UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:
BIOQUÍMICA

MECANISMOS ENVOLVIDOS NA NEUROPROTEÇÃO DA GENISTEÍNA EM MODELOS *IN VITRO* E *IN VIVO* DE TOXICIDADE INDUZIDA PELO PEPTÍDEO BETA-AMILOIDE

FERNANDA DOS SANTOS PETRY

Porto Alegre
2020

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:
BIOQUÍMICA

MECANISMOS ENVOLVIDOS NA NEUROPROTEÇÃO DA GENISTEÍNA EM MODELOS *IN VITRO* E *IN VIVO* DE TOXICIDADE INDUZIDA PELO PEPTÍDEO BETA-AMILOIDE

FERNANDA DOS SANTOS PETRY

Orientadora: Profa. Dra. Vera Maria Treis Trindade
Coorientadora: Profa. Dra. Christianne Gazzana Salbego

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Doutora em Bioquímica.

Porto Alegre
2020

CIP - Catalogação na Publicação

Petry, Fernanda dos Santos
Mecanismos envolvidos na neuroproteção da genisteína em modelos in vitro e in vivo de toxicidade induzida pelo peptídeo beta-amiloide / Fernanda dos Santos Petry. -- 2020.
179 f.
Orientadora: Vera Maria Treis Trindade.

Coorientadora: Christianne Gazzana Salbego.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2020.

1. Doença de Alzheimer. 2. Peptídeo beta-amiloide. 3. Genisteína. 4. Neuroproteção. I. Trindade, Vera Maria Treis, orient. II. Salbego, Christianne Gazzana, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

AGRADECIMENTOS

À minha orientadora, professora Vera Treis Trindade, por ter me guiado durante esses anos com competência e extrema generosidade. Agradeço pela disponibilidade constante para ensinar, conversar e pelo suporte e incentivo sempre presentes. Mais do que isso, agradeço pela grande amizade e parceria que cultivamos durante esse período.

À minha coorientadora, professora Christianne Salbego, pela importante colaboração para a realização deste trabalho, pelos ensinamentos, atenção e disponibilidade para sempre contribuir de forma positiva.

À professora Fátima Guma pela oportunidade de colaboração e pelo incentivo científico nos cultivos celulares.

À professora Cristiane Matté pela disponibilidade de colaboração.

Ao Fernando Kreutz, com quem muito aprendi ainda durante a iniciação científica, agradeço pela amizade e pelos ensinamentos valiosos sobre cultivo celular e experimentação animal.

À Bárbara Coelho, pela amizade e parceria na "nem sempre fácil" tarefa de cultivar as células SH-SY5Y. Agradeço imensamente pela troca de experiências, dicas e ajuda nos experimentos.

À Mariana Gaelzer, pelo incentivo à citometria de fluxo e pela importante ajuda na realização dos experimentos.

Aos amigos do Laboratório 23, Juliana Hoppe, Caroline Klein, Bernardo Gindri, Régis Hözer e Felippo Bifi, pelo apoio e grande parceria na realização dos experimentos envolvendo animais e pela disponibilidade para a troca de ideias e de experiências, além dos sempre divertidos momentos de descontração.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela bolsa concedida.

À UFRGS e ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica pela formação acadêmica de qualidade.

Aos funcionários do Departamento de Bioquímica.

Por fim, agradeço à minha família, pelo constante incentivo, apoio e motivação para que eu sempre busque meus objetivos.

SUMÁRIO

APRESENTAÇÃO

Esta tese está organizada em seções dispostas da seguinte maneira: *Introdução*, *Objetivos*, *Metodologia e Resultados (Capítulos I, II e III)*, *Discussão*, *Conclusões*, *Perspectivas*, *Referências Bibliográficas* e *Anexos*.

A seção *Introdução* apresenta o embasamento teórico que levou à formulação das propostas da tese, as quais estão descritas na seção *Objetivos*.

A seção *Metodologia e Resultados* está organizada em *Capítulos I*, *II* e *III* contendo os artigos científicos publicados e em fase de preparação para submissão, os quais estão apresentados de acordo com os objetivos específicos propostos. Nesta seção, estão detalhados os materiais e métodos e as referências bibliográficas utilizadas em cada artigo. Estes trabalhos foram desenvolvidos no Laboratório de Bioquímica e Biologia Celular de Lipídios e no Laboratório de Neuroproteção e Sinalização Celular, do Departamento de Bioquímica da Universidade Federal do Rio Grande do Sul, sob a orientação da Profa. Dra. Vera Maria Treis Trindade e coorientação da Profa. Dra. Christianne Gazzana Salbego.

A seção *Discussão* contém a interpretação geral dos resultados obtidos nos diferentes artigos científicos. Na seção *Conclusões*, são apresentadas as conclusões gerais obtidas nesta tese. A seção *Perspectivas* aborda os potenciais projetos a serem desenvolvidos a partir da conclusão do presente trabalho, de forma a dar continuidade a esta linha de pesquisa.

A seção *Referências Bibliográficas* lista as referências citadas na *Introdução* e *Discussão* da presente tese.

A seção *Anexos* enumera os prêmios recebidos, os trabalhos apresentados em eventos com os dados desta tese e as publicações em coautoria durante a realização do doutorado.

RESUMO

A doença de Alzheimer (DA), um dos grandes desafios em saúde atualmente, é uma desordem neurodegenerativa marcada pela deterioração progressiva da memória e de outras funções cognitivas. As características histopatológicas da DA incluem o acúmulo extracelular de formas insolúveis do peptídeo beta-amiloide (Aβ) na forma de placas amiloides e a deposição intracelular da proteína Tau hiperfosforilada na forma de emaranhados neurofibrilares, as quais afetam extensas áreas cerebrais com a progressão da doença. O acúmulo do peptídeo Aβ é proposto como um evento precoce na fisiopatologia da DA já que ele desencadeia uma série de alterações moleculares e celulares como perda da função sináptica, alteração na ativação de proteínas intracelulares e hiperfosforilação da Tau, as quais levariam à disfunção e à morte neuronal. Contribuindo em grande parte para o desafio que representa a DA, tem-se a limitada resposta clínica proporcionada pela farmacoterapia atualmente disponível para o manejo sintomático dessa doença. Na busca de estratégias alternativas para a prevenção ou para o tratamento da neurotoxicidade e do processo neurodegenerativo associados à DA, a genisteína, que é uma das isoflavonas predominantes na soja, tem sido sugerida como uma opção promissora por seus efeitos potencialmente neuroprotetores. Entretanto, ainda são necessárias a investigação e a melhor compreensão dos mecanismos responsáveis por seus efeitos. Com isto, o objetivo da presente tese foi avaliar o potencial efeito neuroprotetor do tratamento com genisteína em modelos *in vitro* e *in vivo* de toxicidade induzida pelo peptídeo Aβ. Inicialmente, observou-se que esse composto diminuiu a morte celular provocada pela exposição de células SH-SY5Y ao peptídeo $A\beta_{25-35}$ (25 μM). Além disso, demonstrou-se que esse efeito foi acompanhado pela prevenção da inativação da Akt e da hiperfosforilação da Tau e, ao menos em parte, pela diminuição da ativação da GSK-3β. Para o estudo dos efeitos do tratamento com genisteína (10 mg/kg por 10 dias) sobre o dano cognitivo desencadeado pelo peptídeo Aβ, realizou-se um modelo *in vivo* de toxicidade induzida pela injeção intracerebroventricular de $A\beta_{1-42}$ (2 nmol). A genisteína melhorou significativamente o déficit cognitivo causado pelo peptídeo, atenuando ainda a sinaptotoxicidade, evidenciada pela prevenção da redução dos níveis hipocampais de sinaptofisina, induzida pelo $A\beta_{1-42}$. Além disso, esse composto preveniu a hiperfosforilação da Tau desencadeada pelo $A\beta_{1-42}$ no hipocampo, efeito este que foi acompanhado pela diminuição da ativação da GSK-3β e pela redução parcial da ativação da JNK. Observou-se ainda o efeito protetor da genisteína sobre a inativação da ERK provocada pelo peptídeo. Considerando que o processo neurodegenerativo pode estar associado ao prejuízo das propriedades físico-químicas das membranas, ocasionado por alterações no equilíbrio de sua composição lipídica, avaliou-se os efeitos da injeção intracerebroventricular do $A\beta_{1-42}$ (2 nmol) e do tratamento com genisteína (10mg/kg por 10 dias) sobre os principais lipídios de membranas neurais (fosfolipídios, colesterol e gangliosídios) no córtex frontal de ratos. Observou-se que o peptídeo reduziu o conteúdo total de gangliosídios, diminuindo especificamente os níveis de GD1b e de GQ1b. O tratamento com genisteína, por sua vez, foi capaz de prevenir essas alterações, sugerindo que esse composto possa proteger contra o dano induzido pelo peptídeo à membrana celular. Em conjunto, estes resultados confirmam e reforçam o efeito neuroprotetor da genisteína sobre a toxicidade induzida pelo peptídeo Aβ e, sobretudo, ampliam o conhecimento atual acerca dos mecanismos relacionados à sua neuroproteção.

ABSTRACT

Alzheimer's disease (AD), one of the major health challenges today, is a neurodegenerative disorder marked by the progressive deterioration of memory and other cognitive functions. The histopathological features of AD include the extracellular accumulation of insoluble forms of amyloid-beta (Aβ) peptide in amyloid plaques and the intracellular deposition of hyperphosphorylated Tau protein in neurofibrillary tangles, which affect extensive brain areas as the disease progresses. The accumulation of Aβ peptide is proposed as an early event in the pathophysiology of AD as it triggers a series of molecular and cellular alterations such as loss of synaptic function, changes in the activation of intracellular proteins, and hyperphosphorylation of Tau, which could lead to neuronal dysfunction and death. Contributing in a large part for the challenge that represents AD, there is the limited clinical response provided by the pharmacotherapy currently available for the symptomatic management of this disease. In the search of alternative strategies for the prevention or the treatment of the neurotoxicity and the neurodegenerative process associated with AD, genistein that is one of the predominant isoflavones in soy has been suggested as a promising option for its potentially neuroprotective effects. However, the investigation and a better understanding of the mechanisms responsible for its effects are still needed. So, the objective of the present thesis was to evaluate the potential neuroprotective effect of the treatment with genistein in *in vitro* and *in vivo* models of Aβ peptide-induced toxicity. Initially, it was observed that this compound reduced the cell death caused by the exposure of SH-SY5Y cells to the $A\beta_{25-35}$ peptide (25 μM). Moreover, it has been shown that this effect was accompanied by the prevention of Akt inactivation and Tau hyperphosphorylation and, at least in part, by the decrease of GSK-3β activation. For the study of the effects of treatment with genistein (10 mg/kg for 10 days) on the cognitive impairment triggered by the Aβ peptide, an *in vivo* model of intracerebroventricular infusion of $A\beta_{1-42}$ (2 nmol)-induced toxicity was performed. Genistein significantly improved the cognitive deficit caused by the peptide and also attenuated the synaptotoxicity, as evidenced by the prevention of reduction of hippocampal levels of synaptophysin induced by $A\beta_{1-42}$. In addition to that, this compound prevented Tau hyperphosphorylation triggered by $A\beta_{1-42}$ in the hippocampus, an effect that was accompanied by the decrease of GSK-3β activation and partial reduction of JNK activation. It was also observed the protective effect of genistein on ERK inactivation induced by the peptide. Considering that the neurodegenerative process may be associated with the impairment of the physicochemical properties of the membranes, caused by changes in the balance of their lipid composition, the effects of intracerebroventricular infusion of $A\beta_{1-42}$ (2 nmol) and treatment with genistein (10 mg/kg for 10 days) on the main membrane lipids (phospholipids, cholesterol, and gangliosides) were evaluated in the frontal cortex of rats. It was observed that the peptide decreased the total ganglioside content, specifically reducing the levels of GD1b and GQ1b. Treatment with genistein, in turn, was able to prevent these changes, suggesting that this compound may protect against the damage induced by the peptide to the cell membrane. Taken together, these results confirm and reinforce the neuroprotective effect of genistein on the Aβ peptide-induced toxicity and, mainly, expand the current knowledge concerning the mechanisms related to its neuroprotection.

LISTA DE ABREVIATURAS

Obs.: Abreviaturas presentes na introdução e na discussão.

Aβ	Beta-amiloide
ADAM10	Desintegrina A e metaloproteinase 10
AICD	Domínio intracelular da APP
Akt/PKB	Proteína cinase B
ApoE	Apolipoproteína E
APP	Proteína precursora amiloide
BACE1	Enzima 1 de clivagem do sítio beta da proteína precursora amiloide
BDNF	Fator neurotrófico derivado do cérebro
CaMKII	Cinase II dependente de cálcio/calmodulina
Cdk5	Cinase 5 dependente de ciclina
CK1/2	Caseína cinase-1 e 2
CREB	Proteína de ligação ao elemento de resposta a AMPc
DA	Doença de Alzheimer
ERK	Cinase regulada por sinal extracelular
GSK-3β	Glicogênio sintase cinase-3β
JNK	Cinase c-Jun N-terminal
MAPK	Proteínas cinases ativadas por mitógeno
NMDA	N-metil D-aspartato
PET	Tomografia por emissão de pósitrons
PI3K	Fosfatidilinositol 3-cinase
PKA	Proteína cinase A
PLCγ	Fosfolipase Cγ
PP2A	Proteína fosfatase 2A
PSD-95	Proteína de densidade pós-sináptica 95
PTEN	Fosfatase homóloga à tensina
SAPK	Proteínas cinases ativadas por estresse
sAPPα	Ectodomínio APP α solúvel
sAPPβ	Ectodomínio APP β solúvel

LISTA DE FIGURAS

INTRODUÇÃO

CAPÍTULO I

CAPÍTULO II

CAPÍTULO III

DISCUSSÃO

LISTA DE TABELAS

CAPÍTULO II

CAPÍTULO III

1. INTRODUÇÃO

1.1. Doença de Alzheimer

A doença de Alzheimer (DA) foi descrita pela primeira vez, em 1906, como uma doença peculiar do córtex cerebral pelo psiquiatra e neuropatologista alemão Alois Alzheimer em uma conferência em Tübingen, Alemanha (Alzheimer, 1907). Nessa ocasião, foi apresentado o caso da paciente Auguste Deter, de 51 anos, admitida no Hospital Psiquiátrico de Frankfurt, em 1901, com manifestações clínicas que incluíam alterações de comportamento, distúrbio progressivo de memória, desorientação espacial e temporal, confusão e afasia. Com a deterioração do quadro clínico, a paciente veio a óbito em 1906. A partir da realização de autópsia cerebral, Alzheimer identificou a atrofia generalizada dessa estrutura e, a nível histológico, a perda massiva de células, além de alterações peculiares observadas pelo depósito de substâncias não identificadas nos neurônios e na forma de placas no córtex cerebral, sendo considerada uma patologia ainda desconhecida (Dahm, 2006; Hippius e Neundörfer, 2003). Entre 1907 e 1908, o médico acompanhou outros três casos com sintomas e alterações cerebrais semelhantes (Alzheimer, 1911; Perusini, 1909) e, em 1910, o termo para essa doença foi proposto por Emil Kraepelin, um dos psiquiatras mais influentes do início do século XX (Dahm, 2006; Hippius e Neundörfer, 2003).

A DA é uma desordem neurodegenerativa crônica caracterizada pela deterioração progressiva da memória e de outras funções cognitivas (Querfurth e LaFerla, 2010). Atualmente, considera-se essa patologia como um dos grandes desafios em saúde do século XXI, sendo inclusive reconhecida pela Organização Mundial da Saúde como prioridade global de saúde pública (Lane, Hardy e Schott, 2018; Scheltens *et al.*, 2016). A DA corresponde à principal forma de demência relacionada à idade, sendo responsável por 50 a 75% dos casos (Lane, Hardy e Schott, 2018). Com prevalência geral de 10 a 30% na população acima de 65 anos (Masters *et al.*, 2015), estima-se que essa doença afete mais de 40 milhões de pessoas no mundo (Selkoe e Hardy, 2016) e cerca de 1,2 milhão no Brasil (Associação Brasileira de Alzheimer, 2020). Embora a DA não possa ser considerada um desfecho normal do envelhecimento, sua incidência tende a aumentar com o crescimento da população idosa nos próximos anos (Alzheimer's Association, 2019). Estima-se que o risco de desenvolver a doença dobre a cada cinco anos após os 65 anos de idade (Querfurth e LaFerla, 2010).

Duas formas para a DA são descritas, as quais são comparáveis no que se refere aos aspectos clínicos e fisiopatológicos (Masters *et al.*, 2015). A grande maioria dos

pacientes apresenta a forma esporádica da doença que se caracteriza pelo surgimento tardio dos sintomas, a partir dos 65 anos de idade. Uma pequena parcela dos indivíduos (menos de 1%), no entanto, apresenta a forma familiar da DA, associada a mutações herdadas em genes que codificam a proteína precursora amiloide (APP) e as presenilinas 1 e 2, e manifestam mais precocemente os sintomas (entre 30 e 50 anos de idade) (Alzheimer's Association, 2019; Lane, Hardy e Schott, 2018). Como fator de risco genético para a forma esporádica da DA, tem-se o polimorfismo do gene da apolipoproteína E (ApoE), especificamente a presença do alelo ε4 como o mais significativo (Riedel, Thompson e Brinton, 2016). Sabe-se, contudo, que essa é uma desordem multifatorial que envolve a interação complexa entre influências ambientais e genéticas (Lane, Hardy e Schott, 2018). Além da idade, fator de risco mais significativamente associado a essa patologia, outros fatores potencialmente modificáveis, como diabetes mellitus, hipertensão, obesidade, sedentarismo, dano cerebral prévio e baixa escolaridade podem ser considerados (Alzheimer's Association, 2019; Lane, Hardy e Schott, 2018; Silva *et al.*, 2019).

Três estágios clínicos são descritos para a DA: fase pré-clínica, comprometimento cognitivo leve (ou DA prodrômica) e demência, sendo que os sintomas estão presentes somente nos dois últimos (Alzheimer's Association, 2019). Atualmente, sabe-se que os processos patológicos associados à DA podem iniciar cerca de 20 anos antes do aparecimento evidente de sintomas. Nesse sentido, o estágio pré-clínico é definido pela presença dessas alterações em indivíduos cognitivamente normais (Dubois *et al.*, 2016). É importante salientar que nem todos nesse estágio evoluirão para os seguintes (Alzheimer's Association, 2019). O quadro de comprometimento cognitivo leve pode ser considerado quando há evidência de declínio, centrado na memória episódica, mas que ainda não interfere significativamente nas atividades diárias do paciente. Na medida em que a doença progride, os sintomas se tornam mais evidentes e variados, afetando não só a memória, mas também o comportamento do indivíduo (Alzheimer's Association, 2019; Lane, Hardy e Schott, 2018). Esse estágio de demência pode ser classificado como leve, moderado e severo conforme os sintomas apresentados, os quais variam com o passar dos anos e refletem o grau de comprometimento de diferentes regiões cerebrais. Na fase final da doença, o indivíduo torna-se bastante debilitado, podendo apresentar dificuldades de mobilidade e de deglutição. A sobrevivência dos pacientes após o diagnóstico de demência é, em média, de 4 a 8 anos (Alzheimer's Association, 2019).

As características histopatológicas da DA incluem o acúmulo extracelular de formas insolúveis do peptídeo beta-amiloide (Aβ) na forma de placas amiloides ou senis, bem como a deposição intracelular da proteína Tau hiperfosforilada na forma de emaranhados neurofibrilares. Inicialmente, as placas amiloides afetam os lobos frontal e temporal, o hipocampo e o sistema límbico, enquanto que os emaranhados neurofibrilares se originam no lobo temporal medial e no hipocampo. Com a progressão da doença, essas lesões se espalham gradualmente em taxas que variam consideravelmente entre os indivíduos, afetando áreas extensas do neocórtex (Figura 1) (Masters *et al.*, 2015). Adicionalmente, observa-se a presença de neuritos distróficos, perda de neurônios e de substância branca, astrogliose, microgliose, atrofia macroscópica e angiopatia congofílica (Lane, Hardy e Schott, 2018; Querfurth e LaFerla, 2010).

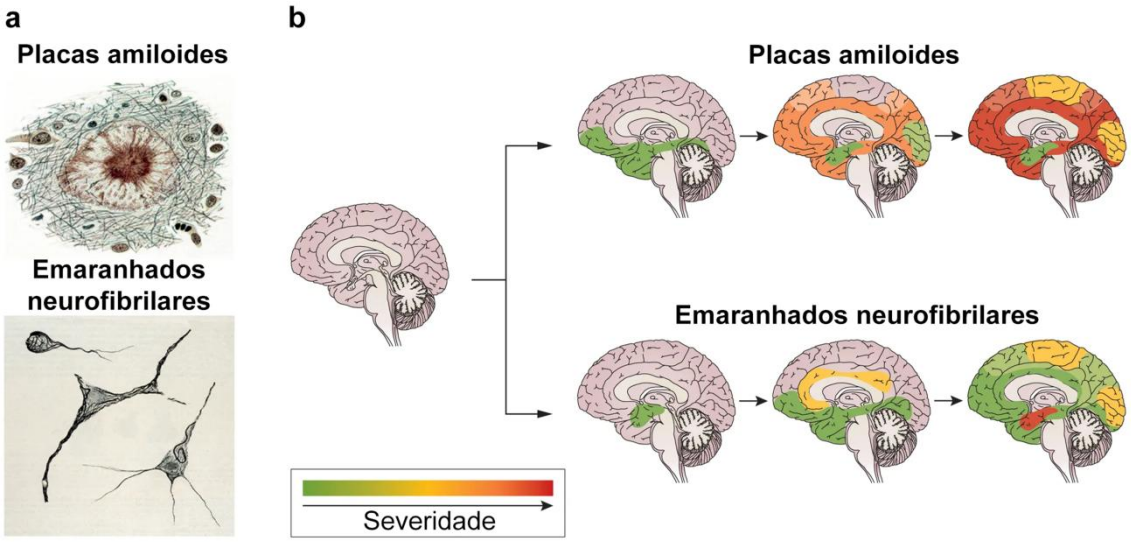


Figura 1. Evolução patológica da doença de Alzheimer. (a) Placas amiloides extracelulares compostas pelo acúmulo do peptídeo Aβ e emaranhados neurofibrilares intracelulares compostos pela proteína Tau hiperfosforilada. **(b)** Áreas cerebrais afetadas pela deposição de placas amiloides e de emaranhados neurofibrilares conforme a progressão da doença (adaptado de Masters *et al.*, 2015).

A base do diagnóstico para a DA envolve a avaliação clínica do paciente para descartar outras possíveis causas para os sintomas e exame cognitivo (Lane, Hardy e Schott, 2018). Além disso, como suporte para o diagnóstico clínico, podem ser utilizadas técnicas de imagem, como a ressonância magnética para a identificação de atrofia cerebral e a tomografia por emissão de pósitrons (PET) para a avaliação do

metabolismo cerebral da glicose, indicativo de atividade sináptica (Johnson *et al.*, 2012). Embora o diagnóstico definitivo ainda exija confirmação histopatológica das lesões características da doença (Lane, Hardy e Schott, 2018), o avanço no desenvolvimento de biomarcadores de imagem (PET com a utilização de radiofármacos específicos para a identificação de acúmulo cerebral de placas amiloides) ou de líquido cefalorraquidiano (níveis de $A\beta_{42}$, Tau total e Tau fosforilada) são ferramentas de grande valor especialmente em estudos clínicos (Jack *et al.*, 2018; Lee *et al.*, 2019; Leuzy *et al.*, 2018; Schilling *et al.*, 2016).

1.1.1. Peptídeo beta-amiloide

O acúmulo extracelular do peptídeo Aβ é proposto como um evento precoce na fisiopatologia da DA. O peptídeo Aβ, constituído por 39-43 aminoácidos, é produzido pela clivagem proteolítica da proteína precursora amiloide (APP) (Picone *et al.*, 2020). A APP é uma glicoproteína transmembrana do tipo 1, expressa em diferentes tipos celulares, que apresenta um domínio de membrana com uma extremidade amino-terminal (N-terminal) glicosilada extracelular e uma extremidade carboxi-terminal (C-terminal) citoplasmática mais curta (Chen *et al.*, 2017). Oito a onze isoformas dessa proteína, com diferentes números de aminoácidos, podem ser geradas por *splicing* alternativo. Entre as isoformas atualmente conhecidas, a forma mais abundante no cérebro (APP695) é produzida principalmente por neurônios, mas outras isoformas (APP751 e APP770) são também expressas por células gliais (Wilkins e Swerdlow, 2017).

O processamento da APP, dependente da ação sequencial de enzimas secretases, resulta na formação de produtos que são secretados para o meio extracelular ou que permanecem dentro da célula ou associados a ela (Wilkins e Swerdlow, 2017). Duas vias para o processamento da APP podem ser consideradas: a via não amiloidogênica e a amiloidogênica (Figura 2). No processamento não amiloidogênico, a APP é inicialmente alvo de uma enzima α-secretase, cuja principal representante em neurônios é a ADAM10, que cliva a APP na região transmembrana, dentro do domínio Aβ, levando à liberação para o meio extracelular do ectodomínio APP α solúvel (sAPPα), o qual apresenta propriedades neuroprotetoras (Chasseigneaux e Allinquant, 2012; Tackenberg e Nitsch, 2019). A seguir, o fragmento C-terminal da APP com 83 resíduos

de aminoácidos (C83), que permanece incorporado à membrana, é alvo da clivagem pela γ-secretase, um complexo enzimático que tem as presenilinas 1 e 2 formando seu domínio catalítico. Como resultado, tem-se a liberação do peptídeo não amiloidogênico p3 (3 kDa) para o meio extracelular e do domínio intracelular da APP (AICD) para o citoplasma.

Por outro lado, o processamento amiloidogênico da APP inicia com a clivagem mediada por uma β-secretase, cuja principal representante é a BACE1, liberando assim o ectodomínio APP β solúvel (sAPPβ) para o meio extracelular. O fragmento C-terminal da APP com 99 resíduos de aminoácidos (C99) é, então, alvo da clivagem intramembrana pela γ-secretase. Como resultado, tem-se a liberação do peptídeo Aβ (4 kDa) para o meio extracelular e do AICD, o qual é direcionado para o núcleo, onde pode regular a expressão gênica (Barage e Sonawane, 2015; Chen *et al.*, 2017; Wilkins e Swerdlow, 2017).

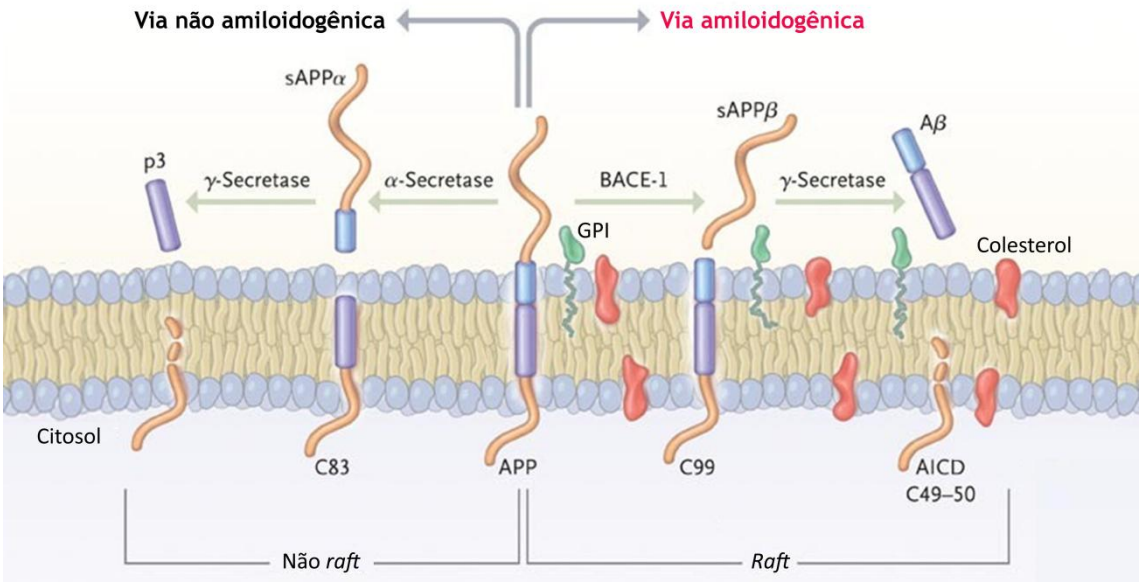


Figura 2. Processamento da APP pelas vias não amiloidogênica e amiloidogênica. O processamento não amiloidogênico envolve a clivagem sequencial da APP por enzimas α e γ-secretases, liberando para o meio extracelular o ectodomínio sAPPα e o fragmento p3. Por outro lado, o processamento amiloidogênico é determinado pela clivagem sequencial da APP por enzimas β e γ-secretases, gerando como principal produto o peptídeo Aβ (adaptado de Querfurth e LaFerla, 2010).

O tamanho do peptídeo Aβ produzido varia em sua extremidade C-terminal de acordo com o padrão de clivagem sofrido pela APP (Barage e Sonawane, 2015). Os

peptídeos constituídos por 40 e 42 aminoácidos, $A\beta_{40}$ e $A\beta_{42}$ respectivamente, são as formas mais predominantes. Em condições fisiológicas, cerca de 80% do peptídeo produzido corresponde ao $A\beta_{40}$. Entretanto, em condições patológicas, há aumento da produção e acúmulo de $A\beta_{42}$, forma mais hidrofóbica e com maior propensão a formar fibrilas insolúveis e, por isso, mais abundante em placas amiloides (Barage e Sonawane, 2015; Picone *et al.*, 2020). A liberação de monômeros de Aβ, inicialmente com estrutura em alfa-hélice, é um processo relativamente não patogênico (Leong *et al.*, 2020). Entretanto, a progressiva transição para conformações em folha-beta leva à agregação em múltiplos arranjos coexistentes: oligômeros, protofibrilas e fibrilas, as quais se arranjam, formando as placas amiloides avançadas (Figura 3) (Barage e Sonawane, 2015; Querfurth e LaFerla, 2010). Oligômeros solúveis são considerados atualmente como as espécies de maior toxicidade. A formação de fibrilas insolúveis e de placas se, por um lado, pode ser considerada como um mecanismo de proteção celular pelo sequestro de espécies tóxicas do peptídeo, por outro, pode atuar como um reservatório a partir do qual oligômeros são liberados. Além disso, sua deposição também prejudica a captação adequada de nutrientes necessários às células (Lane, Hardy e Schott, 2018; Picone *et al.*, 2020).

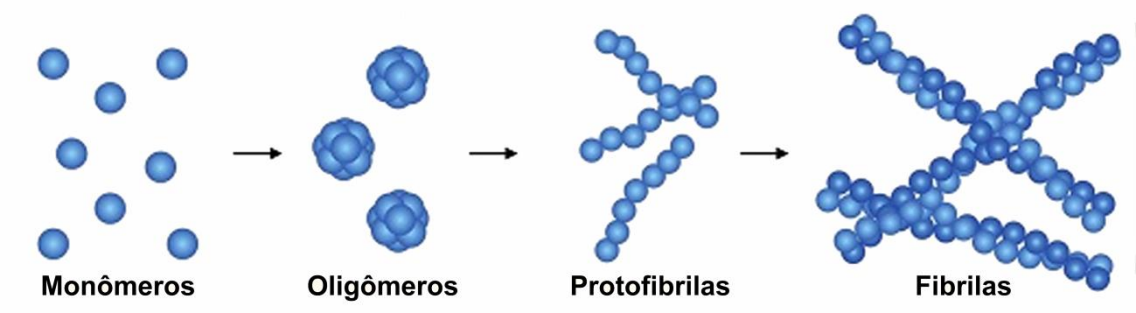


Figura 3. Processo de agregação do peptídeo Aβ. Monômeros de Aβ, inicialmente produzidos, sofrem progressiva agregação formando múltiplos arranjos coexistentes: oligômeros, protofibrilas e fibrilas insolúveis (adaptado de Giorgetti *et al.*, 2018).

Um desequilíbrio na produção ou nos diferentes mecanismos para a remoção do Aβ produzido pode levar ao acúmulo desse peptídeo, o que parece ser um evento precoce e distante da evidência de sintomas clínicos. Essa deposição, contudo, levaria a uma série de alterações moleculares e celulares que desencadeariam, por sua vez, a disfunção neuronal observada (Selkoe e Hardy, 2016). Essas alterações incluem estresse

oxidativo (Butterfield e Boyd-Kimball, 2018; Cheignon *et al.*, 2018; Chiurchiù, Orlacchio e Maccarrone, 2016), neuroinflamação (Calsolaro e Edison, 2016; Minter, Taylor e Crack, 2016; Steardo *et al.*, 2015), alterações na homeostasia do cálcio (Galla *et al.*, 2020; Magi *et al.*, 2016; Tong *et al.*, 2018), disfunção sináptica (Busche e Konnerth, 2016; Ferreira e Klein, 2011; Mucke e Selkoe, 2012; Tu *et al.*, 2014), alteração da ativação de proteínas intracelulares, hiperfosforilação da proteína Tau e, finalmente, morte celular (Majd, Power e Grantham, 2015; Perluigi *et al.*, 2016).

1.1.2. Proteína Tau

Os emaranhados neurofibrilares, característicos da histopatologia da DA, são agregados intraneuronais compostos pela proteína Tau em sua forma hiperfosforilada. A Tau é uma proteína associada a microtúbulos presente em seis diferentes isoformas no cérebro humano, as quais se distinguem em sua região N-terminal e no número de repetições (3 ou 4) no domínio de ligação ao microtúbulo localizado próximo à porção C-terminal, sendo variavelmente expressas ao longo do desenvolvimento e em diferentes Tauopatias (Alonso *et al.*, 2018; Khan e Bloom, 2016).

Em condições fisiológicas, a Tau é uma proteína solúvel, presente predominantemente nos axônios, que promove o arranjo e a estabilização dos microtúbulos ao se ligar às unidades de tubulina, contribuindo de maneira importante para o transporte intracelular de vesículas e de organelas (Alonso *et al.*, 2018; Querfurth e LaFerla, 2010). No entanto, em condições patológicas como a DA, essa proteína se torna anormalmente hiperfosforilada, o que leva à perda de sua afinidade de ligação aos microtúbulos, se desprende e se arranja em filamentos helicoidais pareados formando, por fim, os emaranhados neurofibrilares (Figura 4) (Khan e Bloom, 2016; Querfurth e LaFerla, 2010). Com isso, ocorre a desestabilização do citoesqueleto neuronal e o prejuízo do transporte axonal, o que contribui para a disfunção sináptica associada a essa patologia. Nesse caso, a distribuição da Tau pode se alterar de modo que essa proteína se distribui e se acumula de forma aberrante no compartimento somatodendrítico (Pooler, Noble e Hanger, 2014).

A Tau pode sofrer uma série de modificações pós-traducionais que são capazes de interferir em sua ligação aos microtúbulos, sendo a fosforilação a principal delas (Congdon e Sigurdsson, 2018). Entre os 85 sítios de fosforilação que são encontrados

nessa proteína, 28 são associados exclusivamente à DA, 16 a condições fisiológicas e à DA, 31 a condições fisiológicas e, ainda, 10 sítios putativos sem cinases identificadas (Martin *et al.*, 2013a, 2013b).

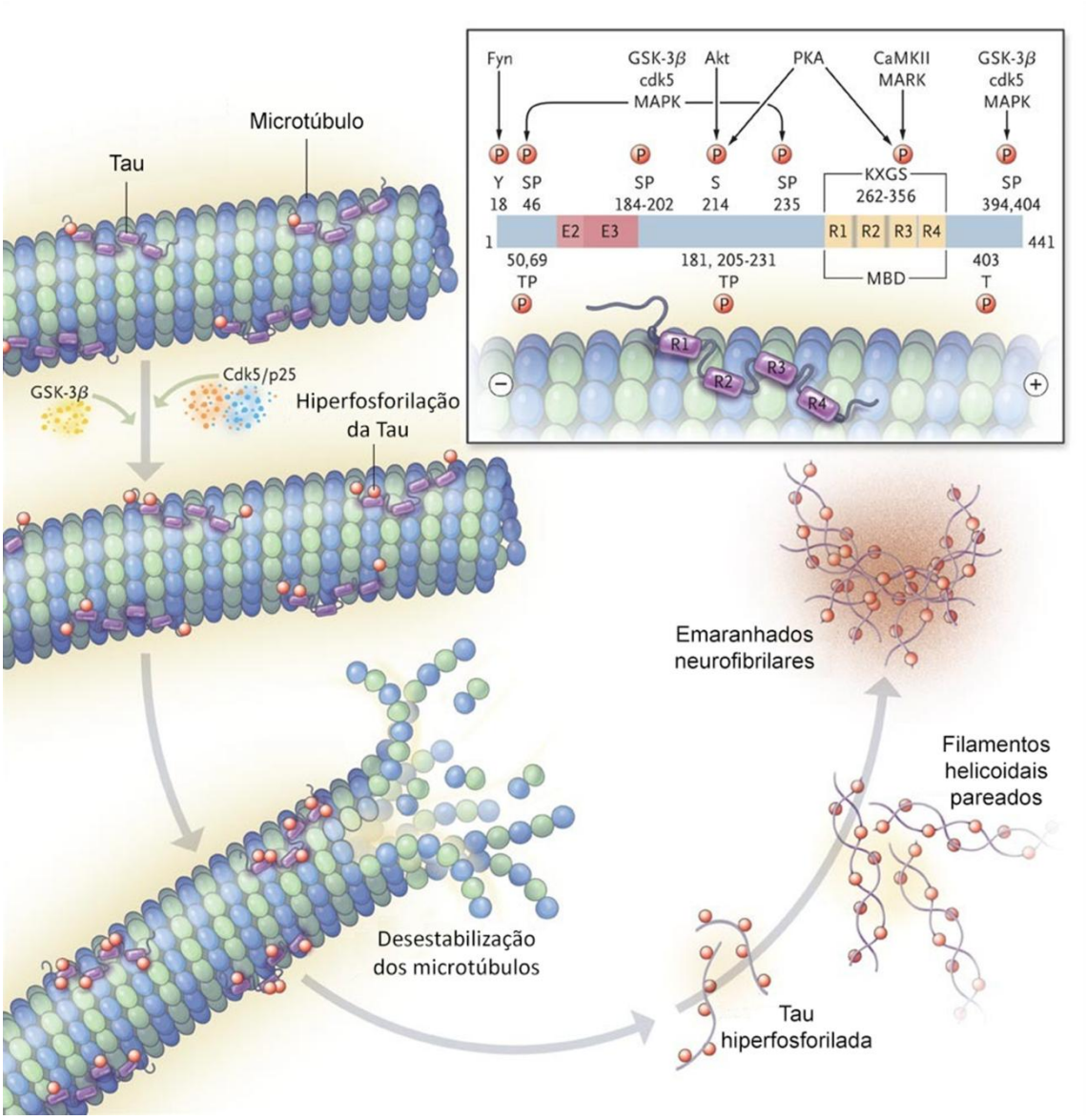


Figura 4. Hiperfosforilação da proteína Tau e formação dos emaranhados neurofibrilares. A Tau promove o arranjo e a estabilização dos microtúbulos. A atividade excessiva de cinases ou reduzida de fosfatases causa a hiperfosforilação dessa proteína que se desprende dos microtúbulos, levando à desestabilização dessas estruturas. Com isso, tem-se a formação dos filamentos helicoidais pareados e dos emaranhados neurofibrilares. No quadro superior são mostradas as regiões que compõem a Tau, sítios de fosforilação e respectivas cinases (adaptado de Querfurth e LaFerla, 2010).

