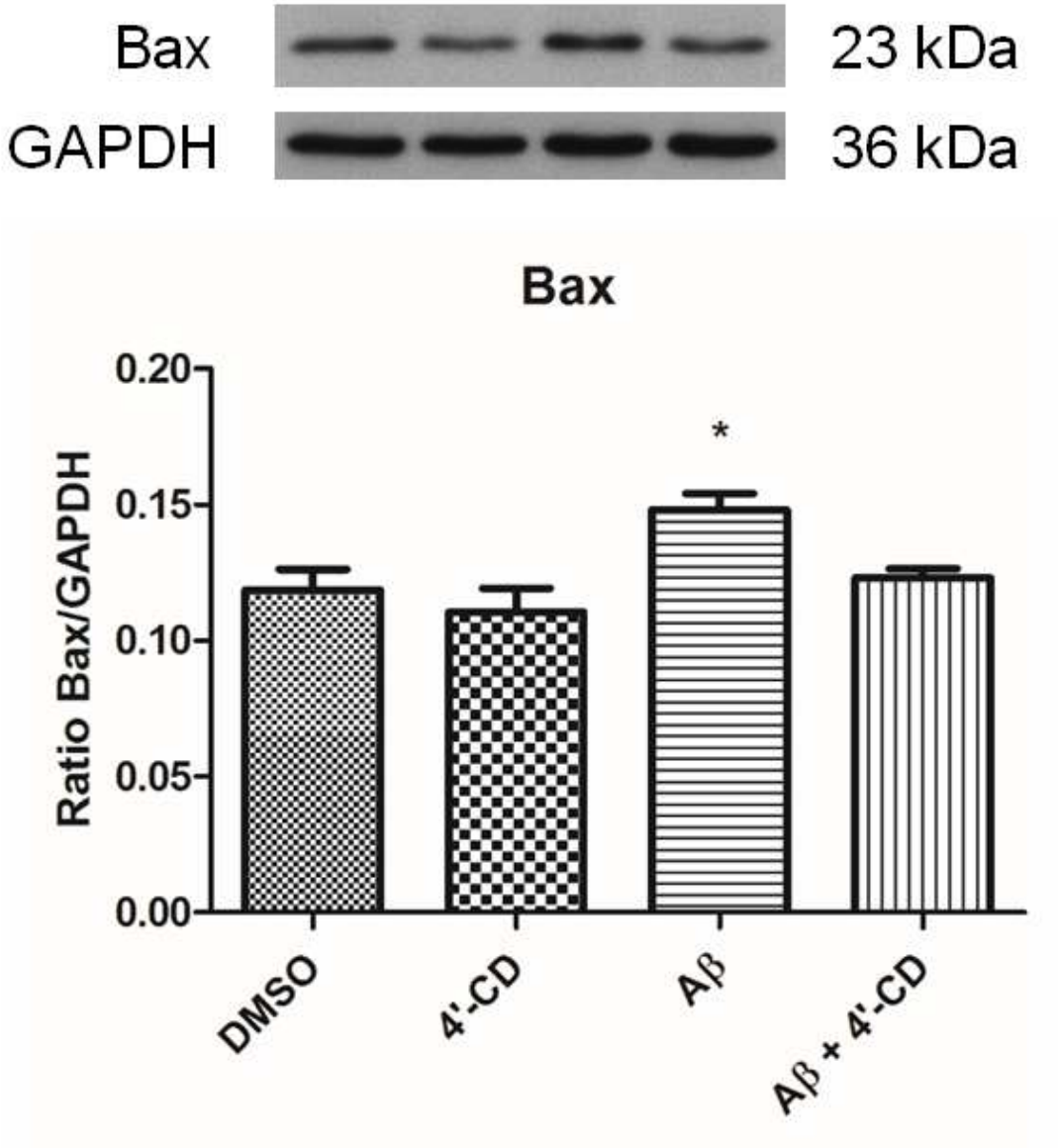
Aβ 12 μM
Aβ + 4'-CD 1nM
Aβ + 4'-CD 10nM
Aβ + 4'-CD 100nM
Aβ + 4'-CD 1 μM

Figure 2


Bax
23 kDa
GAPDH
36 kDa
Bax
Ratio Bax/GAPDH
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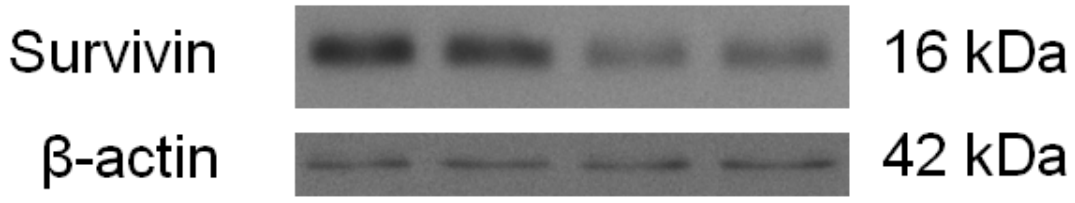
0.15
0.10
0.05
0.00
*
DMSO
4'-CD
Aβ
Aβ + 4'-CD

Figure 3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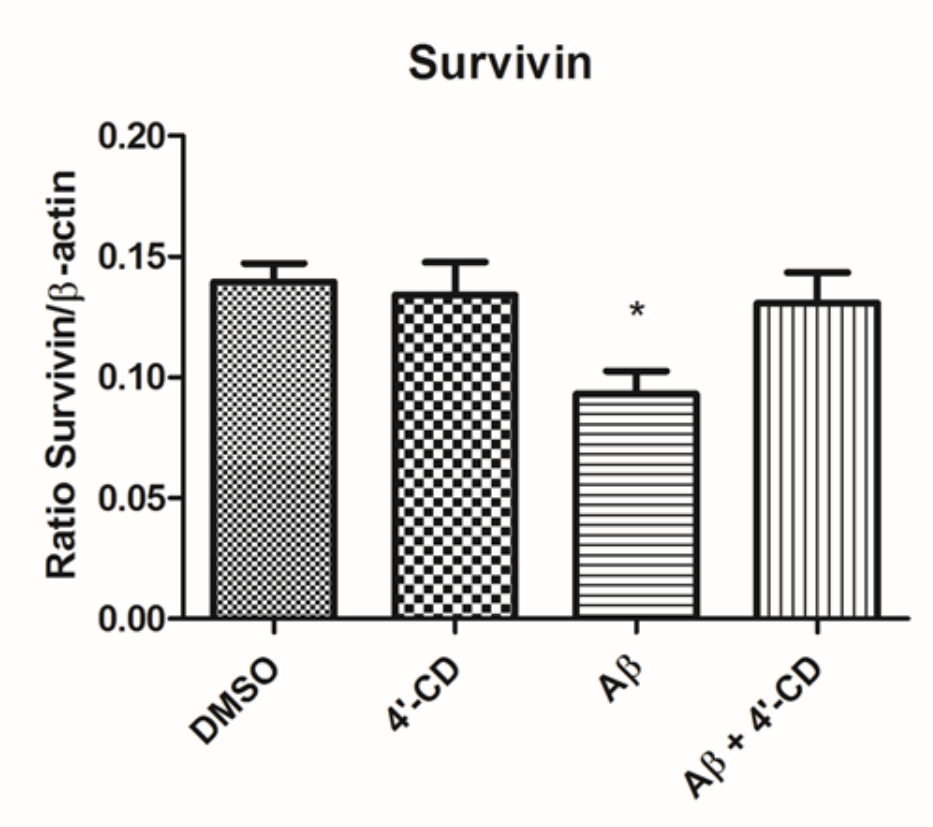
β-actin
42 kDa


Survivin
Ratio Survivin/β-actin
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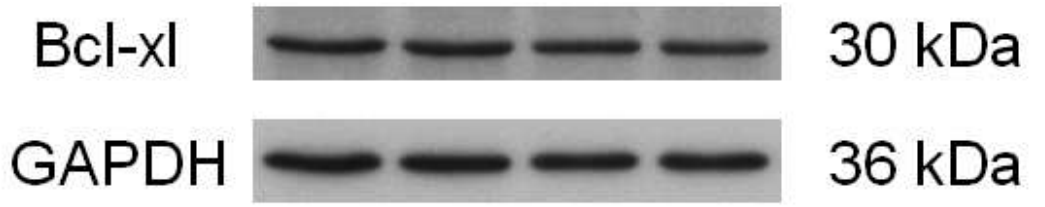
0.15
0.10
0.05
0.00
*
DMSO
4'-CD
Aβ
Aβ + 4'-CD

Figure 4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GAPDH
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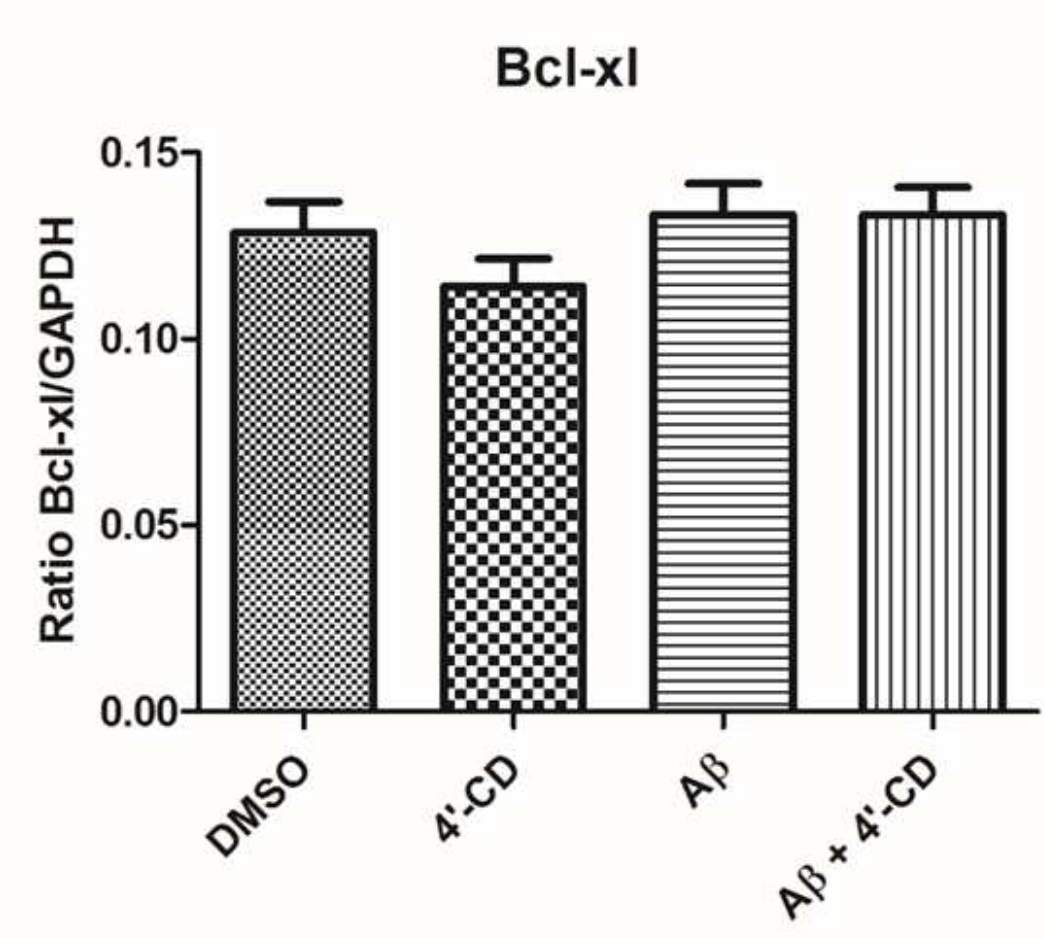
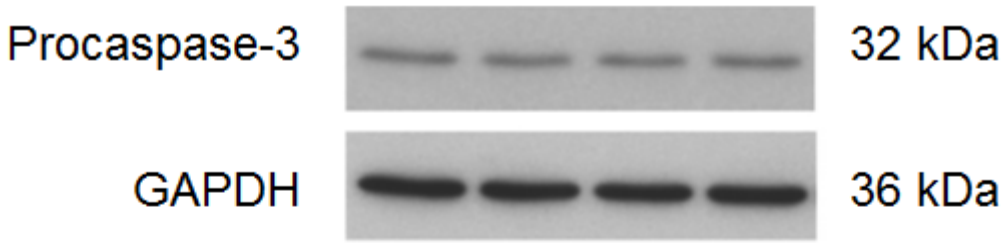

Bcl-xl
Ratio Bcl-xl/GAPDH
0.15
0.10
0.05
0.00
DMSO
4'-CD
Aβ
Aβ + 4'-CD

Figure 5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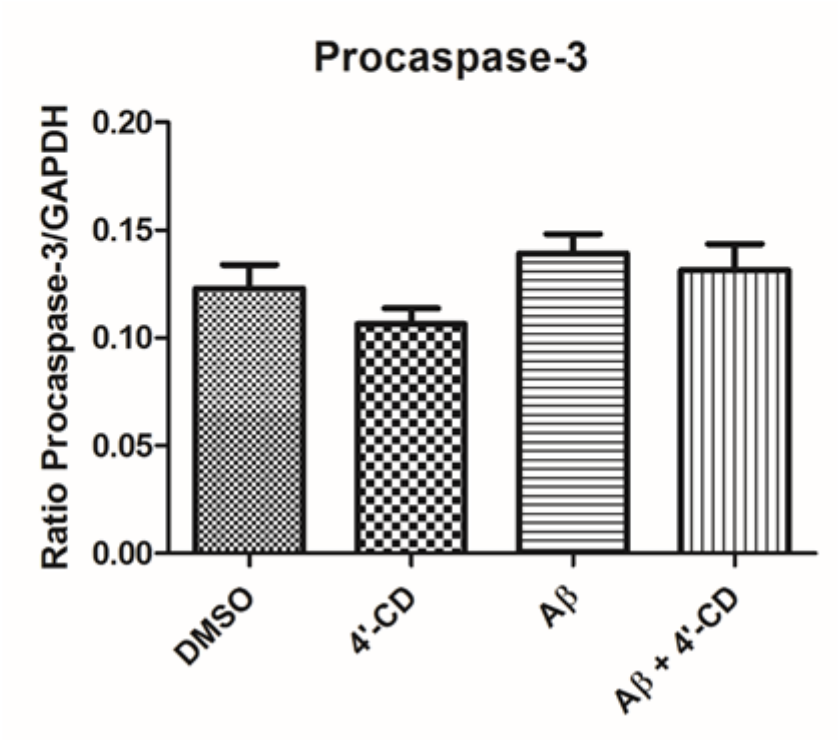
GAPDH
36 kDa


Procaspase-3
Ratio Procaspase-3/GAPDH
0.20
0.15
0.10
0.05
0.00
DMSO
4'-CD
Aβ
Aβ + 4'-CD

7 CAPÍTULO III

Artigo: 4’-Chlorodiazepam is neuroprotective against amyloid-beta in organotypic hippocampal slices

Status: A ser submetido para o periódico Brain Research

4'-Chlorodiazepam is neuroprotective against amyloid-beta in organotypic hippocampal slices

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Abstract

The translocator protein of 18 kDa (TSPO) is located in the outer mitochondrial membrane and is involved in the cholesterol transport into the mitochondria and in the regulation of steroidogenesis, mitochondrial permeability transition pore opening and apoptosis. TSPO ligands have been investigated as therapeutic agents that promote neuroprotective effects in experimental models of brain injury and neurodegenerative diseases. In a previous study, our group showed that 4'-chlorodiazepam (4'-CD), a TSPO ligand, was neuroprotective against amyloid-beta (Aβ) in SHSY-5Y neuroblastoma cells. The aim of this study was to identify the neuroprotective effects of 4'-CD against Aβ in organotypic hippocampal slices and its mechanisms of action. Aβ decreased the cell viability of organotypic hippocampal slices, while 4'-CD had a neuroprotective effect at the doses of 100 nM and 1 μM. The neuroprotective effects of 4'-CD against Aβ were associated with an increased expression of superoxide dismutase (SOD). In summary, our findings indicate that 4'-CD is neuroprotective against Aβ-induced neurotoxicity by a mechanism that may involve the regulation of SOD protein expression.

Keywords: translocator protein (TSPO); steroids; apoptosis; Alzheimer's disease, oxidative stress, superoxide dismutase (SOD).

Introduction

The increase in the life expectancy of the world population is accompanied by an exponential increase in the number of people affected by neurodegenerative diseases related to aging. Alzheimer's disease (AD) is the most common neurodegenerative disease and the major cause of dementia in people over 60 years-old, affecting around 35 million people over the world (Querfurth e LaFerla, 2010). Data from 2013 show that whereas the mortality rate related to cardiovascular diseases and stroke decreased by 14% and 23%, respectively, the mortality rate related to AD increased by 71%, making it the sixth leading cause of death in the USA and the fifth leading cause of death in individuals over 65 years-old (Alzheimer's Association, 2015). The costs related to the treatment and care of AD patients in the USA were estimated in 226 billion dollars in 2015 (Alzheimer's Association, 2015), while the expenses related to the care of patients suffering from dementia in Europe were estimated in 105 billion euros in 2010 (Gustavsson et al. 2011). However, despite the large economic impact of AD, there are no effective treatments for this disease. Currently, the drugs approved for the treatment of AD are only intended to treat some of the symptoms of this disease, and there is no treatment able to prevent its progression through the inhibition of its pathogenic mechanisms (Sugino et al. 2015).

AD pathological hallmarks include the presence of extracellular senile plaques mainly composed of amyloid-beta (Aβ) peptide and intracellular neurofibrillary tangles formed by hyper-phosphorylated aggregates of tau, which is a microtubule-associated protein (Cavalucci et al. 2012). Aβ accumulation causes neuronal damage in AD through the activation of pro-apoptotic pathways (Nikolaev et al. 2009; Vohra et al. 2010; Zhou et al. 2011) and contributes to the formation of intracellular neurofibrillary tangles, which further accelerates neuronal loss and causes the symptoms of dementia (Takata and

Kitamura, 2012). In addition, AD is also related to oxidative stress, manifested by protein oxidation, lipid peroxidation and DNA oxidation (Butterfield et al. 2013). Aβ accumulation has been associated with increased levels of reactive oxygen species (ROS) and decreased levels and activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase (Turunc Bayrakdar et al. 2014; Liu et al. 2015; Zhang et al. 2015).

Translocator protein (TSPO) is an 18 kDa protein located at contact sites between the outer and the inner mitochondrial membranes, which was previously known as peripheral benzodiazepine receptor (PBR), due to its property to bind diazepam (Papadopoulos et al. 2006; Batarseh and Papadopoulos, 2010). This protein is arranged in five transmembrane alpha helices and presents a cholesterol recognition amino acid consensus (CRAC) domain at its C-terminal region, presenting high-affinity for cholesterol (Li and Papadopoulos, 1998; Jaremko et al., 2014). Functionally, TSPO is thought to mediate the translocation of cholesterol across the aqueous mitochondrial intermembrane space, and due to this function, this protein was renamed in 2006 as TSPO (Papadopoulos et al. 2006). The transport of cholesterol into the mitochondria is the rate-limiting step in steroidogenesis. In the mitochondria, the biosynthesis of steroids is initiated with the enzymatic cleavage of the side chain of cholesterol by the cytochrome P450 side chain cleavage enzyme (CYP11A1), which forms the first steroid, pregnenolone (Miller and Auchus, 2011). Pregnenolone then leaves the mitochondria towards the endoplasmic reticulum, where it undergoes further enzymatic transformations that will form the final steroid products, including progesterone, testosterone and estradiol (Lacapère and Papadopoulos, 2003).

In addition to its relationship with the regulation of cholesterol transport and steroidogenesis, TSPO is thought to mediate other mitochondrial functions, such as mitochondrial respiration and cell proliferation and differentiation (Hirsch et al. 1989; Corsi et al. 2008). Moreover, TSPO has been associated with the control of the mitochondrial permeability transition pore (MPTP) opening, therefore regulating cytochrome C release, caspase activation and apoptosis (Zamzami and Kroemer, 2001; Azarashvili et al. 2007; Sileikyte et al. 2011).

Some studies have shown that TSPO ligands may be neuroprotective in different experimental models of neurodegenerative diseases, brain injuries and psychiatric disorders (see Arbo et al. (2015) for review). Specifically regarding AD, it was observed that 4'-CD exerts neuroprotective actions in a mouse model of AD, reducing hippocampal Aβ-40 accumulation and gliosis, and improving the functional outcomes of 3xTgAD mice (Barron et al. 2013). In addition, a previous study of our group showed that 4'-CD is neuroprotective against Aβ administration in SH-SY5Y neuroblastoma cells through the modulation of the protein expression of survivin and Bax (Arbo et al. 2016). Therefore, due to the need to clarify the mechanisms of action of 4'-CD against Aβ-induced neurotoxicity, the aim of this study was to investigate the neuroprotective effects of 4'-CD against Aβ in organotypic hippocampal slices and its mechanisms of action.

Material and Methods

Chemicals

Dimethyl sulfoxide (DMSO), 4'-CD and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Culture mediums were purchased from Gibco (Carlsbad, CA, USA). $A\beta_{1-42}$ was supplied by Bachem (Bubendorf, Switzerland).

Organotypic Hippocampal Cultures

Hippocampal slices were obtained from 8-day-old male Wistar rats as previously described (Hoppe et al. 2013). Briefly, the animals were euthanized, their brains were removed, the hippocampi were dissected and transverse hippocampal slices (400 μm thickness) were obtained by using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Guilford, UK). The slices were placed on membrane inserts (Millicell®-CM 0.4μm, Millipore) in six-well plates. Each well contained 1 ml of culture medium consisting of 50% minimum essential medium (MEM), 25% Hank's balanced salt solution (HBSS), 25% horse serum supplemented with glucose (36 mM), HEPES (25 mM), $NaHCO_3$ (4 mM), fungizone (1%) and gentamicin 0.100 $mg.ml^{-1}$. Cultures were incubated at 37ºC in an atmosphere of 5% of CO_2 for 25 days *in vitro* (DIV) prior to use. Culture medium was changed twice a week.

Drugs and Treatments

4'-CD was dissolved in DMSO and stored at -20ºC until use. $A\beta_{1-42}$ was incubated in Milli-Q water at 37ºC for 72h prior to use. On DIV 25, cultures were treated with $A\beta_{1-42}$ (5 μM) plus 4'CD in three different doses (10 nM, 100 nM and 1 μM) or its vehicle for 72h.

Quantification of cellular death

Cell damage was assessed by fluorescent image analysis of propidium iodide (PI, Calbiochem, San Diego, CA, USA) uptake. 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. Seventy-one hours after treatments, the slices were stained with PI (5 mM) for 1h. PI fluorescence was observed by an inverted fluorescence microscope (Nikon Eclipse TE 300). Images were captured using a CCD camera (Visitron Systems, Puchheim, Germany), stored and subsequently analyzed by using Scion Image software. The amount of PI fluorescence was determined densitometrically after transforming the red values into grey values. For quantification of neural damage, the percentage of area expressing PI fluorescence above background level was calculated in relation to the total area of each slice. PI intensity, meaning cell death, was expressed as a percentage of cell damage (Hoppe et al., 2013). Data are presented as % of PI incorporation.