A hiperfosforilação da Tau reflete a perda do equilíbrio entre as atividades de proteínas cinases e fosfatases, as quais podem se encontrar aumentadas e diminuídas,

respectivamente, em cérebros de indivíduos com a DA (Congdon e Sigurdsson, 2018; Martin *et al.*, 2013a, 2013b). Entre as cinases, tem-se: glicogênio sintase cinase-3β (GSK-3β), caseína cinase-1 e 2 (CK1/2), proteína cinase A (PKA), p38, cinases 1 e 2 reguladas por sinal extracelular (ERK1/2), cinase c-Jun N-terminal (JNK), cinase 5 dependente de ciclina (Cdk5), cinase II dependente de cálcio/calmodulina (CaMKII), entre outras. A GSK-3β pode ser considerada como uma das principais entre elas, já que atua sobre 42 sítios de fosforilação na Tau, número maior em comparação com as demais (Martin *et al.*, 2013b). Em relação às enzimas que medeiam a desfosforilação da Tau, tem-se a proteína fosfatase 2A (PP2A) como a principal (Liu *et al.*, 2005). Além dela, a PP1, PP2B, PP5 e a fosfatase homóloga à tensina (PTEN) também atuam nesse processo, embora em grau consideravelmente menor (Congdon e Sigurdsson, 2018; Martin *et al.*, 2013a).

Evidências experimentais indicam que a deposição do peptídeo Aβ preceda e acelere a formação dos emaranhados neurofibrilares (Götz *et al.*, 2001; Lewis *et al.*, 2001; Oddo *et al.*, 2003a, 2003b). A maneira exata como isso aconteceria não é completamente conhecida, mas sugerem-se quatro mecanismos pelos quais o Aβ promoveria a hiperfosforilação da Tau, a saber: ativação de cinases específicas (como, por exemplo, a GSK-3β); indução de resposta inflamatória massiva com a produção de citocinas pró-inflamatórias que indiretamente modulariam esse processo; redução da degradação da Tau pelo proteassoma; e prejuízo no transporte axonal, levando à localização inadequada da Tau nos neurônios (Blurton-Jones e LaFerla, 2006; Silva *et al.*, 2019).

1.1.3. Alterações na ativação de proteínas intracelulares

Alterações na ativação de diferentes proteínas intracelulares podem ser desencadeadas em resposta ao progressivo acúmulo do peptídeo Aβ. Nesse sentido, a desregulação de algumas cinases envolvidas no controle de funções neuronais pode iniciar uma cascata de eventos neurotóxicos contribuindo, assim, para a maior vulnerabilidade e morte neuronal (Majd, Power e Grantham, 2015; Perluigi *et al.*, 2016).

1.1.3.1. Proteína cinase B (Akt)

A Akt é uma proteína amplamente expressa em diferentes tipos de células e tecidos onde regula processos como crescimento, proliferação, sobrevivência e metabolismo celular e apoptose (Hemmings e Restuccia, 2012; Manning e Toker, 2017; Wu *et al.*, 2010). Nos neurônios, tem papel também na plasticidade sináptica necessária para a formação da memória (Manning e Toker, 2017). A ativação da Akt, pela fosforilação dos resíduos Ser473 e Thr308, se dá em uma sequência de eventos iniciados pela ativação da fosfatidilinositol 3-cinase (PI3K) a partir da autofosforilação de receptores tirosina cinases, induzida por diferentes ligantes, ou ainda da estimulação de receptores acoplados à proteína G (Manning e Toker, 2017; Rai *et al.*, 2019). Uma vez ativada, a Akt pode fosforilar seus substratos citoplasmáticos ou, ainda, se deslocar para o núcleo (Hemmings e Restuccia, 2012; Rai *et al.*, 2019).

Foi demonstrado que a ativação da Akt pode estar diminuída na DA, sendo esse um dos mecanismos pelos quais o peptídeo Aβ exerce sua toxicidade (Hoppe *et al.*, 2013; Magrané *et al.*, 2005; Rai *et al.*, 2019; Suwanna, Thangnipon e Soi-ampornkul, 2014; Wu *et al.*, 2010). A ativação da Akt promove a sobrevivência celular ao inibir as proteínas apoptóticas Bad e caspase-9 e, ainda, os fatores de transcrição Forkhead (Manning e Toker, 2017; Rai *et al.*, 2019; Wu *et al.*, 2010). Dessa forma, estando menos ativa, a Akt poderia contribuir diretamente para a perda neuronal observada na DA. Além disso, essa alteração pode refletir em maior ativação da proteína GSK-3β, um dos substratos da Akt, o que contribuiria também para a neurotoxicidade desencadeada pelo peptídeo Aβ (Rai *et al.*, 2019; Wu *et al.*, 2010).

1.1.3.2. Glicogênio sintase cinase-3β (GSK-3β)

A GSK-3β, cinase abundantemente expressa no sistema nervoso central, participa da regulação de processos celulares como proliferação, diferenciação celular e apoptose. Embora seja constitutivamente ativa, a GSK-3β pode ser regulada por fosforilação inibitória em Ser9, a qual pode ser catalisada por diversas cinases, entre elas a Akt. Dessa forma, quando essa proteína se encontra menos ativa ocorre falha na repressão adequada da GSK-3β e ela se torna anormalmente ativa (Cai, Zhao e Zhao, 2012; Hernández *et al.*, 2010; Lauretti, Dincer e Praticò, 2020).

Aumento na forma ativa da GSK-3β foi observado em cérebro de pacientes com a DA (Leroy, Yilmaz e Brion, 2007). Sabe-se que essa proteína exerce papel central na patogênese da DA, contribuindo de diferentes maneiras para os mecanismos associados ao desencadeamento e à progressão da doença. A GSK-3β é capaz de modular as proteínas que participam do processamento da APP e, dessa forma, favorecer a produção e o acúmulo do peptídeo Aβ (Cai, Zhao e Zhao, 2012; Lauretti, Dincer e Praticò, 2020; Llorens-Martín *et al.*, 2014). Além disso, o próprio acúmulo do Aβ levaria à maior ativação da GSK-3β, o que contribuiria para a toxicidade induzida pelo peptídeo (Frozza *et al.*, 2013; Hoppe *et al.*, 2013; Kreutz *et al.*, 2011). A GSK-3β é uma das principais cinases que atuam sobre a Tau, sendo capaz de fosforilar essa proteína em 42 sítios (Martin *et al.*, 2013b). Portanto, a ativação da GSK-3β contribui diretamente para a hiperfosforilação da Tau e, como consequência, para a formação dos emaranhados neurofibrilares e para a neurodegeneração decorrente. Além disso, a hiperativação da GSK-3β pode contribuir para o processo neuroinflamatório (ao regular a resposta microglial e promover a produção de moléculas pró-inflamatórias), para o déficit de memória (ao inibir a potenciação de longa duração hipocampal necessária para a formação da memória) e para a supressão da neurogênese hipocampal adulta (Cai, Zhao e Zhao, 2012; Lauretti, Dincer e Praticò, 2020; Llorens-Martín *et al.*, 2014; Medina e Avila, 2013).

1.1.3.3. Cinase c-Jun N-terminal (JNK)

A JNK pertence à família de cinases ativadas por mitógeno (MAPK). Ela é também conhecida como proteína cinase ativada por estresse (SAPK) e responde a uma variedade de estímulos estressores tanto extra quanto intracelulares (Mehan *et al.*, 2011). No sistema nervoso central, essa proteína regula processos fisiológicos como o desenvolvimento e a regeneração neuronal. No entanto, a hiperativação da JNK é associada à indução de neuroinflamação e de morte neuronal (Mehan *et al.*, 2011; Zeke *et al.*, 2016). A ativação dessa proteína se dá pela fosforilação dupla em resíduos de tirosina e treonina, o que permite sua atuação direta sobre fatores de transcrição nucleares, regulando a expressão gênica, ou ainda sobre substratos citoplasmáticos (Yarza *et al.*, 2016).

Análises *post mortem* revelaram aumento na forma ativa dessa proteína em cérebros de pacientes com a DA (Shoji *et al.*, 2000; Zhu *et al.*, 2001). Sabe-se que a JNK está envolvida nos processos de neurodegeneração e de morte neuronal associados tanto ao peptídeo Aβ quanto aos emaranhados neurofibrilares (Mehan *et al.*, 2011; Yarza *et al.*, 2016). Evidências experimentais indicam que a exposição ao Aβ desencadeie a ativação da JNK, contribuindo para a apoptose neuronal induzida por esse peptídeo (Frozza *et al.*, 2013; Morishima *et al.*, 2001; Suwanna, Thangnipon e Soi-ampornkul, 2014; Yao, Nguyen e Pike, 2005). Além disso, por ser uma das cinases envolvidas na fosforilação da Tau, o aumento na ativação da JNK está diretamente associado à hiperfosforilação dessa proteína e à formação dos emaranhados neurofibrilares (Martin *et al.*, 2013b; Mehan *et al.*, 2011; Yarza *et al.*, 2016).

1.1.3.4. Cinase regulada por sinal extracelular (ERK)

A ERK, membro da família das MAPK, exerce funções na regulação da sobrevivência e da diferenciação de diferentes tipos celulares. Nos neurônios, atua também como componente essencial dos mecanismos de transdução de sinal e de plasticidade sináptica necessários à consolidação da memória (Sweatt, 2004). A completa ativação dessa proteína se dá a partir de uma sequência de eventos que culminam na sua fosforilação dupla em resíduos de tirosina e de treonina. Uma vez ativa, essa cinase pode fosforilar substratos citoplasmáticos ou fatores de transcrição nucleares (Franco *et al.*, 2017; Rai *et al.*, 2019).

Alterações na ativação da ERK estão envolvidas na patogênese da DA (Rai *et al.*, 2019). Estudos experimentais sugerem a ativação crônica da ERK como um mecanismo de neurotoxicidade subjacente à exposição ao peptídeo Aβ (Kirouac *et al.*, 2017). Nesse sentido, estando mais ativa e sendo uma das cinases responsáveis pela fosforilação da Tau, ela poderia contribuir para a hiperfosforilação dessa proteína (Martin *et al.*, 2013b). Por outro lado, alguns estudos indicam que o peptídeo Aβ desencadeie diminuição da ativação da ERK (Fan *et al.*, 2017; Suwanna, Thangnipon e Soi-ampornkul, 2014). Considerando a participação dessa proteína na plasticidade sináptica em diferentes regiões cerebrais, sua menor ativação poderia contribuir para o desencadeamento de déficit cognitivo (Franco *et al.*, 2017; Sweatt, 2004).

1.1.4. Alterações nos níveis de fator neurotrófico derivado do cérebro (BDNF)

O fator neurotrófico derivado do cérebro (BDNF) pertence a uma família de neurotrofinas que apresentam papel crucial na sobrevivência e na diferenciação neuronal durante o desenvolvimento do sistema nervoso central. No cérebro adulto, o BDNF desempenha função importante na regulação da transmissão sináptica excitatória e inibitória e também da plasticidade sináptica, sendo parte essencial dos mecanismos celulares necessários para a formação e a manutenção da memória (Cunha, Brambilla e Thomas, 2010; Miranda *et al.*, 2019). Tanto a nível pré e pós sináptico, o BDNF se liga e ativa o receptor tirosina cinase TrkB com alta afinidade e o receptor $p75^{NTR}$ com baixa afinidade. Os efeitos sinápticos dessa neurotrofina são atribuídos à ativação de TrkB, o que inicia uma cascata de sinalização pelas vias PI3K/Akt, ERK e fosfolipase Cγ (PLCγ), levando à fosforilação e à ativação da proteína de ligação ao elemento de resposta a AMPc (CREB) que regula a transcrição de genes essenciais para esse processo (Cunha, Brambilla e Thomas, 2010). Por esse mesmo mecanismo, o BDNF pode aumentar a sua própria transcrição (Cunha, Brambilla e Thomas, 2010; Finkbeiner *et al.*, 1997).

Disfunções na sinalização por BDNF/TrkB são associadas ao processo de envelhecimento, mas também a condições patológicas como a DA e, nesse caso, podem contribuir para a disfunção sináptica e para a perda de memória que são observadas (Miranda *et al.*, 2019; Tanila, 2017; Tejeda e Díaz-Guerra, 2017). Análises *post mortem* indicaram redução nos níveis proteico e de mRNA do BDNF em diferentes áreas cerebrais de pacientes com essa patologia (Connor *et al.*, 1997; Fahnestock *et al.*, 2002; Ferrer *et al.*, 1999; Garzon, Yu e Fahnestock, 2004; Hock *et al.*, 2000; Holsinger *et al.*, 2000; Narisawa-Saito *et al.*, 1996; Phillips *et al.*, 1991). Essas alterações podem já estar presentes no estágio de comprometimento cognitivo leve (Peng *et al.*, 2005) e, a partir de evidências experimentais, sugere-se que elas sejam subjacentes ao acúmulo do peptídeo Aβ, embora provavelmente ocorram antes da formação de placas amiloides (Peng *et al.*, 2009). O Aβ poderia levar à diminuição da ativação de vias de sinalização intracelulares, como PI3K/Akt e ERK, com a consequente diminuição da ativação de CREB e da transcrição gênica mediada por ele (Caccamo *et al.*, 2010; Pugazhenthi *et al.*, 2011; Tong *et al.*, 2004). Esse peptídeo pode também inibir a conversão de pro-BDNF a BDNF maduro e interferir tanto em seu transporte axonal quanto no transporte axonal retrógrado do complexo BDNF-TrkB (Poon *et al.*, 2013; Ramser *et al.*, 2013;

Zheng, Sabirzhanov e Keifer, 2010). Além disso, a própria diminuição da sinalização por BDNF, com a consequente inibição da via PI3K/Akt e aumento da ativação da GSK-3β por diminuição de sua fosforilação inibitória, pode favorecer a hiperfosforilação da Tau e a formação dos emaranhados neurofibrilares (Figura 5) (Elliott *et al.*, 2005).

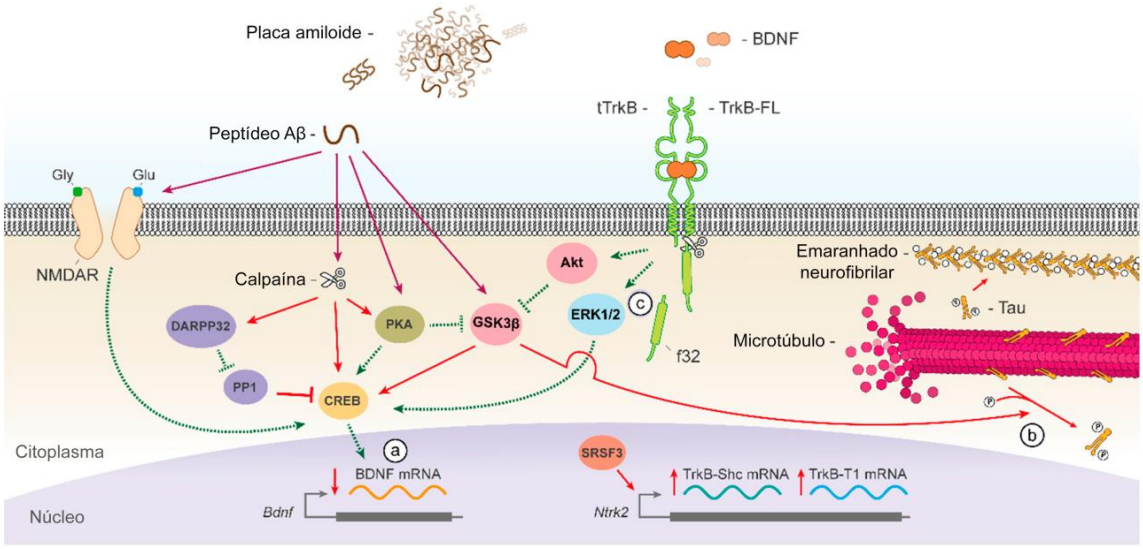


Figura 5. Disfunção da sinalização por BDNF na doença de Alzheimer. (a) Nesta patologia, pode haver redução dos níveis de BDNF em diferentes áreas cerebrais devido à diminuição de sua expressão gênica. **(b)** A redução na sinalização neurotrófica pode levar à supressão da via PI3K/Akt com consequente ativação da GSK-3β, o que contribui para a hiperfosforilação da Tau. **(c)** Ainda, pode ocorrer a supressão da via MAPK/ERK. Setas vermelhas indicam os efeitos desencadeados pelo peptídeo Aβ. Setas verdes indicam a sinalização ativada por BDNF (adaptado de Tejeda e Díaz-Guerra, 2017).

1.1.5. Alterações na composição lipídica da membrana celular

Lipídios são componentes celulares essenciais que desempenham papéis importantes como a estabilização e a sinalização celular. Diferentes tipos de células, organelas e tecidos apresentam variação em sua composição lipídica, o que sugere que essas características sejam necessárias para a realização de diferentes funções. Dessa forma, alterações na composição ou na organização lipídica podem ter efeitos profundos sobre as funções celulares, entre elas a transdução de sinal e a plasticidade e o transporte de membrana (Santos e Preta, 2018). Dentre os principais lipídios que compõem as membranas neurais, têm-se os fosfolipídios, o colesterol e os glicoesfingolipídios

(Figura 6) (Sastry, 1985). Fosfolipídios correspondem aos componentes mais abundantes nas membranas e tem como estrutura uma cabeça polar contendo um grupo fosfato e duas caudas hidrocarbonadas hidrofóbicas (Alberts *et al.*, 2017). Esses lipídios exercem funções estruturais na membrana e participam também da sinalização celular (Yang, Lee e Fairn, 2018). O colesterol, lipídio esteroidal com estrutura em anel rígida que se liga a um único grupo hidroxila polar e a uma pequena cauda hidrocarbonada apolar, é um componente essencial para a estrutura e para a função das membranas celulares. Além de modular as propriedades físico-químicas da membrana, esses lipídios contribuem também para a formação dos *rafts* lipídicos que correspondem a microdomínios de membrana especializados que compartimentalizam processos celulares (Alberts *et al.*, 2017; Lim, Martins e Martins, 2014). Gangliosídios são glicoesfingolipídios compostos por uma ceramida e por uma cadeia oligossacarídica à qual um ou mais resíduos de ácido siálico estão ligados (Magistretti *et al.*, 2019). Esses lipídios participam de funções biológicas, entre as quais: reconhecimento célula-célula, adesão, crescimento e diferenciação celular, sinalização intercelular e modulação de canais iônicos (Ariga, 2017). Além disso, por sua influência sobre o estabelecimento e a estabilização de sinapses funcionais e de circuitos neuronais, atribui-se aos gangliosídios um papel importante no processo de formação da memória. Estando presentes principalmente nos terminais sinápticos, eles também desempenham função na excitabilidade neuronal e na transmissão sináptica ao modularem canais iônicos e a sinalização por receptores (Palmano *et al.*, 2015).

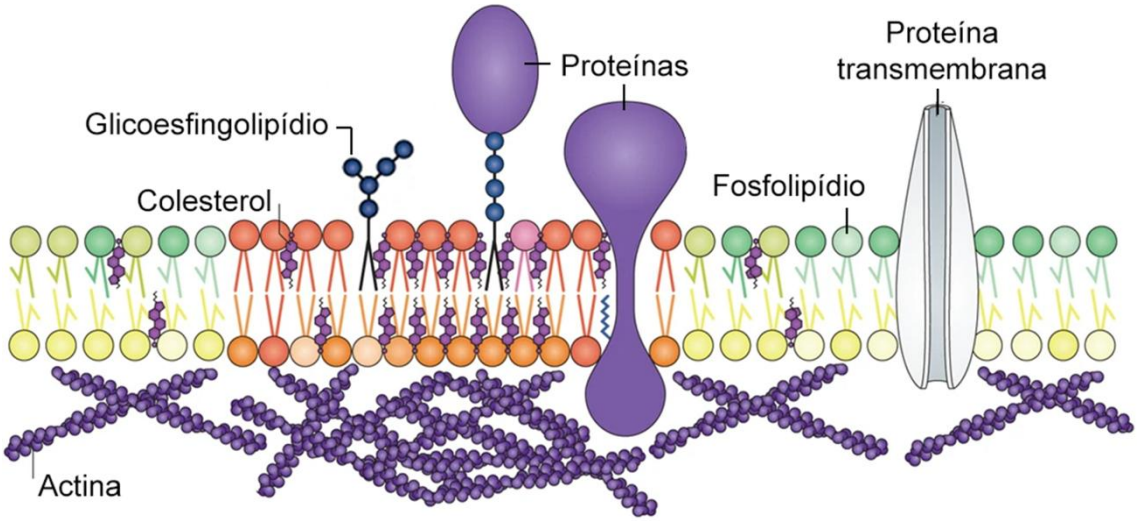


Figura 6. Composição da membrana celular. Como principais lipídios que compõem as membranas neurais, têm-se os fosfolipídios, o colesterol e os glicoesfingolipídios. Além disso, estão representadas também as proteínas de membrana (adaptado de Sezgin *et al.*, 2017).

Sabe-se que o processo neurodegenerativo pode estar associado ao prejuízo das propriedades físico-químicas das membranas, ocasionado por alterações no equilíbrio de sua composição lipídica, resultando em prejuízo da função neuronal (Yadav e Tiwari, 2014). Com isso, alterações em nível de membrana são frequentemente envolvidas na patogênese da DA (Ariga, 2017; Ariga, Wakade e Yu, 2011; Kosicek e Hecimovic, 2013; Magistretti *et al.*, 2019; van Echten-Deckert e Walter, 2012; Williamson e Sutherland, 2011; Yadav e Tiwari, 2014). Por um lado, os próprios lipídios de membrana são capazes de regular a amiloidogênese, ao interferirem na localização e na atividade proteolítica de proteínas de membrana como a APP, a BACE1 e as presenilinas. Além disso, eles podem modular o potencial patogênico do peptídeo Aβ ao afetarem seu processo de agregação. Por outro lado, o peptídeo Aβ desencadeia sua neurotoxicidade primariamente ao interagir com a membrana celular e, com isso, pode induzir ao prejuízo de sua integridade, fluidez e propriedades sinalizadoras (Di Paolo e Kim, 2011; Wong *et al.*, 2017; Zhu *et al.*, 2015).

1.1.6. Terapêutica

Apesar de décadas de pesquisa, ainda há ausência de sucesso na farmacoterapia para a DA. As opções de tratamento disponíveis não apresentam efeito curativo ou são capazes de retardar a progressão da doença (Alzheimer's Association, 2019; Ibrahim *et al.*, 2020; Long e Holtzman, 2019). Atualmente, quatro drogas são aprovadas para o manejo sintomático da DA, as quais incluem três inibidores da colinesterase (donepezil, rivastigmina e galantamina) e um antagonista do receptor N-metil D-aspartato (NMDA), a memantina (Long e Holtzman, 2019). Inibidores da colinesterase, utilizados principalmente nos estágios leve a moderado da doença, atuam aumentando a neurotransmissão colinérgica por meio da inibição da enzima acetilcolinesterase, o que diminui a hidrólise da acetilcolina na fenda sináptica e aumenta sua disponibilidade (Kumar, Singh e Ekavali, 2015; Long e Holtzman, 2019). A memantina é um antagonista não competitivo de NMDA que atua sobre o sistema glutamatérgico ao se ligar a esse receptor e inibir a neurotoxicidade mediada pelo glutamato, a qual aumenta com a progressão da DA (Long e Holtzman, 2019). Essa droga pode oferecer benefícios para pacientes nos estágios moderado e severo da doença, tanto como monoterapia ou em combinação com um inibidor da colinesterase (Lane, Hardy e Schott, 2018;

Scheltens *et al.*, 2016). A magnitude da resposta clínica que esses medicamentos oferecem é, no entanto, bastante limitada. Embora uma pequena parte dos pacientes apresente melhora cognitiva, a maioria deles tem apenas o adiamento da perda adicional de cognição por tempo restrito após a introdução da terapia (Di Santo *et al.*, 2013; Takeda *et al.*, 2006).

Nenhum novo tratamento foi aprovado para a DA desde 2003 (Cummings, Morstorf e Zhong, 2014). Nos últimos anos, mais de 20 compostos foram testados em ensaios clínicos randomizados controlados de fase 3 em coortes de pacientes em vários estágios da DA e nenhum deles demonstrou eficácia em diminuir o declínio cognitivo. Com base nesses dados, novas estratégias terapêuticas que sejam capazes de prevenir, retardar ou tratar os sintomas da DA são urgentemente necessárias (Cummings, Morstorf e Zhong, 2014; Long e Holtzman, 2019).

1.2. Genisteína

Na busca de estratégias alternativas para a prevenção ou para o tratamento da neurotoxicidade e do processo neurodegenerativo associados à DA, diferentes compostos naturais tem sido investigados pelos efeitos neuroprotetores que apresentam (Almeida *et al.*, 2016; Costa *et al.*, 2016; Fei *et al.*, 2020; Hussain *et al.*, 2018; Lakey-Beitia *et al.*, 2015; Pinho *et al.*, 2013; Swaminathan e Jicha, 2014; Williams e Spencer, 2012).

As isoflavonas, grupo da categoria flavonoide de compostos polifenólicos que são abundantes em vegetais, plantas e frutas, tem sido foco de crescente interesse motivado por suas ações benéficas sobre a função cerebral (Lee, Lee e Sohn, 2005; Moosavi *et al.*, 2015; Vauzour, 2012). Concentrações relativamente altas desses compostos estão presentes em leguminosas e, particularmente, na soja. Em países asiáticos, onde o consumo de alimentos à base de soja é bastante comum, a ingestão média de isoflavonas na dieta pode variar de 25 a 50 mg/dia, enquanto que em países ocidentais estima-se que esse valor seja de aproximadamente 1 mg/dia (Messina, Nagata e Wu, 2006; van Erp-Baart *et al.*, 2003).

A genisteína (4',5,7-trihidroxiisoflavona) (Figura 7) corresponde a uma das isoflavonas predominantes na soja, cujas concentrações podem variar de 0,2 a 1 mg/g, principalmente na forma de conjugados glicosídicos (Ganai e Farooqi, 2015; Jaiswal *et*

al., 2019; Polkowski e Mazurek, 2000). Essa biomolécula foi isolada pela primeira vez em 1899, a partir da espécie *Genista tinctoria* de onde seu nome comum foi derivado (Perkin e Newbury, 1899). No entanto, o estímulo que despertou o interesse da comunidade científica sobre a genisteína ocorreu somente em 1987, quando foi demonstrada sua ação sobre a inibição de proteínas tirosina cinases. Com esse achado, o número de publicações acerca de seus efeitos aumentou significativamente. Em 1989, descreveu-se também sua ação sobre a inibição da topoisomerase II, enzima nuclear envolvida na replicação celular (Polkowski e Mazurek, 2000). Desde então, sabe-se que essa biomolécula apresenta uma variedade de propriedades farmacológicas, tendo ações descritas sobre: regulação da apoptose, da diferenciação, da proliferação e da progressão do ciclo celular, inibição da angiogênese e supressão da função osteoclástica e da proliferação e ativação linfocítica (Dixon e Ferreira, 2002; Ganai e Farooqi, 2015; Polkowski e Mazurek, 2000). Ainda, são atribuídos a ela importantes efeitos antioxidantes e anti-inflamatórios. Em vista disso, já foram descritos efeitos positivos da genisteína na profilaxia ou no tratamento de diferentes tipos de câncer, de doenças cardiovasculares, da osteoporose e da perda óssea associadas à pós-menopausa, entre outros (Altavilla *et al.*, 2004; Russo *et al.*, 2016; Si e Liu, 2007; Spagnuolo *et al.*, 2015; Taylor *et al.*, 2009; Thangavel *et al.*, 2019; Tuli *et al.*, 2019).

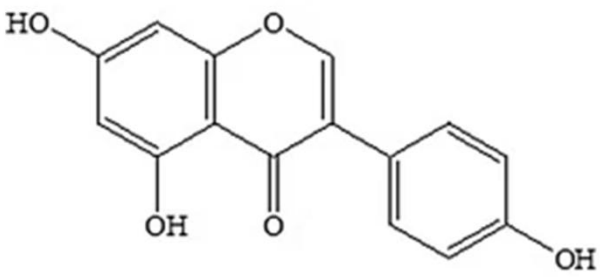


Figura 7. Estrutura química da genisteína (adaptado de Devi *et al.*, 2017).

Assim como outras isoflavonas, a genisteína apresenta estrutura difenólica similar à dos estrógenos endógenos, podendo se ligar diretamente aos receptores estrogênicos, e por isso é classificada como um fitoestrógeno (Dixon e Ferreira, 2002; Ganai e Farooqi, 2015; Jaiswal *et al.*, 2019). Devido ao seu efeito estrogênico, a genisteína já foi inclusive sugerida como uma possível alternativa à terapia de reposição hormonal convencional por atenuar sintomas associados ao período da pós-menopausa (Chen, Ko

e Chen, 2019; Messina, 2014; Thangavel *et al.*, 2019). Análises epidemiológicas mostram que casos de DA são mais frequentes em mulheres do que em homens, principalmente no período de pós-menopausa, o que sugere uma possível relação entre a diminuição da sinalização estrogênica e a maior suscetibilidade a essa doença (Brinton *et al.*, 2015; Uddin *et al.*, 2020). A genisteína é um agonista relativamente seletivo de receptores estrogênicos β, os quais são mais expressos em regiões cerebrais associadas aos processos de aprendizado e memória como o hipocampo e o córtex. Considerando que essa biomolécula cruza a barreira hematoencefálica, sua ligação a esses receptores poderia também contribuir para seus potenciais efeitos neuroprotetores (Devi *et al.*, 2017).

Embora estudos em humanos mostrem resultados discrepantes em relação à melhora cognitiva com a utilização de isoflavonas da soja (Gleason *et al.*, 2008, 2015; John *et al.*, 2014; Kreijkamp-Kaspers *et al.*, 2004; Thorp *et al.*, 2009), estudos experimentais *in vitro* e *in vivo* têm demonstrado efeitos neuroprotetores bastante promissores da genisteína sobre os processos patogênicos associados à DA (Devi *et al.*, 2017; Uddin e Kabir, 2019).

1.2.1. Potencial neuroprotetor da genisteína em modelos *in vitro* para o estudo da DA

Estudos utilizando diversos modelos *in vitro* têm investigado os efeitos da genisteína sobre os mecanismos neurotóxicos associados à DA. Em diferentes linhagens celulares ou em culturas primárias de neurônios corticais ou hipocampais expostas ao peptídeo Aβ, a genisteína mostrou-se capaz de reduzir a morte celular (Bang *et al.*, 2004; Ding *et al.*, 2011; Ma *et al.*, 2010; Vallés *et al.*, 2008; Xu *et al.*, 2019; You *et al.*, 2017; Yu *et al.*, 2009; Zeng, Chen e Zhao, 2004) e a expressão de proteínas apoptóticas (Yu *et al.*, 2009), diminuir a produção de espécies reativas de oxigênio (Ding *et al.*, 2011; Ma *et al.*, 2010; Vallés *et al.*, 2008; Zeng, Chen e Zhao, 2004) e, ainda, inibir a toxicidade sináptica (Wang *et al.*, 2019; Xi *et al.*, 2016) e o desequilíbrio nos níveis de receptores para neurotransmissores (Xi *et al.*, 2016; Xu *et al.*, 2019). Em cultura primária de astrócitos, a genisteína apresentou também efeito anti-inflamatório ao proteger contra a liberação de mediadores inflamatórios subjacente à exposição das células ao peptídeo Aβ (Valles *et al.*, 2010). Ainda, de forma bastante relevante, foi demonstrado que a genisteína pode atuar sobre o processo de agregação do peptídeo

$A\beta_{42}$, inibindo a transição conformacional de monômeros para estruturas em folha β e reduzindo, dessa maneira, a formação de agregados amorfos, protofibrilas e fibrilas maduras (Ren *et al.*, 2018).

1.2.2. Potencial neuroprotetor da genisteína em modelos *in vivo* para o estudo da DA

Ainda são poucos os estudos a explorarem o potencial neuroprotetor da genisteína em modelos animais que mimetizam a DA. Ainda assim, os resultados demonstrados até agora são promissores. Alguns estudos demonstraram que a administração de genisteína em animais que receberam injeções intrahipocampais de Aβ proporcionou melhora no déficit cognitivo causado pelo peptídeo (Bagheri *et al.*, 2011; Wang *et al.*, 2016; Ye *et al.*, 2017), além de proteger contra a toxicidade sobre o hipocampo ao reduzir a astrogliose (Bagheri *et al.*, 2012), a formação de agregados do peptídeo (Bagheri *et al.*, 2012), a via apoptótica mitocondrial e a perda neuronal nessa região (Bagheri *et al.*, 2012; Wang *et al.*, 2016; Ye *et al.*, 2017). Ainda, um estudo utilizando modelo de injeção intracerebroventricular de estreptozotocina sugeriu que a administração de uma alta dose de genisteína (150 mg/kg) poderia ativar o processo de autofagia, levando à redução dos níveis cerebrais de $A\beta_{40}$ e $A\beta_{42}$, com a consequente melhora do dano cognitivo (Pierzynowska *et al.*, 2019). Por fim, em estudo utilizando modelo trangênico, foi demonstrado que a genisteína, administrada em uma baixa dose (0,022 mg/kg), pode diminuir a área e o número de placas amiloides no córtex cerebral e também os níveis cerebrais totais de $A\beta_{40}$ e $A\beta_{42}$, sendo esse mecanismo relacionado ao aumento da liberação da ApoE mediada por PPARγ (Bonet-Costa *et al.*, 2016).

2. OBJETIVOS

2.1. Objetivo geral

O objetivo geral desta tese foi avaliar mecanismos possivelmente envolvidos no efeito neuroprotetor do tratamento com genisteína em modelos *in vitro* e *in vivo* de toxicidade induzida pelo peptídeo beta-amiloide.

2.2. Objetivos específicos

2.2.1. Investigar o efeito da genisteína sobre a morte celular induzida pelo peptídeo $A\beta_{25-35}$ em células de neuroblastoma humano SH-SY5Y e o possível envolvimento das proteínas Akt, GSK-3β e Tau como mecanismo relacionado à sua neuroproteção;

2.2.2. Avaliar o efeito do tratamento com genisteína sobre o dano cognitivo induzido pelo peptídeo $A\beta_{1-42}$ em ratos e elucidar possíveis mecanismos relacionados à sua neuroproteção no hipocampo;

2.2.3. Avaliar o efeito da injeção intracerebroventricular do peptídeo $A\beta_{1-42}$ e do tratamento com genisteína sobre a composição lipídica (gangliosídios, fosfolipídios e colesterol) de membranas neurais em córtex frontal de ratos.

3. METODOLOGIA E RESULTADOS

A metodologia utilizada e os resultados obtidos nesta tese estão organizados em *Capítulos I*, *II* e *III*, os quais correspondem a artigos científicos publicados e em fase de preparação para submissão em revista da área.

3.1. CAPÍTULO I

Genistein protects against amyloid-beta-induced toxicity in SH-SY5Y cells by regulation of Akt and Tau phosphorylation

Fernanda dos Santos Petry, Bárbara Paranhos Coelho, Mariana Maier Gaelzer, Fernando Kreutz, Fátima Theresinha Costa Rodrigues Guma, Christianne Gazzana Salbego, Vera Maria Treis Trindade

Status: Publicado no periódico *Phytotherapy Research*
(disponível em https://doi.org/10.1002/ptr.6560)

Genistein protects against amyloid-beta-induced toxicity in SH-SY5Y cells by regulation of Akt and Tau phosphorylation

Fernanda dos Santos Petry[1], Bárbara Paranhos Coelho[1], Mariana Maier Gaelzer[1], Fernando Kreutz[1,2], Fátima Theresinha Costa Rodrigues Guma[1,3], Christianne Gazzana Salbego[1,3], Vera Maria Treis Trindade [1,3,*]

[1]Programa de Pós-Graduação em Ciências Biológicas:Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[2]Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[3]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[*]Corresponding author:

Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 - Anexo, CEP 90035-003, Porto Alegre, RS, Brazil.

E-mail address: vmtt@ufrgs.br (V. M. T. Trindade)

Phone number: 55 51 3308 5550

ORCID: 0000-0001-5602-6013

Short title: Genistein protects against Aβ-induced toxicity

ABSTRACT

Alzheimer's disease is a neurodegenerative disorder characterized by extracellular deposition of amyloid-β (Aβ) peptide and hyperphosphorylation of Tau protein, which ultimately leads to the formation of intracellular neurofibrillary tangles and cell death. Increasing evidence indicates that genistein, a soy isoflavone, has neuroprotective effects against Aβ-induced toxicity. However, the molecular mechanisms involved in its neuroprotection are not well understood. In this study, we have established a neuronal damage model using retinoic-acid differentiated SH-SY5Y cells treated with different concentrations of $A\beta_{25–35}$ to investigate the effect of genistein against Aβ-induced cell death and the possible involvement of protein kinase B (PKB, also termed Akt), glycogen synthase kinase 3β (GSK-3β), and Tau as an underlying mechanism to this neuroprotection. Differentiated SH-SY5Y cells were pre-treated for 24 h with genistein (1 and 10 nM) and exposed to $A\beta_{25-35}$ (25 μM), and we found that genistein partially inhibited Aβ induced cell death, primarily apoptosis. Furthermore, the protective effect of genistein was associated with the inhibition of Aβ-induced Akt inactivation and Tau hyperphosphorylation. These findings reinforce the neuroprotective effects of genistein against Aβ toxicity and provide evidence that its mechanism may involve regulation of Akt and Tau proteins.

Keywords

Alzheimer's disease; amyloid-beta; genistein; neuroprotection; necrosis; signaling

Introduction

Alzheimer's disease (AD), the most common form of dementia, is a neurodegenerative disorder characterized by the progressive and irreversible impairment of memory and other cognitive functions (Masters et al., 2015; Querfurth & LaFerla, 2010). Although the pathogenesis of this complex disease has not been completely elucidated, it is well established that extracellular deposition of amyloid-β (Aβ) peptide and formation of intracellular neurofibrillary tangles (NFT) of hyperphosphorylated Tau protein are associated with the disease development and neuronal damage. Aβ peptide originates from cleavage of the amyloid precursor protein (APP) by β- and γ-secretases enzymes. This peptide can aggregate into oligomers or insoluble fibrils, the latter being responsible for the formation of advanced senile plaques. Aβ accumulation may induce oxidative stress, neuroinflammation, synaptic dysfunction, alteration of intracellular signaling pathways, and eventually, cell death (Jeong, 2017; Masters et al., 2015; Querfurth & LaFerla, 2010).