Western Blot

Organotypic slices were homogenized in lysis buffer containing 4% sodium dodecyl sulfate (SDS), 2 mM EDTA, 50 mM Tris, 1% mammalian protease inhibitor (Roche, San Francisco, CA, USA) at 4ºC and stored at -20ºC until use. Equal amounts of protein (50 μg) were resolved and immunodetected as previously described (Arbo et al., 2014). The membranes were processed for immunodetection using rabbit polyclonal antibodies for SOD1 (16 kDa) (1:500 dilution), p-Akt (60 kDa) (1:500 dilution), Akt (60 kDa) (1:500 dilution) and procaspase-3 (32 kDa) (1:500 dilution). After washing with TTBS, the membranes were incubated for 2 h at room temperature with goat anti-rabbit

or goat anti-mouse peroxidase-conjugated secondary antibodies (1:10000 dilution) (Millipore, Billerica, MA, USA) and washed with TBS (20 mM Tris-HCl, 140 mM NaCl, pH= 7.4). The blots were revealed for chemiluminescence followed by apposition of the membranes to autoradiographic films (Hyperfilm ECL, Amersham). The films were scanned and the digitalized images analysed using the software ImageJ (NIH, Bethesda, MD, USA). The results from each membrane were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (36 kDa) (1:500 dilution) (Millipore). The average optical density for the control group was designated as 100%. To minimize interassay variations, samples from all experimental groups were processed in parallel. Protein expression values were calculated as arbitrary densitometric units.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 5.0 software (La Jolla, CA, USA). A one-way analysis of variance (one-way ANOVA) was performed to evaluate cell viability and protein expression between different groups. When appropriate, ANOVA was followed by the Student-Newman-Keuls (SNK) *post hoc* test. All results were expressed as mean ± standard error (SEM). The level of statistical significance was set at $P< 0.05$.

Results

4'-CD is neuroprotective against Aβ induced neurotoxicity

First, to establish a model of Aβ-induced neurotoxicity, we evaluated the effect of Aβ (5 μM) treatment for 24, 48 and 72 hours in the viability of organotypical hippocampal slices. It was observed that the administration of Aβ decreased the viability of hippocampal slices after 72h, while its administration for 24 or 48h did not affect the

viability of the slices ($F_{(3,23)}$= 51.36, P<0.0001) (Figure 1A and B). Based on these results, we selected to use the Aβ at the dose of 5 μM for 72h in the other experiments. Later, we evaluated if 4'-CD was neuroprotective against Aβ administration for 72h in rat organotypic hippocampal slices. It was observed that 4'-CD was neuroprotective against Aβ when administered at 100 nM or 1 μM ($F_{(5,48)}$= 61.24, P<0.0001) (Figure 1C and D).

The neuroprotective effects of 4'-CD against Aβ could be related with the modulation of the protein expression of SOD

After the identification of the neuroprotective effect elicited by the administration of 4'-CD against Aβ-induced neurotoxicity, we studied the expression of SOD1, one of the major antioxidant enzymes expressed in the CNS, and of some proteins implicated in the regulation of cell survival and apoptosis, such as Akt and procaspase-3, which could mediate the neuroprotective effects of 4'-CD. We observed that the administration of 4'-CD in all the doses tested increased the protein expression of SOD1 after the administration of Aβ ($F_{(5,53)}$= 3.228, P=0.0136) (Figure 2). No differences were found in the protein expression of Akt (Figure 3) and procaspase-3 (Figure 4) between the experimental groups.

Discussion

In the last decade, several studies have assessed the neuroprotective effects of TSPO ligands in different experimental models, reporting that these compounds exert neuroprotective effects in experimental models of neurodegenerative diseases, brain injuries and psychiatric disorders (Arbo et al. 2015). Specifically regarding the neuroprotective effects of TSPO ligands in experimental models of AD, a previous study by our group showed that 4'-CD is neuroprotective against Aβ in SH-SY5Y

neuroblastoma cells through the modulation of survivin and bax protein expression (Arbo et al. 2016). Corroborating these data, in this study, we showed that 4'-CD was neuroprotective against Aβ in organotypic hippocampal slices. Since glial cells could be an important site for the action of TSPO ligands as TSPO expression is generally higher in these cells than in neurons (Veenman et al. 2007; Chen and Guilarte, 2008), we chose to study the neuroprotective effects of 4'-CD against Aβ in organotypic hippocampal slices, as they preserve the connections between neurons and glial cells and could help us to better understand the mechanisms involved in the neuroprotective actions of 4'-CD against Aβ-induced neurotoxicity.

It is known that AD is associated with oxidative damage and that Aβ accumulation has been associated with increased levels of reactive oxygen species (ROS) and decreased levels and activities of antioxidant enzymes, including SOD, catalase and glutathione peroxidase (Turunc Bayrakdar et al. 2014; Liu et al. 2015; Zhang et al. 2015). In this study, we showed that the treatment with 4'-CD after Aβ administration was able to increase the expression of SOD1 in comparison with the Aβ-only treated group. SOD is an enzyme that catalyzes the dismutation of the superoxide radical, which is produced as a by-product of the oxygen metabolism and may cause many types of cell damage (Bresciani et al. 2015). Oxidative stress that occurs within the bilayer, hypothesized in the Aβ-induced oxidative stress hypothesis in which $A\beta_{1-42}$ inserts as oligomers into the bilayer and serves as a source of ROS, has been shown to initiate lipid peroxidation (Butterfield et al. 2013). Studies show that Met-35 of Aβ peptides is critical for Aβ-associated toxicity and oxidative stress. The oxidation of Met-35 to form methionine sulfoxide plays an important role in the regulation of protein function and cellular defense, and may lead to lipid or protein oxidation (Stadtman, 2004; Butterfield et al. 2013). Studies have reported increased levels of oxidative stress markers for protein

oxidation/nitration and nucleic acid oxidation in brains from patients in the early stages of AD (Gabbita et al. 1998; Wang et al. 2006; Barone et al. 2011, 2012). In addition, Murakami et al. (2011) have shown that SOD1 deficiency drives Aβ oligomerization and memory loss in a mouse model of AD, while other study showed that human neuroblastoma SH-SY5Y cells overexpressing SOD1 are less susceptible to Aβ insult (Celsi et al. 2004), corroborating the idea that SOD could have a crucial role in the defense against Aβ-induced damage. Therefore, the stimulation of the synthesis and activity of antioxidant enzymes could be a mechanism for the action of neuroprotective agents against Aβ-induced neurotoxicity. Indeed, several studies have shown that the neuroprotective actions of different compounds against Aβ involve the modulation of the expression of antioxidant enzymes in brain cells (Liu et al. 2015; Yu et al. 2015; Zhang et al. 2015). Moreover, some studies have shown that 4'-CD exerts antioxidant effects in different experimental models. Mehta et al. (2010) showed that 4'-CD attenuates the cognitive impairment promoted by the administration of a carbamate pesticide through an increase in the expression of antioxidant enzymes. In addition, other authors have also associated the antioxidant effects of 4'-CD with its cardioprotective effects in different experimental models (Jaiswal et al. 2010; Xiao et al. 2010; Paradis et al. 2013). Therefore, all together, these data suggest that the modulation of SOD protein expression could be related with the neuroprotective actions of 4'-CD against Aβ-induced neurotoxicity.

In addition to its oxidative damage, it is known that Aβ may affect neuronal survival through the modulation of the balance between pro-apoptotic and anti-apoptotic proteins (Paradis et al. 1996; Hoppe et al. 2013; Arbo et al. 2016). Therefore, we studied the expression of some proteins involved in the regulation of cell survival to verify their involvement in the neuroprotective effects of 4'-CD against Aβ-induced neurotoxicity. In this study, we did not find any difference in the expression of Akt and procaspase-3

between our experimental groups. Akt is a serine/threonine kinase which is part of the phosphoinositide-3 kinase (PI3K) pro-survival pathway. Although previous studies showed that Akt may be involved in the Aβ-induced cell damage (Chen et al. 2009; Yin et al. 2011), Hoppe et al. (2013) did not show any difference in the expression of Akt after Aβ administration in rat organotypical hippocampal slices in a protocol similar to that used in this paper. However, we cannot rule out the involvement of PI3K/Akt pathway in the effects of both Aβ of 4'-CD in this experimental model, as changes in the expression and activation of Akt could be happening in the first hours after treatments, but could be abolished after 72h. In addition to the absence of differences in Akt expression, we did not find any difference in the expression of procaspase-3 in our experimental groups. Procaspase-3 is the inactive precursor of caspase-3, which is considered the central and final apoptotic effector responsible for apoptosis. Previous studies have suggested that Aβ neurotoxicity may be caspase-independent (Selznick et al. 2000; Dumanchin-Njock et al. 2001; Yu et al. 2010, 2011). In addition, another recent study from our group showed that the neuroprotective effects of 4'-CD against Aβ in SH-SY5Y cells do not involve the regulation of the expression of procaspase-3 (Arbo et al. 2016). Therefore, our data corroborate previous studies and indicate that Aβ-induced neurotoxicity may be caspase-independent.

As previously mentioned, functionally, TSPO is thought to regulate cholesterol transport and steroidogenesis (Papadopoulos et al. 2006; Arbo et al. 2015). Therefore, one of the hypothesis regarding the neuroprotective effects of TSPO ligands against Aβ is that the activation of TSPO could be related with the stimulation of the biosynthesis of neurosteroids, which could mediate the neuroprotective effects of TSPO ligands. Regarding this aspect, it is known that some steroid hormones are neuroprotective against Aβ in different experimental models and modulate oxidative stress in AD. Studies show

that estradiol is neuroprotective against Aβ in SH-SY5Y cells and hippocampal neurons, decreasing ROS levels, lipid peroxidation and oxidative injury after Aβ administration (Goodman et al. 1996; Shea and Ortiz, 2003). In addition, Qian et al. (2015) showed that allopregnanolone attenuates Aβ-induced neurotoxicity in PC12 cells by reducing the intracellular ROS generation and lipid peroxidation and increasing the SOD activity. These data are corroborated by other studies showing that neuroactive steroids are able to exert antioxidant effects in the brain in different experimental conditions (Barron et al. 2006; Ozacmak and Sayan, 2009; Webster et al. 2015). Therefore, as 4'-CD elicit neuroprotection against Aβ through similar mechanisms than neurosteroids, including the modulation of the brain redox status and the activity of antioxidant enzymes, it is possible that these effects could be mediated by increased neurosteroidogenesis, and this hypothesis should be tested by further studies.

In summary, our findings indicate that 4'-CD is neuroprotective against Aβ-induced neurotoxicity by a mechanism that may involve the regulation of SOD protein expression. Further studies may investigate if the neuroprotective effects of 4'-CD against Aβ are mediated by increased neurosteroidogenesis related to the stimulation of TSPO activity and if they involve the modulation of other oxidative stress parameters.

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Figure Legends

Figure 1. Effect of different doses of 4'-CD in the viability of rat organotypic hippocampal slices exposed to Aβ. First, to establish a model of Aβ-induced neurotoxicity, we evaluated the effect of Aβ (5 μM) treatment for 24, 48 and 72 hours in the viability of organotypical hippocampal slices. It was observed that the administration of Aβ decreased the viability of hippocampal slices after 72h, while its administration for 24 or 48h did not affect the viability of the slices (Panels A and B) (***Different from control 72h, ANOVA/SNK, $P<0.001$) (n= 3-12/group). Later, we evaluated if 4'-CD was neuroprotective against Aβ administration for 72h in rat organotypic hippocampal slices. Cultures were treated with vehicle or 4'-CD (10 nM, 100 nM and 1 μM) and exposed to Aβ (1-42) (5 μM) for 72h (Panels C and D) (#Different from Control; ***Different from Aβ, ANOVA/SNK, $P<0.001$) (n= 9/group). Data represent the mean ± SEM for the PI incorporation.

Figure 2. Effect of 4'-CD in the protein expression of SOD in organotypic hippocampal slices exposed to Aβ. Cultures were treated with vehicle or 4'-CD (10 nM, 100 nM and 1 μM) and exposed to Aβ (1-42) (5 μM) for 72h. Data represent the mean ± SEM. *Different from Aβ (n= 8-10/group; ANOVA/SNK, $P=0.0046$).

Figure 3. Effect of 4'-CD in the protein expression of Akt in organotypic hippocampal slices exposed to Aβ. Cultures were treated with vehicle or 4'-CD (10 nM, 100 nM and 1 μM) and exposed to Aβ (1-42) (5 μM) for 72h. Data represent the mean ± SEM (n= 8-10/group).

Figure 4. Effect of 4'-CD in the protein expression of procaspase-3 in organotypic hippocampal slices exposed to Aβ. Cultures were treated with vehicle or 4'-CD (10 nM, 100 nM and 1 μM) and exposed to Aβ (1-42) (5 μM) for 72h. Data represent the mean ± SEM (n= 8-10/group).

Figure 1

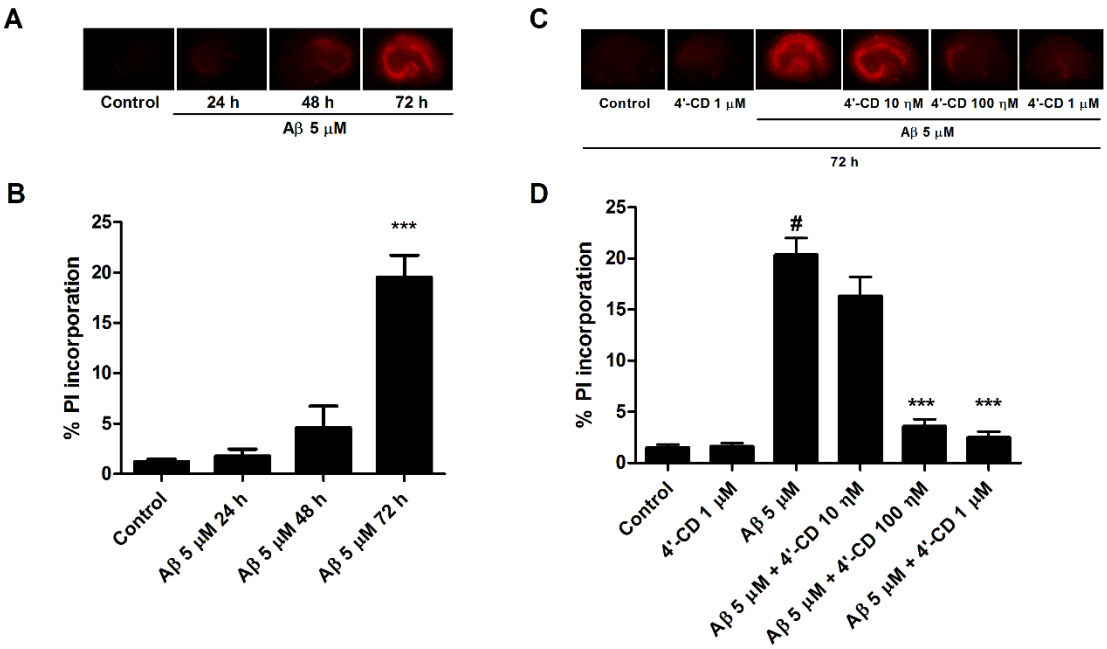

A
Control
24 h
48 h
72 h
Aβ 5 μM
C
Control
4'-CD 1 μM
4'-CD 10 ηM
4'-CD 100 ηM
4'-CD 1 μM
Aβ 5 μM
72 h
B
% PI incorporation
***
Control
Aβ 5 μM 24 h
Aβ 5 μM 48 h
Aβ 5 μM 72 h
D
% PI incorporation
#
***
***
Control
4'-CD 1 μM
Aβ 5 μM
Aβ 5 μM + 4'-CD 10 ηM
Aβ 5 μM + 4'-CD 100 ηM
Aβ 5 μM + 4'-CD 1 μM

Figure 2

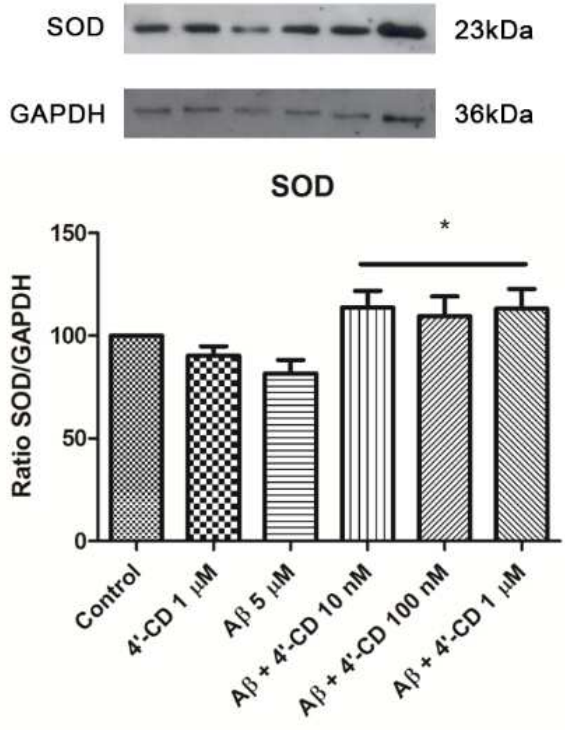

SOD
23kDa
GAPDH
36kDa
SOD
Ratio SOD/GAPDH
150
100
50
0
*
Control
4'-CD 1 μM
Aβ 5 μM
Aβ + 4'-CD 10 nM
Aβ + 4'-CD 100 nM
Aβ + 4'-CD 1 μM

Figure 3

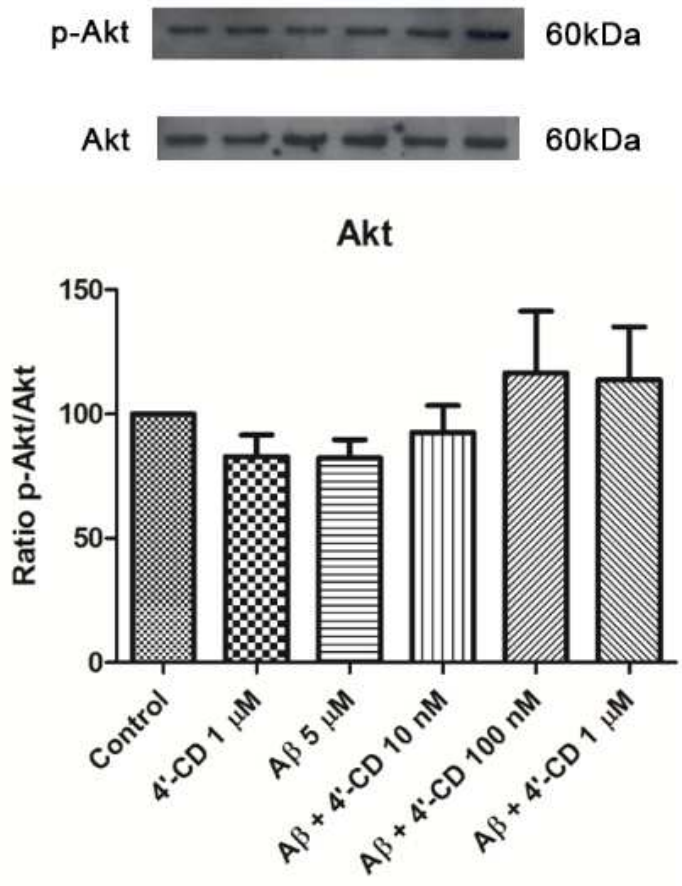
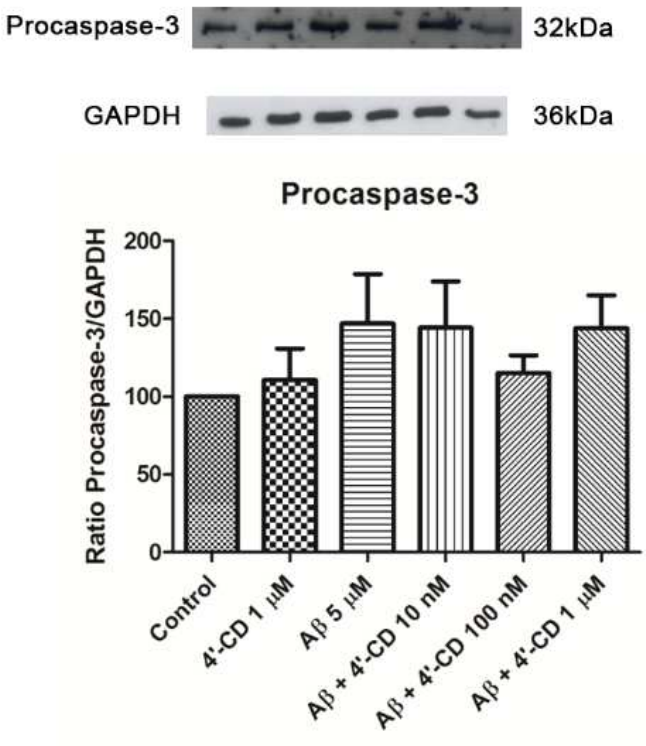

p-Akt
60kDa
Akt
60kDa
Akt
Ratio p-Akt/Akt
150
100
50
0
Control
4'-CD 1 μM
Aβ 5 μM
Aβ + 4'-CD 10 nM
Aβ + 4'-CD 100 nM
Aβ + 4'-CD 1 μM

Figure 4


Procaspase-3
32kDa
GAPDH
36kDa
Procaspase-3
Ratio Procaspase-3/GAPDH
200
150
100
50
0
Control
4'-CD 1 μM
Aβ 5 μM
Aβ + 4'-CD 10 nM
Aβ + 4'-CD 100 nM
Aβ + 4'-CD 1 μM

8 CAPÍTULO IV

Artigo: 4’-Chlorodiazepam modulates the development of primary hippocampal neurons in a sex-dependent manner

Status: A ser submetido para o periódico Biology of Sex Differences

4'-Chlorodiazepam modulates the development of primary hippocampal neurons in a sex-dependent manner

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Abstract

The translocator protein 18 kDa (TSPO) is located in the outer mitochondrial membrane and is involved in the cholesterol transport into the mitochondria and in the regulation of steroidogenesis and other mitochondrial functions. Studies show that some neurodegenerative diseases are related with the inhibition of the neurogenesis, and that the stimulation of this process could be a mechanism for the action of neuroprotective agents against these diseases. TSPO ligands have been shown to promote neuroprotective effects in experimental models of brain injury and neurodegenerative diseases, however, the effects of TSPO ligands during neuronal development are not known. Therefore, the aim of this study was to identify the developmental effects of 4'-chlorodiazepam (4'-CD), a TSPO ligand, in primary cultures of male and female mouse hippocampal neurons. We observed a sex difference between the control groups, with female cultures showing a smaller number of neural progenitors and a higher number of mature neurons than male cultures. In addition, it was verified that female neurons presented a bigger neuritic arbour than males. Moreover, it was identified that 4'-CD administration accelerated the development of male hippocampal neurons, decreasing the number of neural progenitors and increasing the number of mature neurons, without changing the development of female neurons. Also, it was observed that 4'-CD treatment increased the neuritic branching of male but not from female hippocampal neurons. In summary, we showed that there are sex differences in the development of primary hippocampal neurons and that 4'-CD modulates the development of these cells in a sex-dependent manner, stimulating the development of male but not from female neurons.

Keywords: translocator protein (TSPO); gender differences; steroids; steroidogenesis; neuritogenesis; neuronal morphology.

Introduction

Translocator protein (TSPO) is an 18 kDa protein located at contact sites between the outer and the inner mitochondrial membranes, which was previously known as peripheral benzodiazepine receptor (PBR), due to its property to bind to diazepam (Papadopoulos et al. 2006, Batarseh and Papadopoulos, 2010). This protein is arranged in five transmembrane alpha helices and presents high-affinity for cholesterol due to the presence of a cholesterol recognition amino acid consensus (CRAC) domain at its C-terminal region (Li and Papadopoulos, 1998; Jaremko et al. 2014). Functionally, TSPO is thought to mediate the translocation of cholesterol across the aqueous mitochondrial intermembrane space, and due to this function, this protein was renamed in 2006 as TSPO (Papadopoulos et al. 2006).