One of the cell signaling pathways that can be affected by Aβ deposition is the phosphatidylinositol 3-kinase (PI3K) pathway (Hoppe, Frozza, Pires, Meneghetti, & Salbego, 2013). Aβ activates glycogen synthase kinase 3β (GSK-3β) by preventing the inhibitory phosphorylation of this enzyme, which can be phosphorylated on serine residues by several kinases including protein kinase B (PKB, also termed Akt). Aβ accumulation can inhibit these upstream signaling proteins, which in turn fail to inactivate the apoptotic protein GSK-3β. The abnormal activation of this enzyme contributes to oxidative stress, neuroinflammation, and apoptosis related to AD (Cai, Zhao, & Zhao, 2012; Llorens-Marítin, Jurado, Hernández, & Ávila, 2014). GSK-3β can also be considered as a molecular link between the two main hallmarks of AD, Aβ-composed plaques and NFT, as its hyperactivation induces the hyperphosphorylation of

Tau protein, a microtubule-associated protein that stabilizes the cytoskeleton, and thus, impairs its binding to tubulin residues, which ultimately results in microtubule destabilization and NFT formation (Martin et al., 2013).

Genistein (4',5,7-trihydroxyisoflavone) is one of the major isoflavones present in soybeans and soy products and has received special attention for its potential neuroprotective effects in different experimental models of Aβ-induced toxicity (de Oliveira, 2016; Devi, Shanmuganathan, Manayi, Nabavi, & Nabavi, 2017). It has been reported in previous *in vitro* studies that genistein protects against cell death (Bang et al., 2004; Vallés et al., 2008; Xu et al., 2019; You et al., 2017; Yu et al., 2009), improves oxidative stress damages (Janicki & Schupf, 2010; Vallés et al., 2008), inhibits synaptic toxicity (Wang et al., 2019; Xi et al., 2016), and prevents release of inflammatory mediators (Valles et al., 2010), thus, the events that are known to be induced by Aβ peptide. *In vivo* studies also have shown that genistein prevents cognitive deficits (Bagheri, Joghataei, Mohseni, & Roghani, 2011; Wang et al., 2016), formation of Aβ deposits, and astrogliosis (Bagheri, Roghani, Joghataei, & Mohseni, 2012) induced by intracerebral injection of the Aβ peptide. Genistein has also been shown to reduce neuronal loss in hippocampus and inhibit mitochondrial apoptotic pathway (Wang et al., 2016). Although these effects of genistein are promising, the molecular mechanisms involved in its neuroprotection are not totally understood. Thus, the objectives of the present study were to evaluate whether genistein can protect SH-SY5Y cells against Aβ-induced cell death and to explore the underlying mechanisms, focusing specially on Akt, GSK-3β, and Tau proteins.

Material and Methods

Chemicals and reagents

$A\beta_{25-35}$, genistein, dimethyl sulfoxide (DMSO), [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (MTT), propidium iodide (PI), and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture media and amphotericin B were supplied by Gibco (Carlsbad, CA, USA). Gentamicin was from Schering-Plough (São Paulo, SP, Brazil). Fetal bovine serum (FBS) was obtained from Cripion Biotecnologia Ltda. (Andradina, SP, Brazil). All-*trans* retinoic acid (RA) was from Enzo Life Sciences (Farmingdale, NY, USA). All primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies and Hoechst 33342 (HO) were obtained from Gibco-Invitrogen (Grand Island, NY, USA).

Cell culture and differentiation

SH-SY5Y human neuroblastoma cell line was obtained from Cell Bank of Rio de Janeiro (Duque de Caxias, RJ, Brazil). Cells were cultured in a mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 medium (DMEM/F-12, 1:1) supplemented with 10 % (*v/v*) fetal bovine serum (FBS), 50 µg/mL gentamicin, and 0.25 µg/mL amphotericin B. Cells were incubated at 37 °C, in a humidified atmosphere of 5 % CO_2. Cells were not used after 25 passages.

Cells were seeded into 96-well plates (5×10^4 cells/well) for MTT assay and cell death assessment, or into 6-well plates (5×10^5 cells/well) for flow cytometry analysis. For induction of neuronal differentiation, 24 h after seeding, cells were maintained in culture medium with 1 % FBS and 10 µM RA for 7 days (Lopes et al., 2010), replacing the medium every 3 days.

Preparation of Aβ peptide and genistein

$A\beta_{25-35}$ was prepared in sterilized bi-distilled water (1 mg/mL) and stored at -20 °C. Prior to use, aliquots of the stock solution were incubated at 37 °C for 96 h in order to obtain aggregated Aβ (Kreutz et al., 2011). Genistein was dissolved in DMSO (10 mg/L) and stored at -20 ºC until use. Aliquots were further diluted in culture medium and added to the cells to provide the required final concentrations.

Cell treatments

For the initial evaluation of Aβ-induced toxicity, undifferentiated and differentiated cells were exposed on the 1st and 7th day after seeding, respectively, to different concentrations of $A\beta_{25-35}$ (7.5, 25, and 50 µM) for 72 h.

In order to investigate the effect of genistein on Aβ-induced toxicity, differentiated cells were pre-treated for 24 h with genistein (1 and 10 nM; Bang et al., 2004) or DMSO (0.1 %) and were then exposed to $A\beta_{25-35}$ (25 µM) plus DMSO or genistein with the same concentrations as the pre-treatment. Seventy-two and 24 h after incubation, the analysis of cell death and flow cytometry evaluation of signaling proteins was performed, respectively.

MTT assay

Cell viability was determined by the quantification of [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (MTT) reduction by mitochondrial dehydrogenases of viable cells. MTT substrate was prepared in phosphate-buffered saline (PBS, 5 mg/mL). After Aβ treatment for 72 h, cells were incubated with 0.5 mg/mL MTT for 1 h at 37 ºC. The medium was discarded and DMSO was added to solubilize the formazan crystals. Absorbance was measured at 560 and 630 nm using a

SoftMax Pro Microplate Reader (Molecular Devices, USA). Control groups were considered as 100 % of cell viability.

Propidium iodide and Hoechst 33342 staining

Cell damage was assessed by propidium iodide (PI) uptake, which is an indicative of significant cell membrane injury. After Aβ treatment, cells were incubated with PI (5 µM) for 30 min and the fluorescence was observed under an inverted fluorescence microscope (Nikon Eclipse TE 300) and the captured images were analyzed using ImageJ software (version 1.52a, National Institutes of Health, USA). Cell death was expressed as a percentage of PI stained cells from the total number of cells observed in 3 fields from each well.

For some experiments, cells were incubated with PI (5 µM) and Hoechst 33342 (HO) (5 µg/mL), which enables the analysis of cell nuclei morphology, for 30 min. Cells were observed as mentioned above and two different images (PI and HO stained cells) were captured and analyzed by Image J software. After the overlay of the images, cells were classified as: viable (low blue fluorescent cells with round and regular nuclei stained only by HO), apoptotic (cells with condensed and/or fragmented nuclei stained by HO), and necrotic (cells with red spherical or vesicular nuclei and red cytoplasm stained by PI). Cells in early apoptosis (bright blue nuclei) as well as in late apoptosis (bright blue and red nuclei – purple in the merged images) were included in the analysis. The percentage of viable, apoptotic, and necrotic cells was established for different experimental groups, taking into account the cell morphology and staining.

Flow cytometry

For analysis of neuronal nuclei protein (NeuN), Akt, p-Akt, GSK-3β, p-GSK-3β, Tau, and p-Tau levels, cells were trypsinized and centrifuged at 400 *g* for 5 min. Cells were suspended in PBS containing 0.1 % Triton X-100 and the following primary antibodies (1:50) for 40 min: anti-NeuN, anti-Akt, anti-p-Akt (Ser473), anti-GSK-3β, anti-p-GSK-3β (Ser9), anti-Tau, and anti-p-Tau (Ser396). Next, Alexa Fluor 488 anti-rabbit or Alexa Fluor 555 anti-mouse secondary antibodies (1:100) were added and, after incubation for 30 min, fluorescence intensity was analyzed by flow cytometry. Data acquisition was done using a FACSCalibur flow cytometer and Cell Quest software (BD Biosciences, Mountain View, CA, USA). A total of 10,000 events were acquired. Data obtained was analyzed with FCS Express 4 Software (De Novo Software, Los Angeles, CA, USA).

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). All results are representative of at least three independent experiments. Student's *t* test or two-way analysis of variance (ANOVA), with cell phenotype and Aβ concentration as factors, were used for experiments comparing undifferentiated and differentiated cells. For all other experiments, two-way ANOVA, with genistein and Aβ as factors, was applied to determine statistical differences between the experimental groups. Post hoc comparisons were performed using the Tukey test. Differences were considered statistically significant at $p < 0.05$. All analyses were done using the IBM SPSS Statistics 18 software.

Results

Retinoic acid-differentiated SH-SY5Y cells show morphological alterations and increased NeuN levels

In this study, we first investigated the effects of retinoic acid (RA) on differentiation of neuroblastoma cells. We evaluated cell morphology and neuronal nuclei protein (NeuN) levels in the differentiated cells. As shown in Fig. 1a, the differentiated cells showed extensive neurite outgrowth and branching. Moreover, we also observed an increase in NeuN levels after 7 days of RA treatment ($p < 0.01$, Fig. 1b).

Differentiated SH-SY5Y cells are more susceptible to Aβ toxicity

In order to establish a model of Aβ-induced toxicity, undifferentiated and differentiated SH-SY5Y cells were exposed to different concentrations of $A\beta_{25-35}$ (7.5, 25, and 50 μM) for 72 h. We examined the cell viability and cell damage using MTT assay and PI uptake, respectively (Fig. 2).

As shown in Fig. 2a, a significant impact of cell phenotype and Aβ concentration on cell viability was detected [$F(3,68) = 5.96$; $p < 0.001$]. Post hoc analysis indicated that Aβ, in all used concentrations, reduced the viability of differentiated cells in comparison to respective control group ($p < 0.01$ for 7.5 μM Aβ; $p < 0.001$ for 25 μM Aβ; $p < 0.01$ for 50 μM). However, no difference in cell viability was observed between the three different concentrations used ($p > 0.05$). In undifferentiated cells, Aβ had no effect on viability when compared to respective control ($p > 0.05$). We also observed a significant difference between undifferentiated and differentiated cells exposed to the same Aβ concentration ($p < 0.001$ for 25 and 50 μM Aβ, undifferentiated vs. differentiated cells).

Statistical analysis showed a significant interaction between the factors in cell damage [$F(3,40) = 212.96$; $p < 0.001$]. As shown in Fig. 2b, Aβ induced cell damage in a concentration dependent manner to differentiated cells in comparison to respective control ($p < 0.001$ for all Aβ concentrations; $p < 0.001$ for 7.5 μM Aβ vs. 25 μM Aβ; $p < 0.001$ for 25 μM Aβ vs. 50 μM Aβ). None of the Aβ concentrations were able to induce a significant alteration in undifferentiated cells when compared to respective control ($p > 0.05$). A significant difference was also observed upon comparing undifferentiated and differentiated cells exposed to the same Aβ concentration ($p < 0.001$ for 7.5, 25 and 50 μM Aβ, undifferentiated vs. differentiated cells).

These data suggest that, in 72 h of exposure, $A\beta_{25-35}$ only induced toxicity in the differentiated cells. Based on these findings, we decided to use differentiated cells and 25 μM Aβ concentration in further experiments.

Genistein partially prevents Aβ-induced cell death

We then evaluated if genistein could be neuroprotective against Aβ-induced toxicity in differentiated cells. Thus, cells were pre-treated for 24 h with genistein (1 and 10 nM), and then $A\beta_{25-35}$ (25 μM) was added to the culture medium. Genistein was maintained in the culture medium during 72 h of peptide incubation. Cell death was assessed by PI and Hoechst 33342 (HO) co-staining (Fig. 3a).

Statistical analysis indicated a significant interaction between genistein and Aβ on the number of viable cells [$F(2,28) = 44.24$; $p < 0.001$]. It was observed that Aβ significantly reduced the number of viable cells ($p < 0.001$) when compared to control group (Fig. 3b). Genistein, when co-incubated with Aβ, increased the number of viable cells at 1 nM ($p < 0.001$) and 10 nM ($p < 0.001$) when compared to Aβ group. No difference was observed between the groups treated with the two tested concentrations

of this drug ($p > 0.05$). However, the number of viable cells in genistein and Aβ-co-treated groups was less from control ($p < 0.001$ for both the genistein concentrations), indicating partial protection from cell death.

Regarding the number of necrotic cells, statistical analysis indicated a significant interaction between the factors [$F(2,28) = 5.84$; $p < 0.01$]. Aβ induced an increase in the number of necrotic cells ($p < 0.001$) when compared to control (Fig. 3c). Genistein treatment, at both concentrations, significantly reduced this effect of the peptide ($p < 0.05$ for Aβ + Gen 1 nM; $p < 0.01$ for Aβ + Gen 10 nM), and no significant difference was observed in the number of necrotic cells from the control group ($p > 0.05$). In relation to late apoptosis, a significant interaction between genistein and Aβ was detected [$F(2,28) = 35.44$; $p < 0.001$]. It was observed that Aβ also increased the number of cells in late apoptosis ($p < 0.001$) when compared to control group (Fig. 3d). Genistein reduced the number of late apoptotic cells, induced by Aβ, at 1 nM ($p < 0.001$) and 10 nM ($p < 0.001$) compared to Aβ group. However, no significant difference was detected between the two concentrations of the drug ($p > 0.05$). Moreover, although genistein could attenuate the Aβ effect, this protection was not complete, since the number of late apoptotic cells in genistein and Aβ-co-treated group was higher than the control group ($p < 0.001$ for Aβ + Gen 1 nM and Aβ + Gen 10 nM). For early apoptosis analysis, a significant effect of genistein [$F(3,28) = 5.51$; $p < 0.01$] and Aβ [$F(1,28) = 9.85$; $p < 0.01$] was observed. The Aβ exposure did not affect the number of early apoptotic cells ($p > 0.05$, Fig. 3e). Yet, an increase in early apoptotic cells was observed upon pre-treatment with 1 or 10 nM genistein, followed by Aβ exposure ($p < 0.01$ vs. control). However, the difference was not significant when compared to Aβ group ($p > 0.05$). Moreover, no significant difference was observed between the different drug concentrations ($p > 0.05$).

Genistein prevents Aβ-induced decrease in p-Akt and Tau hyperphosphorylation

We next explored possible mechanisms that could be involved in the protective effect of genistein and analyzed the modulation of Akt, GSK-3β, and Tau proteins. Differentiated cells were pre-treated for 24 h with genistein (10 nM), followed by $A\beta_{25-35}$ (25 μM), which was added to the culture medium for another 24 h. Genistein was maintained in the culture medium during the incubation with the peptide. Levels of different phosphorylated proteins were analyzed by flow cytometry.

We did not observe a significant difference in total Akt levels between the groups [$F(1,12) = 1.82$; $p > 0.05$] (Fig. 4a). However, a significant effect of genistein [$F(2,14) = 20.05$; $p < 0.001$] and Aβ [$F(1,14) = 27.82$; $p < 0.001$] on p-Akt levels was observed. As shown in Fig. 4b, genistein, per se, increased p-Akt levels when compared to control ($p < 0.01$). Furthermore, Aβ treatment inactivated Akt by decreasing its phosphorylation ($p < 0.05$), while genistein prevented this effect ($p < 0.01$). We next investigated the levels of GSK-3β protein and a significant effect of genistein [$F(2,14) = 4.77$; $p < 0.05$] was verified. None of the treatments had an effect on total protein levels when compared to control group, but a significant increase in GSK-3β was found in the cells exposed to Aβ and treated with genistein when compared to cells exposed to Aβ only ($p < 0.05$, Fig. 4c). Furthermore, it was detected a significant effect of Aβ [$F(1,15) = 11.99$; $p < 0.01$] on p-GSK-3β levels. As shown in Fig. 4d, the Aβ exposure activated GSK-3β by decreasing the levels of p-GSK-3β, when compared to control ($p < 0.05$, Fig. 4d). Interestingly, genistein and peptide treated cells showed no difference from the cells exposed only to Aβ ($p > 0.05$), or the control group ($p > 0.05$). This finding suggests that genistein treatment partially prevented GSK-3β activation induced by the peptide. We next investigated the levels of total Tau and found a significant interaction between genistein and Aβ [$F(1,25) = 12.03$; $p < 0.01$]. Aβ induced a significant

decrease in this protein levels ($p < 0.05$, Fig. 4e) when compared to control group. We also observed a significant interaction between the factors [$F(1,13) = 16.51$; $p < 0,001$] for p-Tau levels. As shown in Fig. 4f, genistein, per se, reduced Tau phosphorylation when compared to control ($p < 0.05$). Aβ induced a significant increase in p-Tau levels ($p < 0.05$) and genistein was able to inhibit this effect ($p < 0.001$). Tau phosphorylation in cells treated with genistein and exposed to Aβ was even lower than the control group ($p < 0.01$).

Discussion

Current therapies for AD, though are effective in attenuating the symptoms of the disease, are unable to affect the underlying disease process or to delay its progression. For this reason, it is imperative to investigate new alternative strategies that can target this disease at a molecular level. In the present study, we have examined the potential neuroprotective effects of genistein against Aβ peptide-induced toxicity in SH-SY5Y cell line.

Since there are several studies using this cell line as an *in vitro* model for neurodegenerative disorders (Liu et al., 2018; Murillo et al., 2017; Xicoy, Wieringa, & Martens, 2017), particularly for investigation of Aβ toxicity and drug screening (Jämsä, Belda, Edlund, & Lindström, 2011; Lattanzio, Carboni, Carretta, Candeletti, & Romualdi, 2016; Yuan et al., 2017), our first objective was to compare the effect of the peptide on undifferentiated and differentiated cells. SH-SY5Y are neuroblast-like cells that can be differentiated into a mature neuronal phenotype using several differentiating agents, including RA, phorbol esters, and neurotrophins (Kovalevich & Langford, 2013). Cells under RA treatment have shown to develop long and extensively branched neurites, express neuronal cell markers (NeuN, tyrosine hydroxylase, neuron specific

enolase, synaptophysin, post-synaptic associated protein-97, and β-III tubulin) (Cheung et al., 2009; Kunzler et al., 2017; Lopes et al., 2010), and exhibit mature excitability (Toselli, Tosetti, & Taglietti, 1996; Tosetti, Taglietti, & Toselli, 1998). We observed an alteration in cell morphology, as well as an increase in NeuN levels (a marker for mature neurons) after RA treatment, as previously reported (Cheung et al., 2009; Lopes et al., 2010). Various studies have described the characteristics of differentiated cells, and they seem to be an appropriate model to study Aβ peptide-induced toxicity (Bang et al., 2004; da Rocha, da Cruz e Silva, & Vieira, 2015; Gill, Kaur, Kaur, Dhiman, & Mantha, 2017). Here we have used $A\beta_{25-35}$ peptide, which has similar toxic effects with respect to cell death, as well as on cell signaling pathway, as the $A\beta_{1-42}$ in *in vitro* models (Frozza et al., 2009). In this study, we observed a difference in the susceptibility of these two cell phenotypes to the Aβ peptide. None of the tested Aβ concentrations affected the viability of undifferentiated cells. On the other hand, differentiated cells were susceptible even to the lowest concentration of the peptide, therefore, we used this cell phenotype in further experiments.

Natural compounds have gained attention in recent times as possible alternative strategies for prevention or treatment of AD, as they have shown to inhibit pathogenic pathways related to the disease development (Almeida, Alves, Sousa, Oliveira, & Silva, 2016; Costa et al., 2016; Frozza et al., 2013; Hoppe et al., 2013; Hussain et al., 2018; Lakey-Beitia, Berrocal, Rao, & Durant, 2015; Pasinetti, Wang, Ho, Zhao, & Dubner, 2015; Pinho, Ferreres, Valentão, & Andrade, 2013; Swaminathan & Jicha, 2014). Genistein has emerged as a potential drug that shows promising effects against the neuronal damage associated with Aβ peptide (de Oliveira, 2016; Devi et al., 2017). In order to further investigate the neuroprotective roles of this drug, we initially evaluated its effect on cell death triggered by the peptide exposure. Our results showed that Aβ

induced cell death primarily by apoptosis, and to a lesser extent, by necrosis. Genistein treatment, initiated before and maintained during the exposure of cells to the peptide, was able to partially prevent apoptosis and to completely avert necrosis. Interestingly, we observed a higher number of cells in early apoptosis when cells were exposed to the peptide and treated with genistein. This may be due to slowing down of cell death that was triggered by the peptide, considering that genistein decreased the number of cells in late apoptosis and increased the number of cells in early apoptosis.

Several studies have already shown the role of genistein in preventing the cell death triggered by Aβ peptide using *in vitro* models (Bagheri et al., 2012; Bang et al., 2004; Wang et al., 2019; Xu et al., 2019; You et al., 2017; Yu et al., 2009; Zeng, Chen, & Zhao, 2004). Some of these studies highlight the hypothesis that this drug has different mechanisms of neuroprotection at nanomolar and micromolar concentrations. Zeng et al. (2004) suggested that at nanomolar concentrations genistein exerts its effects against Aβ-induced damage via the estrogen receptor-mediated pathway; and at micromolar concentrations, its effect could be due to its antioxidant properties. It is well established that AD affects women and men differently, being more prevalent in women. This elevated risk of disease could be explained by the reduced levels of estrogens in postmenopausal women (Craig & Murphy, 2009, 2010; Janicki & Schupf, 2010). Estrogen replacement therapy seems to reduce this risk, but it is associated with high risk of breast and endometrial cancers (Carroll & Rosario, 2012). For this reason, phytoestrogens, such as genistein, which are natural compounds with similar structure as the endogenous estrogens and can bind to its receptors, are being studied as an alternative therapy for preventing or retarding neurodegenerative disorders. Bang et al. (2004) also showed that genistein, at nanomolar levels, protects SH-SY5Y cells against Aβ-induced cell death in a way similar to 17β-estradiol. Moreover, the specific estrogen

receptor antagonist ICI 182,780 blocked the neuroprotective effects of genistein and 17β-estradiol, suggesting a role of estrogen receptors. Studies using micromolar concentrations of this drug have demonstrated that it can prevent, at least partially, Aβ-induced apoptosis by reducing the expression of p53, Bax, and caspase-3, and by increasing the expression of Bcl-2 (Yu et al., 2009), and also by attenuation of oxidative stress and maintenance of redox balance (Ma et al., 2010). Besides, Xu et al. (2019) attributed genistein neuroprotection at micromolar concentration to the regulation of apoptosis-related proteins and Ca^{2+} influx through ionotropic glutamate receptors.

As previously mentioned, Aβ deposition triggers aberrant phosphorylation of several proteins and alteration of intracellular signaling pathways. To investigate a possible mechanism involved in genistein protective effects against Aβ peptide-induced toxicity, we analyzed Akt, GSK-3β, and Tau proteins. Akt is a serine/threonine kinase that regulates a wide range of processes, including cell survival, cell growth, and apoptosis (Hemmings & Restuccia, 2012; Wu, Wang, Fei, Santanam, & Blough, 2010). Previous studies have reported that Aβ peptide may decrease Akt phosphorylation, thus, inhibiting its activation (Hoppe et al., 2013; Magrané et al., 2005). Furthermore, it was recently demonstrated that Akt could be a therapeutic target for memory impairment associated with AD, since the pharmacological activation of this protein was able to rescue memory impairment and aberrant synaptic plasticity in Aβ-injected mice as well as in a transgenic AD mouse model (Yi et al., 2018). Reduced activation of Akt is known to decrease phosphorylation of GSK-3β, which in turn induces Tau hyperphosphorylation, and eventually, cell death (Hemmings & Restuccia, 2012). In a previous study, Park, Jang, & Kwon (2009) demonstrated that genistein, at nanomolar concentrations, protects SH-SY5Y cells against cell death mediated by endoplasmic reticulum stress through inactivation of GSK-3β and attenuation of Tau

hyperphosphorylation. Here we found that Aβ peptide decreased the phosphorylation of Akt and GSK-3β, and increased Tau phosphorylation. Genistein treatment, in turn, was able to prevent Akt inactivation, Tau hyperphosphorylation and, at least in part, GSK-3β activation. Using an *in vivo* model of Aβ toxicity, Ye et al. (2017) showed that genistein administration significantly reduced Tau hyperphosphorylation in the hippocampus of rats injected with $A\beta_{25-35}$.

In conclusion, our results provide further evidence for the neuroprotective effects of genistein against Aβ peptide-induced toxicity and indicate that the mechanism involves prevention of Akt inactivation and Tau hyperphosphorylation, as summarized in Fig. 5.

Acknowledgements

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil), Instituto Nacional de Ciência e Tecnologia - INCT (EN 465671/2014-4)/CNPq-Brazil.

Conflict of interest

There are no conflicts of interest to declare.

References

Almeida, S., Alves, M. G., Sousa, M., Oliveira, P. F., & Silva, B. M. (2016). Are Polyphenols Strong Dietary Agents Against Neurotoxicity and Neurodegeneration? *Neurotoxicity Research*, *30*(3), 345–366. https://doi.org/10.1007/s12640-015-9590-4

Bagheri, M., Joghataei, M.-T., Mohseni, S., & Roghani, M. (2011). Genistein

ameliorates learning and memory deficits in amyloid β(1–40) rat model of Alzheimer's disease. *Neurobiology of Learning and Memory*, *95*(3), 270–276. https://doi.org/10.1016/J.NLM.2010.12.001

Bagheri, M., Roghani, M., Joghataei, M.-T., & Mohseni, S. (2012). Genistein inhibits aggregation of exogenous amyloid-beta1–40 and alleviates astrogliosis in the hippocampus of rats. *Brain Research*, *1429*, 145–154. https://doi.org/10.1016/J.BRAINRES.2011.10.020

Bang, O. Y., Hong, H. S., Kim, D. H., Kim, H., Boo, J. H., Huh, K., & Mook-Jung, I. (2004). Neuroprotective effect of genistein against beta amyloid-induced neurotoxicity. *Neurobiology of Disease*, *16*(1), 21–28. https://doi.org/10.1016/J.NBD.2003.12.017

Carroll, J. C., & Rosario, E. R. (2012). The Potential Use of Hormone-Based Therapeutics for the Treatment of Alzheimers Disease. *Current Alzheimer Research*, *9*(1), 18–34. https://doi.org/10.2174/156720512799015109

Cai, Z., Zhao, Y., & Zhao, B. (2012). Roles of Glycogen Synthase Kinase 3 in Alzheimer's Disease. *Current Alzheimer Research*, *9*(7), 864–879. https://doi.org/10.2174/156720512802455386

Cheung, Y.-T., Lau, W. K.-W., Yu, M.-S., Lai, C. S.-W., Yeung, S.-C., So, K.-F., & Chang, R. C.-C. (2009). Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. *NeuroToxicology*, *30*(1), 127–135. https://doi.org/10.1016/J.NEURO.2008.11.001

Costa, S. L., Silva, V. D. A., dos Santos Souza, C., Santos, C. C., Paris, I., Muñoz, P., & Segura-Aguilar, J. (2016). Impact of Plant-Derived Flavonoids on Neurodegenerative Diseases. *Neurotoxicity Research*, *30*(1), 41–52. https://doi.org/10.1007/s12640-016-9600-1

Craig, M. C., & Murphy, D. G. M. (2009). Alzheimer's disease in women. *Best Practice & Research Clinical Obstetrics & Gynaecology*, *23*(1), 53–61. https://doi.org/10.1016/J.BPOBGYN.2008.10.004

Craig, M. C., & Murphy, D. G. M. (2010). Estrogen therapy and Alzheimer's dementia. *Annals of the New York Academy of Sciences*, *1205*(1), 245–253. https://doi.org/10.1111/j.1749-6632.2010.05673.x

da Rocha, J. F., da Cruz e Silva, O. A. B., & Vieira, S. I. (2015). Analysis of the amyloid precursor protein role in neuritogenesis reveals a biphasic SH-SY5Y neuronal cell differentiation model. *Journal of Neurochemistry*, *134*(2), 288–301. https://doi.org/10.1111/jnc.13133

de Oliveira, M. R. (2016). Evidence for genistein as a mitochondriotropic molecule. *Mitochondrion*, *29*, 35–44. https://doi.org/10.1016/j.mito.2016.05.005

Devi, K. P., Shanmuganathan, B., Manayi, A., Nabavi, S. F., & Nabavi, S. M. (2017). Molecular and Therapeutic Targets of Genistein in Alzheimer's Disease. *Molecular Neurobiology*, *54*(9), 7028–7041. https://doi.org/10.1007/s12035-016-0215-6

Frozza, R. L., Bernardi, A., Hoppe, J. B., Meneghetti, A. B., Matté, A., Battastini, A. M. O., … Salbego, C. (2013). Neuroprotective Effects of Resveratrol Against Aβ Administration in Rats are Improved by Lipid-Core Nanocapsules. *Molecular Neurobiology*, *47*(3), 1066–1080. https://doi.org/10.1007/s12035-013-8401-2

Frozza, R.L., Horn, A. P., Hoppe, J. B., Simão, F., Gerhardt, D., Comiran, R. A., & Salbego, C. G. (2009). A Comparative Study of β-Amyloid Peptides Aβ1-42 and Aβ25-35 Toxicity in Organotypic Hippocampal Slice Cultures. *Neurochemical Research*, *34*(2), 295–303. https://doi.org/10.1007/s11064-008-9776-8

Gill, I., Kaur, S., Kaur, N., Dhiman, M., & Mantha, A. K. (2017). Phytochemical

Ginkgolide B Attenuates Amyloid-β1-42 Induced Oxidative Damage and Altered Cellular Responses in Human Neuroblastoma SH-SY5Y Cells. *Journal of Alzheimer's Disease*, *60*(s1), S25–S40. https://doi.org/10.3233/JAD-161086

Hemmings, B. A., & Restuccia, D. F. (2012). PI3K-PKB/Akt pathway. *Cold Spring Harbor Perspectives in Biology*, *4*(9), a011189. https://doi.org/10.1101/cshperspect.a011189

Hoppe, J. B., Coradini, K., Frozza, R. L., Oliveira, C. M., Meneghetti, A. B., Bernardi, A., Salbego, C. G. (2013). Free and nanoencapsulated curcumin suppress β-amyloid-induced cognitive impairments in rats: Involvement of BDNF and Akt/GSK-3β signaling pathway. *Neurobiology of Learning and Memory*, *106*, 134–144. https://doi.org/10.1016/J.NLM.2013.08.001

Hoppe, J. B., Frozza, R. L., Pires, E. N. S., Meneghetti, A. B., & Salbego, C. (2013). The curry spice curcumin attenuates beta-amyloid-induced toxicity through beta-catenin and PI3K signaling in rat organotypic hippocampal slice culture. *Neurological Research*, *35*(8), 857–866. https://doi.org/10.1179/1743132813Y.0000000225

Hussain, G., Zhang, L., Rasul, A., Anwar, H., Sohail, M. U., Razzaq, A., Sun, T. (2018). Role of Plant-Derived Flavonoids and Their Mechanism in Attenuation of Alzheimer's and Parkinson's Diseases: An Update of Recent Data. *Molecules (Basel, Switzerland)*, *23*(4). https://doi.org/10.3390/molecules23040814

Jämsä, A., Belda, O., Edlund, M., & Lindström, E. (2011). BACE-1 inhibition prevents the γ-secretase inhibitor evoked Aβ rise in human neuroblastoma SH-SY5Y cells. *Journal of Biomedical Science*, *18*(1), 76. https://doi.org/10.1186/1423-0127-18-76

Janicki, S. C., & Schupf, N. (2010). Hormonal influences on cognition and risk for Alzheimer's disease. *Current Neurology and Neuroscience Reports*, *10*(5), 359–

366. https://doi.org/10.1007/s11910-010-0122-6

Jeong, S. (2017). Molecular and Cellular Basis of Neurodegeneration in Alzheimer's Disease. *Molecules and Cells*, *40*(9), 613–620. https://doi.org/10.14348/molcells.2017.0096

Kovalevich, J., & Langford, D. (2013). Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. *Methods in Molecular Biology (Clifton, N.J.)*, *1078*, 9–21. https://doi.org/10.1007/978-1-62703-640-5_2

Kreutz, F., Frozza, R. L., Breier, A. C., de Oliveira, V. A., Horn, A. P., Pettenuzzo, L. F., … Trindade, V. M. T. (2011). Amyloid-β induced toxicity involves ganglioside expression and is sensitive to GM1 neuroprotective action. *Neurochemistry International*, *59*(5), 648–655. https://doi.org/10.1016/J.NEUINT.2011.06.007

Kunzler, A., Zeidán-Chuliá, F., Gasparotto, J., Girardi, C. S., Klafke, K., Petiz, L. L., … Gelain, D. P. (2017). Changes in Cell Cycle and Up-Regulation of Neuronal Markers During SH-SY5Y Neurodifferentiation by Retinoic Acid are Mediated by Reactive Species Production and Oxidative Stress. *Molecular Neurobiology*, *54*(9), 6903–6916. https://doi.org/10.1007/s12035-016-0189-4

Lakey-Beitia, J., Berrocal, R., Rao, K. S., & Durant, A. A. (2015). Polyphenols as Therapeutic Molecules in Alzheimer's Disease Through Modulating Amyloid Pathways. *Molecular Neurobiology*, *51*(2), 466–479. https://doi.org/10.1007/s12035-014-8722-9

Lattanzio, F., Carboni, L., Carretta, D., Candeletti, S., & Romualdi, P. (2016). Treatment with the neurotoxic Aβ (25–35) peptide modulates the expression of neuroprotective factors Pin1, Sirtuin 1, and brain-derived neurotrophic factor in SH-SY5Y human neuroblastoma cells. *Experimental and Toxicologic Pathology*, *68*(5), 271–276. https://doi.org/10.1016/J.ETP.2016.02.001

Liu, Y., Eaton, E. D., Wills, T. E., McCann, S. K., Antonic, A., & Howells, D. W. (2018). Human Ischaemic Cascade Studies Using SH-SY5Y Cells: a Systematic Review and Meta-Analysis. *Translational Stroke Research*, *9*(6), 564–574. https://doi.org/10.1007/s12975-018-0620-4

Llorens-Marítin, M., Jurado, J., Hernández, F., & Ávila, J. (2014). GSK-3β, a pivotal kinase in Alzheimer disease. *Frontiers in Molecular Neuroscience*, *7*, 46. https://doi.org/10.3389/FNMOL.2014.00046

Lopes, F. M., Schröder, R., Júnior, M. L. C. da F., Zanotto-Filho, A., Müller, C. B., Pires, A. S., … Klamt, F. (2010). Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. *Brain Research*, *1337*, 85–94. https://doi.org/10.1016/J.BRAINRES.2010.03.102

Ma, W., Yuan, L., Yu, H., Ding, B., Xi, Y., Feng, J., & Xiao, R. (2010). Genistein as a neuroprotective antioxidant attenuates redox imbalance induced by β-amyloid peptides 25–35 in PC12 cells. *International Journal of Developmental Neuroscience*, *28*(4), 289–295. https://doi.org/10.1016/J.IJDEVNEU.2010.03.003

Magrané, J., Rosen, K. M., Smith, R. C., Walsh, K., Gouras, G. K., & Querfurth, H. W. (2005). *Neurobiology of Disease Intraneuronal-Amyloid Expression Downregulates the Akt Survival Pathway and Blunts the Stress Response*. https://doi.org/10.1523/JNEUROSCI.1723-05.2005

Martin, L., Latypova, X., Wilson, C. M., Magnaudeix, A., Perrin, M.-L., Yardin, C., & Terro, F. (2013). Tau protein kinases: Involvement in Alzheimer's disease. *Ageing Research Reviews*, *12*(1), 289–309. https://doi.org/10.1016/J.ARR.2012.06.003

Masters, C. L., Bateman, R., Blennow, K., Rowe, C. C., Sperling, R. A., & Cummings, J. L. (2015). Alzheimer's disease. *Nature Reviews Disease Primers*, *1*, 15056. https://doi.org/10.1038/nrdp.2015.56

Murillo, J. R., Goto-Silva, L., Sánchez, A., Nogueira, F. C. S., Domont, G. B., & Junqueira, M. (2017). Quantitative proteomic analysis identifies proteins and pathways related to neuronal development in differentiated SH-SY5Y neuroblastoma cells. *EuPA Open Proteomics*, *16*, 1–11. https://doi.org/10.1016/j.euprot.2017.06.001

Park, Y.-J., Jang, Y., & Kwon, Y. H. (2009). Isoflavones Prevent Endoplasmic Reticulum Stress-Mediated Neuronal Degeneration by Inhibiting Tau Hyperphosphorylation in SH-SY5Y Cells. *Journal of Medicinal Food*, *12*(3), 528–535. https://doi.org/10.1089/jmf.2008.1069

Pasinetti, G. M., Wang, J., Ho, L., Zhao, W., & Dubner, L. (2015). Roles of resveratrol and other grape-derived polyphenols in Alzheimer's disease prevention and treatment. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1852*(6), 1202–1208. https://doi.org/10.1016/J.BBADIS.2014.10.006

Pinho, B. R., Ferreres, F., Valentão, P., & Andrade, P. B. (2013). Nature as a source of metabolites with cholinesterase-inhibitory activity: an approach to Alzheimer's disease treatment. *Journal of Pharmacy and Pharmacology*, *65*(12), 1681–1700. https://doi.org/10.1111/jphp.12081

Querfurth, H. W., & LaFerla, F. M. (2010). Alzheimer's disease. *The New England Journal of Medicine*, *362*(4), 329–344. https://doi.org/10.1056/NEJMra0909142

Swaminathan, A., & Jicha, G. A. (2014). Nutrition and prevention of Alzheimer's dementia. *Frontiers in Aging Neuroscience*, *6*, 282. https://doi.org/10.3389/fnagi.2014.00282

Toselli, M., Tosetti, P., & Taglietti, V. (1996). Functional changes in sodium conductances in the human neuroblastoma cell line SH-SY5Y during in vitro differentiation. *Journal of Neurophysiology*, *76*(6), 3920–3927.

https://doi.org/10.1152/jn.1996.76.6.3920

Tosetti, P., Taglietti, V., & Toselli, M. (1998). Functional Changes in Potassium Conductances of the Human Neuroblastoma Cell Line SH-SY5Y During In Vitro Differentiation. *Journal of Neurophysiology*, *79*(2), 648–658. https://doi.org/10.1152/jn.1998.79.2.648

Vallés, S. L., Borrás, C., Gambini, J., Furriol, J., Ortega, A., Sastre, J., … Viña, J. (2008). Oestradiol or genistein rescues neurons from amyloid beta-induced cell death by inhibiting activation of p38. *Aging Cell*, *7*(1), 112–118. https://doi.org/10.1111/j.1474-9726.2007.00356.x

Valles, S. L., Dolz-Gaiton, P., Gambini, J., Borras, C., LLoret, A., Pallardo, F. V., & Viña, J. (2010). Estradiol or genistein prevent Alzheimer's disease-associated inflammation correlating with an increase PPARγ expression in cultured astrocytes. *Brain Research*, *1312*, 138–144. https://doi.org/10.1016/J.BRAINRES.2009.11.044

Wang, Yan, Cai, B., Shao, J., Wang, T., Cai, R., Ma, C., … Du, J. (2016). Genistein suppresses the mitochondrial apoptotic pathway in hippocampal neurons in rats with Alzheimer's disease. *Neural Regeneration Research*, *11*(7), 1153. https://doi.org/10.4103/1673-5374.187056

Wang, Yu-xiang, Xia, Z., Jiang, X., Li, L., An, D., Wang, H., … Liu, Y. (2019). Genistein Inhibits Aβ25–35-Induced Neuronal Death with Changes in the Electrophysiological Properties of Voltage-Gated Sodium and Potassium Channels. *Cellular and Molecular Neurobiology*, 39(6), 809-822. https://doi.org/10.1007/s10571-019-00680-w

Wu, M., Wang, B., Fei, J., Santanam, N., & Blough, E. R. (2010). Important roles of Akt/PKB signaling in the aging process. *Frontiers in Bioscience (Scholar Edition)*,

2, 1169–1188. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20515848

Xi, Y.-D., Zhang, D.-D., Ding, J., Yu, H.-L., Yuan, L.-H., Ma, W.-W., … Xiao, R. (2016). Genistein Inhibits Aβ25–35-Induced Synaptic Toxicity and Regulates CaMKII/CREB Pathway in SH-SY5Y Cells. *Cellular and Molecular Neurobiology*, *36*(7), 1151–1159. https://doi.org/10.1007/s10571-015-0311-6

Xicoy, H., Wieringa, B., & Martens, G. J. M. (2017). The SH-SY5Y cell line in Parkinson's disease research: a systematic review. *Molecular Neurodegeneration*, *12*(1), 10. https://doi.org/10.1186/s13024-017-0149-0

Xu, H., Li, L., Wang, Y., Wang, H., An, D., Heng, B., & Liu, Y. (2019). Genistein inhibits Aβ $_{25-35}$ -induced SH-SY5Y cell damage by modulating the expression of apoptosis-related proteins and Ca $^{2+}$ influx through ionotropic glutamate receptors. *Phytotherapy Research*, *33*(2), 431–441. https://doi.org/10.1002/ptr.6239

Ye, S., Wang, T., Cai, B., Wang, Y., Li, J., Zhan, J., & Shen, G. (2017). Genistein protects hippocampal neurons against injury by regulating calcium/calmodulin dependent protein kinase IV protein levels in Alzheimer's disease model rats. *Neural Regeneration Research*, *12*(9), 1479. https://doi.org/10.4103/1673-5374.215260

Yi, J. H., Baek, S. J., Heo, S., Park, H. J., Kwon, H., Lee, S., … Kim, D. H. (2018). Direct pharmacological Akt activation rescues Alzheimer's disease like memory impairments and aberrant synaptic plasticity. *Neuropharmacology*, *128*, 282–292. https://doi.org/10.1016/J.NEUROPHARM.2017.10.028

You, F., Li, Q., Jin, G., Zheng, Y., Chen, J., & Yang, H. (2017). Genistein protects against Aβ25–35 induced apoptosis of PC12 cells through JNK signaling and modulation of Bcl-2 family messengers. *BMC Neuroscience*, *18*(1), 12. https://doi.org/10.1186/s12868-016-0329-9

Yu, H.-L., Li, L., Zhang, X.-H., Xiang, L., Zhang, J., Feng, J.-F., & Xiao, R. (2009). Neuroprotective effects of genistein and folic acid on apoptosis of rat cultured cortical neurons induced by β-amyloid 31-35. *British Journal of Nutrition*, *102*(5), 655–662. https://doi.org/10.1017/S0007114509243042

Yuan, Z., Luan, G., Wang, Z., Hao, X., Li, J., Suo, Y., … Wang, H. (2017). Flavonoids from *Potentilla parvifolia* Fisch . and Their Neuroprotective Effects in Human Neuroblastoma SH-SY5Y Cells *in vitro*. *Chemistry & Biodiversity*, *14*(6), e1600487. https://doi.org/10.1002/cbdv.201600487

Zeng, H., Chen, Q., & Zhao, B. (2004). Genistein ameliorates β-amyloid peptide (25–35)-induced hippocampal neuronal apoptosis. *Free Radical Biology and Medicine*, *36*(2), 180–188. https://doi.org/10.1016/J.FREERADBIOMED.2003.10.018

Figure legends

Fig 1. Differences in the morphology and levels of NeuN in undifferentiated and differentiated SH-SY5Y cells. **(a)** Representative photomicrographs of undifferentiated cells (left panel) and differentiated cells (right panel). Scale bar 100 µm (top) and 50 µm (bottom). **(b)** Flow cytometry analysis of NeuN levels in the two cell phenotypes. Representative histograms of median fluorescence intensity are shown (Figure on the left). Bar graph values are expressed as mean ± SEM of four independent experiments (n = seven to eight replicates) (Figure on the right). Data were analyzed by Student's *t* test. $^{**}p < 0.01$.