The transport of cholesterol into the mitochondria is the rate-limiting step in steroidogenesis. TSPO forms part of a multiprotein complex, called transduceosome, that also includes other proteins such as the voltage-dependent anion channel (VDAC) and the steroidogenic acute regulatory protein (StAR), which regulates the transport of cholesterol into the mitochondria (Midzak et al. 2011; Rone et al. 2012). In the mitochondria, the biosynthesis of steroid hormones is initiated with the enzymatic cleavage of the side chain of cholesterol by the cytochrome P450 side chain cleavage enzyme (CYP11A1), which forms the first steroid, pregnenolone (Miller and Auchus, 2011). Pregnenolone, then, leaves the mitochondria towards the endoplasmic reticulum, where it undergoes further enzymatic transformations that will form the final steroid products (Lacapère and Papadopoulos, 2003).

Brain expression of TSPO in physiological conditions is low, however, this protein is upregulated at sites of injury and inflammation, as well as in several

neuropathological conditions including stroke and neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis (Gerhard et al. 2006; Yasuno et al. 2008; Rissanen et al. 2015; Zürcher et al. 2015). It is known that some neurodegenerative diseases are associated with the inhibition of the neurogenesis in the hippocampus, resulting in memory and learning deficits, and that the stimulation of this process could be a mechanism for the action of neuroprotective agents against these diseases (Demars et al., 2010; Mu and Gage, 2011; Richetin et al., 2015). Some studies have shown that TSPO ligands, such as the 4'-chlorodiazepam (4'-CD, also known as Ro5-4864) may be neuroprotective in experimental models of different neurodegenerative diseases, as well as in experimental models of brain injuries and psychiatric disorders (as reviewed by Arbo et al. 2015). However, in spite of its use in several experimental models of brain diseases, the effects of 4'-CD on the neuronal development and its modulatory effects in some processes including neuritogenesis and axon development are still not known.

It is known that steroid hormones, such as estradiol, regulate neuritogenesis in the CNS by different mechanisms, including the modulation of the mitogen activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K) and Notch/Neurogenin 3 (Ngn3) pathways (Arevalo et al. 2012). However, although some studies have investigated the effects of estradiol in the neuritogenesis, axon development and synaptogenesis in the hippocampus (von Schassen et al. 2006; Ruiz-Palmero et al. 2011; Fester et al. 2012), none of them have investigated the developmental effects of TSPO ligands in the CNS. In addition, it was recently shown that there are sex differences in the rate of neuronal differentiation in the hypothalamus (Scerbo et al. 2014), and it remains to be elucidated if these same differences are also present in the differentiation of hippocampal neurons.

Thus, there is the need to understand how 4'-CD could affect the neuronal development, as this process is closely associated with neuroprotection, being particularly important to the promotion of neuronal regeneration after injury. Therefore, the aim of this study was to identify the developmental effects of 4'-CD in primary cultures of male and female mouse hippocampal neurons.

Material and Methods

Chemicals

Dimethyl sulfoxide (DMSO), 4'-CD, DNAse I and poly-L-lysine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Culture mediums, B-27 and GlutaMAX I were purchased from Invitrogen (Invitrogen, Crewe, UK). Trypsin was purchased from Worthington Biochemicals (Worthington Biochemicals, Freehold, NJ, USA).

Animals

CD1 mice were raised in Cajal Institute and used to generate embryos for this study. The day of the vaginal plug was defined as the embryonic day 0 (E0). All the protocols used in the present study were approved by our institutional animal care and use committee and were in accordance with the European Commission guidelines (86/609/CEE).

Hippocampal Neuronal Cultures

E17 mouse embryos were sexed by the identification of the testis of the male fetuses under a dissecting microscope and their hippocampus was dissected and dissociated to single cells after digestion with 0.5% Trypsin and DNase I at 37ºC for 15 min (Goslin and Banker, 1989). Cells were washed in Ca^{2+}/Mg^{2+}-free Hank's buffered salt solution and the neurons were counted and plated on glass coverslips coated with poly-L-lysine at a density of 200 neurons/mm^2. Cells were cultured in phenol red-free Neurobasal supplemented with GlutaMAX I and B-27. At these conditions, the level of astrocyte contamination should be less than 5% after 1 day in vitro (DIV) as reported by a previous study of our group (Ruiz-Palmero et al. 2011). After 1DIV, male and female hippocampal neurons were treated with DMSO or 4'-CD at three different doses (10 nM, 100 nM and 1 μM) for 24h.

Immunocytochemistry and morphological analysis

After treatments, cells were fixed for 20 min at room temperature in 4% paraformaldehyde and permeabilized for 4 min with 0.12% Triton-X plus 0.12% gelatin in phosphate-buffered saline (PBS). Cells were then washed with PBS/gelatin and incubated for 1h with anti-Tau rabbit polyclonal antibody (1:500 dilution) (Abcam, Cambridge, UK) and anti-MAP-2 chicken polyclonal antibody (1:4000 dilution) (Abcam) for the staining of the axons and dendrites, respectively. Cells were washed again with PBS/gelatin and incubated for 1h at room temperature with Alexa Fluor® 568 goat anti-rabbit (1:1000 dilution) (Abcam) for the detection of Tau and Fluoroscein (FITC) AffiniPure donkey anti-chicken (1:1000 dilution) (Jackson ImmunoResearch, West Grove, PA, USA) for the detection of MAP-2. Cell nuclei were stained with DAPI.

Images were obtained using a Leica fluorescence microscope equipped with a Leica digital camera (Leica, Heidelberg, Germany) at 40x magnification.

To evaluate the effects of 4'-CD on neuronal development, cells were classified into three different stages of development (I, II and III) in accordance with their morphology (Scerbo et al. 2014). Briefly, in stage I, neurites are not still emerged, in stage II, neurons show short neurites or minor processes, while in stage III, neurons show a long Tau-positive neurite, relatively uniform in diameter, which corresponds to the axon (Scerbo et al. 2014). For each group, at least 100 immunostained neurons were counted in each experiment, and at least three independent cultures were performed. In addition, to assess the effects of 4'-CD on neuronal development, the number of primary neurites from cells in stages II and III was counted and the axon length of the cells in stage III was measured using the ImageJ software (NIH, Bethesda, MD, USA) and expressed as pixel/μm. Also, the neuritic arbour was evaluated by the method of Sholl using the software CellTarget (Garcia-Segura and Perez-Marquez, 2014). Briefly, a grid of seven concentric circles with increasing radius of 20 μm was superimposed to the images, with the innermost circle placed over the perikaryon, and the number of neurites intersecting each circle was counted (Sholl, 1953).

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 5.0 software (La Jolla, CA, USA). A two-way analysis of variance (two-way ANOVA) was performed to compare the neuronal morphology between different groups, using sex and treatment as factors. When appropriate, two-way ANOVA was followed by the Bonferroni *post hoc* test. All

results were expressed as mean ± standard error (SEM). The level of statistical significance was $P< 0.05$.

Results

To study the effects of 4'-CD on neuritogenesis and neuronal development, male and female primary hippocampal neurons were treated with three different doses of 4'-CD and different morphological aspects were analyzed, including the number of neurites, the axon length and the extension of the neuritic arbour. We observed a sex difference regarding the control groups, with female cultures showing a smaller number of stage I cells ($F_{int\ (3,16)}= 4.185$, $P= 0.0229$), represented mostly by neural progenitors, and a higher number of stage III cells ($F_{int\ (3,16)}= 4.036$, $P= 0.0258$) (Figure 1), represented by mature neurons, with well defined dendrites and axons, than male cultures. In addition, we observed that 4'-CD administration at 10, 100 or 1000 nM accelerated the development of male hippocampal neurons, decreasing the number of stage I cells ($F_{int\ (3,16)}= 4.185$, $P= 0.0229$) and increasing the number of stage III cells ($F_{int\ (3,16)}= 4.036$, $P= 0.0258$) (Figure 1), without changing the neuronal development of female cells. Although we did not observe any difference regarding the number of neurites and the axon length of the neurons between our experimental groups, we observed a sex difference and a sex-dependent effect of 4'-CD on the analysis of the neuritic arbour by the method of Sholl. Female neurons showed a higher number of intersections than male neurons, independent of treatment ($F_{sex\ (1,16)}= 11.49$, $P= 0.0037$). The administration of 4'-CD at 10 and 100 nM increased the number of intersections in male but not in female hippocampal neurons ($F_{treatment\ (3,16)}= 3.556$, $P= 0.0382$) (Figure 2).

Discussion

In the last decade, several studies have assessed the neuroprotective actions of TSPO ligands in different experimental models. These compounds, which are currently used for brain imaging of neuroinflammation due to the upregulation of TSPO in reactive microglia and astrocytes, have been shown to be neuroprotective in different experimental models of neurodegenerative diseases, brain injuries and psychiatric disorders (as reviewed by Arbo et al. 2015). However, although several groups have assessed the neuroprotective effects of TSPO ligands in different experimental models, its effects on neuronal development and neuronal morphology are not known. Studies have shown that some neurodegenerative diseases are related with the inhibition of the neurogenesis in the hippocampus, and that therapeutic strategies that enhance neurogenesis could promote neuroprotective effects against these diseases. In this study, it was observed that the TSPO ligand 4'-CD presents sex-dependent effects on the development of primary hippocampal neurons. In cultures of male hippocampal neurons, 4'-CD treatment decreased the number of cells on stage I and increased the number of cells on stage III, indicating that 4'-CD may accelerate the development of male hippocampal neurons. In contrast, as female neurons showed a faster development in comparison with male neurons, 4'-CD was not able to produce the same changes in these cells. These results corroborate previous data showing that female neurons present an increased rate of development in comparison with male neurons in primary cultures of hypothalamus, represented by a higher number of neurons with branched neurites until 5 DIV due to an increased expression of Ngn3 (Scerbo et al. 2014). In the same fashion, although 4'-CD treatment did not change the

number of primary neurites and the axon length, it was observed that 4'-CD increased the neuritic arborization in male but not in female hippocampal neurons.

As previously mentioned, functionally, TSPO is thought to regulate cholesterol transport and steroidogenesis (Midzak et al. 2011; Rone et al. 2012). Therefore, one of the hypothesis regarding the developmental effects of TSPO ligands in hippocampal neurons is that the activation of TSPO could be related with the stimulation of the biosynthesis of neurosteroids, which could mediate the effects of TSPO ligands on neuronal development. Several studies have demonstrated that hippocampal neurons are able to synthesize estradiol in vitro (Fester et al. 2006; von Schassen et al. 2006). Indeed, studies have shown that locally synthesized estradiol has a key role on neurite outgrowth and synaptogenesis, and that the inhibition of its synthesis is associated with the inhibition of neurite outgrowth and synapse loss (von Schassen et al. 2006; Fester et al. 2012). Moreover, it has been shown that cholesterol, which is the precursor for the synthesis of steroid hormones, is able to stimulate synaptogenesis in vitro through its conversion into estradiol, and that the knock-down of StAR, another protein involved in the regulation of cholesterol transport into the mitochondria, is able to inhibit the synaptogenic effects of cholesterol (Fester et al. 2009). Regarding the effects of estradiol on neuritogenesis, previous studies by our group demonstrated that estradiol stimulates the neurite development of non-sexed hippocampal neurons, increasing the number of primary neurites and the neuritic arborization through a mechanism involving an upregulation in Ngn3 expression and the activation of PI3K pathway (Ruiz-Palmero et al. 2011; Ruiz-Palmero et al. 2013). Interestingly, in another study, it was verified that there is a differential expression of Ngn3 in male and female hypothalamic neurons that is involved in the generation of sex differences in the rate of neuronal differentiation in the

hypothalamus, and that estradiol administration stimulates the axonal growth and the neuritic branching of males primary hypothalamic neurons through increasing Ngn3 levels, abolishing sex differences in neuronal development (Scerbo et al. 2014). These findings suggest that the sex-specific effects of 4'-CD may be related with a possible stimulation of the steroidogenesis through the stimulation of TSPO activity, including a stimulation of estradiol biosynthesis, which could be mediating the effects of 4'-CD on neuronal development through the modulation of Notch/Ngn3 and PI3K signaling.