Fig 2. Effects of different Aβ concentrations on viability of undifferentiated and differentiated SH-SY5Y cells. Cells were exposed to $A\beta_{25-35}$ (7.5, 25, and 50 µM) for 72 h, and then cell viability was assessed by MTT assay **(a)** and cell damage was evaluated using propidium iodide (PI) incorporation **(b)**. Results are expressed as means ± SEM of four independent experiments (MTT assay, n = four to twelve replicates; PI incorporation, n= four to nine replicates). Data were analyzed by two-way ANOVA followed by Tukey's post hoc test. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to respective control; $^{\S}p < 0.001$ compared to the same Aβ concentration in undifferentiated cells; $^{\#\#\#}p < 0.001$ compared to indicated Aβ concentration.

Fig 3. Effect of genistein on Aβ-induced cell death. Cells were pre-treated with genistein (1 and 10 nM) or DMSO (0.1 %) for 24 h, and were then exposed to $A\beta_{25-35}$ (25 µM) plus genistein or DMSO in the concentrations mentioned above for 72 h. After the treatments, cells were incubated with Hoechst 33342 and propidium iodide and were

observed under an inverted fluorescence microscope. **(a)** Representative images of cells stained with Hoechst 33342 (blue), propidium iodide (red), and the merged results of the two are shown. The percentage of viable **(b)**, necrotic **(c)**, late apoptotic **(d),** and early apoptotic cells **(e)** was calculated as described in Experimental procedures. Scale bar 100 µm. Results are expressed as means ± SEM of four independent experiments (n = four to nine replicates). Data were analyzed by two-way ANOVA followed by Tukey's post hoc test. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to Aβ group. Gen: genistein.

Fig 4. Effects of genistein treatment and Aβ exposition on levels of Akt **(a)**, p-Akt (Ser473) **(b)**, GSK-3β **(c)**, p-GSK-3β (Ser9) **(d)**, Tau **(e)** and p-Tau (Ser396) **(f)** in differentiated SH-SY5Y cells. Cells were pre-treated with genistein (10 nM) or DMSO (0.1 %) for 24 h, and were then exposed to $A\beta_{25\text{-}35}$ (25 µM) plus genistein or DMSO in the concentrations mentioned above for 24 h. After the treatments, protein levels were analyzed by flow cytometry. Representative histograms of median fluorescence intensity of each protein are shown (Figure on the left). Bar graph values are presented as means ± SEM of three independent experiments for each protein (n = three to seven replicates) (Figure on the right). Data were analyzed by two-way ANOVA followed by Tukey's post hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to Aβ group. Gen: genistein.

Fig 5. Graphical abstract of the effects of Aβ exposition and genistein treatment on Akt, GSK-3β, Tau hyperphosphorylation, and cell death of differentiated SH-SY5Y. **(a)** In summary, Aβ peptide decreases Akt phosphorylation, thus, inhibiting its activation. Reduced activation of Akt decreases the inhibitory phosphorylation of GSK-3β, which

in turn induces Tau hyperphosphorylation, and eventually, cell death. **(b)** Genistein treatment, in turn, is able to prevent Akt inactivation, GSK-3β activation (at least in part), Tau hyperphosphorylation and cell death. Red arrows indicate Aβ effect. Green arrows indicate genistein effect. Black arrows indicate phosphorylation reaction. Gray arrows indicate enzymatic action. Black discontinuous arrows indicate downstream processes leading to cell death. Aβ: amyloid-β peptide; Akt: protein kinase B; GSK-3β: glycogen synthase kinase 3β.

Figure 1

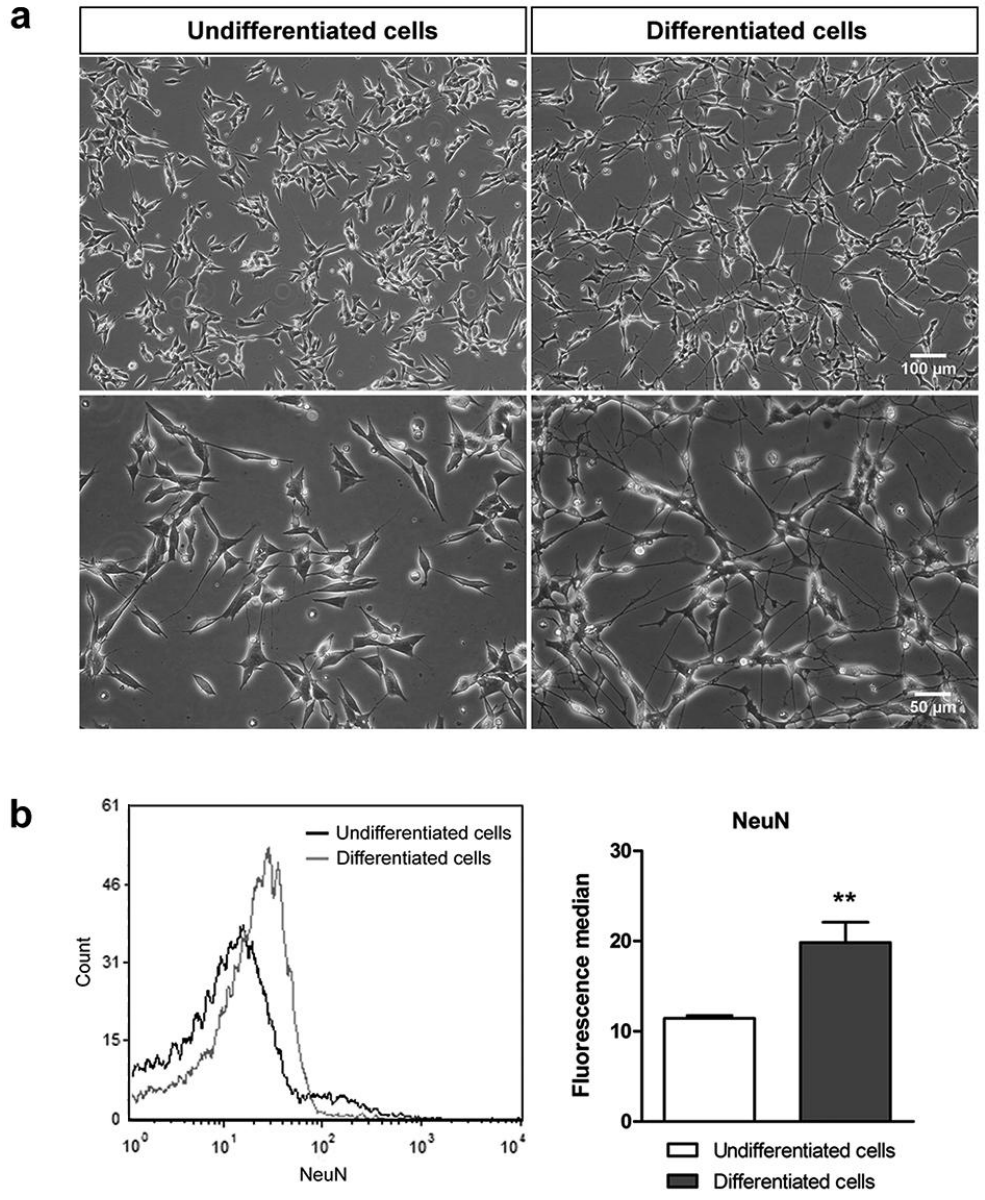

a
Undifferentiated cells
Differentiated cells
100 µm
50 µm
b
Undifferentiated cells
Differentiated cells
Count
61
46
31
15
0
10^0
10^1
10^2
10^3
10^4
NeuN
NeuN
Fluorescence median
30
20
10
0
**
Undifferentiated cells
Differentiated cells

Figure 2

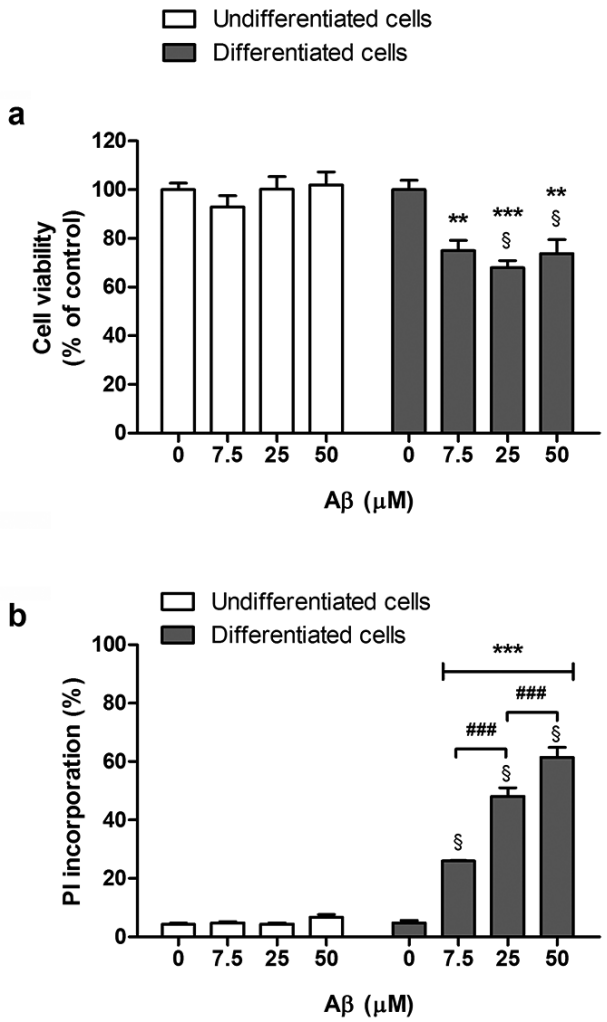

Undifferentiated cells
Differentiated cells
a
Cell viability (% of control)
120
100
80
60
40
20
0
0 7.5 25 50 0 7.5 25 50
Aβ (μM)
**
***
§
**
§
b
Undifferentiated cells
Differentiated cells
PI incorporation (%)
100
80
60
40
20
0
0 7.5 25 50 0 7.5 25 50
Aβ (μM)
***
###
###
§
§
§

Figure 3

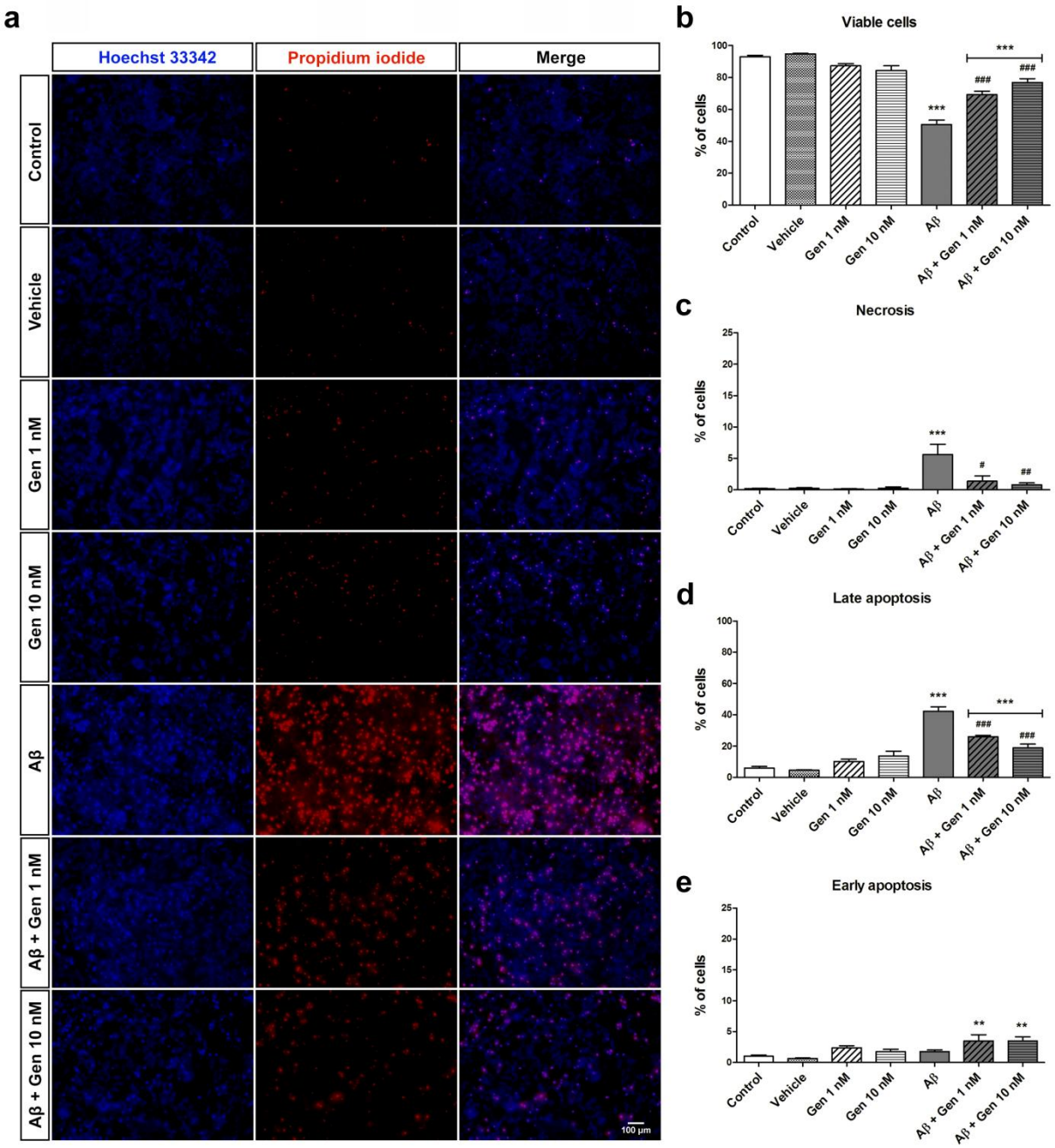

a
Hoechst 33342
Propidium iodide
Merge
Control
Vehicle
Gen 1 nM
Gen 10 nM
Aβ
Aβ + Gen 1 nM
Aβ + Gen 10 nM
100 µm
b
Viable cells
% of cells
c
Necrosis
d
Late apoptosis
e
Early apoptosis
Control
Vehicle
Gen 1 nM
Gen 10 nM
Aβ
Aβ + Gen 1 nM
Aβ + Gen 10 nM

Figure 4

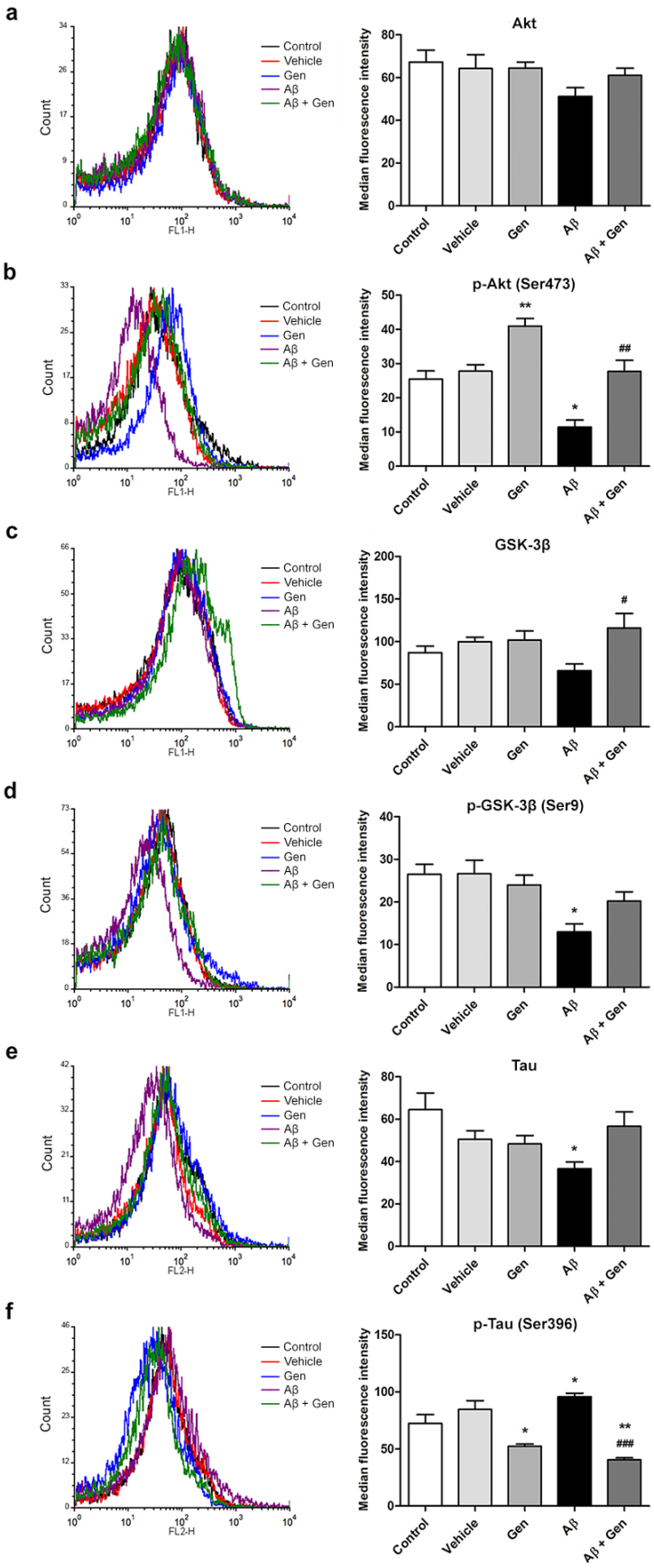
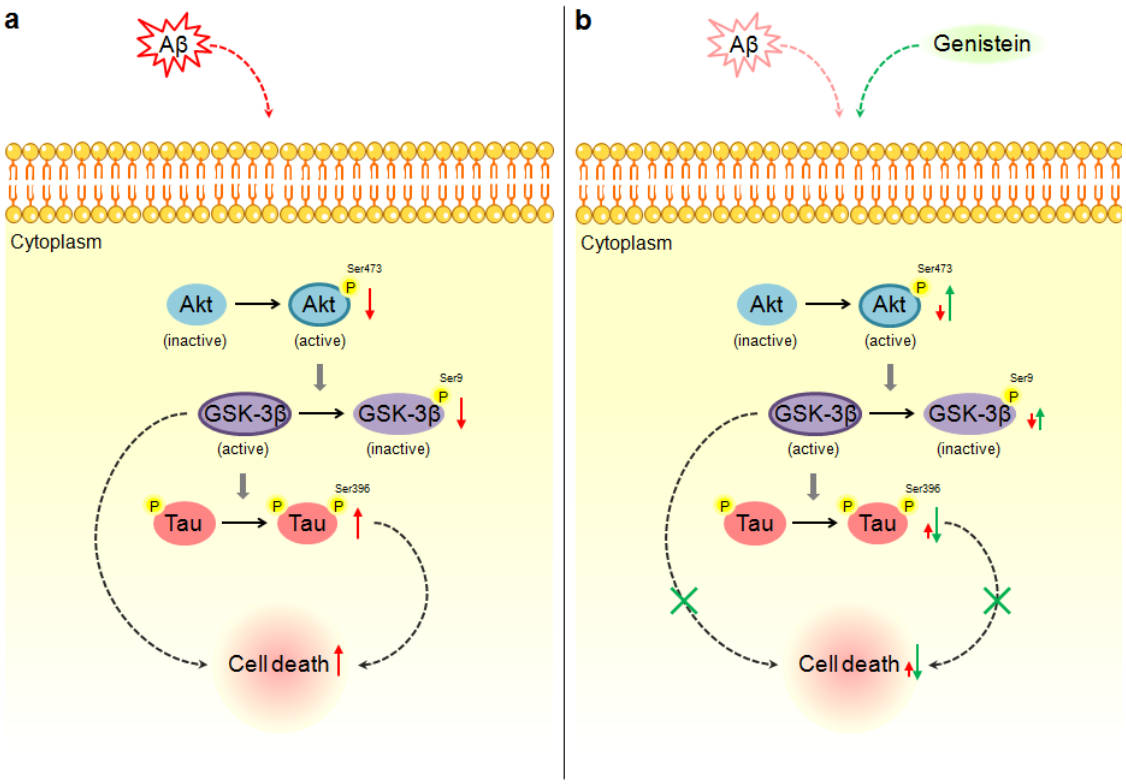

a
Akt
b
p-Akt (Ser473)
c
GSK-3β
d
p-GSK-3β (Ser9)
e
Tau
f
p-Tau (Ser396)
Control
Vehicle
Gen
Aβ
Aβ + Gen
Count
FL1-H
FL2-H
Median fluorescence intensity

Figure 5


a
Aβ
Cytoplasm
Akt
(inactive)
Akt
(active)
Ser473
P
GSK-3β
(active)
GSK-3β
(inactive)
Ser9
P
Tau
Tau
Ser396
P
Cell death
b
Aβ
Genistein
Cytoplasm
Akt
(inactive)
Akt
(active)
Ser473
P
GSK-3β
(active)
GSK-3β
(inactive)
Ser9
P
Tau
Tau
Ser396
P
Cell death

3.2. CAPÍTULO II

Genistein attenuates amyloid-beta-induced cognitive impairment in rats by modulation of hippocampal synaptotoxicity and hyperphosphorylation of Tau

Fernanda dos Santos Petry, Juliana Bender Hoppe, Caroline Peres Klein, Bernardo Gindri dos Santos, Régis Mateus Hözer, Felippo Bifi, Cristiane Matté, Christianne Gazzana Salbego, Vera Maria Treis Trindade

Status: Publicado no periódico *The Journal of Nutritional Biochemistry*
(disponível em https://doi.org/10.1016/j.jnutbio.2020.108525)

Genistein attenuates amyloid-beta-induced cognitive impairment in rats by modulation of hippocampal synaptotoxicity and hyperphosphorylation of Tau

Fernanda dos Santos Petry[1*], Juliana Bender Hoppe[1], Caroline Peres Klein[1], Bernardo Gindri dos Santos[1], Régis Mateus Hözer[1], Felippo Bifi[1], Cristiane Matté[1,2], Christianne Gazzana Salbego[1,2], Vera Maria Treis Trindade[1,2]

[1]Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[2]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

*Corresponding author

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 - Anexo, CEP 90035-003, Porto Alegre, RS, Brazil

E-mail address: fspetry@yahoo.com.br (F. S. Petry)

Tel: 55 51 3308 5549

ORCID: 0000-0002-8216-410X

Running title: Genistein reduces Aβ-induced neurotoxicity in rats

ABSTRACT

Alzheimer's disease is a progressive neurodegenerative disorder characterized by extracellular accumulation of amyloid-beta (Aβ) peptide, which induces synaptic dysfunction, alteration of intracellular signaling pathways, hyperphosphorylation of the Tau protein, and cognitive impairment. Genistein, one of the major isoflavones present in soy and soy products, has been shown to modulate some of the pathogenic events associated with the neurodegeneration process. However, its underlying mechanisms remain to be clarified. Therefore, the objectives of the present study were to evaluate the ability of genistein to protect against $A\beta_{1-42}$-induced cognitive impairment in rats and to elucidate some of the possible mechanisms involved in its neuroprotective effects in the hippocampus. Male Wistar rats received bilateral intracerebroventricular infusions of $A\beta_{1-42}$ (2 nmol) and genistein 10 mg/kg orally for 10 days. The Aβ-infused animals showed significant impairment of memory, which was accompanied by the following neurochemical alterations in the hippocampus: decreased levels of the synaptic proteins synaptophysin and PSD-95, hyperphosphorylation of Tau with increased activation of GSK-3β and JNK, and inactivation of ERK. Treatment with genistein improved Aβ-induced cognitive impairment by attenuation of synaptotoxicity, hyperphosphorylation of Tau, and inactivation of ERK. Furthermore, treatment with this soy isoflavone did not cause systemic toxicity. These findings provide further evidence of the neuroprotective effect of genistein in an *in vivo* model of Aβ toxicity and, importantly, extend the current knowledge concerning the mechanisms associated with the neuroprotective effects of this compound in the hippocampus.

Keywords: Alzheimer's disease; amyloid-beta; genistein; memory; neuroprotection; signaling

1. INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that accounts for most cases of age-related dementia. The sporadic form of this pathology, which is present in the vast majority of cases, is characterized by late onset of noticeable symptoms that manifest initially as a decline in memory and progress gradually to impairment of other cognitive functions [1].

Although AD is a very complex multifactorial disease, the mechanisms of which are not yet fully elucidated, accumulation of amyloid-beta (Aβ) peptide in extracellular amyloid plaques and hyperphosphorylated Tau protein in intracellular neurofibrillary tangles are considered to be its histopathological hallmarks [2]. These lesions may originate in crucial areas for learning and memory processes, such as the hippocampus, and spread progressively throughout the brain during the course of the disease [3].

The Aβ peptide is produced by proteolytic cleavage of the amyloid precursor protein by the sequential actions of β-secretase and γ-secretase enzymes [4]. Spontaneous self-assembly of Aβ into soluble oligomers or insoluble fibrils can initiate a cascade of pathogenic events, including synaptic failure, depletion of neurotrophins, activation or inactivation of intracellular proteins, and hyperphosphorylation of Tau, thereby impairing correct neuronal function and, ultimately, causing loss of neurons [4,5].

Predominantly found in the axons of neurons, Tau is a microtubule-associated protein that promotes the assembly and stability of these structures and plays an important role in synaptic function [6]. Deposition of Aβ peptide may trigger hyperactivation of Tau kinases, such as glycogen synthase kinase-3β (GSK-3β) and c-Jun N-terminal kinase (JNK), which induces hyperphosphorylation of Tau [7–9]. In this case, Tau lacks affinity for microtubules and self-assembles into paired helical filaments, impairing

appropriate synaptic function [10]. The dysfunction and loss of synapses correlates strongly with the cognitive decline observed in patients with AD [6].

Considering the increased prevalence of AD in our aging population and the current lack of disease-modifying treatments, there is strong interest in new therapeutic strategies that can modulate the pathogenic alterations associated with development or progression of this disease. The isoflavones, a group in the flavonoid category of polyphenolic compounds that are abundant in food sources, have been the focus of increasing attention because of their beneficial effects on brain function [11–13]. One of the major isoflavones present in soy and soy products, genistein (4',5,7-trihydroxyisoflavone), has been shown to modulate some of the pathogenic events associated with neurodegeneration [14–16]. Studies in animal models that mimic AD have indicated that this isoflavone has the potential to improve memory impairment [17–20], exert antiapoptotic [19] and anti-amyloidogenic [20] effects, and reduce the deposition of Aβ [18,20,21], astrogliosis [21], and loss of neurons in the hippocampus [19,22]. Moreover, studies of Aβ-induced toxicity in cell models have demonstrated the ability of genistein to modulate intracellular signaling pathways [9,23–25], prevent imbalance of neurotransmitter receptor levels [25,26], and exert antioxidant effects [24,27,28].

Despite the potential ability of genistein to protect against neurodegeneration-related processes, the underlying mechanisms remain to be clarified. Therefore, the objectives of the present study were to evaluate the ability of genistein to protect against Aβ-induced cognitive impairment in rats and to elucidate some of the possible mechanisms involved in its neuroprotective effect in the hippocampus.

2. MATERIAL AND METHODS

2.1. Animals

Adult male Wistar rats (aged 90 days, weight 330-450 g) were obtained from the Centro de Reprodução e Experimentação de Animais de Laboratório (Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul [UFRGS], Porto Alegre, Brazil) and maintained at the Departamento de Bioquímica (Universidade Federal do Rio Grande do Sul). The animals were housed in groups of 3–4 per cage with food and water provided *ad libitum*. They were maintained under optimum light (12/12-h light/dark cycle), temperature (22 ± 1°C), and humidity (50%–60%) conditions. The animals were handled for 2 weeks for adaptation before the experiments were initiated. All experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2011) and the Brazilian Federal Law on Procedures for the Scientific Use of Animals (Law 11.794/2008) and were approved by the institutional Animal Ethics Committee (CEUA/UFRGS) under project number 33552.

2.2. Preparation of $A\beta_{1-42}$ peptide

$A\beta_{1-42}$ peptide (American Peptide Co., Sunnyvale, CA, USA) was dissolved in Tris 0.05 M (pH 7.5), prepared with sterilized bi-distilled water at a concentration of 1 mg/mL, and stored at -20°C. Aliquots of the stock solution were incubated at 37°C for 96 h in order to obtain aggregated Aβ before use [30].

2.3. Surgical procedure

The animals were anesthetized using a mixture of xylazine 10 mg/kg and ketamine 100 mg/kg administered intraperitoneally and placed in a stereotaxic apparatus. After implementation of standard sterilization procedures, a middle sagittal incision was made

in the scalp and holes were drilled bilaterally in the skull over the lateral ventricles using a dental drill. The injection coordinates were chosen according to the Paxinos and Watson rat brain atlas [31]: anteroposterior, -0.8 mm posterior to the bregma; mediolateral, ± 1.5 mm lateral to the sagittal suture; and dorsoventral, -3.5 mm beneath the brain surface. The experimental rats received bilateral intracerebroventricular infusions of $A\beta_{1-42}$ (5 µL; total, 2 nmol in 10 µL) [7]. Bilateral intracerebroventricular infusions of an equal volume of Tris 0.05 M (pH 7.5) were administered to a group of control rats. All microinjections were performed using a 10-µL Hamilton syringe fitted with a 26-gauge needle. All infusions were delivered at a rate of 1 µL/min over a period of 5 min. At the end of infusion, the needle was left in place for a further 3–5 min before being slowly withdrawn to allow diffusion from the tip and prevent reflux of the solution. After the surgical procedure, the scalp was sutured, and the animal was allowed to recover from anesthesia on a heating pad to maintain body temperature at 37.5 ± 0.5°C.

2.4. Drug treatment

A timeline of the experimental design is shown in **Figure 1**. One day after the surgical procedure, the rats were randomly divided into four experimental groups. Control animals that received an intracerebroventricular infusion of vehicle were subdivided into two groups that were treated orally with (1) vehicle (Veh/Veh group) or (2) genistein (Veh/Gen group). Animals that received an intracerebroventricular infusion of $A\beta_{1-42}$ were similarly subdivided into two groups that were treated orally with (3) vehicle (Aβ/Veh group) or (4) genistein (Aβ/Gen group).

Genistein (LKT Laboratories, St. Paul, MN, USA) was dissolved at a concentration of 4 mg/mL in 0.9% NaCl solution containing 50% ethanol (Merck Millipore, Darmstadt,

Germany). This solution was freshly made up immediately before each administration. Genistein (10 mg/kg body weight) [17, 32] was administered via oral gavage daily for 10 consecutive days, starting on the day following the surgical procedure (day 1). The control groups received equivalent volumes of the vehicle. Body weight was checked on alternate days and the genistein dose was adjusted as necessary.

2.5. Behavioral analyses

Behavioral analyses were initiated 24 h after completion of drug treatment and performed sequentially on days 11–15 (**Figure 1**). All behavioral tasks were implemented by the same investigator during the light phase (between 11:00 and 17:00) in a sound-attenuated room under constant illumination. The animals were allowed to habituate to the testing room for one hour before starting the tests. The behavioral data were recorded automatically by a video camera positioned above the center of the arena and analyzed using an ANY-maze video tracking system (Stoelting Co., Wood Dale, IL, USA).

2.5.1. Open field task

Locomotor activity and spontaneous exploratory behavior in a novel environment were evaluated using the open field task [33]. The open field apparatus consisted of a square wooden arena covered with impermeable formica (dimensions, 50 × 50 × 50 cm), with a black floor and walls. The rats were placed individually in the left corner of the arena facing the wall and allowed to explore it for 10 min on two consecutive days (days 11 and 12). Using the video tracking software, the floor of the arena was divided into 16 equal-sized squares, forming two zones consisting of the periphery and the center. For analysis of locomotion, the total distance travelled, average speed, and number of crossings were evaluated. For analysis of anxiety-related behavior, the times spent in the central and peripheral zones were considered. The apparatus was cleaned with 10%

ethanol solution between trials. The same apparatus was used for the novel object recognition task; therefore, the open field task was treated as the habituation phase for that test.

2.5.2. Novel object recognition task

The novel object recognition task is based on the spontaneous tendency of rodents to explore novel objects [34]. It consists of three phases: habituation, training, and test. In the habituation phase, the animals were individually exposed to the apparatus, without exposure to any objects, for 10 min on two consecutive days. On the following day (day 13), in the training phase, two identical objects (A and A') were placed at two opposite corners of the arena and the rats were allowed to explore them for 5 min. After 24 h (day 14), the test phase was performed to evaluate long-term recognition memory. During this session, two dissimilar objects, one familiar and one novel (A and B, respectively), were presented in the arena and the animals were allowed to explore them for 5 min. After each session, the animals were returned to their home cages. Between trials, the objects and apparatus were carefully cleaned with 10% ethanol solution to minimize olfactory cues. All objects used in this task had similar textures and sizes but different shapes and colors. Active exploration was considered when the animal directed its nose to the object at a distance of no more than 2 cm and/or touched the object with the nose or forepaws. Sitting on the object was not considered to be exploratory behavior. The time spent exploring each object was recorded in the training and test phases. The recognition index (RI) was expressed as the ratio of time spent exploring the novel object (T_N) to the total time spent exploring both objects during the test phase (novel + familiar objects, $T_N + T_F$, respectively) [$RI = T_N/(T_N + T_F)$]. An RI >0.5 indicated preferential exploration of the novel object and was considered to be an index of enhanced cognitive performance.