In summary, we showed that there are sex differences in the development of primary hippocampal neurons and that 4'-CD modulates the development of these cells in a sex-dependent manner, stimulating the development of male but not from female neurons. Further studies may investigate if the effects of 4'-CD on neuronal development are mediated by increased neurosteroidogenesis related to the stimulation of TSPO activity.

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Figure 1. Effect of 4'-CD in the development of primary hippocampal neurons. Male and female cells were treated at 1DIV with 4'-CD (10, 100 and 1000 nM) for 24h. Cells were fixed and immunostained for Tau and MAP-2 and classified into three stages of maturation (stages I, II and III) in accordance with their morphology. At least 100 cells were evaluated from each experimental group and three independent cultures were performed. Data represent the mean ± SEM. Panel A – Male DMSO; Panel B – Male 4'-CD 10 nM; Panel C – Female DMSO; Panel D – Female 4'-CD 10 nM. *Different from Males DMSO (ANOVA/Bonferroni, $P<0.05$).

Figure 2. Effect of 4'-CD on the morphology of primary hippocampal neurons. Male and female cells were treated at 1DIV with 4'-CD (10, 100 and 1000 nM) for 24h. Cells were fixed and immunostained for Tau and MAP-2, and it was evaluated the number of primary neurites from cells in stages II and III and the axon length of cells in stage III. In addition, the neuritic arbour of cells in stages II and III was evaluated by the number of intersections measured in the Sholl analysis. Three independent cultures were performed. Data represent the mean ± SEM. Panel A – Male DMSO; Panel B – Male 4'-CD 10 nM; Panel C – Female DMSO; Panel D – Female 4'-CD 10 nM. *Different from Males DMSO; #Different from males (ANOVA/Bonferroni, $P<0.05$).

Figure 0-1

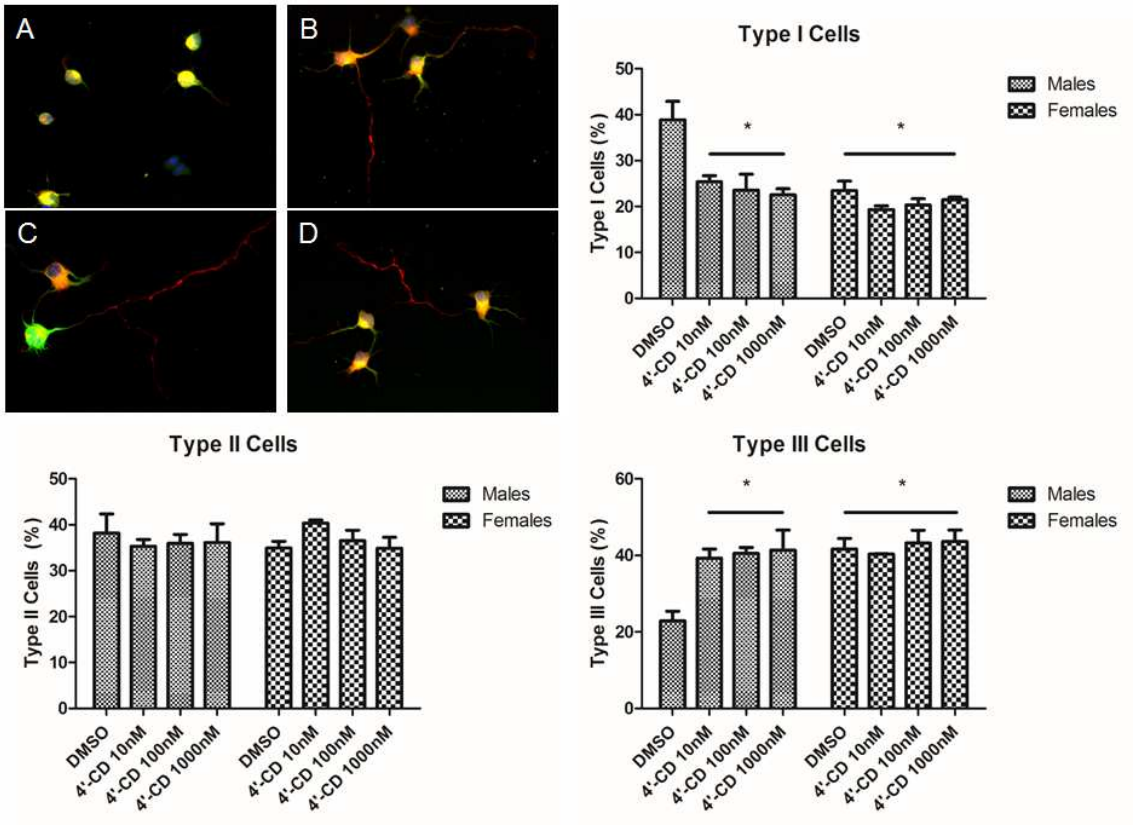
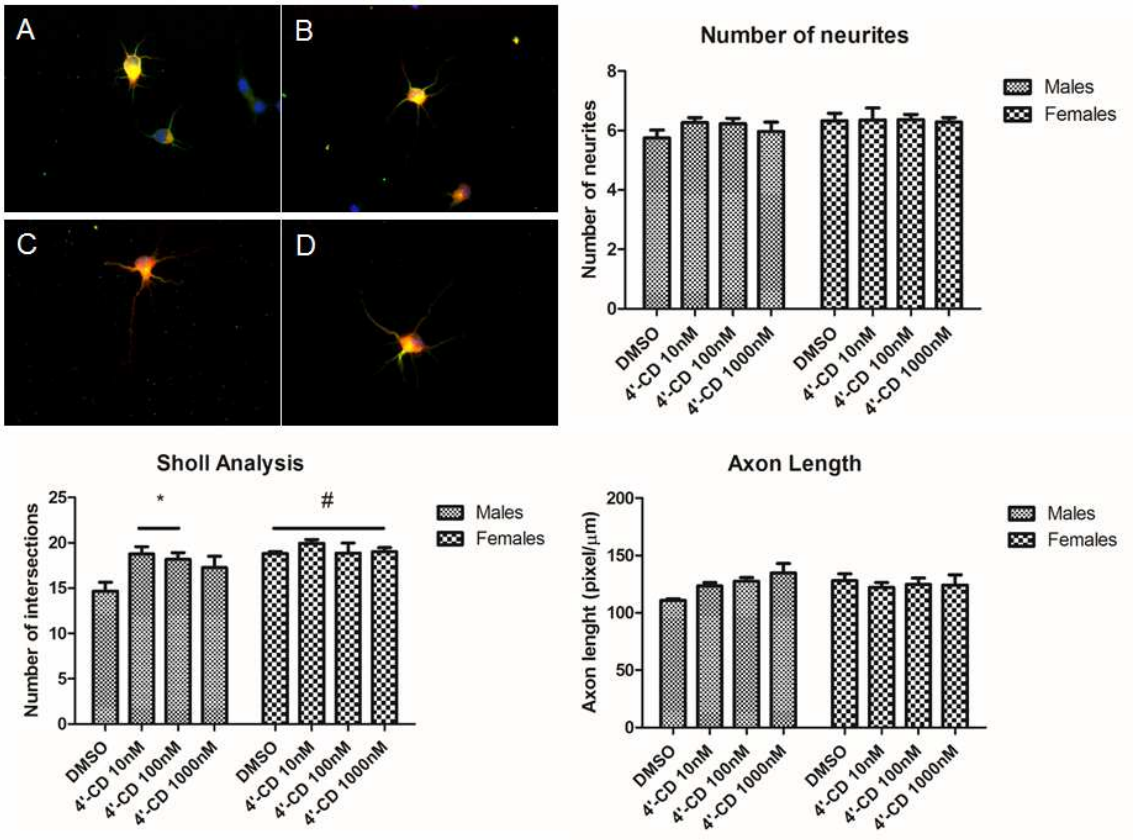

A
B
C
D
Type I Cells
Type I Cells (%)
Males
Females
DMSO
4'-CD 10nM
4'-CD 100nM
4'-CD 1000nM
Type II Cells
Type II Cells (%)
Type III Cells
Type III Cells (%)

Figure 2


A
B
C
D
Number of neurites
Number of neurites
Males
Females
DMSO
4'-CD 10nM
4'-CD 100nM
4'-CD 1000nM
Sholl Analysis
Number of intersections
*
#
Axon Length
Axon lenght (pixel/µm)

9 DISCUSSÃO

O aumento da expectativa de vida da população mundial tem se associado com uma maior prevalência de doenças neurodegenerativas. Dentre as doenças neurodegenerativas, a DA é a que apresenta maior prevalência, de forma que se estima em 35 milhões o número de pessoas em todo o mundo que sofrem desse distúrbio (Querfurth e LaFerla, 2010). Todavia, apesar da sua grande prevalência, que aumenta progressivamente com o aumento da expectativa de vida da população mundial, não existem tratamentos suficientemente eficazes visando atenuar a progressão da DA. Na última década, diversos estudos avaliaram os efeitos neuroprotetores dos ligantes da TSPO em diferentes modelos experimentais. Estes compostos, que são atualmente usados na área de diagnóstico por imagem em quadros de neuroinflamação, devido ao aumento da expressão da TSPO em células gliais reativas, apresentam efeitos neuroprotetores em uma série de modelos experimentais distintos (conforme revisto por Arbo et al., 2015, capítulo I). Além da TSPO se tornar um alvo terapêutico interessante na medida em que sua expressão aumenta em sítios de lesão e/ou neuroinflamação, o uso de ligantes da TSPO ganhou notoriedade na última década devido ao fato de alguns estudos indicarem que o seu uso poderia resultar em efeitos neuroprotetores devido ao estímulo da neuroesteroidogênese em diferentes modelos experimentais (Girard et al., 2008; Papadopoulos e Lecanu, 2009; Giatti et al., 2009; Mitro et al., 2012). O estímulo da síntese de neuroesteroides poderia resultar em ações neuroprotetoras, uma vez que são conhecidos os efeitos benéficos de diversos hormônios esteroides em modelos de lesão neuronal (Arevalo et al., 2015; Arbo et al., 2016; Grimm et al., 2016).

Neste trabalho, foi demonstrado que o 4’-CD exerceu efeitos neuroprotetores contra a neurotoxicidade induzida pelo Aβ em dois modelos experimentais distintos. No primeiro experimento (capítulo II) foi observado que o 4’-CD nas doses de 1 e 10 nM

protegeu células SH-SY5Y contra a morte neuronal induzida pela administração do Aβ (Figura 1 – Capítulo II), enquanto no segundo experimento (capítulo III) observamos um efeito neuroprotetor exercido pelo 4'-CD nas doses de 100 nM e 1 μM contra a administração do Aβ em culturas organotípicas de hipocampo (Figura 1 – Capítulo III). Esses resultados corroboram os dados de um estudo anterior, que mostrou que o 4'-CD exerce efeitos neuroprotetores em um modelo experimental de AD em camundongos, reduzindo o acúmulo de Aβ no hipocampo e a gliose reativa, melhorando os desfechos funcionais dos animais (Barron et al., 2013). É importante mencionarmos que ao passo que no experimento com as células SH-SY5Y (capítulo II) o 4'-CD exerce um efeito neuroprotetor atuando diretamente sobre células neuronais, o efeito neuroprotetor exercido pelo 4'-CD nas culturas organotípicas de hipocampo poderiam envolver não só uma ação direta sobre os neurônios, mas também a modulação de células gliais. Essa observação é importante pois tem-se observado que a astrogliose tem um papel central na patogênese da DA, e a alteração da função astrocitária com a progressão da doença pode resultar em alterações importantes na comunicação neuronal e no seu metabolismo energético (Lian e Zheng, 2016; Osborn et al., 2016). Devido a isso, alguns estudos têm sugerido que tratamentos capazes de atuar sobre as células gliais poderiam representar um avanço interessante para o tratamento da DA (Lopategui Cabezas et al., 2014; Dzamba et al., 2016; Wes et al., 2016). Dessa forma, esses dados indicam que o 4'-CD poderia ser um fármaco em potencial para o combate aos efeitos neurotóxicos ocasionados pelo Aβ.