2.5.3. Y-maze task

The Y-maze task was used to assess spontaneous alternation behavior, which reflects spatial working memory and is based on the natural tendency of rodents to alternate their choices of Y-maze arms on successive opportunities [35]. The apparatus consists of three symmetric arms (40 cm long, 25 cm high, and 10 cm wide; labeled A, B, and C) made of plywood covered with impermeable formica, with a black floor and walls. Each rat was placed at the end of one arm and allowed to freely explore the apparatus for 8 min in a single session (day 15). The number of arm entries and pattern of choices were recorded for each animal. An arm entry was considered when the hind paws of the rat were completely within the arm. Spontaneous alternation behavior was defined as entry into all three arms on consecutive choices in overlapping triplet sets (i.e., ABC, ACB, BAC, BCA, CAB, and CBA). The percentage of alternation was calculated as [number of alternations/(total number of arm entries – 2) × 100]. After performing the task, the animals were returned to their home cages. Between trials, the apparatus was cleaned with 10% ethanol solution.

2.6. Collection of samples

On the day following completion of the behavioral tasks (day 16), the animals were euthanized by decapitation without anesthesia. Blood samples were collected for serum analyses. The brains were removed, and the hippocampi were quickly dissected out on ice and stored immediately at -80°C until the neurochemical analyses.

2.7. Western blotting

The hippocampi were homogenized in ice-cold lysis buffer containing 4% sodium dodecyl sulfate, 2 mM EDTA, 50 mM Tris, and 1% protease inhibitor cocktail. The homogenates were denatured at 100ºC for 5 min and then centrifuged at 10,000 *g* for 30 min. The supernatant containing the cytosolic fraction was collected, the protein

concentration was determined [36], and β-mercaptoethanol was added to a final concentration of 5%. Equal amounts of proteins (30–80 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare Life Sciences, Chicago, IL, USA) using a semi-dry transfer apparatus (Trans-Blot SD, Bio-Rad, Hercules, CA, USA). The membranes were then incubated in blocking solution (Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat dry milk, pH 7.4) for 1 h at room temperature. The following primary antibodies were incubated with the membranes overnight at 4°C: anti-synaptophysin (1:3000, Merck Millipore, Billerica, MA, USA, #AB9272), anti-PSD95 (1:1000, Cell Signaling Technology, Danvers, MA, USA, #2507), anti-phospho-Tau (Ser396) (1:1000, Cell Signaling, #9632), anti-phospho-Tau (Ser199, Ser202) (1:1000, Invitrogen, Waltham, MA, USA, #44768), anti-Tau (1:1000, Cell Signaling, #4019), anti-phospho-GSK-3β (Ser9) (1:1000, Cell Signaling, #9336), anti-GSK-3β (1:1000, Cell Signaling, #9315), anti-phospho-SAPK/JNK (Thr183/Tyr185) (1:1000, Cell Signaling, #9251), anti-SAPK/JNK (1:1000, Cell Signaling, #9252), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (1:1000, Cell Signaling, #9101), anti-p44/42 MAPK (Erk1/2) (1:1000, Cell Signaling, #9102), and anti-β-actin (1:2000, Sigma-Aldrich, St Louis, MO, USA, #A3854). After washing, the membranes were incubated with a secondary antibody containing peroxidase-conjugated anti-rabbit IgG (1:1000, GE Healthcare Life Sciences, #NA934V) or anti-mouse IgG (1:1000, Cell Signaling, #7076) for 1 h at room temperature. The blots were developed using a chemiluminescence ECL kit (Bio-Rad). Chemiluminescence was detected using a digital image system (ImageQuant LAS 4000; GE Healthcare Life Sciences). Densitometric analysis was performed using ImageJ software (version 1.52a, National Institutes of Health, Bethesda, MD, USA). Band intensities were normalized to β-actin

as a loading control. The average optical density for the control group was designated as 100%.

2.8. Measurement of BDNF levels

Hippocampal brain-derived neurotrophic factor (BDNF) levels were determined using a commercial sandwich enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (CYT306, Merck Millipore). Briefly, the samples were homogenized in ice-cold homogenization buffer containing 100 mM Tris/HCl (pH 7), 1 M NaCl, 4 mM disodium EDTA, 2% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride, and the lysates were centrifuged at 14,000 *g* for 30 min at 4°C. The supernatant was collected and diluted 1:10 (*v/v*) in sample diluent. Microtiter plates (96-well, flat-bottom) were coated for 24 h with the samples and the standard curve ranged from 7.8 to 500 pg/mL of BDNF. The plates were then washed four times with wash buffer, and a biotinylated anti-BDNF monoclonal antibody (1:1000) was added to each well and incubated for 3 h at room temperature. After washing, peroxidase-conjugated streptavidin (1:1000) was added to each well and incubated for 1 h at room temperature. After addition of substrate (3,3',5,5'-tetramethylbenzidine) and stop solution, the amount of BDNF was determined by absorbance at 450 nm in a spectrophotometer. The protein concentration was measured according to the method described by Peterson (1977) [36]. The results were expressed as pg BDNF/mg protein.

2.9. Serum analyses

Blood samples were centrifuged at 1,000 *g* for 10 min for separation of serum. Serum urea and creatinine concentrations were considered as parameters of nephrotoxicity. The activity of hepatic enzymes (alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase) was used to assess hepatotoxicity. All serum analyses were performed using specific commercially available kits according to the manufacturers'

instructions (K047, K019, K034, K035, Bioclin, Belo Horizonte, MG, Brazil; #35, Labtest, Lagoa Santa, MG, Brazil).

2.10. Statistical analysis

The data are expressed as the mean ± standard error of the mean. Two-way analysis of variance (ANOVA), with Aβ infusion and treatment with genistein as fixed factors, was applied to determine statistically significant differences between the experimental groups. Body weight data were analyzed using repeated-measures ANOVA. Post hoc comparisons were performed using the Tukey test. For the novel object recognition sessions, a one-sample Student's *t*-test was used to compare the mean exploration time for each object with the fixed value of 50% (chance level). All statistical analyses were performed using IBM SPSS Statistics 18 software (IBM Corp., Armonk, NY, USA). A *p*-value <0.05 was considered statistically significant.

3. RESULTS

3.1. Effects of $A\beta_{1-42}$ infusion and genistein on locomotor and exploratory activities

In order to evaluate locomotor and exploratory activities, the rats were exposed to the open field on two consecutive days, starting one day after completion of treatment with genistein. **Table 1** shows the results and the statistical data for the parameters analyzed in this task. Two-way ANOVA identified no significant differences in total distance travelled, average speed, number of crossings, and the time spent in the periphery or center between the experimental groups on the first or second day of the test (all $p>0.05$). These results indicate that neither Aβ infusion nor treatment with genistein altered the locomotor and exploratory activities or anxiety-related behavior in the animals.

3.2. Effect of treatment with genistein on $A\beta_{1-42}$-induced cognitive impairment

Next, we assessed whether or not treatment with genistein could protect against Aβ-induced cognitive impairment. For this purpose, the rats were sequentially exposed to novel object recognition test to evaluate long-term recognition memory and Y-maze task to assess spontaneous alternation behavior.

The results of the novel object recognition test are shown in **Figure 2**. In the training phase, there was no significant between-group difference in the time spent exploring each object ($p>0.05$; **Figure 2a**). However, in the test phase, which was carried out 24 h later, the rats that received an Aβ infusion followed by oral administration of vehicle spent a similar amount of time exploring the novel object and the familiar one ($p>0.05$) whereas all other groups spent more time exploring the novel object ($p<0.01$ for the Veh/Veh, Veh/Gen, and Aβ/Gen groups, **Figure 2b**). Moreover, the statistical analysis indicated a significant interaction effect of Aβ infusion and treatment with genistein on the recognition index [$F(1,37)=8.961$; $p<0.01$]. As shown in **Figure 2c**, rats that received the Aβ infusion followed by oral administration of vehicle had impaired long-term recognition memory, as reflected in a lower recognition index, when compared with the Veh/Veh group ($p<0.01$). In contrast, the recognition index was higher in Aβ-injected rats that were treated with genistein than in the Aβ/Veh group ($p<0.01$), indicating that genistein protected against the memory deficit induced by the peptide.

Figure 2 also shows the results for the Y-maze test. Infusion of Aβ was observed to have a significant impact [$F(1,35)=4.158$; $p<0.05$] and there was a tendency for an interaction between the factors [$F(1,35)=4.027$; $p=0.053$] with regard to spontaneous alternation behavior. As shown in **Figure 2d**, the percentage of spontaneous alternation was lower in the Aβ-injected rats that received the oral vehicle than in the Veh/Veh group ($p<0.05$), again indicating a cognitive deficit in this group. For this behavioral

parameter, treatment with genistein showed a tendency to prevent the effect of Aβ infusion that did not reach statistical significance (p=0.077). There was no statistically significant difference in spontaneous alternation behavior between the Aβ/Gen group and the Veh/Veh group (p=0.993). As shown in **Figure 2e**, the effect of Aβ on spontaneous alternation behavior was not associated with a decrease in the total number of arm entries in the Y-maze (p>0.05), suggesting no impairment of locomotor or exploratory activities, which was also observed in the open field test.

3.3. Effect of genistein on synaptotoxicity triggered by $A\beta_{1-42}$ in the hippocampus

We also evaluated the levels of the synaptic proteins synaptophysin and postsynaptic density protein 95 (PSD-95) in the rat hippocampus in order to identify the mechanisms potentially involved in the ability of genistein to protect against Aβ-induced cognitive impairment. Aβ infusion [F(1,23)=13.721; p=0.001] and treatment with genistein [F(1,23)=12.262; p=0.002] had a significant effect on the hippocampal immunocontent of synaptophysin. There was a greater decrease in the level of this protein in the rats that received an Aβ infusion followed by oral administration of vehicle than in the Veh/Veh group (p<0.05, **Figure 3a**). The synaptophysin immunocontent was found to be significantly higher in the Aβ-injected rats that received genistein than in the Aβ/Veh group (p<0.05, **Figure 3a**). There was also a significant effect of Aβ infusion [F(1,25)=11.381; p=0.002] on the PSD-95 level in the hippocampus. A reduction in the hippocampal immunocontent of this protein was detected in rats that received an Aβ infusion followed by oral administration of vehicle when compared with the Veh/Veh group (p<0.05, **Figure 3b**). However, in this case, genistein did not protect against the Aβ-induced effect (p>0.05 vs. Aβ/Veh group, **Figure 3b**).

3.4. Effect of genistein on hyperphosphorylation of Tau triggered by $A\beta_{1-42}$ in the hippocampus

Given the reported relationship between accumulation of Aβ and hyperphosphorylation of Tau [8,9], we evaluated the hippocampal immunocontent of the phosphorylated form of this protein at two different sites as well as its total content.

Significant effects of Aβ infusion [$F(1,27)=5.206$; $p=0.031$] and treatment with genistein [$F(1,27)=5.705$; $p=0.024$] on the hippocampal pTau (Ser396)/Tau immunocontent ratio were detected. This ratio was significantly higher in rats that received the Aβ infusion followed by oral administration of vehicle than in the Veh/Veh group ($p<0.05$, **Figure 4a**), suggesting hyperphosphorylation of Tau (Ser 396). This effect was significantly decreased by administration of genistein in Aβ-injected rats when compared with the Aβ/Veh group ($p<0.05$, **Figure 4a**). There was no difference in the total immunocontent of Tau in the hippocampus between the groups (data not shown).

In terms of the ratio of pTau (Ser199/202)/Tau content, there was a significant interaction effect between infusion of Aβ and treatment with genistein [$F(1,24)=10.677$; $p=0.003$]. Phosphorylation of Tau (Ser199/202) was greater in the hippocampus of Aβ-injected rats that received the oral vehicle than in the Veh/Veh group ($p<0.05$, **Figure 4b**). Again, treatment with genistein completely prevented Aβ-induced hyperphosphorylation of Tau ($p<0.01$, **Figure 4b**). No difference was found in the total hippocampal immunocontent of Tau between the groups (data not shown).

3.5. Effect of genistein on activation of GSK-3β and JNK induced by $A\beta_{1-42}$ in the hippocampus

To further explore the neuroprotective effect of genistein on Aβ-induced hyperphosphorylation of Tau, we evaluated GSK-3β and JNK, both of which are Tau kinases that could be involved in the alterations described above.

Statistical analysis showed a significant effect of Aβ infusion [$F(1,25)=5.838$; $p=0.023$] and treatment with genistein [$F(1,25)=9.043$; $p=0.006$] on the ratio of p-GSK-3β/GSK-3β immunocontent in the hippocampus. Aβ infusion followed by oral administration of vehicle induced activation of GSK-3β, as indicated by the decreased p-GSK-3β/GSK-3β ratio when compared with the Veh/Veh group ($p<0.05$, **Figure 4c**). In contrast, administration of genistein in Aβ-injected rats induced a significant increase in the p-GSK-3β/GSK-3β ratio when compared with the Aβ/Veh group ($p<0.05$, **Figure 4c**), suggesting prevention of Aβ-induced activation of GSK-3β. There was no difference in total GSK-3β immunocontent between the groups (data not shown).

We also confirmed a significant effect of Aβ infusion [$F(1,25)=12.609$; $p=0.002$] on the ratio of p-JNK/JNK immunocontent in the rat hippocampus. The p-JNK/JNK ratio was higher, suggesting activation of this protein, in rats that received the Aβ infusion followed by oral administration of vehicle than in the Veh/Veh group ($p<0.05$, **Figure 4d**). There was no significant difference in the ratio of p-JNK/JNK immunocontent between Aβ-injected rats that received genistein and the Aβ/Veh ($p>0.05$) and Veh/Veh ($p>0.05$) groups (**Figure 4d**), suggesting that genistein partially protected against Aβ-induced activation of JNK. No difference in the total hippocampal immunocontent of JNK was found between the groups (data not shown).

3.6. Effects of $A\beta_{1-42}$ and genistein on BDNF levels and ERK activation in the hippocampus

We then investigated BDNF levels and the extracellular signal-regulated kinase (ERK) immunocontent in the rat hippocampus in view of their importance in learning and memory processes.

Neither infusion of Aβ nor treatment with genistein altered the levels of BDNF in the hippocampus (p>0.05, **Figure 5a**). However, there was a significant interaction effect of Aβ infusion and treatment with genistein [F(1,27)=7.735; p=0.010] on the ratio of p-ERK/ERK immunocontent in the hippocampus. Aβ infusion followed by oral administration of vehicle induced inactivation of ERK, as indicated by a lower p-ERK/ERK ratio when compared with the Veh/Veh group (p<0.05, **Figure 5b**). In contrast, administration of genistein in Aβ-injected rats was able to induce a significant increase in the p-ERK/ERK ratio when compared with the Aβ/Veh group (p<0.05, **Figure 5b**), suggesting prevention of Aβ-induced inactivation of ERK. No difference in total ERK immunocontent was found between the groups (data not shown).

3.7. Systemic effects of genistein

Finally, we sought to determine the toxicity profile of genistein. Ten days of treatment with genistein was not associated with mortality or changes in body weight [F(1.91, 80.41)=0.158; p=0.845; **Figure 6a**]. There was no significant between-group difference in the urea [F(1,20)=0.131; p=0.721; **Figure 6b**] or creatinine [F(1,20)=1.005; p=0.328; **Figure 6c**] level, suggesting an absence of nephrotoxicity. Furthermore, there was no significant difference in the alkaline phosphatase [F(1,21)=0.123; p=0.729; **Figure 6d**], aspartate aminotransferase [F(1,21)=0.005; p=0.945; **Figure 6e**], or alanine aminotransferase [F(1,21)=0.607; p=0.445; **Figure 6f**] levels, indicating lack of hepatotoxicity.

4. DISCUSSION

In this study, we investigated the neuroprotective effect of genistein 10 mg/kg administered orally for 10 days in an *in vivo* model of Aβ peptide-induced toxicity, focusing specifically on its effects on the cognitive impairment and neurochemical changes in the hippocampus triggered by bilateral intracerebroventricular injection of $A\beta_{1-42}$ peptide (2 nmol) in adult male rats. We demonstrated that infusion of Aβ induced marked impairment of memory that was accompanied by synaptic dysfunction, disturbances in activation of intracellular signaling proteins, and hyperphosphorylation of Tau. Treatment with genistein successfully attenuated most of these changes.

Given that memory impairment is usually the first clinical manifestation of AD, our first objective was to evaluate this parameter in our experimental groups using two behavioral analyses, both of which are highly dependent on the integrity of the hippocampus [37,38]. We observed that infusion of Aβ followed by oral administration of vehicle caused a significant memory deficit in the experimental animals, as indicated by impairment of both recognition memory and spontaneous alternation behavior. Daily administration of genistein for 10 days, starting one day after induction of the experimental model, completely protected against the decline in recognition memory and at least partially prevented the reduction in spontaneous alternation behavior, indicating that this compound can improve Aβ-induced cognitive impairment. Importantly, we confirmed that neither the damage caused by Aβ infusion nor the protective effect induced by genistein were due to impairment or improvement of locomotor activity, respectively, as observed in the open field test and in the number of arm entries in the Y-maze test. Previous studies have also demonstrated that this soy isoflavone markedly improves behavioral parameters after intrahippocampal injections

of $A\beta_{1-40}$ [17] and $A\beta_{25-35}$ [19], intracerebroventricular injections of streptozotocin [20], and in a transgenic mouse model of AD [18].

In order to elucidate some of the mechanisms that could be involved in the observed ability of genistein to protect against Aβ-induced cognitive impairment, we focused on some pathogenic events that are known to be triggered by this peptide in the hippocampus, a brain region that plays a crucial role in learning and memory processes and is profoundly affected in AD. Given that accumulation of Aβ can lead to a disturbance in the synaptic mechanisms required for learning and memory, and that this dysfunction is among the earliest events associated with the cognitive decline observed in AD [39], we initially evaluated the levels of the synaptic proteins synaptophysin and PSD-95 as indicators of synaptic integrity in the hippocampus. Synaptophysin is the most abundant integral protein in the synaptic vesicle membrane [39] whereas PSD-95 is a postsynaptic scaffolding protein that regulates protein assembly, synaptic development, and neural plasticity. PSD-95 is even important for the activity of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [40]. Previous studies have demonstrated a decrease in levels of one or both of these proteins after exposure of primary cultures of rat hippocampal neurons [39] and organotypic hippocampal slice cultures [41] to $A\beta_{25-35}$ and $A\beta_{1-42}$ peptides, respectively, and also in the rat hippocampus injected with $A\beta_{1-42}$ via the intracerebroventricular route [7,8,42,43]. Here, we also found significant reductions in these two synaptic proteins in the hippocampi of Aβ-injected rats that received the oral vehicle. Treatment with genistein was effective in preventing or restoring the decreased synaptophysin content in Aβ-injected rats but not the PSD-95 content. Our findings are partially in accordance with those of a previous *in vitro* study by Xi *et al.* (2016) [25], in which it was demonstrated that genistein (50 μM) prevented downregulation of the

mRNA and protein content of synaptophysin and PSD-95 in a SH-SY5Y human neuroblastoma cell line exposed to $A\beta_{25-35}$. The differences between the findings as regards the PSD-95 protein in that study and those described here can probably be explained by the different experimental models used.

It is well known that synaptic disturbances may be associated with intracellular accumulation of hyperphosphorylated Tau-composed neurofibrillary tangles. Abnormal phosphorylation of Tau, as a consequence of Aβ deposition, leads to decreased affinity and binding of this protein to the microtubules, which impairs axonal transport and ultimately contributes to synaptic dysfunction [10,44]. For this reason, we then investigated the possible effect of genistein on the hyperphosphorylation of Tau triggered by Aβ in the hippocampus. We observed that infusion of Aβ followed by oral administration of vehicle alone markedly increased the phosphorylation of Tau at the Ser396 and Ser199/202 sites, indicating hyperphosphorylation of this protein. Treatment with genistein completely prevented these effects of Aβ. Previous research has found similar effects using this soy isoflavone, albeit using higher dosages. As demonstrated by Ye *et al.* (2017) [22], genistein at a dosage of 90 mg/kg reduced the phosphorylated Tau immunocontent in the $A\beta_{25-35}$-injected rat hippocampus. Recently, Pierzynowska *et al.* (2019) [20] showed that high-dose genistein (150 mg/kg) was able to not only reduce the levels of phosphorylated Tau (at Ser396) but also the number of paired helical filaments in the cortex and hippocampus of streptozotocin-injected rats.

Given a report that phosphorylation of Tau is regulated by a balance between Tau kinase and phosphatase activity [45], we next investigated the hippocampal immunocontents of two proline-directed protein kinases, GSK-3β and JNK, which are known to be hyperactivated by the Aβ peptide in both *in vitro* and *in vivo* models [7–9,46–48]. We found that infusion of Aβ followed by oral administration of vehicle

induced a significant decrease in phosphorylated GSK-3β and an increase in phosphorylated JNK, indicating increased activation of these proteins and reinforcing our finding of hyperphosphorylation of Tau. In contrast, treatment with genistein was able to completely prevent the increase in GSK-3β activation in Aβ-injected rats but had a partial effect on hyperactivation of JNK. It seems that the observed effect of genistein on activation of GSK-3β is of particular relevance, considering that this kinase phosphorylates the majority of sites in Tau [49]. This protein undergoes phosphorylation at 42 sites by GSK-3 and at 12 sites by JNK [45]. In accordance with the findings described here, we have recently shown that the ability of genistein to protect against $A\beta_{25-35}$-induced death in differentiated SH-SY5Y cells involves prevention of Tau hyperphosphorylation by regulation of the protein kinase B (Akt)/GSK-3β signaling pathway [9]. A similar effect of genistein on GSK-3β and Tau proteins was also found in an *in vitro* model of endoplasmic reticulum stress [50]. Considering that the decreased activity of phosphatases can also contribute to the hyperphosphorylation of Tau, it would be important that future studies explored the effect of treatment with genistein on this parameter.

Learning and memory processes require correct function of molecular and cellular mechanisms that involve neurotrophins, such as BDNF, and activation of intracellular signaling pathways downstream [51]. BDNF has a critical role in the differentiation, maturation, and survival of neurons and is also an important mediator of synaptic plasticity. Binding of BDNF to its receptor (TrkB) leads to activation of intracellular proteins, including ERK. This protein in turn regulates neuronal differentiation and survival by suppression of pro-apoptotic proteins and activation of the cyclic adenosine monophosphate response-element binding protein (CREB) transcription factor to regulate gene expression [51,52]. Considering that AD is accompanied by severe

impairment of the neurotrophic signaling pathways [52], we analyzed the levels of BDNF and the content of both the phosphorylated and total forms of ERK in the hippocampus. Surprisingly, in our experimental model, intracerebroventricular injection of Aβ did not cause a reduction in BDNF levels, at least in the 16th day following the surgical procedure. However, we observed a significant decrease in activation of ERK, as indicated by a reduction in the immunocontent of the phosphorylated form of this protein. Therefore, we cannot rule out the possibility that downregulation of BDNF occurred at an earlier point in time. Treatment with genistein successfully prevented this effect in Aβ-injected rats. Lu *et al.* (2018) [32] demonstrated that administration of genistein at the same dose as that used in the present study improved cognitive performance in rats in a model of scopolamine-induced memory deficit via upregulation of the BDNF/ERK/CREB signaling pathway.

In summary, the present findings provide further evidence for a neuroprotective effect of genistein in an *in vivo* model of Aβ-toxicity. Importantly, they extend the current knowledge concerning the mechanisms associated with neuroprotection by this compound in the hippocampus. We have demonstrated that genistein exerts its neuroprotective effect by attenuation of synaptotoxicity, hyperphosphorylation of Tau, and inactivation of ERK triggered by $A\beta_{1-42}$ peptide in adult male rats, and all these mechanisms possibly contributed to the improvement in memory impairment. The positive effects of this soy isoflavone combined with the absence of systemic toxicity suggest that genistein is a promising alternative strategy for preventing or counteracting the harmful events associated with AD.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil), Instituto Nacional de Ciência e Tecnologia - INCT (EN 465671/2014-4)/CNPq-Brazil.

Conflicts of interest

There are no conflicts of interest to declare.

REFERENCES

[1] Lane CA, Hardy J, Schott JM. Alzheimer's disease. Eur J Neurol 2018;25:59–70. https://doi.org/10.1111/ene.13439.

[2] Scheltens P, Blennow K, Breteler MMB, de Strooper B, Frisoni GB, Salloway S, et al. Alzheimer's disease. Lancet 2016;388:505–17. https://doi.org/10.1016/S0140-6736(15)01124-1.

[3] Masters CL, Bateman R, Blennow K, Rowe CC, Sperling RA, Cummings JL. Alzheimer's disease. Nat Rev Dis Prim 2015;1:15056. https://doi.org/10.1038/nrdp.2015.56.

[4] Querfurth HW, LaFerla FM. Alzheimer's Disease. N Engl J Med 2010;362:329–44. https://doi.org/10.1056/NEJMra0909142.

[5] Selkoe DJ, Hardy J. The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO Mol Med 2016;8:595–608. https://doi.org/10.15252/emmm.201606210.

[6] Pooler AM, Noble W, Hanger DP. A role for tau at the synapse in Alzheimer's disease pathogenesis. Neuropharmacology 2014;76:1–8. https://doi.org/10.1016/J.NEUROPHARM.2013.09.018.

[7] Frozza RL, Bernardi A, Hoppe JB, Meneghetti AB, Matté A, Battastini AMO, et

al. Neuroprotective Effects of Resveratrol Against Aβ Administration in Rats are Improved by Lipid-Core Nanocapsules. Mol Neurobiol 2013;47:1066–80. https://doi.org/10.1007/s12035-013-8401-2.

[8] Hoppe JB, Coradini K, Frozza RL, Oliveira CM, Meneghetti AB, Bernardi A, et al. Free and nanoencapsulated curcumin suppress β-amyloid-induced cognitive impairments in rats: Involvement of BDNF and Akt/GSK-3β signaling pathway. Neurobiol Learn Mem 2013;106:134–44. https://doi.org/10.1016/J.NLM.2013.08.001.

[9] Petry FS, Coelho BP, Gaelzer MM, Kreutz F, Guma FTCR, Salbego CG, Trindade VMT. Genistein protects against amyloid-beta-induced toxicity in SH-SY5Y cells by regulation of Akt and Tau phosphorylation. Phytother Res 2020;34:796–807. https://doi.org/10.1002/ptr.6560.

[10] Naseri NN, Wang H, Guo J, Sharma M, Luo W. The complexity of tau in Alzheimer's disease. Neurosci Lett 2019;705:183–94. https://doi.org/10.1016/J.NEULET.2019.04.022.

[11] Lee YB, Lee HJ, Sohn HS. Soy isoflavones and cognitive function. J Nutr Biochem 2005;16:641–9. https://doi.org/10.1016/J.JNUTBIO.2005.06.010.

[12] Vauzour D. Dietary Polyphenols as Modulators of Brain Functions: Biological Actions and Molecular Mechanisms Underpinning Their Beneficial Effects. Oxid Med Cell Longev 2012;2012: 914273. https://doi.org/10.1155/2012/914273.

[13] Moosavi F, Hosseini R, Saso L, Firuzi O. Modulation of neurotrophic signaling pathways by polyphenols. Drug Des Devel Ther 2015;10:23–42. https://doi.org/10.2147/DDDT.S96936.

[14] Devi KP, Shanmuganathan B, Manayi A, Nabavi SF, Nabavi SM. Molecular and Therapeutic Targets of Genistein in Alzheimer's Disease. Mol Neurobiol

2017;54:7028–41. https://doi.org/10.1007/s12035-016-0215-6.

[15] Oliveira MR. Evidence for genistein as a mitochondriotropic molecule. Mitochondrion 2016;29:35–44. https://doi.org/10.1016/j.mito.2016.05.005.

[16] Uddin MS, Kabir MT. Emerging Signal Regulating Potential of Genistein Against Alzheimer's Disease: A Promising Molecule of Interest. Front Cell Dev Biol 2019;7:197. https://doi.org/10.3389/fcell.2019.00197.

[17] Bagheri M, Joghataei MT, Mohseni S, Roghani M. Genistein ameliorates learning and memory deficits in amyloid β(1–40) rat model of Alzheimer's disease. Neurobiol Learn Mem 2011;95:270–6. https://doi.org/10.1016/J.NLM.2010.12.001.

[18] Bonet-Costa V, Herranz-Pérez V, Blanco-Gandía M, Mas-Bargues C, Inglés M, Garcia-Tarraga P, et al. Clearing amyloid-β through PPARγ/ApoE Activation by Genistein is a Treatment of Experimental Alzheimer's Disease. J Alzheimer's Dis 2016;51:701–11. https://doi.org/10.3233/JAD-151020.

[19] Wang Y, Cai B, Shao J, Wang T, Cai R, Ma C, et al. Genistein suppresses the mitochondrial apoptotic pathway in hippocampal neurons in rats with Alzheimer's disease. Neural Regen Res 2016;11:1153–8. https://doi.org/10.4103/1673-5374.187056.

[20] Pierzynowska K, Podlacha M, Gaffke L, Majkutewicz I, Mantej J, Węgrzyn A, et al. Autophagy-dependent mechanism of genistein-mediated elimination of behavioral and biochemical defects in the rat model of sporadic Alzheimer's disease. Neuropharmacology 2019;148:332–46. https://doi.org/10.1016/J.NEUROPHARM.2019.01.030.

[21] Bagheri M, Roghani M, Joghataei MT, Mohseni S. Genistein inhibits aggregation of exogenous amyloid-beta1–40 and alleviates astrogliosis in the hippocampus of

rats. Brain Res 2012;1429:145–54. https://doi.org/10.1016/J.BRAINRES.2011.10.020.

[22] Ye S, Wang TT, Cai B, Wang Y, Li J, Zhan J, et al. Genistein protects hippocampal neurons against injury by regulating calcium/calmodulin dependent protein kinase IV protein levels in Alzheimer's disease model rats. Neural Regen Res 2017;12:1479–84. https://doi.org/10.4103/1673-5374.215260.

[23] Yu HL, Li L, Zhang XH, Xiang L, Zhang J, Feng JF, et al. Neuroprotective effects of genistein and folic acid on apoptosis of rat cultured cortical neurons induced by β-amyloid 31-35. Br J Nutr 2009;102:655–62. https://doi.org/10.1017/S0007114509243042.

[24] Ma W, Yuan L, Yu H, Ding B, Xi Y, Feng J, et al. Genistein as a neuroprotective antioxidant attenuates redox imbalance induced by β-amyloid peptides 25–35 in PC12 cells. Int J Dev Neurosci 2010;28:289–95. https://doi.org/10.1016/J.IJDEVNEU.2010.03.003.

[25] Xi YD, Zhang DD, Ding J, Yu HL, Yuan LH, Ma WW, et al. Genistein Inhibits Aβ25–35-Induced Synaptic Toxicity and Regulates CaMKII/CREB Pathway in SH-SY5Y Cells. Cell Mol Neurobiol 2016;36:1151–9. https://doi.org/10.1007/s10571-015-0311-6.

[26] Xu H, Li L, Wang Y, Wang H, An D, Heng B, et al. Genistein inhibits Aβ25-35-induced SH-SY5Y cell damage by modulating the expression of apoptosis-related proteins and Ca2+ influx through ionotropic glutamate receptors. Phytother Res 2019;33:431–41. https://doi.org/10.1002/ptr.6239.

[27] Vallés SL, Borrás C, Gambini J, Furriol J, Ortega A, Sastre J, et al. Oestradiol or genistein rescues neurons from amyloid beta-induced cell death by inhibiting activation of p38. Aging Cell 2008;7:112–8. https://doi.org/10.1111/j.1474-

9726.2007.00356.x.

[28] Ding B, Yuan L, Yu H, Li L, Ma W, Bi Y, et al. Genistein and Folic Acid Prevent Oxidative Injury Induced by β-Amyloid Peptide. Basic Clin Pharmacol Toxicol 2011;108:333–40. https://doi.org/10.1111/j.1742-7843.2010.00661.x.

[29] National Research Council (U.S.). Guide for the Care and Use of Laboratory Animals. 8th ed. Washington, DC: National Academies Press; 2011.

[30] Kreutz F, Scherer EB, Ferreira AGK, Petry FS, Pereira CL, Santana F, et al. Alterations on Na+,K+-ATPase and Acetylcholinesterase Activities Induced by Amyloid-β Peptide in Rat Brain and GM1 Ganglioside Neuroprotective Action. Neurochem Res 2013;38:2342–50. https://doi.org/10.1007/s11064-013-1145-6.

[31] Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. 6th ed. San Diego: Academic Press; 2007.

[32] Lu C, Wang Y, Xu T, Li Q, Wang D, Zhang L, et al. Genistein Ameliorates Scopolamine-Induced Amnesia in Mice Through the Regulation of the Cholinergic Neurotransmission, Antioxidant System and the ERK/CREB/BDNF Signaling. Front Pharmacol 2018;9:1153. https://doi.org/10.3389/fphar.2018.01153.

[33] Gould TD, Dao DT, Kovacsics CE. The Open Field Test. In: Gould T, editor. Mood and anxiety related phenotypes in mice: Characterization using behavioral tests, Totowa: Humana Press; 2009, p. 1–20. https://doi.org/10.1007/978-1-60761-303-9_1.

[34] Ennaceur A, Delacour J. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav Brain Res 1988;31:47–59. https://doi.org/10.1016/0166-4328(88)90157-X.

[35] Hughes RN. The value of spontaneous alternation behavior (SAB) as a test of

retention in pharmacological investigations of memory. Neurosci Biobehav Rev 2004;28:497–505. https://doi.org/10.1016/J.NEUBIOREV.2004.06.006.

[36] Peterson GL. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 1977;83:346–56. https://doi.org/10.1016/0003-2697(77)90043-4.

[37] Broadbent NJ, Gaskin S, Squire LR, Clark RE. Object recognition memory and the rodent hippocampus. Learn Mem 2010;17:5–11. https://doi.org/10.1101/lm.1650110.

[38] Albani SH, McHail DG, Dumas TC. Developmental studies of the hippocampus and hippocampal-dependent behaviors: Insights from interdisciplinary studies and tips for new investigators. Neurosci Biobehav Rev 2014;43:183–90. https://doi.org/10.1016/J.NEUBIOREV.2014.04.009.

[39] Liu J, Chang L, Roselli F, Almeida OFX, Gao X, Wang X, et al. Amyloid-β induces caspase-dependent loss of PSD-95 and synaptophysin through NMDA receptors. J Alzheimer's Dis 2010;22:541–56. https://doi.org/10.3233/JAD-2010-100948.

[40] Tu S, Okamoto S, Lipton SA, Xu H. Oligomeric Aβ-induced synaptic dysfunction in Alzheimer's disease. Mol Neurodegener 2014;9:48. https://doi.org/10.1186/1750-1326-9-48.

[41] Hoppe JB, Frozza RL, Pires ENS, Meneghetti AB, Salbego C. The curry spice curcumin attenuates beta-amyloid-induced toxicity through beta-catenin and PI3K signaling in rat organotypic hippocampal slice culture. Neurol Res 2013;35:857–66. https://doi.org/10.1179/1743132813y.0000000225.

[42] Zhang LL, Sui HJ, Liang B, Wang HM, Qu WH, Yu SX, et al. Atorvastatin prevents amyloid-β peptide oligomer-induced synaptotoxicity and memory

dysfunction in rats through a p38 MAPK-dependent pathway. Acta Pharmacol Sin 2014;35:716–26. https://doi.org/10.1038/aps.2013.203.

[43] Li Q, Che H, Wang C, Zhang L, Ding L, Xue C, et al. Cerebrosides from Sea Cucumber Improved Aβ1–42-Induced Cognitive Deficiency in a Rat Model of Alzheimer's Disease. Mol Nutr Food Res 2019;63:1800707. https://doi.org/10.1002/mnfr.201800707.

[44] Khan SS, Bloom GS. Tau: The Center of a Signaling Nexus in Alzheimer's Disease. Front Neurosci 2016;10:31. https://doi.org/10.3389/fnins.2016.00031.

[45] Martin L, Latypova X, Wilson CM, Magnaudeix A, Perrin ML, Yardin C, et al. Tau protein kinases: Involvement in Alzheimer's disease. Ageing Res Rev 2013;12:289–309. https://doi.org/10.1016/J.ARR.2012.06.003.

[46] Hoppe JB, Frozza RL, Horn AP, Comiran RA, Bernardi A, Campos MM, et al. Amyloid-β neurotoxicity in organotypic culture is attenuated by melatonin: Involvement of GSK-3β, tau and neuroinflammation. J Pineal Res 2010;48:230–8. https://doi.org/10.1111/j.1600-079X.2010.00747.x.

[47] Kreutz F, Frozza RL, Breier AC, Oliveira VA, Horn AP, Pettenuzzo LF, et al. Amyloid-β induced toxicity involves ganglioside expression and is sensitive to GM1 neuroprotective action. Neurochem Int 2011;59:648–55. https://doi.org/10.1016/j.neuint.2011.06.007.

[48] Coelho BP, Gaelzer MM, Petry FS, Hoppe JB, Trindade VMT, Salbego CG, et al. Dual Effect of Doxazosin: Anticancer Activity on SH-SY5Y Neuroblastoma Cells and Neuroprotection on an In Vitro Model of Alzheimer's Disease. Neuroscience 2019;404:314–25. https://doi.org/10.1016/J.NEUROSCIENCE.2019.02.005.

[49] Avila J, León-Espinosa G, García E, García-Escudero V, Hernández F, Defelipe

J. Tau phosphorylation by GSK3 in different conditions. Int J Alzheimers Dis 2012;2012:578373. https://doi.org/10.1155/2012/578373.

[50] Park YJ, Jang Y, Kwon YH. Isoflavones Prevent Endoplasmic Reticulum Stress-Mediated Neuronal Degeneration by Inhibiting Tau Hyperphosphorylation in SH-SY5Y Cells. J Med Food 2009;12:528–35. https://doi.org/10.1089/jmf.2008.1069.

[51] Cunha C, Brambilla R, Thomas KL. A simple role for BDNF in learning and memory? Front Mol Neurosci 2010;3:1. https://doi.org/10.3389/neuro.02.001.2010.

[52] Tejeda GS, Díaz-Guerra M. Integral Characterization of Defective BDNF/TrkB Signalling in Neurological and Psychiatric Disorders Leads the Way to New Therapies. Int J Mol Sci 2017;18:268. https://doi.org/10.3390/ijms18020268.