Sabe-se que o Aβ afeta a sobrevivência neuronal através de vários mecanismos distintos, incluindo a modulação do balanço entre proteínas pró e anti-apoptóticas no encéfalo (Paradis et al., 1996; Hoppe et al., 2013). Neste estudo, foi demonstrado que a administração de Aβ em células SH-SY5Y reduziu a expressão proteica da survivina,

enquanto o tratamento com 4'-CD inibiu os efeitos ocasionados pelo Aβ sobre a expressão dessa proteína (Figura 2 – Capítulo II). A survivina é uma componente da via da β-catenina e um membro da família de proteínas inibidoras da apoptose, que exerce efeitos anti-apoptóticos através da inibição da ativação de caspases e das caspases ativadas, incluindo as caspases-3 e -7 (Tamm et al., 1998; Shin et al., 2001). Apesar de não terem sido observadas alterações na expressão da procaspase-3 entre os grupos experimentais (Figura 5 – Capítulo II), a survivina poderia estar atuando modulando a sua clivagem em caspase-3, cuja expressão não foi detectada nos *Western-blots* realizados. Na verdade, estudos sugerem que apesar da survivina se ligar com alta afinidade às caspases ativas como as caspases-3 e -7, ela pode não se ligar aos seus precursores inativos, atuando principalmente através da inibição das caspases ativadas e regulando a ativação das caspases através da inibição da clivagem dos seus precursores (Tamm et al., 1998). Dessa forma, isso poderia explicar o fato de que mudanças nos níveis da survivina não se correlacionaram com mudanças significativas nos níveis da procaspase-3. Além disso, deve ser destacado que o papel da caspase-3 na morte neuronal induzida pelo Aβ ainda é controverso, e estudos têm sugerido que a neurotoxicidade ocasionada pelo Aβ poderia ser independente da ativação das caspases (Selznick et al., 2000; Dumanchin-Njock et al., 2001; Yu et al., 2010, 2011).

Além da modulação da expressão proteica da survivina, os efeitos neuroprotetores ocasionados pelo 4'-CD contra a administração do Aβ em células SH-SY5Y se associaram com uma redução na expressão proteica da Bax (Figura 3 – Capítulo II). Alguns estudos já demonstraram que o dano neuronal causado pelo Aβ está relacionado com o aumento da expressão da Bax e com uma redução na expressão das proteínas anti-apoptóticas da família Bcl-2 (Paradis et al., 1996; Selznick et al., 2000; Clementi et al.,

2006). A Bax é uma proteína pró-apoptótica da família Bcl-2 que é encontrada principalmente no citoplasma das células na forma de monômeros solúveis, e, em menor escala, frouxamente associada à mitocôndria (Dejean et al., 2006). A atividade da Bax é controlada por membros pró-sobrevivência da família Bcl-2, incluindo a Bcl-2 e a Bcl-xl, que se heterodimerizam com a Bax e com outras proteínas pró-apoptóticas, inibindo a sua ativação (Chipuk et al., 2010). Após um estímulo apoptótico, a Bax sofre uma alteração conformacional e é translocada do citoplasma para a mitocôndria, onde regula a formação de poros e leva à permeabilização da membrana mitocondrial externa, resultando na liberação de citocromo c e de outros fatores pró-apoptóticos da mitocôndria, que levam à ativação de caspases e à apoptose (Gillies et al., 2015). Dessa forma, os efeitos neuroprotetores do 4'-CD contra o Aβ poderiam estar relacionados com uma redução na expressão da Bax, que poderia estar associada com uma redução na liberação de citocromo c e com um aumento da viabilidade celular. Apesar das mudanças na expressão da Bax não estarem associadas com alterações concomitantes na expressão proteica da Bcl-xl (Figura 4 – Capítulo II), outros membros da família Bcl-2 como a proteína Bcl-2 poderiam estar envolvidos na regulação da expressão da Bax após a administração do 4'-CD.

Além da modulação do balanço entre proteínas pró e anti-apoptóticas, sabe-se que o acúmulo do Aβ também se associa com um aumento nos níveis de EROs e com uma redução na expressão e na atividade de enzimas antioxidantes, incluindo a SOD, a catalase e a glutationa peroxidase (Turunc Bayrakdar et al., 2014; Liu et al., 2015; Zhang et al., 2015). No presente estudo, foi demonstrado que o efeito neuroprotetor ocasionado pela administração do 4'-CD em culturas organotípicas de hipocampo se associou com um aumento na expressão proteica da SOD1 nos grupos tratados com o 4'-CD em todas as

doses testadas após a administração do Aβ em relação ao grupo tratado apenas com o Aβ (Figura 2 – Capítulo III). A SOD é uma enzima que catalisa a dismutação do radical superóxido, que é produzido como um subproduto do metabolismo do oxigênio e que pode causar muitos tipos de dano celular (Bresciani et al., 2015). Segundo a hipótese do estresse oxidativo induzido pelo Aβ, o estresse oxidativo que ocorre na bicamada lipídica deve-se à inserção de oligômeros do $A\beta_{1-42}$ nesse local, onde atuam como uma fonte de EROs, que estão envolvidas no início do processo de lipoperoxidação (Butterfield et al., 2013). Estudos mostram que a Met-35 dos peptídeos Aβ é fundamental para a toxicidade e os danos oxidativo associados ao Aβ. A oxidação da Met-35 para a formação de metionina sulfóxido exerce um papel importante na regulação da função proteica e da defesa celular, e pode levar à oxidação lipídica ou proteica (Stadtman, 2004; Butterfield et al. 2013). Alguns estudos já mostraram a presença de níveis elevados de marcadores de oxidação e nitração proteica e de oxidação de ácidos nucleicos no encéfalo de pacientes em estágios iniciais da DA (Gabbita et al. 1998; Wang et al. 2006; Barone et al. 2011, 2012). Além disso, Murakami et al. (2011) mostraram que a deficiência da SOD1 leva à oligomerização do Aβ e a perda de memória em um modelo de DA em camundongos, enquanto outro estudo mostrou que células de neuroblastoma humano SH-SY5Y que superexpressam SOD1 são menos suscetíveis aos danos ocasionados pelo Aβ (Celsi et al., 2004), corroborando a ideia de que a SOD teria um papel fundamental na defesa contra os danos ocasionados pelo Aβ. Dessa forma, o estímulo da síntese e da atividade de enzimas antioxidantes poderia ser um mecanismo para a ação de agentes neuroprotetores contra a toxicidade induzida pelo Aβ. Dentro desse contexto, diversos estudos mostram que as ações neuroprotetoras de diferentes compostos contra o Aβ envolvem a modulação da expressão de enzimas antioxidantes no encéfalo (Liu et al., 2015; Yu et al., 2015; Zhang et al., 2015). Além disso, alguns estudos mostram que o 4'-

CD exerce efeitos antioxidantes em outros modelos experimentais. Mehta et al. (2010) mostraram que o 4'-CD atenua os déficits cognitivos produzidos pela administração de um pesticida carbamato através de um aumento na expressão de enzimas antioxidantes. Ainda, outros estudos têm associado os efeitos antioxidantes do 4'-CD com seus efeitos cardioprotetores em diferentes modelos experimentais (Jaiswal et al. 2010; Xiao et al. 2010; Paradis et al. 2013). Dessa forma, esses dados indicam que a modulação da expressão proteica da SOD poderia estar relacionada com as ações neuroprotetoras do 4'-CD contra a neurotoxicidade induzida pelo Aβ. Outras enzimas antioxidantes também poderiam ser moduladas dentro das ações neuroprotetoras exercidas pelo 4'-CD, sendo que o estudo da expressão dessas enzimas permanece como uma perspectiva para a sequência desse trabalho.

Devido aos resultados obtidos nos experimentos apresentados no capítulo II, indicando que a modulação da expressão de proteínas pró e anti-apoptóticas poderia ser um mecanismo de ação importante envolvido nos efeitos neuroprotetores do 4'-CD, foram avaliadas as expressões das proteínas Akt e procaspase-3 nas culturas organotípicas de hipocampo afim de verificarmos se os efeitos do 4'-CD poderiam estar relacionados com alterações na expressão dessas proteínas. Nestes experimentos, não foram encontradas diferenças significativas na expressão da Akt (Figura 3 – Capítulo III) e da procaspase-3 (Figura 4 – Capítulo III) entre os grupos experimentais. A Akt é uma serina/treonina cinase que faz parte da via pró-sobrevivência da fosfoinositídeo-3-cinase (PI3K). Embora alguns estudos tenham mostrado que o dano celular induzido pelo Aβ envolve uma redução na expressão da Akt (Chen et al., 2009; Yin et al., 2011), Hoppe et al. (2013) não encontraram diferenças na expressão da Akt após a administração do Aβ em culturas organotípicas de hipocampo, em um protocolo de tratamento semelhante ao

usado neste trabalho. Entretanto, o envolvimento da via da PI3K/Akt nos efeitos tanto do Aβ quanto do 4'-CD nesse modelo experimental não pode ser descartado, uma vez que mudanças na expressão e na ativação da Akt poderiam ocorrer nas primeiras horas após a administração dos tratamentos, e então deixar de existir após 72h. Além da ausência de diferenças na expressão da Akt, não foram encontradas diferenças significativas na expressão da procaspase-3 entre os grupos experimentais. Conforme previamente dito, alguns estudos têm sugerido que a morte neuronal induzida pelo Aβ poderia ser independente da ativação das caspases (Selznick et al., 2000; Dumanchin-Njock et al., 2001; Yu et al., 2010, 2011), de forma que o resultado encontrado é semelhante ao resultado dos experimentos realizados com as células SH-SY5Y, em que tampouco foram observadas diferenças na expressão da procaspase-3. Infelizmente, por não termos em nosso laboratório os anticorpos anti-survivina e anti-Bax que utilizamos no estudo envolvendo as células SH-SY5Y (que foi realizado na Espanha), não foi possível voltarmos a estudar a expressão dessas proteínas nas culturas organotípicas de hipocampo. Dessa forma, nossos dados corroboram os resultados obtidos por estudos anteriores e sugerem que a neurotoxicidade induzida pelo Aβ pode ser independente das caspases.

Funcionalmente, acredita-se que a TSPO poderia estar relacionada com o transporte do colesterol e com a regulação da esteroidogênese (Papadopoulos et al., 2006; Rone et al., 2012; Arbo et al., 2015). Dessa forma, uma das hipóteses para a explicação dos efeitos neuroprotetores ocasionados pelos ligantes da TSPO contra o Aβ é de que a ativação da TSPO poderia estar relacionada com a estimulação da biossíntese de neuroesteroides, que poderiam mediar os efeitos neuroprotetores dos ligantes da TSPO. Em relação a este aspecto, sabe-se que alguns hormônios esteroides são neuroprotetores

contra os efeitos causados pelo Aβ em diferentes modelos experimentais, modulando o balanço entre a expressão de proteínas pró e anti-apoptóticas, assim como parâmetros de estresse oxidativo. Estudos mostram que o estradiol é capaz de proteger neurônios corticais e hipocampais da toxicidade induzida pelo Aβ através da regulação da expressão de proteínas da família Bcl-2 e reduzindo a liberação mitocondrial de citocromo c (Nilsen et al., 2006; Yao et al., 2007). Esses dados são corroborados por resultados de outro estudo mais recente, que mostrou que o estradiol protege células granulares cerebelares contra o Aβ através da modulação da expressão das proteínas Bax e Bcl-xl, inibindo a liberação mitocondrial de citocromo c e a apoptose (Napolitano et al., 2014). Também, Qin et al. (2015) mostraram que a progesterona é neuroprotetora conta a toxicidade induzida pelo Aβ em culturas primárias de neurônios corticais de ratos, inibindo a via da c-Jun N-terminal cinase (JNK) e a via apoptótica mitocondrial. Além disso, Grimm et al. (2016) mostraram que diferentes neuroesteroides como a progesterona, o estradiol e a testosterona são capazes de recuperar o déficit bioenergético induzido pela superexpressão do Aβ em células SH-SY5Y. Corroborando esses dados, outro estudo mostrou que o estradiol e a progesterona são capazes de regular a expressão de fatores de depuração do Aβ em culturas primárias de neurônios e no encéfalo de ratas (Jayaraman et al., 2012), indicando um possível papel exercido pelos neuroesteroides na regulação das ações do Aβ no SNC. Por outro lado, em relação aos efeitos modulatórios de neuroesteroides sobre parâmetros de estresse oxidativo em modelos de DA, alguns estudos mostram que o estradiol protege células SH-SY5Y e neurônios hipocampais contra a toxicidade induzida pelo Aβ através da redução dos níveis de EROS, da lipoperoxidação e do dano oxidativo causados pela administração do Aβ (Goodman et al., 1996; Shea e Ortiz, 2003). Além disso, Qian et al. (2015) mostraram que a alopregnanolona atenua os efeitos tóxicos ocasionados pela administração de Aβ em

células PC12 através da redução da produção intracelular de EROS e da lipoperoxidação, e do aumento da atividade da SOD. Esses dados são corroborados por outros estudos indicando que os esteroides neuroativos são capazes de exercer efeitos antioxidantes em outros modelos experimentais (Barron et al., 2006; Ozacmak e Sayan, 2009; Webster et al., 2015). Por outro lado, sabe-se que a neuroesteroidogênese pode ser afetada pela administração de Aβ (Schaeffer et al., 2008), e que camundongos 3xTg-AD (que representam um modelo experimental de DA) apresentam alterações nos níveis de esteroides neuroativos relacionadas com a idade e com o desenvolvimento da doença (Caruso et al., 2013), corroborando a existência de uma possível relação importante entre os efeitos causados pelo Aβ e os neuroesteroides. Dessa forma, na medida em que o 4'-CD exerce efeitos neuroprotetores contra o Aβ através de mecanismos de ação semelhantes aos utilizados pelos neuroesteroides, incluindo a modulação do balanço entre os níveis de proteínas pró e anti-apoptóticas, assim como a modulação de parâmetros de estresse oxidativo e da expressão de enzimas antioxidantes, é possível que esses efeitos estejam relacionados com um aumento da síntese desses hormônios após a estimulação da TSPO, de forma que essa hipótese deveria ser testada por outros estudos. Apesar de inicialmente termos estabelecido como um dos objetivos do estudo a quantificação dos níveis dos hormônios esteroides após o tratamento com o 4'-CD, a fim de verificarmos como o tratamento poderia modular o nível desses hormônios, essa análise envolveria o uso da técnica de cromatografia líquida de alto desempenho acoplado à espectrometria de massas (HPLC-MS), e não foi possível viabilizarmos nenhuma parceria durante esse período que nos possibilitasse realizar tal avaliação.

O desenvolvimento e a maturação neuronal são processos essenciais dentro da fisiopatologia de doenças neurodegenerativas e da regeneração do SNC após os danos

(Royo et al., 2003; Liu et al., 2011; Winner et al., 2011; Doron-Mandel et al., 2015). Estudos têm mostrado que a inibição da neurogênese poderia anteceder as lesões características observadas no cérebro de pacientes portadores da DA, exercendo um papel importante no desencadeamento e na progressão da doença (Lazarov e Marr, 2010; Demars et al., 2010; Perry et al., 2012; Gomez-Nicola et al., 2014; Ekonomou et al., 2015). Devido a isso, uma possibilidade de abordagem terapêutica para o combate à DA seriam tratamentos ou outras intervenções capazes de aumentar a neurogênese no hipocampo e melhorar os déficits de memórias associados à DA (Schaeffer et al., 2009; Valero et al., 2011; Richetin et al., 2015). Embora os efeitos neuroprotetores dos ligantes da TSPO em diferentes modelos experimentais venham sendo estudados nos últimos anos, ainda não se sabe de que forma esses compostos modulam o desenvolvimento e a morfologia neuronal. Dessa forma, um dos objetivos desse estudo foi avaliar os efeitos do 4'-CD sobre o desenvolvimento de culturas primárias de neurônios hipocampais de camundongos machos e fêmeas. Nesse estudo, foi observado que o 4'-CD apresentou efeitos dependentes do sexo sobre o desenvolvimento de culturas primárias de neurônios hipocampais. Nas culturas dos neurônios hipocampais de machos, o 4'-CD reduziu o número de células em estágio I e aumentou o número de células em estágio III, indicando que o 4'-CD poderia estimular o desenvolvimento de neurônios hipocampais de machos. Por outro lado, como os neurônios hipocampais de fêmeas apresentaram um desenvolvimento naturalmente mais rápido do que o dos machos, o 4'-CD não foi capaz de alterar a taxa de desenvolvimento dessas células. Esses resultados são semelhantes aos resultados encontrados por Scerbo et al. (2014), que mostraram que culturas primárias de neurônios hipotalâmicos de fêmeas apresentam um desenvolvimento mais rápido do que de machos, representado por um número maior de neurônios com ramificações neuríticas até 5DIV devido a um aumento na expressão da neurogenina 3 (Ngn3). Da mesma forma,

apesar do 4’-CD não ter alterado o número de neuritos primários nem o comprimento axonal, ele aumentou a ramificação neurítica nas culturas primárias de neurônios hipocampais de machos, mas não de fêmeas. Outros estudos devem avaliar se os efeitos modulatórios do 4’-CD sobre o desenvolvimento neuronal poderiam estar associados às suas ações neuroprotetoras em diferentes modelos experimentais de doenças neurodegenerativas, incluindo a DA.

Assim como mencionado no que diz respeito a seus efeitos neuroprotetores, uma das hipóteses que poderia explicar os efeitos do 4’-CD sobre o desenvolvimento neuronal envolve uma possível estimulação da neuroesteroidogênese devido à ativação da TSPO, de forma que os efeitos do 4’-CD poderiam ser mediados pelos esteroides sintetizados a partir desse processo. Dentro desse contexto, alguns estudos já mostraram que neurônios hipocampais cultivados *in vitro* são capazes de sintetizar estradiol (Fester et al., 2006; von Schassen et al., 2006). Além disso, sabe-se que o estradiol sintetizado localmente possui um papel fundamental no crescimento dendrítico e na sinaptogênese, e que a inibição desse processo se associa com a inibição do crescimento neurítico e com perda sináptica (von Schassen et al. 2006; Fester et al., 2012). Também, já foi demonstrado que o colesterol, que é a molécula precursora para a síntese dos hormônios esteroides, é capaz de estimular a sinaptogênese *in vitro* através da sua conversão em estradiol, e que a deleção da StAR, uma outra proteína envolvida na regulação do transporte do colesterol para o interior da mitocôndria, é capaz de inibir os efeitos sinaptogênicos do colesterol (Fester et al., 2009). Em relação aos efeitos de hormônios esteroides sobre a neuritogênese, estudos anteriores do nosso grupo mostram que o estradiol estimula o desenvolvimento neurítico de culturas de neurônios hipocampais não-sexadas, aumentando o número de neuritos primários e a ramificação neurítica através de um

mecanismo envolvendo um aumento na expressão da Ngn3 e a ativação da via da PI3K (Ruiz-Palmero et al., 2011; Ruiz-Palmero et al., 2013). Além disso, em outro estudo, foi verificado que existe uma diferença sexual na expressão da Ngn3 em neurônios hipotalâmicos que está envolvida na geração de diferenças sexuais na taxa de diferenciação neuronal no hipotálamo, e que a administração de estradiol estimula o crescimento axonal e a ramificação neurítica de culturas primárias de neurônios hipotalâmicos de camundongos machos através de um aumento da expressão da Ngn3, eliminando as diferenças sexuais na taxa de diferenciação neuronal (Scerbo et al., 2014). Esses resultados sugerem que os efeitos dependentes do sexo exercidos pelo 4'-CD poderiam estar relacionados com uma possível estimulação da neuroesteroidogênese através da ativação da TSPO, incluindo a estimulação da síntese de estradiol que poderia mediar os efeitos do 4'-CD sobre o desenvolvimento neuronal através da modulação das vias da Ngn3 e da PI3K.

10 CONCLUSÕES

1 – O 4’-CD apresentou efeitos neuroprotetores contra a toxicidade induzida pelo Aβ tanto em células SH-SY5Y de neuroblastoma humano como em culturas organotípicas de hipocampo de ratos, apresentando-se como um fármaco em potencial para o tratamento da DA (Capítulos II e III).

2 – Os efeitos neuroprotetores exercidos pelo 4’-CD nas células SH-SY5Y envolveram a inibição do aumento da expressão da Bax e da redução da expressão da survivina provocados pela administração do Aβ, enquanto seus efeitos neuroprotetores nas culturas organotípicas de hipocampo envolveram um efeito antioxidante representado por um aumento na expressão proteica da SOD (Capítulos II e III).

3 – Existem diferenças sexuais entre o desenvolvimento de culturas primárias de neurônios hipocampais de camundongos machos e fêmeas, com os neurônios hipocampais de fêmeas apresentando um desenvolvimento mais precoce do que os de machos (Capítulo IV).

4 – O 4’-CD exerce efeitos dependentes do sexo sobre o desenvolvimento de culturas primárias de neurônios hipocampais, estimulando o desenvolvimento de neurônios hipocampais de camundongos machos e aumentando a sua ramificação dendrítica, sem exercer os mesmos efeitos nos neurônios de fêmeas. Esse efeito poderia ser um outro mecanismo envolvido nas ações protetoras exercidas pelo 4’-CD em modelos experimentais de doenças neurodegenerativas, incluindo a DA (Capítulo IV).

11 PERSPECTIVAS

1 – Avaliar se os efeitos neuroprotetores e sobre o desenvolvimento neuronal causados pelo 4’-CD envolvem a modulação da neuroesteroidogênese, incluindo um estudo visando identificar a possível participação de enzimas da rota da esteroidogênese e de receptores de hormônios esteroides nos efeitos exercidos pelo 4’-CD.

2 – Estudar o envolvimento de outras vias de sinalização intracelular nos efeitos neuroprotetores ocasionados pelo 4’-CD.

3 – Aprofundar o estudo do impacto dos efeitos antioxidantes exercidos pelo 4’-CD em seus efeitos neuroprotetores contra o Aβ, estudando seus efeitos sobre outros parâmetros de estresse oxidativo.

4 – Estudar as proteínas e vias de sinalização envolvidas nos efeitos do 4’-CD no desenvolvimento neuronal, e avaliar se existem diferenças sexuais nos efeitos modulatórios exercidos pelo 4’-CD sobre essas vias.

5 – Reproduzir os efeitos neuroprotetores do 4’-CD contra a toxicidade induzida pelo Aβ *in vitro* em um modelo de toxicidade induzida pelo Aβ *in vivo*, visando avaliar a viabilidade do uso do 4’-CD como um possível fármaco para o tratamento da DA.

6 – Avaliar se os efeitos do 4’-CD sobre o desenvolvimento e a maturação neuronal poderiam estar envolvidos em suas ações protetoras em modelos animais de DA.

7 – Investigar os efeitos do 4’-CD sobre a modulação da função astrocitária e qual sua participação nas ações neuroprotetoras exercidas pelo 4’-CD em modelos experimentais de DA.

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Anexo A – Carta de Aprovação da Comissão de Ética no Uso de Animais

(CEUA/UFRGS)

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: 24118
Título: Mecanismos neuroprotetores da indução da neuroesteroidogênese em diferentes estruturas do sistema nervoso central

Pesquisadores:

Equipe UFRGS:

MARIA FLAVIA MARQUES RIBEIRO - coordenador desde 01/02/2013
Bruno Dutra Arbo - Aluno de Doutorado desde 01/02/2013

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 04/03/2013 - Sala de Reuniões do 2º andar do Prédio da Reitoria - Campus Central., em seus aspectos éticos e metolodológicos, para a utilização de 330 ratos Wistar de 7 dias de idade, 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, Quarta-Feira, 29 de Maio de 2013

BRUNO CASSEL NETO
Vice Pró-Reitor de Pesquisa