FIGURE LEGENDS

Fig 1. Timeline of the experimental procedures. Male Wistar rats (aged 90 days at the start of the experiments) received bilateral intracerebroventricular infusions of $A\beta_{1-42}$ (2 nmol) or vehicle. The rats were treated daily with genistein 10 mg/kg via oral gavage for 10 consecutive days, starting on day 1 after the surgical procedure. The control groups received equivalent volumes of vehicle solution. Behavioral analyses were initiated 24 h after completion of drug treatment and performed sequentially on days 11–15. On the day following completion of the behavioral tests, the hippocampi and blood samples were collected for neurochemical and serum analyses, respectively. Aβ, amyloid-β peptide; Gen, genistein; Veh, vehicle

Fig 2. Effects of $A\beta_{1-42}$ infusion and treatment with genistein on recognition memory and spontaneous alternation behavior. Rats were injected with $A\beta_{1-42}$ (2 nmol, intracerebroventricular route) and treated daily with genistein 10 mg/kg for 10 days. The novel object recognition task was performed 13 and 14 days after infusion of Aβ. The percentage of time spent exploring each object in the training **(a)** and test **(b)** sessions are shown. $^{\&}p<0.01$ vs. a 50% chance level (one-sample Student's *t*-test). **(c)** Recognition index for each experimental group in the test session. The Y-maze task was performed 15 days after infusion of Aβ. **(d)** Percentage of spontaneous alternation. **(e)** Total number of arm entries. The data are expressed as the mean ± standard error of the mean (9–11 animals/group). $^{*}p<0.05$ vs Veh/Veh group, $^{**}p<0.01$ vs Veh/Veh group; $^{\#\#}p<0.01$ vs Aβ/Veh group (two-way analysis of variance followed by Tukey's post hoc test). Aβ, amyloid-β peptide; Gen, genistein; Veh, vehicle

Fig 3. Effect of treatment with genistein on synaptotoxicity triggered by $A\beta_{1-42}$ in the hippocampus. Western blot analyses of synaptophysin **(a)** and PSD-95 **(b)** were performed 16 days after infusion of $A\beta_{1-42}$ (2 nmol, intracerebroventricular route) and daily treatment with genistein 10 mg/kg for 10 days. Representative Western blot bands are shown in the panels above the graphs. The band intensities were normalized to β-actin as a loading control. The values shown represent the mean ± standard error of the mean (6–9 animals/group). $^{*}p<0.05$ vs Veh/Veh group; $^{\#}p<0.05$ vs Aβ/Veh group (two-way analysis of variance followed by Tukey's post hoc test). Aβ, amyloid-β peptide; Gen, genistein; Veh, vehicle

Fig 4. Effect of treatment with genistein on hyperphosphorylation of Tau triggered by $A\beta_{1-42}$ in the hippocampus. Western blot analyses were performed 16 days after infusion of $A\beta_{1-42}$ (2 nmol, intracerebroventricular route) and daily treatment with genistein 10 mg/kg for 10 days. **(a)** Ratio of pTau (Ser396)/Tau immunocontent; 6–10 animals/group. **(b)** Ratio of pTau (Ser199/202)/Tau immunocontent; 6–8 animals/group. **(c)** Ratio of pGSK-3β (Ser9)/GSK-3β immunocontent; 6–10 animals/group. **(d)** Ratio of pJNK (Thr183/Tyr185)/JNK immunocontent; 6–9 animals/group. Representative Western blot bands are shown in the panels above the graphs. The band intensities were normalized to β-actin as a loading control. The values shown represent the mean ± standard error of the mean. $^{*}p<0.05$ vs Veh/Veh group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs Aβ/Veh group (two-way analysis of variance followed by Tukey's post hoc test). Aβ, amyloid-β peptide; Gen, genistein; Veh, vehicle

Fig 5. Effects of $A\beta_{1-42}$ and treatment with genistein on BDNF levels and activation of ERK in the hippocampus. Enzyme-linked immunosorbent assay and Western blot

analyses were performed 16 days after infusion of $A\beta_{1-42}$ (2 nmol, intracerebroventricular route) and daily treatment with genistein 10 mg/kg for 10 days. **(a)** BDNF levels; 5–7 animals/group. **(b)** Ratio of pERK (Thr202/Tyr204)/ERK immunocontent; 7–9 animals/group. Representative Western blot bands are shown in the panel above the graph. Band intensities were normalized to β-actin as a loading control. The values shown represent the mean ± standard error of the mean. $^{*}p<0.05$ vs. the Veh/Veh group; $^{\#}p<0.05$ vs. the Aβ/Veh group (two-way analysis of variance followed by Tukey's post hoc test). Aβ, amyloid-β peptide; Gen, genistein; Veh, vehicle

Fig 6. Body weight, serum urea and creatinine levels, and serum alkaline phosphatase, AST and ALT activity after 10 days of treatment with genistein in rats. (a) Body weight (11–12 animals/group). The results were evaluated by repeated-measures analysis of variance. **(b, c)** Nephrotoxicity was assessed by serum urea and creatinine levels (6 animals/group). **(d-f)** Hepatotoxicity was evaluated by alkaline phosphatase, AST and ALT activity levels (6–7 animals/group). The data are expressed as the mean ± standard error of the mean. Two-way analysis of variance showed no significant differences. Aβ, amyloid-β peptide; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Gen, genistein; Veh, vehicle

Fig 7. Effects of infusion of $A\beta_{1-42}$ and treatment with genistein in a rodent model. **(a)** Intracerebroventricular infusion of $A\beta_{1-42}$ led to a decrease in levels of the synaptic proteins synaptophysin and PSD-95, increased activation of GSK-3β and JNK, hyperphosphorylation of Tau, inactivation of ERK, and, finally, significant cognitive impairment. **(b)** Treatment with genistein attenuated these effects. Red arrows indicate the effect of Aβ. Green arrows and crossed lines indicate the effect of genistein. Gray

arrows indicate the action of enzymes. The discontinuous black arrows indicate processes leading to cognitive impairment.

Figure 1

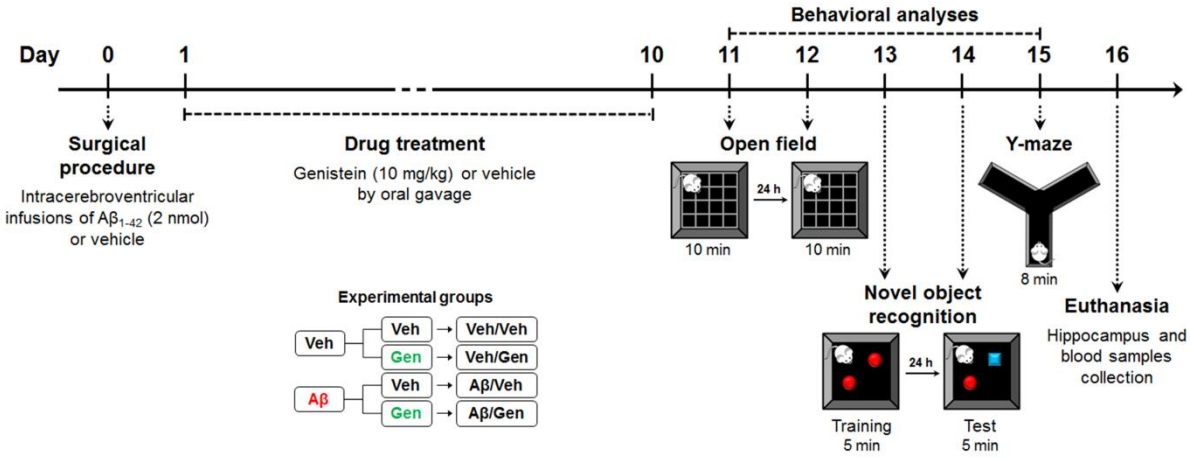

Behavioral analyses
Day
0
1
10
11
12
13
14
15
16
Surgical procedure
Intracerebroventricular infusions of Aβ1-42 (2 nmol) or vehicle
Drug treatment
Genistein (10 mg/kg) or vehicle by oral gavage
Open field
24 h
10 min
10 min
Y-maze
8 min
Novel object recognition
24 h
Training 5 min
Test 5 min
Euthanasia
Hippocampus and blood samples collection
Experimental groups
Veh
Veh
Veh/Veh
Gen
Veh/Gen
Aβ
Veh
Aβ/Veh
Gen
Aβ/Gen

Figure 2

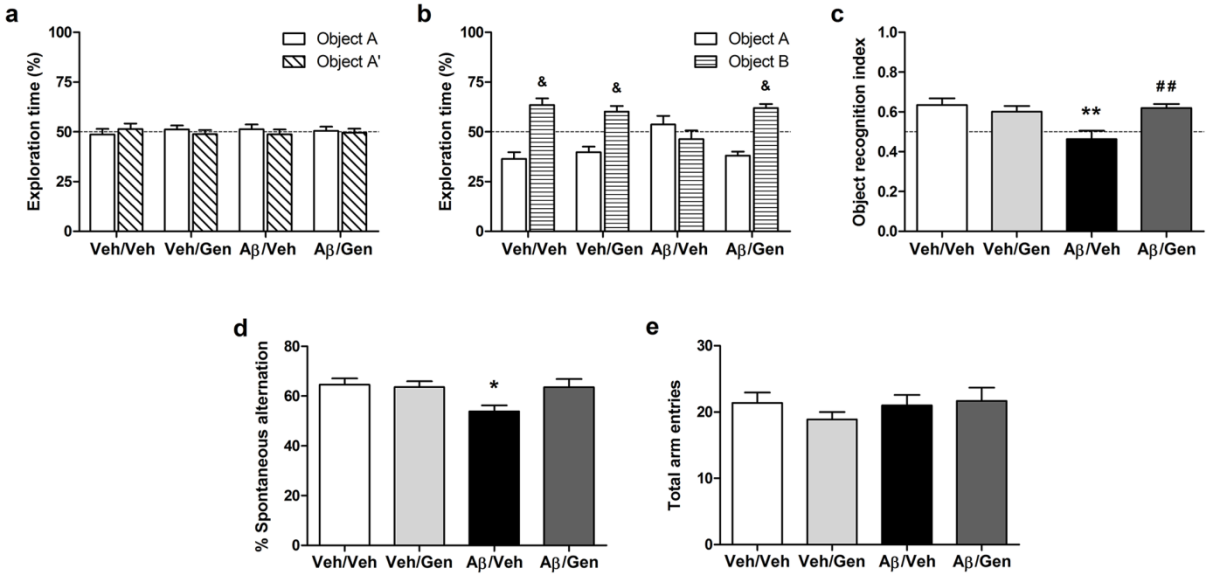

a
Exploration time (%)
Object A
Object A'
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen
b
Exploration time (%)
Object A
Object B
&
c
Object recognition index
**
##
d
% Spontaneous alternation
*
e
Total arm entries

Figure 3

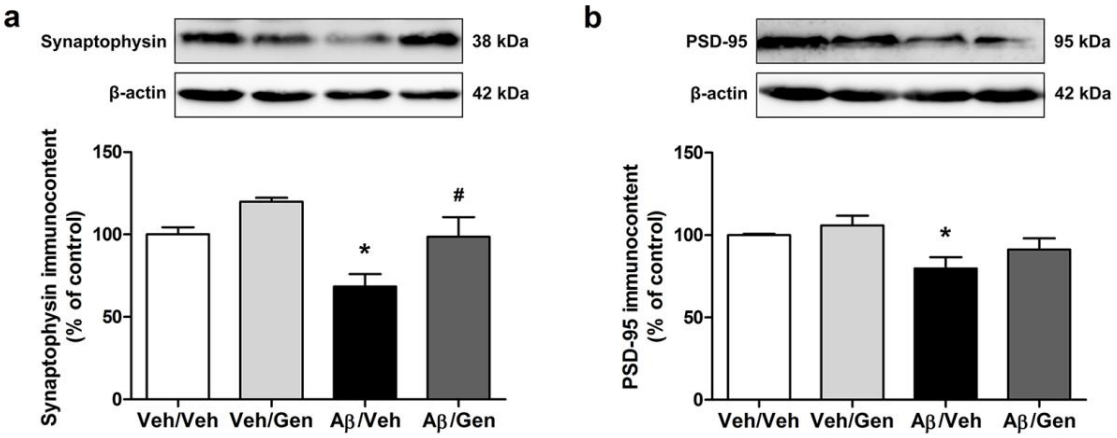

a
Synaptophysin
38 kDa
β-actin
42 kDa
Synaptophysin immunocontent (% of control)
150
100
50
0
*
#
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen
b
PSD-95
95 kDa
β-actin
42 kDa
PSD-95 immunocontent (% of control)
150
100
50
0
*
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen

Figure 4

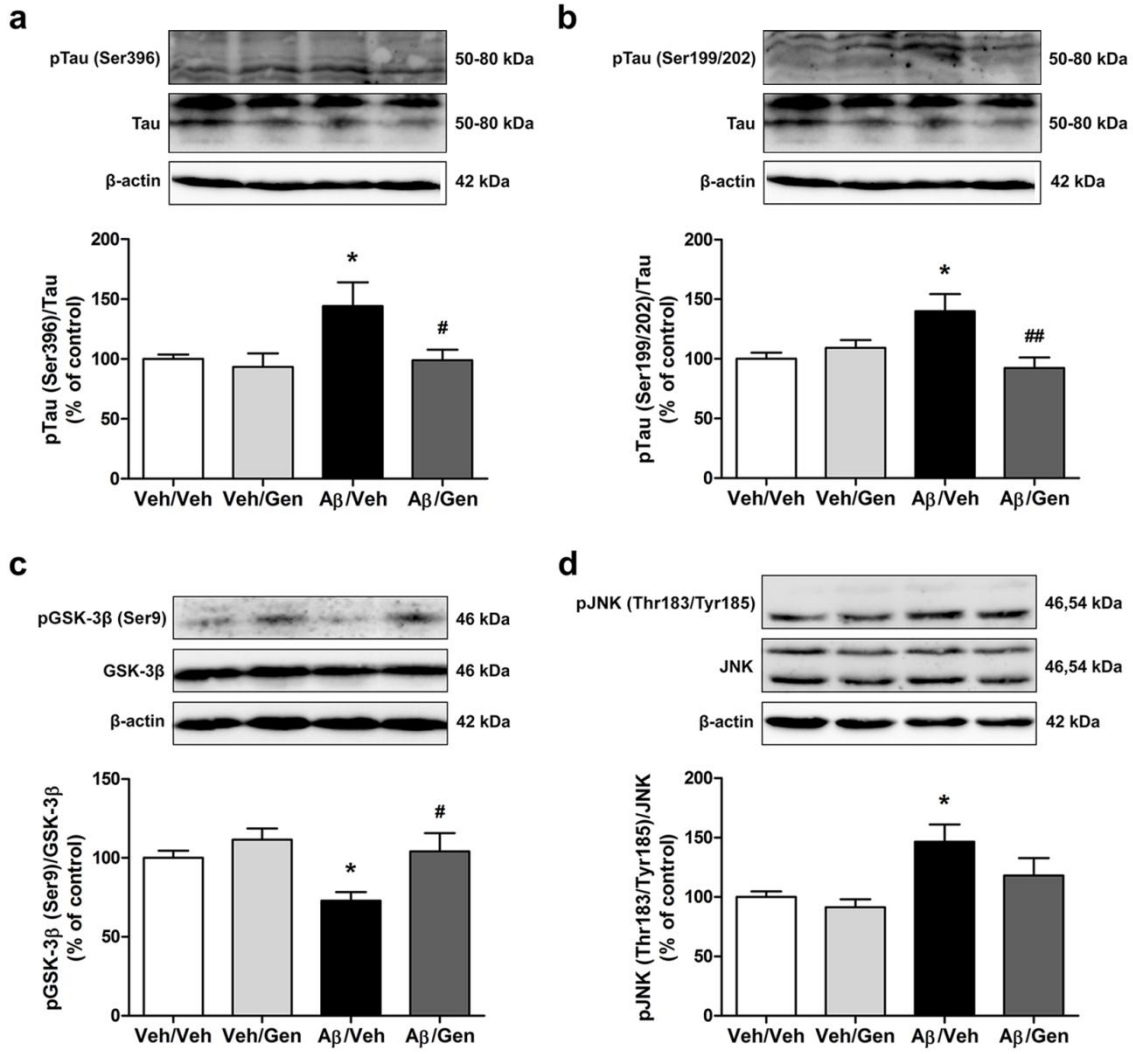

a
pTau (Ser396)
50-80 kDa
Tau
50-80 kDa
β-actin
42 kDa
pTau (Ser396)/Tau (% of control)
200
150
100
50
0
*
#
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen
b
pTau (Ser199/202)
50-80 kDa
Tau
50-80 kDa
β-actin
42 kDa
pTau (Ser199/202)/Tau (% of control)
200
150
100
50
0
*
##
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen
c
pGSK-3β (Ser9)
46 kDa
GSK-3β
46 kDa
β-actin
42 kDa
pGSK-3β (Ser9)/GSK-3β (% of control)
150
100
50
0
*
#
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen
d
pJNK (Thr183/Tyr185)
46,54 kDa
JNK
46,54 kDa
β-actin
42 kDa
pJNK (Thr183/Tyr185)/JNK (% of control)
200
150
100
50
0
*
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen

Figure 5

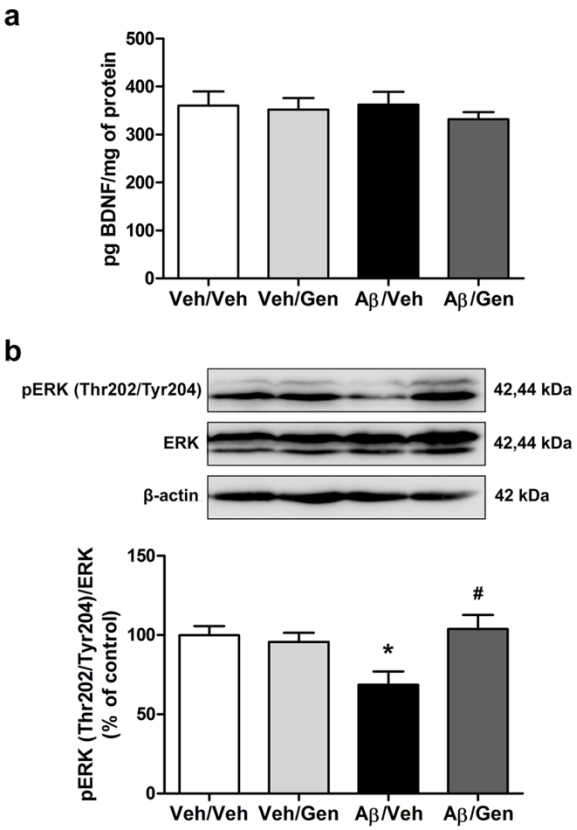

a
pg BDNF/mg of protein
500
400
300
200
100
0
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen
b
pERK (Thr202/Tyr204)
42,44 kDa
ERK
42,44 kDa
β-actin
42 kDa
pERK (Thr202/Tyr204)/ERK
(% of control)
150
100
50
0
*
#
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen

Figure 6

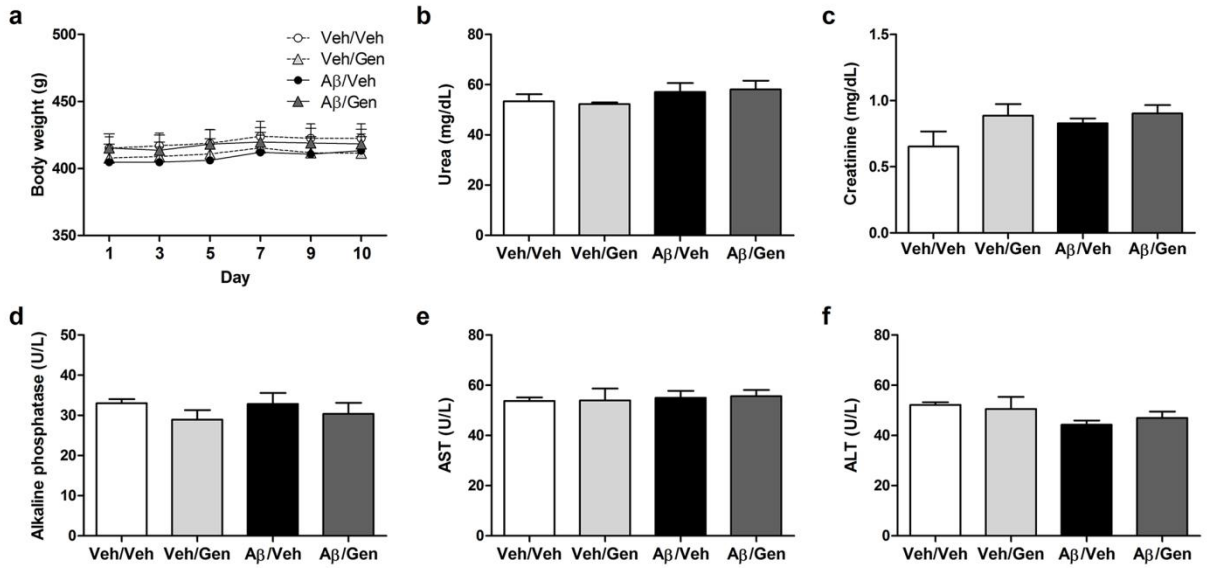

a
Body weight (g)
500
450
400
350
1
3
5
7
9
10
Day
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen
b
Urea (mg/dL)
80
60
40
20
0
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen
c
Creatinine (mg/dL)
1.5
1.0
0.5
0.0
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen
d
Alkaline phosphatase (U/L)
50
40
30
20
10
0
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen
e
AST (U/L)
80
60
40
20
0
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen
f
ALT (U/L)
80
60
40
20
0
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen

Figure 7

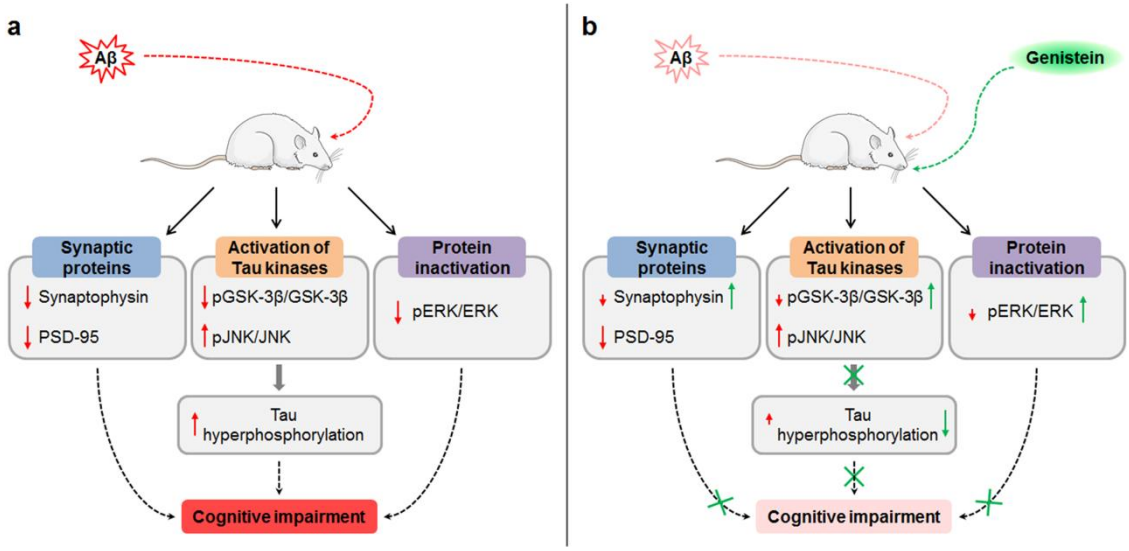

a
Aβ
Synaptic proteins
Synaptophysin
PSD-95
Activation of Tau kinases
pGSK-3β/GSK-3β
pJNK/JNK
Protein inactivation
pERK/ERK
Tau hyperphosphorylation
Cognitive impairment
b
Aβ
Genistein
Synaptic proteins
Synaptophysin
PSD-95
Activation of Tau kinases
pGSK-3β/GSK-3β
pJNK/JNK
Protein inactivation
pERK/ERK
Tau hyperphosphorylation
Cognitive impairment

Table 1. Effects of infusion of $A\beta_{1-42}$ and treatment with genistein on the performance of rats in the open field task.

	Veh/Veh	Veh/Gen	Aβ/Veh	Aβ/Gen	*F* value	*p* value
Day 1						
Total distance travelled (m)	22.7 ± 1.4	19.0 ± 2.0	25.4 ± 2.3	22.7 ± 2.3	(1,37) = 0.052	0.820
Average speed (m/min)	2.3 ± 0.1	1.9 ± 0.2	2.5 ± 0.2	2.3 ± 0.2	(1,37) = 0.052	0.821
Number of crossings	302.3 ± 19.6	256.8 ± 20.9	338.1 ± 22.4	299.4 ± 24.0	(1,37) = 0.024	0.878
Time in the periphery (s)	555. 6 ± 10.6	532.9 ± 18.0	549.9 ± 10.5	521.9 ± 22.7	(1,37) = 0.026	0.874
Time in the center (s)	44.4 ± 10.6	67.1 ± 18.0	50.1 ± 10.5	78.1 ± 22.7	(1,37) = 0.026	0.874
Day 2						
Total distance travelled (m)	16.1 ± 1.6	15.9 ± 2.2	20.0 ± 2.2	19.8 ± 2.5	(1,37) = 0.001	0.999
Average speed (m/min)	1.6 ± 0.2	1.6 ± 0.2	2.0 ± 0.2	1.98 ± 0.3	(1,37) = 0.001	0.999
Number of crossings	201.8 ± 19.2	209.1 ± 26.0	243.6 ± 23.5	248.5 ± 29.4	(1.37) = 0.002	0.963
Time in the periphery (s)	587.4 ± 4.3	572.4 ± 6.6	576.8 ± 5.4	571.5 ± 7.2	(1,37) = 0.645	0.427
Time in the center (s)	12.6 ± 4.3	27.6 ± 6.6	23.2 ± 5.4	28.5 ± 7.2	(1,37) = 0.645	0.427

The results are expressed as the mean ± standard error of the mean for 9–11 animals in each group. The data were analyzed by two-way analysis of variance. Aβ, amyloid-β peptide; Gen, genistein; Veh, vehicle

3.3. CAPÍTULO III

Ganglioside, phospholipid, and cholesterol contents in the frontal cortex of rats after Aβ infusion and treatment with genistein

Fernanda dos Santos Petry, Juliana Bender Hoppe, Caroline Peres Klein, Bernardo Gindri dos Santos, Régis Mateus Hözer, Cristiane Matté, Christianne Gazzana Salbego, Vera Maria Treis Trindade

Status: A ser submetido para publicação

Ganglioside, phospholipid, and cholesterol contents in the frontal cortex of rats after Aβ infusion and treatment with genistein

Fernanda dos Santos Petry[1], Juliana Bender Hoppe[1], Caroline Peres Klein[1], Bernardo Gindri dos Santos[1], Régis Mateus Hözer[1], Cristiane Matté[1,2], Christianne Gazzana Salbego[1,2], Vera Maria Treis Trindade[1,2*]

[1]*Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*

[2]*Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*

[*]Corresponding author:
Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 - Anexo, CEP 90035-003, Porto Alegre, RS, Brazil.
E-mail address: vmtt@ufrgs.br (V.M. T. Trindade)
Phone number: 55 51 3308 5550
ORCID: 0000-0001-5602-6013

Ganglioside, phospholipid, and cholesterol contents in the frontal cortex of rats after Aβ infusion and treatment with genistein

Alzheimer's disease is a neurodegenerative disorder in which synaptic dysfunction, memory damage, imbalance on lipid composition of cell membranes and neuronal death can be observed. In this study, an *in vivo* model of Aβ toxicity was used to investigate the effects of this peptide and the treatment with genistein on the lipid composition in the frontal cortex. Male Wistar rats received bilateral intracerebroventricular infusions of $A\beta_{1-42}$ (2 nmol) and genistein 10 mg/kg orally for 10 days. The Aβ-infused animals showed a significant decrease of ganglioside concentration and relative reduction of GD1b and GQ1b species. Treatment with genistein prevented the decrease in ganglioside levels. Phospholipid and cholesterol contents did not show significant differences. Considering ganglioside roles on neuronal function, findings described here reinforce the neuroprotection of genistein against Aβ toxicity *in vivo* and provide a novel view in the multifaceted scenario associated with its beneficial effects.

Keywords: Alzheimer’s disease; amyloid-beta; genistein; neuroprotection; lipids; cell membrane

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by progressive cognitive decline affecting mainly the memory and also other cognitive functions, whose prevalence increases with the aging (Lane et al. 2018). It is considered that the extracellular accumulation of amyloid-β peptide (Aβ) and the formation of intracellular neurofibrillary tangles (composed of hyperphosphorylated and aggregated Tau protein) are associated with the development of this disease (Chen & Mobley 2019). Aβ peptides (39 to 43 aminoacids) are products of the amyloid precursor protein (APP) proteolysis by the sequential enzymatic actions of β- and γ-secretases. These peptides spontaneously aggregate into soluble oligomers and insoluble fibrils, which accumulate leading to the formation of amyloid plaques (Picone et al. 2020). As a

consequence of this deposition, several pathogenic processes can occur such as triggering of synaptic dysfunction, depletion of neurotrophins and cell death (Selkoe & Hardy 2016; Li et al. 2018).

The pathogenesis of neurodegenerative diseases is closely related to changes in the lipid homeostasis, which can severely affect the neuronal function (Piccinini et al. 2010; Yadav & Tiwari 2014). With the aging process, the physicochemical properties of cell membranes can be altered as a result of an imbalance in their lipid composition (Piccinini et al. 2010). Gangliosides are sialic acid-containing glycosphingolipids, present in high concentrations in neuronal cell membranes, which play important roles in the formation of memories, neuritogenesis and synaptic transmission. Moreover, they are related to functions such as cell differentiation and growth, ion channel modulation, and intercellular signaling (Ariga 2017). Phospholipids exert structural functions in the membrane, also participating in cell signaling. Cholesterol, in turn, is an essential component for the structure and function of cell membranes, modulating their physicochemical properties and contributing to the formation of lipid rafts, specialized membrane microdomains that compartmentalize cellular processes (Santos & Preta 2018). The lipid composition of cell membranes can be related to the pathogenesis of AD in different ways. Glycosphingolipids, especially GM1 ganglioside, can modulate the pathogenic potential of the Aβ peptide by inducing its aggregation and its direct interaction with the cell membrane. On the other hand, the Aβ peptide can also affect the biophysical properties of the membrane, altering its composition and structure, which impairs correct cellular functions (Zhu et al. 2015).

Currently, there is still no effective cure for AD and the existing treatment options act only in the mitigation of symptoms (Lane et al. 2018). In this way, it is necessary to investigate new therapeutic approaches that could enable its treatment or

even its prevention. Genistein (4',5,7-trihydroxyisoflavone), one of the most abundant isoflavones in soy, has been shown to be able to modulate some of the pathogenic processes underlying AD (Oliveira 2016; Devi et al. 2017; Uddin & Kabir 2019). In this field, previous studies have demonstrated that the administration of this compound was capable to ameliorate cognitive deficits (Bagheri et al. 2011; Bonet-Costa et al. 2016; Wang et al. 2016; Pierzynowska et al. 2019), prevent the astrogliosis (Bagheri et al. 2012) and formation of Aβ aggregates (Bagheri et al. 2012; Bonet-Costa et al. 2016; Ren et al. 2018; Pierzynowska et al. 2019), and reduce the neuronal loss in the hippocampus (Wang et al. 2016; Ye et al. 2017).

There are still few studies in the literature focusing on the ability of genistein to protect against the toxicity triggered by the Aβ peptide in animal models (Bagheri et al. 2011; Bagheri et al. 2012; Wang et al. 2016; Ye et al. 2017). Therefore, it is necessary to deepen the evaluation of its potential neuroprotection as well as its underlying mechanisms. In this study, an *in vivo* model of Aβ toxicity was used to investigate the effects of the $A\beta_{1-42}$ peptide and the treatment with genistein on the lipid composition (gangliosides, phospholipids and cholesterol) in the frontal cortex of rats, one of the brain regions most affected in AD.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (aged 90 days, weight 330-450 g) were obtained from the Centro de Reprodução e Experimentação de Animais de Laboratório (Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul [UFRGS], Porto Alegre, Brazil) and maintained at the Departamento de Bioquímica (Universidade Federal do Rio Grande do Sul). The animals were housed in groups of 3–4 per cage with food and

water provided *ad libitum*. They were maintained under optimum light (12/12-h light/dark cycle), temperature (22 ± 1°C), and humidity (50%–60%) conditions. The animals were handled for 2 weeks for adaptation before the experiments were initiated. All experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2011) and the Brazilian Federal Law on Procedures for the Scientific Use of Animals (Law 11.794/2008) and were approved by the institutional Animal Ethics Committee (CEUA/UFRGS) under project number 33552.

2.2. Preparation of $A\beta_{1-42}$ peptide

$A\beta_{1-42}$ peptide (American Peptide Co., Sunnyvale, CA, USA) was dissolved in Tris 0.05 M (pH 7.5), prepared with sterilized bi-distilled water at a concentration of 1 mg/mL, and stored at -20°C. Aliquots of the stock solution were incubated at 37°C for 96 h in order to obtain aggregated Aβ before use (Kreutz et al. 2013).

2.3. Surgical procedure

The animals were anesthetized using a mixture of xylazine 10 mg/kg and ketamine 100 mg/kg administered intraperitoneally and placed in a stereotaxic apparatus. After implementation of standard sterilization procedures, a middle sagittal incision was made in the scalp and holes were drilled bilaterally in the skull over the lateral ventricles using a dental drill. The injection coordinates were chosen according to the Paxinos and Watson rat brain atlas (2007): anteroposterior, -0.8 mm posterior to the bregma; mediolateral, ± 1.5 mm lateral to the sagittal suture; and dorsoventral, -3.5 mm beneath the brain surface. The experimental rats received bilateral intracerebroventricular infusions of $A\beta_{1-42}$ (5 µL; total, 2 nmol in 10 µL) (Frozza et al. 2013). Bilateral intracerebroventricular infusions of an equal volume of Tris 0.05 M (pH 7.5) were

administered to a group of control rats. All microinjections were performed using a 10-µL Hamilton syringe fitted with a 26-gauge needle. All infusions were delivered at a rate of 1 µL/min over a period of 5 min. At the end of infusion, the needle was left in place for a further 3–5 min before being slowly withdrawn to allow diffusion from the tip and prevent reflux of the solution. After the surgical procedure, the scalp was sutured, and the animal was allowed to recover from anesthesia on a heating pad to maintain body temperature at 37.5 ± 0.5°C.

2.4. Drug treatment

One day after the surgical procedure, the rats were randomly divided into four experimental groups. Control animals that received an intracerebroventricular infusion of vehicle were subdivided into two groups that were treated orally with (1) vehicle (Veh/Veh group) or (2) genistein (Veh/Gen group). Animals that received an intracerebroventricular infusion of $A\beta_{1-42}$ were similarly subdivided into two groups that were treated orally with (3) vehicle (Aβ/Veh group) or (4) genistein (Aβ/Gen group).

Genistein (LKT Laboratories, St. Paul, MN, USA) was dissolved at a concentration of 4 mg/mL in 0.9% NaCl solution containing 50% ethanol (Merck Millipore, Darmstadt, Germany). This solution was freshly made up immediately before each administration. Genistein (10 mg/kg body weight) (Lu et al. 2018) was administered via oral gavage daily for 10 consecutive days, starting on the day following the surgical procedure (day 1). The control groups received equivalent volumes of the vehicle. Body weight was checked on alternate days and the genistein dose was adjusted as necessary.

2.5. Collection of samples

Six days after completion of drug treatment (day 16), the animals were euthanized by

decapitation without anesthesia. The brains were removed, and the frontal cortex were quickly dissected out on ice and stored immediately at -80°C until the neurochemical analyses. The neuroprotective effect of genistein against Aβ-induced cognitive impairment was confirmed between days 11-15 (unpublished data).

2.6. Lipid extraction

Frontal cortex were weighed, homogenized in a 2:1 mixture of chloroform:methanol (C:M, 2:1, v/v) to a 20-fold dilution of tissue mass, and centrifuged at 800 *g* for 10 min. The pellet was re-homogenized in C:M (1:2) to a 10-fold dilution of original sample mass (Folch et al. 1957). The C:M extracts were combined and this pool was used for the following determinations.

2.7. Ganglioside evaluation

Aliquots corresponding to the eighth part of the total lipid extract were evaporated and used for total ganglioside determination by the N-acetyl-neuraminic acid (NeuAc) quantification, according to the resorcinol-hydrochloric acid method (Svennerholm 1957), with an adaptation to aqueous medium using dimethyl sulphoxide (Fragoso and Trindade 2015). The absolute total quantity of gangliosides was expressed as nmol NeuAc/mg protein.

Ganglioside species were analyzed by thin layer chromatography (TLC) performed on TLC Silica gel 60 Aluminium sheets (Merck Millipore, Darmstadt, Germany) using a developing tank described by Nores *et al.* (1994). Aliquots of the total lipid extracts containing 60 nmol of NeuAc suspended in 10 µL C:M (1:1) were spotted on 8 mm lanes. TCL was developed, sequentially, with two mixtures of solvents, firstly C:M (4:1, v/v) and secondly C:M:0.25% $CaCl_2$ (60:36:8, v/v/v). Ganglioside profile was visualized with resorcinol reagent (Svennerholm 1957; Lake &

Goodwin 1976). The chromatographic bands were scanned and densitometric analysis was performed using ImageJ software (version 1.52a, National Institutes of Health, Bethesda, MD, USA).

2.8. Phospholipid quantification

Aliquots corresponding to the fourth part of the total lipid extract were evaporated and used for phospholipid determination by the Bartlett method (Bartlett 1959). The absolute total quantity of phospholipids-inorganic phosphorus was expressed as nmol Pi/mg protein.

2.9. Cholesterol quantification

Aliquots of 100 µL of total lipid extract were evaporated, suspended in isopropanol, and quantified according to the Trinder enzymatic technique (Röschlau et al. 1974), using a commercial kit (Bioclin, Belo Horizonte, MG, Brazil). Cholesterol concentrations were expressed as µg cholesterol/mg protein.

2.10. Protein quantification

The protein sediments obtained after lipid extractions were dissolved with NaOH 1 N. Protein concentration was determined according to the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

2.11. Statistical analysis

The data are expressed as the mean ± standard error of the mean. Two-way analysis of variance (ANOVA), with Aβ infusion and treatment with genistein as fixed factors, was applied to determine statistically significant differences between the experimental groups. Post hoc comparisons were performed using the Newman-Keuls test. All

statistical analyses were performed using GraphPad Prism software (version 5.01, San Diego, CA, USA). A *p*-value <0.05 was considered statistically significant.

3. Results and discussion

3.1. Effects of $A\beta_{1-42}$ infusion and treatment with genistein on total lipid content in the frontal cortex of rats

Table 1 presents data relative to total ganglioside, phospholipid, and cholesterol contents in the frontal cortex of rats of the different experimental groups. The statistical analysis showed a reduction of total ganglioside content in rats that received the Aβ infusion followed by oral administration of vehicle in comparison to Veh/Veh group ($p<0.05$). Treatment with genistein in Aβ-injected rats, in contrast, induced a significant increase of total ganglioside content when compared with the Aβ/Veh group ($p<0.05$). It was not observed significant statistical differences in total phospholipid and cholesterol contents between the experimental groups in this cerebral structure.

Disturbances in the lipid composition of cell membranes have been strongly associated with the AD pathogenesis. As to ganglioside levels, previous studies have already demonstrated the significant reduction of total concentration of these glycosphingolipids in postmortem brain tissues of patients with familial or sporadic AD cases, including the frontal and temporal cortices and also the hippocampus (Svennerholm 1994; Svennerholm & Gottfries 1994; Fukami et al. 2017). It seems that not only the pathogenic process underlying AD could be associated with this imbalance but also the aging. Total ganglioside levels may undergo an age-related decline as observed in 12 and 24 months old control mices when compared to 3 or 6 months old ones (Barrier et al. 2007). In addition to that, similar results have been found in other models of diseases affecting the central nervous system, such as inborn errors of

metabolism, estrogen deprivation and ischemia (Monteiro et al. 2005; Stefanello et al. 2007; Simão et al. 2013).

Concerning phospholipid levels, the findings seem to vary considerably between postmortem brain tissues of familial or sporadic AD cases. Svennerholm and Gottfries (1994) demonstrated a significant decrease of its total content in frontal and temporal cortices and also in the hippocampus of familial cases of the disease but not in the sporadic type. The same study evaluated cholesterol concentration in the same brain regions and no significant differences were found in familial or sporadic AD patients. Similar results were observed in the whole tissue of frontal and temporal cortices from AD patients compared to healthy controls (Molander-Melin et al. 2005).

Here, we aimed to evaluate the possible effect of treatment with genistein on probable Aβ-induced alterations on lipid composition of cell membrane. Daily administration of this soy isoflavone for 10 days, starting one day after the induction of the experimental model, successfully protected against the decrease of ganglioside content in the frontal cortex of rats after the infusion of the Aβ peptide.

3.2. Effects of $A\beta_{1-42}$ infusion and treatment with genistein on profile of ganglioside species in the frontal cortex of rats

To further explore the effects of $A\beta_{1-42}$ infusion and treatment with genistein on ganglioside species, we performed TLC to evaluate the profile of these glycosphingolipids. This methodology allowed the detection of the main gangliosides present in the central nervous system of adult rats: GM1, GD1a, GD1b, GT1b and GQ1b (**Figure 1**). **Figure 2** displays the individual ganglioside species, as a percentage of the control, for the different experimental groups. There were significant reductions of GD1b ($p<0.05$) and GQ1b ($p<0.05$) in the frontal cortex of rats that received the Aβ

infusion followed by oral administration of vehicle when compared with the Veh/Veh group. In contrast, administration of genistein in Aβ-injected rats induced a significant increase of both these gangliosides, to a value similar to the control, when compared with the Aβ/Veh group ($p<0.05$).

In addition to the quantitative alterations of gangliosides that can be associated with AD pathogenesis, specific qualitative changes were also detected in postmortem AD brain tissues. Previous works demonstrated that complex gangliosides (GM1, GD1a, GD1b, and GT1b) were significantly reduced in frontal and temporal cortices of AD patients when compared with controls, while simple gangliosides (GM2, GM3, and GM4) were found to be increased (Kracun et al. 1990; Kracun et al. 1991; Kracun et al. 1992). These disturbances in ganglioside homeostasis were suggested to be correlated with the degeneration of cortical neurons and the accelerated lysossomal degradation of these glycosphingolipids, astrogliosis, and neuronal death, respectively (Kracun et al. 1990).

In our experimental model, we observed the specific decrease of GD1b and GQ1b species in the frontal cortex of rats. Similar results have also been found in the cortex of APP^{SL} mice, a transgenic model of AD (Barrier et al. 2007). In addition to that, a specific reduction of GD1b was already observed after the exposure of organotypic hippocampal slice cultures to $A\beta_{25-35}$ peptide (Kreutz et al. 2011). Although GQ1b is a minor ganglioside among the main glycosphingolipids expressed in central nervous system, evidence indicate that it plays an important role in the regulation of cognitive function. The exogenous administration of this ganglioside was able to improve the spatial learning and memory performance of rats (Jung et al. 2008). Moreover, in a transgenic mouse model of AD, GQ1b ameliorated the cognitive impairment and also inhibited some underlying alterations in the hippocampus, such as

deposition of Aβ plaques, hyperphosphorylation of Tau protein, activation of glycogen synthase kinase-3β, and reduction of BDNF expression (Shin et al. 2019). *In vitro* models also demonstrated that this ganglioside was capable to prevent the Aβ-induced death of rat primary cortical neurons (Shin et al. 2019), exert neuritogenic (Nagai 1995) and synaptogenic activities (Mizutani et al. 1996), regulate BDNF expression via the NMDA receptor signaling (Shin et al. 2014), induce long-term potentiation (LTP) (Furuse et al. 1998) and enhance ATP-induced LTP in hippocampal neurons (Fujii et al. 2002).

Here, we found a significant increase in GD1b and GQ1b gangliosides in the frontal cortex of Aβ-injected rats that received the oral administration of genistein. Taking into account the above mentioned roles of mainly GQ1b on cognitive function, we consider that the findings described here could contribute to the neuroprotective effect exerted by this isoflavone against the Aβ-induced toxicity.

4. Conclusion

To the best of our knowledge, this is the first study to focus on the possible effect of genistein treatment on Aβ-induced alterations on lipid composition of cell membranes. In conclusion, we observed that in the *in vivo* model of Aβ toxicity used there was a reduction in the concentration of total gangliosides in the frontal cortex and that genistein was able to completely prevent this alteration. In addition, this compound protected against the specific decreases of GD1b and GQ1b gangliosides. Considering the pivotal roles of gangliosides for neuronal function, the findings described here reinforce the neuroprotection of genistein against Aβ toxicity *in vivo* and provide a novel view in the multifaceted scenario associated with its beneficial effects.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil), Instituto Nacional de Ciência e Tecnologia - INCT (EN 465671/2014-4)/CNPq-Brazil.

Conflicts of interest

There are no conflicts of interest to declare.

References

Ariga T. 2017. The pathogenic role of ganglioside metabolism in Alzheimer's disease-Cholinergic neuron-specific gangliosides and neurogenesis. Mol Neurobiol. 54(1):623–638.

Bagheri M, Joghataei MT, Mohseni S, Roghani M. 2011. Genistein ameliorates learning and memory deficits in amyloid β(1–40) rat model of Alzheimer's disease. Neurobiol Learn Mem. 95(3):270–276.

Bagheri M, Roghani M, Joghataei MT, Mohseni S. 2012. Genistein inhibits aggregation of exogenous amyloid-beta1–40 and alleviates astrogliosis in the hippocampus of rats. Brain Res. 1429:145–154.

Barrier L, Ingrand S, Damjanac M, Bilan AR, Hugon J, Page G. 2007. Genotype-related changes of ganglioside composition in brain regions of transgenic mouse models of Alzheimer's disease. Neurobiol Aging. 28(12):1863–1872.

Bartlett GR. 1959. Phosphorus assay in column chromatography. J Biol Chem. 234(3):466–468.

Bonet-Costa V, Herranz-Pérez V, Blanco-Gandía M, Mas-Bargues C, Inglés M, Garcia-Tarraga P, Rodriguez-Arias M, Miñarro J, Borras C, Garcia-Verdugo JM, Viña J. 2016. Clearing amyloid-β through PPARγ/ApoE activation by genistein is a treatment of experimental Alzheimer's disease. J Alzheimer's Dis. 51(3):701–711.

Chen XQ, Mobley WC. 2019. Alzheimer disease pathogenesis: Insights from molecular and cellular biology studies of oligomeric Aβ and tau species. Front Neurosci. 13:659.

Devi KP, Shanmuganathan B, Manayi A, Nabavi SF, Nabavi SM. 2017. Molecular and therapeutic targets of genistein in Alzheimer's disease. Mol Neurobiol. 54(9):7028–7041.

Folch J, Lees M, Sloane Stanley GH. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem. 226(1):497–509.

Fragoso ALR, Trindade VMT. 2015. Resorcinol-HCl method adaption for N-acetyl neuraminic acid measurement in an aqueous medium. Oral and poster communication at: XXVII Salão de Iniciação Científica da UFRGS; Oct 19-23; Porto Alegre, RS, Brazil. http://hdl.handle.net/10183/136988

Frozza RL, Bernardi A, Hoppe JB, Meneghetti AB, Matté A, Battastini AMO, Pohlmann AR, Guterres SS, Salbego C. 2013. Neuroprotective effects of resveratrol against Aβ administration in rats are improved by lipid-core nanocapsules. Mol Neurobiol. 47(3):1066–1080.

Fujii S, Igarashi K, Sasaki H, Furuse H, Ito KI, Kaneko K, Kato H, Inokuchi JI, Waki H, Ando S. 2002. Effects of the mono-and tetrasialogangliosides GM1 and GQ1b on ATP-induced long-term potentiation in hippocampal CA1 neurons. Glycobiology. 12(5):339-344.

Fukami Y, Ariga T, Yamada M, Yuki N. 2017. Brain gangliosides in Alzheimer's disease: increased expression of cholinergic neuron-specific gangliosides. Curr Alzheimer Res. 14(6):586–591.

Furuse H, Waki H, Kaneko K, Fujii S, Miura M, Sasaki H, Ito KI, Kato H, Ando S. 1998. Effect of the mono- and tetra-sialogangliosides, GM1 and GQ1b, on long-term potentiation in the CA1 hippocampal neurons of the guinea pig. Exp brain Res. 123(3):307–314.

Jung WR, Kim HG, Kim KL. 2008. Ganglioside GQ1b improves spatial learning and memory of rats as measured by the Y-maze and the Morris water maze tests. Neurosci Lett. 439(2):220–225.

Kracun I, Kalanj S, Cosovic C, Talan-Hranilovic J. 1990. Brain gangliosides in Alzheimer's disease. J Hirnforsch. 31(6):789–793.

Kracun I, Kalanj S, Talan-Hranilovic J, Cosovic C. 1992. Cortical distribution of gangliosides in Alzheimer's disease. Neurochem Int. 20(3):433–438.

Kracun I, Rosner H, Drnovsek V, Heffer-Lauc M, Cosovic C, Lauc G. 1991. Human brain gangliosides in development, aging and disease. Int J Dev Biol. 35(3):289–295.

Kreutz F, Frozza RL, Breier AC, Oliveira VA, Horn AP, Pettenuzzo LF, Netto CA, Salbego CG, Trindade VMT. 2011. Amyloid-β induced toxicity involves ganglioside expression and is sensitive to GM1 neuroprotective action. Neurochem Int. 59(5):648–655.

Kreutz F, Scherer EB, Ferreira AGK, Petry FS, Pereira CL, Santana F, Wyse ATS, Salbego CG, Trindade VMT. 2013. Alterations on Na+,K+-ATPase and acetylcholinesterase activities induced by amyloid-β peptide in rat brain and GM1 ganglioside neuroprotective action. Neurochem Res. 38(11):2342–2350.

Lake BD, Goodwin HJ. 1976. Lipids. In: Smith I, Seakings JWT, editors. Chromatographic and eletrophoretic techniques. Paper and thin layer chromatography, vol 1. 4th ed. London: William Heinemann Medical Books Ltd.; p. 345–366.

Lane CA, Hardy J, Schott JM. 2018. Alzheimer's disease. Eur J Neurol. 25(1):59–70.

Li K, Wei Q, Liu FF, Hu F, Xie A, Zhu LQ, Liu D. 2018. Synaptic dysfunction in Alzheimer's disease: Aβ, Tau, and epigenetic alterations. Mol Neurobiol. 55(4):3021–3032.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem. 193(1):265–275.

Lu C, Wang Y, Xu T, Li Q, Wang D, Zhang L, Fan B, Wang F, Liu X. 2018. Genistein ameliorates scopolamine-induced amnesia in mice through the regulation of the cholinergic neurotransmission, antioxidant system and the ERK/CREB/BDNF signaling. Front Pharmacol. 9:1153.

Mizutani A, Kuroda Y, Muramoto K, Kobayashi K, Yamagishi K, Inokuchi J. 1996. Effects of glucosylceramide synthase inhibitor and ganglioside GQ1b on synchronous oscillations of intracellular Ca2+ in cultured cortical neurons. Biochem Biophys Res Commun. 222(2):494–498.

Molander-Melin M, Blennow K, Bogdanovic N, Dellheden B, Mansson JE, Fredman P. 2005. Structural membrane alterations in Alzheimer brains found to be associated with regional disease development; increased density of gangliosides GM1 and GM2 and loss of cholesterol in detergent-resistant membrane domains. J Neurochem. 92(1):171–

182.

Monteiro SC, Stefanello FM, Vianna LP, Matté C, Barp J, Belló-Klein A, Trindade VMT, Wyse ATS. 2005. Ovariectomy enhances acetylcholinesterase activity but does not alter ganglioside content in cerebral cortex of female adult rats. Metab Brain Dis. 20(1):35–44.

Nagai Y. 1995. Functional roles of gangliosides in bio-signalling. Behav Brain Res. 66(1–2):99–104.

National Research Council (U.S.). 2011. Guide for the care and use of laboratory animals. Washington, DC: National Academies Press.

Nores GA, Mizutamari RK, Kremer DM. 1994. Chromatographic tank designed to obtain highly reproducible high-performance thin-layer chromatograms of gangliosides and neutral glycosphingolipids. J Chromatogr A. 686(1):155–157.

Oliveira MR. 2016. Evidence for genistein as a mitochondriotropic molecule. Mitochondrion. 29:35–44.

Paxinos G, Watson C. 2007. The rat brain in stereotaxic coordinates. San Diego: academic Press.

Piccinini M, Scandroglio F, Prioni S, Buccinnà B, Loberto N, Aureli M, Chigorno V, Lupino E, DeMarco G, Lomartire A, et al. 2010. Deregulated sphingolipid metabolism and membrane organization in neurodegenerative disorders. Mol Neurobiol. 41:314–340.

Picone P, Nuzzo D, Giacomazza D, Di Carlo M. 2020. β-amyloid peptide: the cell compartment multi-faceted interaction in Alzheimer's disease. Neurotox Res. 37(2):250–263.

Pierzynowska K, Podlacha M, Gaffke L, Majkutewicz I, Mantej J, Węgrzyn A, Osiadły M, Myślińska D, Węgrzyn G. 2019. Autophagy-dependent mechanism of genistein-mediated elimination of behavioral and biochemical defects in the rat model of sporadic Alzheimer's disease. Neuropharmacology. 148:332–346.

Ren B, Liu Y, Zhang Y, Cai Y, Gong X, Chang Y, Xu L, Zheng J. 2018. Genistein: a dual inhibitor of both amyloid β and human islet amylin peptides. ACS Chem Neurosci. 9(5):1215–1224.

Röschlau P, Bernt E, Gruber W. 1974. Cholesterol and esterified cholesterol. In: Bergmeyer HU, editor. Methods of Enzymatic Analysis. 2nd ed. New York: Academic Press; p. 1890-1893.

Santos AL, Preta G. 2018. Lipids in the cell: organisation regulates function. Cell Mol Life Sci. 75(11):1909–1927.

Selkoe DJ, Hardy J. 2016. The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO Mol Med. 8(6):595–608.

Shin MK, Jung WR, Kim HG, Roh SE, Kwak CH, Kim CH, Kim SJ, Kim KL. 2014. The ganglioside GQ1b regulates BDNF expression via the NMDA receptor signaling pathway. Neuropharmacology. 77:414–421.

Shin MK, Choi MS, Chae HJ, Kim JW, Kim HG, Kim KL. 2019. Ganglioside GQ1b ameliorates cognitive impairments in an Alzheimer's disease mouse model, and causes reduction of amyloid precursor protein. Sci Rep. 9(1):8512.

Simão F, Matté A, Breier AC, Kreutz F, Trindade VMT, Netto CA, Salbego CG. 2013. Resveratrol prevents global cerebral ischemia-induced decrease in lipid content. Neurol Res. 35(1):59–64.

Stefanello FM, Kreutz F, Scherer EBS, Breier AC, Vianna LP, Trindade VMT, Wyse ATS. 2007. Reduction of gangliosides, phospholipids and cholesterol content in cerebral cortex of rats caused by chronic hypermethioninemia. Int J Dev Neurosci. 25(7):473–477.

Svennerholm L. 1957. Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. Biochim Biophys Acta. 24(3):604–611.

Svennerholm L. 1963. Chromatographic separation of human brain gangliosides. J Neurochem. 10(9):613–623.

Svennerholm L. 1994. Ganglioside loss is a primary event in Alzheimer disease Type I. Prog Brain Res. 101(C):391–404.

Svennerholm L, Gottfries CG. 1994. Membrane lipids, selectively diminished in Alzheimer brains, suggest synapse loss as a primary event in early-onset form (type I) and demyelination in late-onset form (type II). J Neurochem. 62(3):1039–1047.

Uddin MS, Kabir MT. 2019. Emerging signal regulating potential of genistein against

Alzheimer’s disease: a promising molecule of interest. Front Cell Dev Biol. 7:197.

Wang Y, Cai B, Shao J, Wang T, Cai R, Ma C, Han T, Du J. 2016. Genistein suppresses the mitochondrial apoptotic pathway in hippocampal neurons in rats with Alzheimer’s disease. Neural Regen Res. 11(7):1153–1158.

Yadav RS, Tiwari NK. 2014. Lipid integration in neurodegeneration: an overview of Alzheimer’s disease. Mol Neurobiol. 50(1):168–176.

Ye S, Wang TT, Cai B, Wang Y, Li J, Zhan J, Shen GM. 2017. Genistein protects hippocampal neurons against injury by regulating calcium/calmodulin dependent protein kinase IV protein levels in Alzheimer’s disease model rats. Neural Regen Res. 12(9):1479–1484.

Zhu D, Bungart BL, Yang X, Zhumadilov Z, Lee JCM, Askarova S. 2015. Role of membrane biophysics in Alzheimer’s-related cell pathways. Front Neurosci. 9:186.

Table 1. Effects of $A\beta_{1-42}$ infusion and treatment with genistein on lipid content in the frontal cortex of rats.

	Veh/Veh	Veh/Gen	Aβ/Veh	Aβ/Gen
Gangliosides (nmol NeuAc/mg protein)	43.29 ± 0.81	44.28 ± 1.51	40.44 ± 0.24*	44.73 ± 0.98#
Phospholipids (nmol Pi/mg protein)	340.23 ± 21.78	379.49 ± 30.03	348.06 ± 21.62	356.24 ± 15.56
Cholesterol (µg cholesterol/mg protein)	176.40 ± 10.35	170.44 ± 10.95	160.48 ± 8.04	151.64 ± 7.40

The results are expressed as the mean ± standard error of the mean for 6-8 animals in each group. *p<0.05 vs Veh/Veh group, #p<0.05 vs Aβ/Veh group (two-way analysis of variance followed by Newman-Keuls post hoc test). Aβ, amyloid-β peptide; Gen, genistein; Veh, vehicle

Figure 1. Representative chromatogram of frontal cortex of rats after $A\beta_{1-42}$ infusion and treatment with genistein. TLC was developed and the ganglioside bands were revealed as described in experimental procedures. The positions of co-chromatographed ganglioside standards (GM1, GD1a, GD1b, GT1b, and GQ1b) are indicated. Gangliosides were named as recommended by Svennerholm (1963). O, origin; 1, Veh/Veh; 2, Veh/Gen; 3, Aβ/Veh; 4, Aβ/Gen. This is a representative experiment from three independent TLC migrations.

Figure 2. Effects of $A\beta_{1-42}$ infusion and treatment with genistein on ganglioside profile in the frontal cortex of rats. Ganglioside species were analyzed by TLC 16 days after infusion of $A\beta_{1-42}$ (2 nmol, intracerebroventricular route) and daily treatment with genistein 10 mg/kg for 10 days. The results correspond to the densitometric analysis of chromatographic bands as shown in Fig. 1. The values represent the mean ± standard error of the mean for individual gangliosides as a percentage of control (5 animals/group). $^{*}p<0.05$ vs. Veh/Veh group; $^{\#}p<0.05$ vs. Aβ/Veh group (two-way analysis of variance followed by Newman-Keuls post hoc test). Aβ, amyloid-β peptide; Gen, genistein; Veh, vehicle

Figure 1

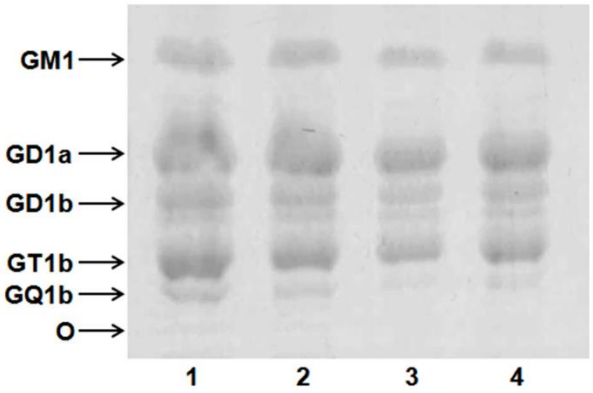
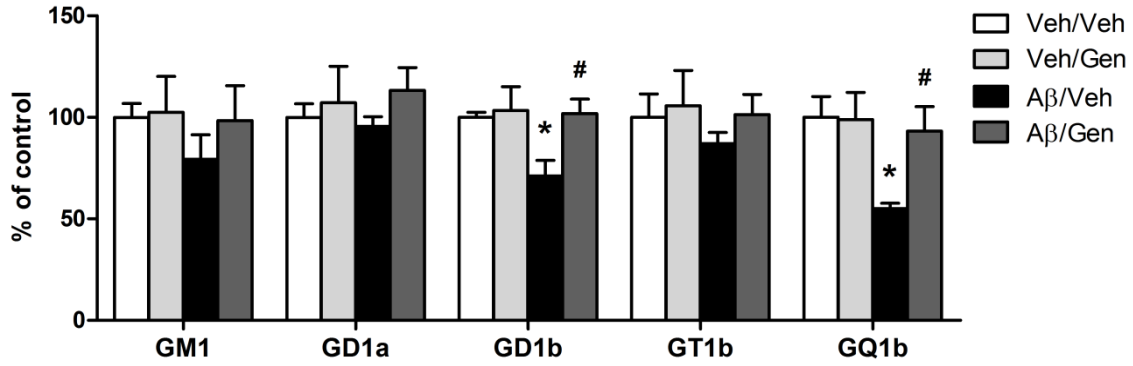
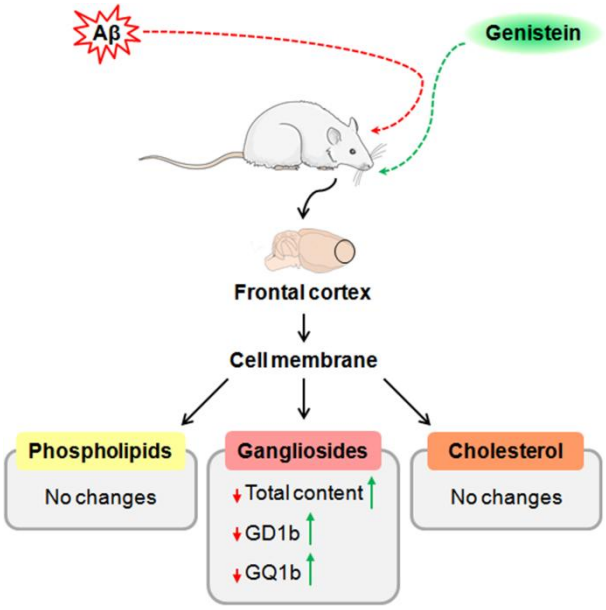


Figure 2


150
100
50
0
% of control
GM1
GD1a
GD1b
GT1b
GQ1b
*
#
*
#
Veh/Veh
Veh/Gen
Aβ/Veh
Aβ/Gen

Graphical abstract



4. DISCUSSÃO

A DA, principal forma de demência relacionada à idade, permanece como um dos grandes desafios em saúde desse século apesar de ter sido descrita há mais de cem anos. Sendo uma desordem neurodegenerativa que se apresenta com deterioração progressiva da memória e das demais funções cognitivas, essa patologia leva à significativa diminuição da qualidade de vida dos pacientes (Alzheimer's Association, 2019; Lane, Hardy e Schott, 2018). Atualmente, um número expressivo de indivíduos é acometido pela DA no Brasil e no mundo (Associação Brasileira de Alzheimer, 2020; Selkoe e Hardy, 2016). Com a tendência de aumento da expectativa de vida e com o consequente crescimento da população idosa global nos próximos anos, projeções da Organização Mundial da Saúde (2019) indicam que o número de indivíduos que desenvolvam alguma forma de demência possa triplicar até 2050, ultrapassando a marca de 150 milhões de casos. Contribuindo em grande parte para o desafio que representa a DA, tem-se a ausência de sucesso na farmacoterapia atualmente disponível, já que as limitadas opções de tratamento oferecem apenas a atenuação dos sintomas por tempo restrito e não são capazes de interferir na evolução do processo patogênico em curso (Alzheimer's Association, 2019; Ibrahim *et al.*, 2020; Long e Holtzman, 2019). Por esses motivos, inúmeros esforços têm sido realizados na tentativa de identificar novas estratégias terapêuticas que possam prevenir, tratar ou retardar com maior sucesso a DA.

O acúmulo extracelular do peptídeo Aβ é considerado um evento precoce na fisiopatologia da DA, embora distante da evidência de sintomas clínicos. No entanto, o acúmulo de Aβ poderia induzir uma série de alterações bioquímicas e celulares, as quais contribuiriam, por sua vez, para a disfunção neuronal e para a morte celular observadas (Selkoe e Hardy, 2016). Na busca de estratégias alternativas que sejam capazes de prevenir ou ainda de interferir nesses processos, as isoflavonas, que fazem parte da categoria flavonoide de compostos polifenólicos abundantes na soja, têm recebido crescente atenção por seus efeitos potencialmente benéficos para a função cerebral (Lee, Lee e Sohn, 2005; Vauzour, 2012). A genisteína, uma das isoflavonas predominantes na soja, tem reconhecidas propriedades antioxidante, anti-inflamatória e estrogênica cujos efeitos têm sido muito explorados na profilaxia ou no tratamento do câncer, de doenças cardiovasculares, de alterações decorrentes do período de pós-menopausa, dentre outros (Altavilla *et al.*, 2004; Chen, Ko e Chen, 2019; Messina, 2014; Russo *et al.*, 2016; Si e Liu, 2007; Spagnuolo *et al.*, 2015; Taylor *et al.*, 2009; Thangavel *et al.*, 2019; Tuli *et al.*, 2019). Nos últimos anos, também, têm sido demonstrados seus efeitos benéficos

sobre a neurotoxicidade e a neurodegeneração em diferentes modelos para o estudo da DA (Devi *et al.*, 2017; Uddin e Kabir, 2019). Entretanto, embora as evidências indiquem que a genisteína seja um composto potencialmente neuroprotetor, ainda são necessárias a investigação e a melhor compreensão dos mecanismos responsáveis por seus efeitos nessa área. Tendo isso em vista, os objetivos centrais desta tese foram avaliar o potencial efeito neuroprotetor do tratamento com genisteína sobre a toxicidade induzida pelo peptídeo Aβ e elucidar alguns dos mecanismos possivelmente relacionados, utilizando para isso modelos experimentais *in vitro* e *in vivo*.

O Capítulo I desta tese teve como objetivo investigar, em modelo *in vitro*, o efeito da genisteína sobre a morte celular induzida pelo peptídeo $A\beta_{25-35}$ e o envolvimento das proteínas Akt, GSK-3β e Tau como possível mecanismo relacionado à sua neuroproteção. Para isso, foram utilizadas células SH-SY5Y que correspondem a um modelo *in vitro* bem estabelecido para o estudo de dano celular decorrente da exposição a agentes neurotóxicos e também para a avaliação de compostos neuroprotetores (Cheung *et al.*, 2009; Lopes *et al.*, 2010; Xicoy, Wieringa e Martens, 2017). Essas células são neuroblastos que podem ser diferenciados ao fenótipo neuronal maduro a partir da exposição a agentes de diferenciação como o ácido retinoico (Kovalevich e Langford, 2013). Com isso, as células adquirem morfologia característica, com o desenvolvimento de neuritos extensos e ramificados, expressam marcadores neuronais específicos e apresentam excitabilidade madura (Cheung *et al.*, 2009; Kunzler *et al.*, 2017; Lopes *et al.*, 2010; Toselli, Tosetti e Taglietti, 1996; Tosetti, Taglietti e Toselli, 1998). Células SH-SY5Y, tanto em seu fenótipo não diferenciado quanto em seu fenótipo diferenciado, têm sido extensivamente utilizadas para o estudo da toxicidade induzida por diferentes fragmentos do peptídeo Aβ (Arbo *et al.*, 2016; Bang *et al.*, 2004; Huang, Xu e Jiang, 2012; Lattanzio *et al.*, 2016; Xi *et al.*, 2016; Xu *et al.*, 2019). Considerando que o processo de diferenciação leva à significativa alteração no perfil bioquímico das células e que isso poderia influenciar na sua suscetibilidade a agentes neurotóxicos, o objetivo inicial deste trabalho foi avaliar a viabilidade e o dano sofrido por células não diferenciadas e diferenciadas após a exposição a diferentes concentrações (7,5, 25 e 50 μM) do peptídeo $A\beta_{25-35}$ por 72h. Observou-se diferença na suscetibilidade desses dois fenótipos celulares, já que somente células diferenciadas tiveram significativa redução de sua viabilidade e aumento do dano celular após a exposição às três concentrações utilizadas de $A\beta_{25-35}$. A partir desses resultados, optou-se por utilizar células diferenciadas expostas à concentração intermediária de $A\beta_{25-35}$ (25

μM) para os experimentos posteriores. Conforme demonstrado anteriormente em modelo *in vitro*, esse fragmento do peptídeo induz efeitos tóxicos comparáveis aos desencadeados pelo $A\beta_{1-42}$ no que se refere à morte celular e à ativação de proteínas apoptóticas, sendo considerada uma ferramenta viável para a investigação dos mecanismos neurotóxicos envolvidos na DA (Frozza *et al.*, 2009). Para todos os experimentos, o peptídeo $A\beta_{25-35}$ foi submetido ao processo de agregação conforme descrito previamente (Frozza *et al.*, 2009; Kreutz *et al.*, 2011).

Com o objetivo de confirmar o já sugerido efeito neuroprotetor da genisteína sobre a morte celular induzida pelo peptídeo Aβ (Bang *et al.*, 2004; Wang *et al.*, 2019; Xu *et al.*, 2019; You *et al.*, 2017; Yu *et al.*, 2009; Zeng, Chen e Zhao, 2004), células diferenciadas foram pré-tratadas por 24h com genisteína (1 e 10 nM) e, então, expostas ao $A\beta_{25-35}$ (25 μM) por 72h, período durante o qual a genisteína foi mantida no meio de cultivo. As concentrações de genisteína foram escolhidas com base nas menores concentrações descritas na literatura com efeito significativo sobre a redução da viabilidade celular causada pelo Aβ (Bang *et al.*, 2004). Corroborando os resultados descritos anteriormente, observou-se que esse composto, nas duas concentrações testadas, foi capaz de proteger contra a morte celular induzida pelo $A\beta_{25-35}$, reduzindo em parte a apoptose e totalmente a necrose. A fim de explorar alguns mecanismos que poderiam estar envolvidos nesse efeito, procedeu-se à avaliação dos níveis das proteínas Akt, GSK-3β e Tau, nas formas total e fosforilada, após o tratamento com genisteína (10 nM) e a exposição ao $A\beta_{25-35}$ (25 μM) por 24h. Sabe-se que a Akt, proteína envolvida na regulação da sobrevivência celular e da apoptose, pode estar menos ativa na DA, sendo esse um dos mecanismos de toxicidade desencadeados pelo Aβ (Hoppe *et al.*, 2013; Magrané *et al.*, 2005). Como consequência direta disso, tem-se a diminuição da capacidade da Akt em realizar a fosforilação inibitória da GSK-3β em Ser9, o que torna essa proteína anormalmente ativa (Cai, Zhao e Zhao, 2012; Hernández *et al.*, 2010; Lauretti, Dincer e Praticò, 2020). Por ser uma das principais cinases responsáveis pela fosforilação da Tau, o aumento da ativação da GSK-3β pode contribuir diretamente para a hiperfosforilação dessa proteína e também para a morte celular induzida pelo peptídeo Aβ (Martin *et al.*, 2013b). Dessa forma, drogas que sejam capazes de modular a ativação da Akt e da GSK-3β e, ainda, de prevenir a hiperfosforilação da Tau podem potencialmente contribuir para a diminuição do dano e da morte celular induzidos pelo peptídeo Aβ. Conforme descrito no Capítulo I, observou-se que o peptídeo desencadeou

diminuição nos níveis de Akt fosforilada (ativa) e de GSK-3β fosforilada (inativa), além de aumento nos níveis de Tau fosforilada. O tratamento com genisteína, por sua vez, foi capaz de prevenir a inativação da Akt e a hiperfosforilação da Tau e, ao menos em parte, de diminuir a ativação da GSK-3β desencadeada pelo $A\beta_{25-35}$. Com esses resultados, foi possível confirmar o efeito neuroprotetor da genisteína sobre a morte celular induzida pelo peptídeo $A\beta_{25-35}$ e, ainda, sugerir que a prevenção da inativação da Akt e da hiperfosforilação da Tau desencadeadas por esse composto possa contribuir para esse efeito.

Como já mencionado na introdução desta tese, são poucos os trabalhos na literatura que relatam os efeitos da genisteína em modelos experimentais *in vivo* para o estudo da DA. Considerando os resultados obtidos no Capítulo I e, ainda, com o intuito de aprofundar a investigação da neuroproteção desencadeada por esse composto, no Capítulo II foi realizado um modelo *in vivo* para avaliar o efeito do tratamento com genisteína sobre o dano cognitivo induzido pelo peptídeo $A\beta_{1-42}$ e para elucidar alguns dos mecanismos relacionados à sua possível neuroproteção no hipocampo. Entre os modelos experimentais *in vivo* utilizados para o estudo da DA, o modelo de intervenção a partir da administração intracerebral do peptídeo Aβ em roedores é bem estabelecido para a investigação dos mecanismos envolvidos na neurotoxicidade e na neurodegeneração induzidas por esse peptídeo, bem como para a avaliação pré-clínica de drogas candidatas com alvo de atuação nesses processos. Embora a complexidade da doença observada em humanos não seja completamente reproduzida nesse modelo, ele permite mimetizar aspectos característicos da patologia, como o déficit de aprendizado e de memória, além de alterações neuropatológicas em determinadas regiões cerebrais (Laurijssens, Aujard e Rahman, 2013; Van Dam e De Deyn, 2011). Neste trabalho, foi utilizado o modelo de infusão intracerebroventricular de $A\beta_{1-42}$ (2 nmol) fibrilado em ratos *Wistar* machos adultos, conforme trabalhos prévios (Frozza *et al.*, 2013; Hoppe *et al.*, 2013; Kreutz *et al.*, 2013). As seguintes tarefas comportamentais foram realizadas sequencialmente do 11º ao 15º dia após a indução do modelo experimental: campo aberto, para avaliação da atividade locomotora e do comportamento exploratório espontâneo; reconhecimento de objetos, para análise da memória de reconhecimento de longo prazo; e Y-*maze*, para avaliação do comportamento de alternância espontânea que reflete a memória espacial de trabalho. Corroborando resultados anteriores (Frozza *et al.*, 2013; Hoppe *et al.*, 2013; Kreutz *et al.*, 2013), demonstrou-se que a infusão intracerebroventricular do peptídeo $A\beta_{1-42}$ induziu dano cognitivo conforme

evidenciado pelo prejuízo da memória de reconhecimento e do comportamento de alternância espontânea sem, no entanto, alterar a atividade locomotora dos animais. Para a avaliação do efeito da genisteína sobre o déficit de memória induzido pelo $A\beta_{1-42}$, 24h após a indução do modelo experimental, foi iniciada a administração, por gavagem, desse composto na concentração de 10 mg/kg, a qual foi realizada por 10 dias. Como a literatura descreve a administração de genisteína em concentrações muito variáveis, a concentração aqui escolhida foi baseada em trabalho prévio em que foi demonstrado o efeito positivo desse composto em modelo de amnésia induzida pela administração de escopolamina (Lu *et al.*, 2018). Conforme descrito no capítulo II, observou-se que o tratamento com genisteína foi capaz de melhorar o desempenho cognitivo dos animais ao prevenir o prejuízo da memória de reconhecimento e ao impedir, ao menos em parte, a diminuição do comportamento de alternância espontânea induzidos pelo peptídeo $A\beta_{1-42}$.

Com o intuito de elucidar alguns dos mecanismos que poderiam estar envolvidos no efeito neuroprotetor desencadeado pela genisteína, seguiu-se à avaliação de alguns parâmetros neuroquímicos hipocampais conhecidamente alterados pela toxicidade causada pelo peptídeo $A\beta_{1-42}$. O hipocampo é uma estrutura com papel crucial nos processos de aprendizado e de formação da memória, sendo primariamente afetado na DA (Masters *et al.*, 2015). Conforme apresentado na introdução desta tese, inúmeros eventos patogênicos subjacentes ao acúmulo do peptídeo Aβ podem levar ao prejuízo progressivo da função sináptica. Sugere-se inclusive que a disfunção e a perda sináptica sejam as alterações mais bem correlacionadas com a manifestação de déficit cognitivo (Pooler, Noble e Hanger, 2014). Em vista disso, como parâmetros indicativos da integridade sináptica, foram avaliados os imunoconteúdos da sinaptofisina, proteína abundante na membrana da vesícula sináptica, e também da proteína de densidade pós-sináptica 95 (PSD-95). Observou-se que o peptídeo $A\beta_{1-42}$ induziu significativa redução nos níveis hipocampais dessas duas proteínas. A administração de genisteína atenuou a sinaptotoxicidade causada pelo $A\beta_{1-42}$ ao prevenir ou reverter a redução do imunoconteúdo de sinaptofisina sem, no entanto, ser efetiva sobre os níveis de PSD-95.

Considera-se que a disfunção sináptica na DA esteja intimamente relacionada à hiperfosforilação da proteína Tau e à formação dos emaranhados neurofibrilares (Naseri *et al.*, 2019; Pooler, Noble e Hanger, 2014). Conforme mencionado anteriormente, o acúmulo do peptídeo Aβ pode desencadear a hiperfosforilação da Tau, levando à perda de sua afinidade pelos microtúbulos e à desestabilização dessas estruturas, o que

culmina no comprometimento do transporte axonal e, por consequência, na disfunção sináptica (Khan e Bloom, 2016; Pooler, Noble e Hanger, 2014). No presente estudo, demonstrou-se que a infusão intracerebroventricular do peptídeo $A\beta_{1-42}$ provocou a hiperfosforilação da Tau conforme evidenciado pelo aumento da forma fosforilada dessa proteína em diferentes sítios (Ser396 e Ser199/202). O tratamento com genisteína, assim como demonstrado no modelo *in vitro*, preveniu essa alteração induzida pelo peptídeo. A fim de ainda explorar esses efeitos, e considerando o aumento na ativação de cinases atribuído ao acúmulo do peptídeo Aβ que acaba por contribuir para a hiperfosforilação da Tau, decidiu-se por avaliar duas das cinases que poderiam estar envolvidas nessa alteração. Conforme relatado anteriormente, no modelo *in vitro* inicialmente utilizado (Capítulo I) demonstrou-se o efeito parcial da genisteína sobre a ativação da GSK-3β induzida pela exposição ao peptídeo Aβ. Dessa forma, além de confirmar se esse efeito seria também observado no modelo *in vivo*, decidiu-se por ampliar a investigação das cinases da Tau possivelmente envolvidas, avaliando também a JNK. Corroborando o resultado encontrado no modelo *in vitro*, observou-se que a infusão intracerebroventricular do peptídeo $A\beta_{1-42}$ induziu ativação da GSK-3β. Além disso, o peptídeo provocou também o aumento na ativação da JNK, conforme evidenciado pelo aumento da forma fosforilada dessa proteína. A administração de genisteína, nesse caso, foi completamente eficaz na prevenção da hiperativação da GSK-3β, embora tenha tido efeito parcial sobre a ativação da JNK.

Com o objetivo ainda de investigar os mecanismos envolvidos na neuroproteção exercida pela genisteína sobre o dano cognitivo desencadeado pelo peptídeo $A\beta_{1-42}$, foram avaliados os níveis de BDNF e também da proteína ERK. O BDNF, a partir da ligação a seu receptor TrkB com a consequente ativação de proteínas intracelulares, como a ERK, desempenha função importante na sobrevivência neuronal e na plasticidade sináptica, participando também dos mecanismos necessários à formação e à manutenção da memória (Cunha, Brambilla e Thomas, 2010; Miranda *et al.*, 2019). Sugere-se que o acúmulo do peptídeo Aβ possa levar à redução nos níveis dessa neurotrofina (Hoppe *et al.*, 2013; Peng *et al.*, 2009). Surpreendentemente, neste trabalho não foi observada alteração nos níveis hipocampais de BDNF após a infusão intracerebroventricular de $A\beta_{1-42}$. Entretanto, detectou-se que o peptídeo provocou diminuição na ativação da ERK, conforme evidenciado pela redução do imunoconteúdo da forma fosforilada dessa proteína. A administração de genisteína mostrou-se capaz de prevenir esse efeito. Dessa forma, não se pode excluir a possibilidade de que tenha

ocorrido precocemente a diminuição nos níveis de BDNF e que, por um mecanismo compensatório, seus níveis tenham sido restabelecidos. Essa possibilidade foi sugerida em estudo a partir da avaliação de cérebros de pacientes com DA em que foi demonstrado aumento na concentração de BDNF no hipocampo e no córtex parietal, o que se atribuiu a um mecanismo de compensação em resposta ao processo de neurodegeneração (Durany *et al.*, 2000). Com base nos resultados descritos nesse capítulo, foi possível concluir que o tratamento com genisteína foi capaz de melhorar o dano cognitivo induzido pela infusão intracerebroventricular do peptídeo $A\beta_{1-42}$ e que esse efeito possivelmente esteja relacionado à atenuação da sinaptotoxicidade, à diminuição da hiperfosforilação da Tau e à prevenção da inativação da ERK no hipocampo.

No Capítulo III desta tese, dando seguimento à avaliação da neuroproteção da genisteína em modelo *in vivo* de toxicidade induzida pelo peptídeo $A\beta_{1-42}$, levantou-se a hipótese de que esse composto, além de desencadear efeitos intracelulares (conforme descrito nos Capítulos I e II), poderia também atuar em nível de membrana celular. Sabe-se que o processo neurodegenerativo pode estar associado ao prejuízo das propriedades físico-químicas das membranas, ocasionado por alterações no equilíbrio de sua composição lipídica (Yadav e Tiwari, 2014). Além disso, a neurotoxicidade induzida pelo Aβ se dá primariamente pela interação desse peptídeo com a membrana celular, o que pode afetar sua integridade, fluidez e propriedades sinalizatórias (Di Paolo e Kim, 2011; Wong *et al.*, 2017; Zhu *et al.*, 2015). Sendo assim, alterações na composição lipídica dos principais componentes das membranas neurais (fosfolipídios, colesterol e gangliosídios) podem afetar não só a estrutura, mas também funções celulares como o transporte de membrana e a sinalização, o que contribuiria para o prejuízo da transmissão sináptica e do processo de formação da memória (Ariga, 2017; Lim, Martins e Martins, 2014; Santos e Preta, 2018; Yang, Lee e Fairn, 2018). Além disso, não havia até o presente momento, trabalho na literatura que tenha avaliado o efeito da genisteína sobre esses parâmetros. Diante disso, o Capítulo III desta tese teve como objetivo avaliar o efeito da infusão intracerebroventricular do peptídeo $A\beta_{1-42}$ e do tratamento com genisteína sobre a composição lipídica (gangliosídios, fosfolipídios e colesterol) de membranas neurais em córtex frontal de ratos, outra região cerebral significativamente afetada na DA. Observou-se que o peptídeo $A\beta_{1-42}$ provocou diminuição no conteúdo total de gangliosídios sem, no entanto, afetar o conteúdo de colesterol ou de fosfolipídios. Por outro lado, a administração de genisteína (10 mg/kg),

durante 10 dias, foi capaz de prevenir a alteração no conteúdo de gangliosídios desencadeada pelo $A\beta_{1-42}$.

Com o intuito de explorar os efeitos acima descritos e, ainda, considerando que se sugere que a degeneração de neurônios corticais possa estar relacionada à redução específica de gangliosídios complexos como GM1, GD1a, GD1b, GT1b e GQ1b, decidiu-se por avaliar o perfil desses glicoesfingolipídios nos diferentes grupos experimentais. Observou-se a redução específica de GD1b e de GQ1b após a infusão do peptídeo $A\beta_{1-42}$, corroborando achados prévios em modelo transgênico da DA e em modelo *in vitro* de toxicidade do peptídeo $A\beta_{25-35}$ (Barrier *et al.*, 2007; Kreutz *et al.*, 2011). A administração de genisteína, por sua vez, foi efetiva na prevenção dessas alterações. Neste capítulo, então, foi demonstrado que o tratamento com genisteína foi capaz de prevenir a redução no conteúdo total de gangliosídios, bem como a diminuição específica de GD1b e de GQ1b, observadas no córtex frontal de ratos após a infusão intracerebroventricular do peptídeo $A\beta_{1-42}$. Certamente mais estudos são necessários para a melhor compreensão do efeito protetor desse composto sobre as membranas neurais. No entanto, considerando o papel crucial exercido pelos gangliosídios para a adequada função celular, os efeitos descritos para a genisteína neste capítulo podem contribuir para a neuroproteção desencadeada por esse composto sobre a toxicidade induzida pelo peptídeo $A\beta_{1-42}$ *in vivo*.

Finalizando, nesta tese foram utilizados dois modelos experimentais de toxicidade induzida pelo peptídeo Aβ com o intuito de explorar e de melhor compreender o potencial neuroprotetor atribuído à genisteína. Os resultados aqui demonstrados permitiram confirmar e reforçar os efeitos neuroprotetores da genisteína sobre a morte celular (modelo *in vitro)* e sobre o dano cognitivo induzido (modelo *in vivo*) pelo peptídeo Aβ e, sobretudo, ampliar o conhecimento atual acerca dos mecanismos relacionados à sua neuroproteção (Figura 1).

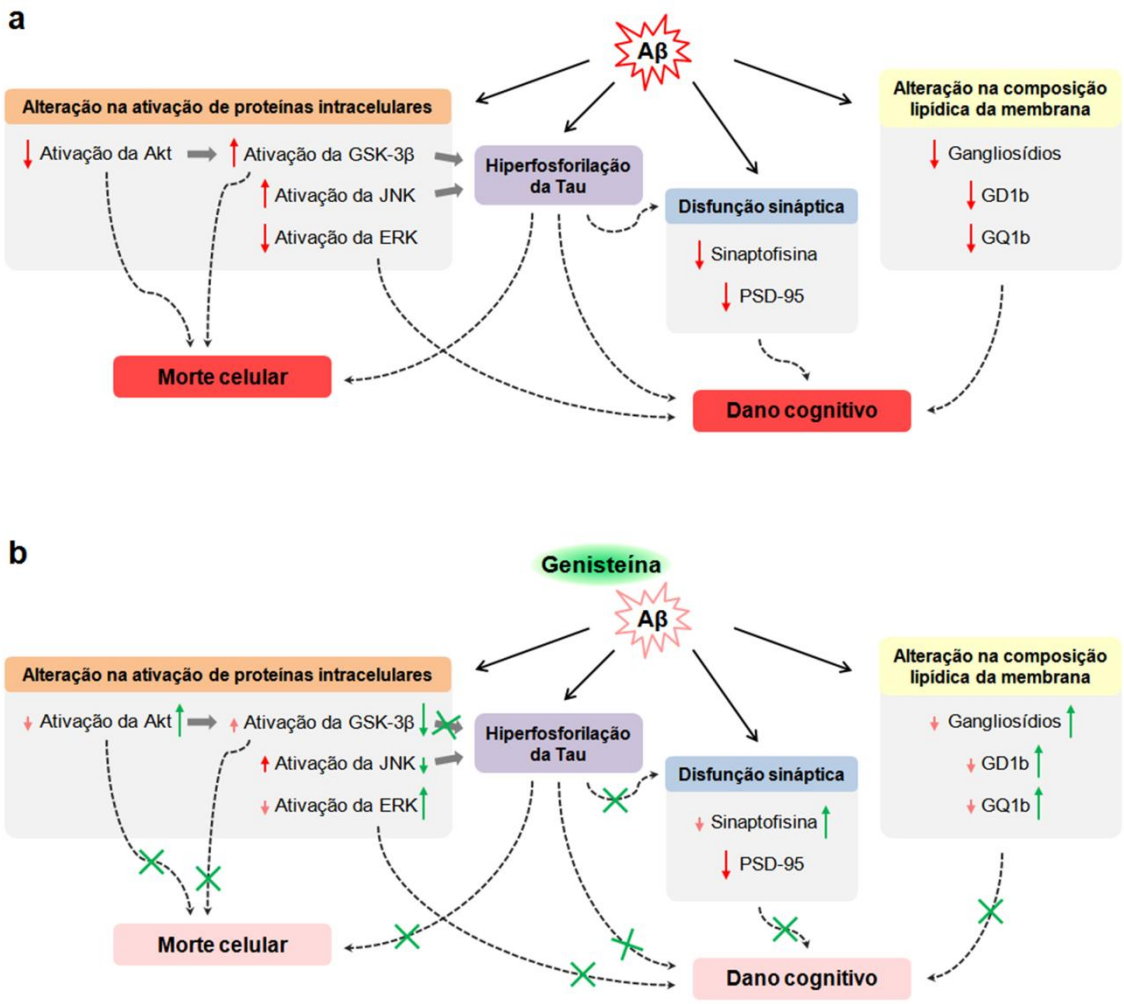


Figura 1. Principais mecanismos envolvidos na neuroproteção da genisteína, observada em modelos *in vitro* e *in vivo*, sobre a morte celular e sobre o dano cognitivo induzidos pelo peptídeo Aβ. Setas vermelhas indicam o efeito do peptídeo Aβ. Setas e linhas cruzadas verdes indicam o efeito da genisteína. Setas contínuas cinzas indicam ação enzimática. Setas tracejadas representam processos subjacentes levando à morte celular e ao dano cognitivo.

5. CONCLUSÕES

Nesta tese foram apresentados os resultados obtidos no estudo dos mecanismos envolvidos na neuroproteção da genisteína em modelos *in vitro* e *in vivo* de toxicidade induzida pelo peptídeo Aβ.

Os resultados aqui descritos permitiram concluir que a genisteína apresenta um importante efeito neuroprotetor sobre os danos induzidos pelo peptídeo Aβ, conforme evidenciado pelos seguintes resultados:

- O tratamento com genisteína diminui a morte celular desencadeada pelo peptídeo Aβ ao prevenir a inativação da Akt e a hiperfosforilação da Tau e, ao menos em parte, a ativação da GSK-3β;

- A administração de genisteína melhora o dano cognitivo induzido pelo peptídeo Aβ em ratos, além de atenuar a sinaptotoxicidade e prevenir a hiperfosforilação da Tau e a inativação da ERK no hipocampo;

- A genisteína previne alterações na composição lipídica das membranas neurais (diminuição nos níveis de gangliosídios) provocadas pelo peptídeo Aβ no córtex frontal de ratos.

Dessa forma, os resultados aqui demonstrados permitiram confirmar e reforçar os efeitos neuroprotetores da genisteína sobre a toxicidade induzida pelo peptídeo Aβ e, sobretudo, ampliar o conhecimento atual acerca dos mecanismos relacionados à sua neuroproteção.

6. PERSPECTIVAS

As principais perspectivas de estudo que surgem ao término desta tese são as seguintes:

- Aprofundar a investigação relativa ao efeito protetor da genisteína sobre o dano à membrana induzido pelo peptídeo Aβ por meio da avaliação da integridade dos microdomínios de membrana em modelos *in vitro* e *in vivo*;

- Investigar, em modelos *in vitro* e *in vivo*, o possível efeito antiamiloidogênico da genisteína ao influenciar a localização das proteínas APP e BACE1 nos microdomínios *rafts*;

- Explorar os efeitos da genisteína sobre a inflamação desencadeada pelo peptídeo Aβ em modelos *in vitro* e *in vivo*.

7. REFERÊNCIAS BIBLIOGRÁFICAS

ALBERTS, B.; JOHNSON, A.; LEWIS, J.; MORGAN, D.; RAFF, M.; ROBERTS, K.; WALTER, P.; WILSON, J.; HUNT, T. **Biologia Molecular da Célula**. 6. ed. Porto Alegre: Artmed, 2017.

ALMEIDA, S.; ALVES, M. G.; SOUSA, M.; OLIVEIRA, P. F.; SILVA, B. M. Are Polyphenols Strong Dietary Agents Against Neurotoxicity and Neurodegeneration? **Neurotoxicity Research**, v. 30, n. 3, p. 345–366, 2016.

ALONSO, A. D.; COHEN, L. S.; CORBO, C.; MOROZOVA, V.; ELIDRISSI, A.; PHILLIPS, G.; KLEIMAN, F. E. Hyperphosphorylation of Tau Associates With Changes in Its Function Beyond Microtubule Stability. **Frontiers in Cellular Neuroscience**, v. 12, p. 338, 2018.

ALTAVILLA, D.; CRISAFULLI, A.; MARINI, H.; ESPOSITO, M.; D'ANNA, R.; CORRADO, F.; BITTO, A.; SQUADRITO, F. Cardiovascular Effects of the Phytoestrogen Genistein. **Current Medicinal Chemistry-Cardiovascular & Hematological Agents**, v. 2, n. 2, p. 179–186, 2004.

ALZHEIMER, A. Über eine eigenartige Erkrankung der Hirnrinde. **Allgemeine Zeitschrift fur Psychiatrie und Psychisch-Gerichtlich Medizin**, v. 64, n. 1-2, p. 146-148, 1907.

ALZHEIMER, A. Über eigenartige Krankheitsfälle des späteren Alters. **Zeitschrift für die gesamte Neurologie und Psychiatrie**, v. 4, n. 1, p. 356-385, 1911.

ALZHEIMER'S ASSOCIATION. 2019 Alzheimer's disease facts and figures. **Alzheimer's & Dementia**, v. 15, n. 3, p. 321–387, 2019.

ARBO, B. D.; MARQUES, C. V.; RUIZ-PALMERO, I.; ORTIZ-RODRIGUEZ, A.; GHORBANPOOR, S.; AREVALO, M. A.; GARCIA-SEGURA, L. M.; RIBEIRO, M. F. 4′-Chlorodiazepam is neuroprotective against amyloid-beta through the modulation of survivin and bax protein expression in vitro. **Brain Research**, v. 1632, p. 91–97, 2016.

ARIGA, T. The Pathogenic Role of Ganglioside Metabolism in Alzheimer's Disease-Cholinergic Neuron-Specific Gangliosides and Neurogenesis. **Molecular Neurobiology**, v. 54, n. 1, p. 623–638, 2017.

ARIGA, T.; WAKADE, C.; YU, R. K. The pathological roles of ganglioside metabolism in Alzheimer's disease: Effects of gangliosides on neurogenesis. **International Journal of Alzheimer's Disease**, v. 2011, 2011.

ASSOCIAÇÃO BRASILEIRA DE ALZHEIMER – ABRAz. Disponível em: http://abraz.org.br/web/. Acesso em: 15 mai. 2020.

BAGHERI, M.; JOGHATAEI, M. T.; MOHSENI, S.; ROGHANI, M. Genistein ameliorates learning and memory deficits in amyloid β(1–40) rat model of Alzheimer's disease. **Neurobiology of Learning and Memory**, v. 95, n. 3, p. 270–276, 2011.

BAGHERI, M.; ROGHANI, M.; JOGHATAEI, M. T.; MOHSENI, S. Genistein inhibits aggregation of exogenous amyloid-beta1–40 and alleviates astrogliosis in the hippocampus of rats. **Brain Research**, v. 1429, p. 145–154, 2012.

BANG, O. Y.; HONG, H. S.; KIM, D. H.; KIM, H.; BOO, J. H.; HUH, K.; MOOK-JUNG, I. Neuroprotective effect of genistein against beta amyloid-induced neurotoxicity. **Neurobiology of Disease**, v. 16, n. 1, p. 21–28, 2004.

BARAGE, S. H.; SONAWANE, K. D. Amyloid cascade hypothesis: Pathogenesis and therapeutic strategies in Alzheimer's disease. **Neuropeptides**, v. 52, p. 1–18, 2015.

BARRIER, L.; INGRAND, S.; DAMJANAC, M.; BILAN, A. R.; HUGON, J.; PAGE, G. Genotype-related changes of ganglioside composition in brain regions of transgenic mouse models of Alzheimer's disease. **Neurobiology of Aging**, v. 28, n. 12, p. 1863–1872, 2007.

BLURTON-JONES, M.; LAFERLA, F. Pathways by which Aβ facilitates Tau pathology. **Current Alzheimer Research**, v. 3, n. 5, p. 437–448, 2006.

BONET-COSTA, V.; HERRANZ-PÉREZ, V.; BLANCO-GANDÍA, M.; MAS-BARGUES, C.; INGLÉS, M.; GARCIA-TARRAGA, P.; RODRIGUEZ-ARIAS, M.; MIÑARRO, J.; BORRAS, C.; GARCIA-VERDUGO, J. M.; VIÑA, J. Clearing amyloid-β through PPARγ/ApoE Activation by Genistein is a Treatment of Experimental Alzheimer's Disease. **Journal of Alzheimer's Disease**, v. 51, n. 3, p. 701–711, 2016.

BRINTON, R. D.; YAO, J.; YIN, F.; MACK, W. J.; CADENAS, E. Perimenopause as a neurological transition state. **Nature Reviews Endocrinology**, v. 11, n. 7, p. 393–405, 2015.

BUSCHE, M. A.; KONNERTH, A. Impairments of neural circuit function in Alzheimer's disease. **Philosophical transactions of the Royal Society of London. Series B, Biological sciences**, v. 371, n. 1700, 2016.

BUTTERFIELD, D. A.; BOYD-KIMBALL, D. Oxidative Stress, Amyloid-β Peptide, and Altered Key Molecular Pathways in the Pathogenesis and Progression of Alzheimer's Disease. **Journal of Alzheimer's Disease**, v. 62, n. 3, p. 1345–1367, 2018.

CACCAMO, A.; MALDONADO, M. A.; BOKOV, A. F.; MAJUMDER, S.; ODDO, S. CBP gene transfer increases BDNF levels and ameliorates learning and memory deficits in a mouse model of Alzheimer's disease. **Proceedings of the National Academy of Sciences of the United States of America**, v. 107, n. 52, p. 22687–22692, 2010.

CAI, Z.; ZHAO, Y.; ZHAO, B. Roles of Glycogen Synthase Kinase 3 in Alzheimer's Disease. **Current Alzheimer Research**, v. 9, n. 7, p. 864–879, 2012.

CALSOLARO, V.; EDISON, P. Neuroinflammation in Alzheimer's disease: Current evidence and future directions. **Alzheimer's & Dementia**, v. 12, n. 6, p. 719–732, 2016.

CHASSEIGNEAUX, S.; ALLINQUANT, B. Functions of Aβ, sAPPα and sAPPβ: Similarities and differences. **Journal of Neurochemistry**, v. 120, n. Suppl. 1, p. 99–108, 2012.

CHEIGNON, C.; TOMAS, M.; BONNEFONT-ROUSSELOT, D.; FALLER, P.; HUREAU, C.; COLLIN, F. Oxidative stress and the amyloid beta peptide in Alzheimer's disease. **Redox Biology**, v. 14, p. 450–464, 2018.

CHEN, G.; XU, T.; YAN, Y.; ZHOU, Y.; JIANG, Y.; MELCHER, K.; XU, H. E. Amyloid beta: structure, biology and structure-based therapeutic development. **Acta Pharmacologica Sinica**, v. 38, n. 9, p. 1205–1235, 2017.

CHEN, L. R.; KO, N. Y.; CHEN, K. H. Isoflavone supplements for menopausal women: A systematic review. **Nutrients**, v. 11, n. 11, p. 2649, 2019.

CHEUNG, Y. T.; LAU, W. K. W.; YU, M. S.; LAI, C. S. W.; YEUNG, S. C.; SO, K. F.; CHANG, R. C. C. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. **NeuroToxicology**, v. 30, n. 1, p. 127–135, 2009.

CHIURCHIÙ, V.; ORLACCHIO, A.; MACCARRONE, M. Is Modulation of Oxidative Stress an Answer? The State of the Art of Redox Therapeutic Actions in Neurodegenerative Diseases. **Oxidative Medicine and Cellular Longevity**, v. 2016, p. 1–11, 2016.

CONGDON, E. E.; SIGURDSSON, E. M. Tau-targeting Therapies for Alzheimer Disease. **Nature Reviews Neurology**, v. 14, n. 7, p. 399-415, 2018.

CONNOR, B.; YOUNG, D.; YAN, Q.; FAULL, R. L. M.; SYNEK, B.; DRAGUNOW, M. Brain-derived neurotrophic factor is reduced in Alzheimer's disease. **Molecular Brain Research**, v. 49, n. 1–2, p. 71–81, 1997.

COSTA, S. L.; SILVA, V. D. A.; SOUZA, C. S.; SANTOS, C. C.; PARIS, I.; MUÑOZ, P.; SEGURA-AGUILAR, J. Impact of Plant-Derived Flavonoids on Neurodegenerative Diseases. **Neurotoxicity Research**, v. 30, n. 1, p. 41–52, 2016.

CUMMINGS, J. L.; MORSTORF, T.; ZHONG, K. Alzheimer's disease drug-development pipeline: Few candidates, frequent failures. **Alzheimer's Research and Therapy**, v. 6, n. 4, p. 37, 2014.

CUNHA, C.; BRAMBILLA, R.; THOMAS, K. L. A simple role for BDNF in learning and memory? **Frontiers in Molecular Neuroscience**, v. 3, p. 1, 2010.

DAHM, R. Alzheimer's discovery. **Current Biology**, v. 16, n. 21, p. R906–R910, 2006.

DEVI, K. P.; SHANMUGANATHAN, B.; MANAYI, A.; NABAVI, S. F.; NABAVI, S. M. Molecular and Therapeutic Targets of Genistein in Alzheimer's Disease. **Molecular Neurobiology**, v. 54, n. 9, p. 7028–7041, 2017.

DI PAOLO, G.; KIM, T. W. Linking lipids to Alzheimer's disease: cholesterol and beyond. **Nature Reviews Neuroscience**, v. 12, n. 5, p. 284–296, 2011.

DI SANTO, S. G.; PRINELLI, F.; ADORNI, F.; CALTAGIRONE, C.; MUSICCO, M. A meta-analysis of the efficacy of donepezil, rivastigmine, galantamine, and memantine in relation to severity of Alzheimer's disease. **Journal of Alzheimer's Disease**, v. 35, n. 2, p. 349–361, 2013.

DING, B.; YUAN, L.; YU, H.; LI, L.; MA, W.; BI, Y.; FENG, J.; XIAO, R. Genistein and Folic Acid Prevent Oxidative Injury Induced by β-Amyloid Peptide. **Basic & Clinical Pharmacology & Toxicology**, v. 108, n. 5, p. 333–340, 2011.

DIXON, R. A.; FERREIRA, D. Genistein. **Phytochemistry**, v. 60, n. 3, p. 205–211, 2002.

DUBOIS, B. *et al.* Preclinical Alzheimer's disease: Definition, natural history, and diagnostic criteria. **Alzheimer's & Dementia**, v. 12, n. 3, p. 292–323, 2016.

DURANY, N.; MICHEL, T.; KURT, J.; CRUZ-SÁNCHEZ, F. F.; CERVÓS-NAVARRO, J.; RIEDERER, P. Brain-derived neurotrophic factor and neurotrophin-3 levels in Alzheimer's disease brains. **International Journal of Developmental Neuroscience**, v. 18, n. 8, p. 807–813, 2000.

ELLIOTT, E.; ATLAS, R.; LANGE, A.; GINZBURG, I. Brain-derived neurotrophic factor induces a rapid dephosphorylation of tau protein through a PI-3Kinase signalling mechanism. **European Journal of Neuroscience**, v. 22, n. 5, p. 1081–1089, 2005.

FAHNESTOCK, M.; GARZON, D.; HOLSINGER, R. M. D.; MICHALSKI, B. Neurotrophic factors and Alzheimer's disease: Are we focusing on the wrong molecule? **Journal of Neural Transmission, Supplementa**, n. 62, p. 241–252, 2002.

FAN, C. D.; LI, Y.; FU, X. T.; WU, Q. J.; HOU, Y. J.; YANG, M. F.; SUN, J. Y.; FU, X. Y.; ZHENG, Z. C.; SUN, B. L. Reversal of Beta-Amyloid-Induced Neurotoxicity in PC12 Cells by Curcumin, the Important Role of ROS-Mediated Signaling and ERK Pathway. **Cellular and Molecular Neurobiology**, v. 37, n. 2, p. 211–222, 2017.

FEI, F.; SU, N.; LI, X.; FEI, Z. Neuroprotection mediated by natural products and their chemical derivatives. **Neural Regeneration Research**, v. 15, n. 11, p. 2008–2015, 2020.

FERREIRA, S. T.; KLEIN, W. L. The Aβ oligomer hypothesis for synapse failure and memory loss in Alzheimer's disease. **Neurobiology of Learning and Memory**, v. 96, n. 4, p. 529–543, 2011.

FERRER, I.; MARÍN, C.; REY, M. J.; RIBALTA, T.; GOUTAN, E.; BLANCO, R.; TOLOSA, E.; MARTÍ, E. BDNF and Full-length and Truncated TrkB Expression in Alzheimer Disease. Implications in Therapeutic Strategies. **Journal of Neuropathology and Experimental Neurology**, v. 58, n. 7, p. 729–739, 1999.

FINKBEINER, S.; TAVAZOIE, S. F.; MALORATSKY, A.; JACOBS, K. M.; HARRIS, K. M.; GREENBERG, M. E. CREB: A major mediator of neuronal neurotrophin responses. **Neuron**, v. 19, n. 5, p. 1031–1047, 1997.

FRANCO, R.; MARTÍNEZ-PINILLA, E.; NAVARRO, G.; ZAMARBIDE, M. Potential of GPCRs to modulate MAPK and mTOR pathways in Alzheimer's disease. **Progress in Neurobiology**, v. 149–150, p. 21–38, 2017.

FROZZA, R. L.; HORN, A. P.; HOPPE, J. B.; SIMÃO, F.; GERHARDT, D.; COMIRAN, R. A.; SALBEGO, C. G. A Comparative Study of β-Amyloid Peptides Aβ1-42 and Aβ25-35 Toxicity in Organotypic Hippocampal Slice Cultures. **Neurochemical Research**, v. 34, n. 2, p. 295–303, 2009.

FROZZA, R. L.; BERNARDI, A.; HOPPE, J. B.; MENEGHETTI, A. B.; MATTÉ, A.; BATTASTINI, A. M. O.; POHLMANN, A. R.; GUTERRES, S. S.; SALBEGO, C. Neuroprotective Effects of Resveratrol Against Aβ Administration in Rats are Improved by Lipid-Core Nanocapsules. **Molecular Neurobiology**, v. 47, n. 3, p. 1066–1080, 2013.

GALLA, L.; REDOLFI, N.; POZZAN, T.; PIZZO, P.; GREOTTI, E. Intracellular Calcium Dysregulation by the Alzheimer's Disease-Linked Protein Presenilin 2. **International Journal of Molecular Sciences**, v. 21, n. 3, p. 770, 2020.

GANAI, A. A.; FAROOQI, H. Bioactivity of genistein: A review of in vitro and in vivo studies. **Biomedicine and Pharmacotherapy**, v. 76, p. 30–38, 2015.

GARZON, D.; YU, G.; FAHNESTOCK, M. A new brain-derived neurotrophic factor transcript and decrease in brain-derived neurotrophic factor transcripts 1, 2 and 3 in Alzheimer's disease parietal cortex. **Journal of Neurochemistry**, v. 82, n. 5, p. 1058–1064, 2004.

GIORGETTI, S.; GRECO, C.; TORTORA, P.; APRILE, F. Targeting Amyloid Aggregation: An Overview of Strategies and Mechanisms. **International Journal of Molecular Sciences**, v. 19, n. 9, p. 2677, 2018.

GLEASON, C. E.; CARLSSON, C. M.; BARNET, J. H.; MEADE, S. A.; SETCHELL, K. D. R.; ATWOOD, C. S.; JOHNSON, S. C.; RIES, M. L.; ASTHANA, S. A preliminary study of the safety, feasibility and cognitive efficacy of soy isoflavone supplements in older men and women. **Age and Ageing**, v. 38, n. 1, p. 86–93, 2008.

GLEASON, C. E.; FISCHER, B. L.; DOWLING, N. M.; SETCHELL, K. D. R.; ATWOOD, C. S.; CARLSSON, C. M.; ASTHANA, S. Cognitive effects of soy isoflavones in patients with Alzheimer's disease. **Journal of Alzheimer's Disease**, v. 47, n. 4, p. 1009–1019, 2015.

GÖTZ, J.; CHEN, F.; VAN DORPE, J.; NITSCH, R. M. Formation of neurofibrillary tangles in P301L tau transgenic mice induced by Aβ42 fibrils. **Science**, v. 293, n. 5534, p. 1491–1495, 2001.

HEMMINGS, B. A.; RESTUCCIA, D. F. PI3K-PKB/Akt pathway. **Cold Spring Harbor Perspectives in Biology**, v. 4, n. 9, p. a011189, 2012.

HERNÁNDEZ, F.; BARREDA, E. G.; FUSTER-MATANZO, A.; LUCAS, J. J.; AVILA, J. GSK3: A possible link between beta amyloid peptide and tau protein. **Experimental Neurology**, v. 223, n. 2, p. 322–325, 2010.

HIPPIUS, H.; NEUNDÖRFER, G. The discovery of Alzheimer's disease. **Dialogues in Clinical Neuroscience**, v. 5, n. 1, p. 101–108, 2003.

HOCK, C.; HEESE, K.; HULETTE, C.; ROSENBERG, C.; OTTEN, U. Region-specific neurotrophin imbalances in Alzheimer disease: Decreased levels of brain-derived neurotrophic factor and increased levels of nerve growth factor in hippocampus and cortical areas. **Archives of Neurology**, v. 57, n. 6, p. 846–851, 2000.

HOLSINGER, R. M. D.; SCHNARR, J.; HENRY, P.; CASTELO, V. T.; FAHNESTOCK, M. Quantitation of BDNF mRNA in human parietal cortex by competitive reverse transcription-polymerase chain reaction: decreased levels in Alzheimer's disease. **Molecular Brain Research**, v. 76, n. 2, p. 347–354, 2000.

HOPPE, J. B.; CORADINI, K.; FROZZA, R. L.; OLIVEIRA, C. M.; MENEGHETTI, A. B.; BERNARDI, A.; PIRES, E. S.; BECK, R. C. R.; SALBEGO, C. G. Free and nanoencapsulated curcumin suppress β-amyloid-induced cognitive impairments in rats: Involvement of BDNF and Akt/GSK-3β signaling pathway. **Neurobiology of Learning and Memory**, v. 106, p. 134–144, 2013.

HUANG, H. C.; XU, K.; JIANG, Z. F. Curcumin-mediated neuroprotection against amyloid-β-induced mitochondrial dysfunction involves the inhibition of GSK-3β. **Journal of Alzheimer's Disease**, v. 32, n. 4, p. 981–996, 2012.

HUSSAIN, G.; ZHANG, L.; RASUL, A.; ANWAR, H.; SOHAIL, M. U.; RAZZAQ, A.; AZIZ, N.; SHABBIR, A.; ALI, M.; SUN, T. Role of Plant-Derived Flavonoids and Their Mechanism in Attenuation of Alzheimer's and Parkinson's Diseases: An Update of Recent Data. **Molecules**, v. 23, n. 4, p. 814, 2018.

IBRAHIM, N. H.; YAHAYA, M. F.; MOHAMED, W.; TEOH, S. L.; HUI, C. K.; KUMAR, J. Pharmacotherapy of Alzheimer's Disease: Seeking Clarity in a Time of Uncertainty. **Frontiers in Pharmacology**, v. 11, p. 261, 2020.

JACK, C. R. *et al.* NIA-AA Research Framework: Toward a biological definition of Alzheimer's disease. **Alzheimer's and Dementia**, v. 14, n. 4, p. 535-562, 2018.

JAISWAL, N.; AKHTAR, J.; SINGH, S. P.; AHSAN, F. An Overview on Genistein and its Various Formulations. **Drug Research**, v. 69, n. 6, p. 305-313, 2019.

JOHN, J. A. S.; HENDERSON, V. W.; HODIS, H. N.; KONO, N.; MCCLEARY, C. A.; FRANKE, A. A.; MACK, W. J. Associations between urine excretion of isoflavonoids and cognition in postmenopausal women in the Women's Isoflavone Soy Health clinical trial. **Journal of the American Geriatrics Society**, v. 62, n. 4, p. 629–635, 2014.

JOHNSON, K. A.; FOX, N. C.; SPERLING, R. A.; KLUNK, W. E. Brain imaging in Alzheimer disease. **Cold Spring Harbor Perspectives in Medicine**, v. 2, n. 4, p. a006213, 2012.

KHAN, S. S.; BLOOM, G. S. Tau: The Center of a Signaling Nexus in Alzheimer's Disease. **Frontiers in Neuroscience**, v. 10, p. 31, 2016.

KIROUAC, L.; RAJIC, A. J.; CRIBBS, D. H.; PADMANABHAN, J. Activation of Ras-ERK signaling and GSK-3 by amyloid precursor protein and amyloid beta facilitates neurodegeneration in Alzheimer's disease. **eNeuro**, v. 4, n. 2, 2017.

KOSICEK, M.; HECIMOVIC, S. Phospholipids and Alzheimer's Disease: Alterations, Mechanisms and Potential Biomarkers. **International Journal of Molecular Sciences**, v. 14, n. 1, p. 1310–1322, 2013.

KOVALEVICH, J.; LANGFORD, D. Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. *In:* **Neuronal Cell Culture**. Totowa: Humana Press, 2013. p. 9-21.

KREIJKAMP-KASPERS, S.; KOK, L.; GROBBEE, D. E.; HAAN, E. H. F.; ALEMAN, A.; LAMPE, J. W.; SCHOUW, Y. T. Effect of Soy Protein Containing Isoflavones on Cognitive Function, Bone Mineral Density, and Plasma Lipids in Postmenopausal Women. **JAMA**, v. 292, n. 1, p. 65–74, 2004.

KREUTZ, F.; FROZZA, R. L.; BREIER, A. C.; OLIVEIRA, V. A.; HORN, A. P.; PETTENUZZO, L. F.; NETTO, C. A.; SALBEGO, C. G.; TRINDADE, V. M. T. Amyloid-β induced toxicity involves ganglioside expression and is sensitive to GM1 neuroprotective action. **Neurochemistry International**, v. 59, n. 5, p. 648–655, 2011.

KREUTZ, F.; SCHERER, E. B.; FERREIRA, A. G. K.; PETRY, F. S.; PEREIRA, C. L.; SANTANA, F.; WYSE, A. T. S.; SALBEGO, C. G.; TRINDADE, V. M. T. Alterations on Na+,K+-ATPase and Acetylcholinesterase Activities Induced by

Amyloid-β Peptide in Rat Brain and GM1 Ganglioside Neuroprotective Action. **Neurochemical Research**, v. 38, n. 11, p. 2342–2350, 2013.

KUMAR, A.; SINGH, A.; EKAVALI. A review on Alzheimer's disease pathophysiology and its management: an update. **Pharmacological Reports**, v. 67, n. 2, p. 195–203, 2015.

KUNZLER, A. *et al.* Changes in Cell Cycle and Up-Regulation of Neuronal Markers During SH-SY5Y Neurodifferentiation by Retinoic Acid are Mediated by Reactive Species Production and Oxidative Stress. **Molecular Neurobiology**, v. 54, n. 9, p. 6903–6916, 2017.

LAKEY-BEITIA, J.; BERROCAL, R.; RAO, K. S.; DURANT, A. A. Polyphenols as Therapeutic Molecules in Alzheimer's Disease Through Modulating Amyloid Pathways. **Molecular Neurobiology**, v. 51, n. 2, p. 466–479, 2015.

LANE, C. A.; HARDY, J.; SCHOTT, J. M. Alzheimer's disease. **European Journal of Neurology**, v. 25, n. 1, p. 59–70, 2018.

LATTANZIO, F.; CARBONI, L.; CARRETTA, D.; CANDELETTI, S.; ROMUALDI, P. Treatment with the neurotoxic Aβ (25–35) peptide modulates the expression of neuroprotective factors Pin1, Sirtuin 1, and brain-derived neurotrophic factor in SH-SY5Y human neuroblastoma cells. **Experimental and Toxicologic Pathology**, v. 68, n. 5, p. 271–276, 2016.

LAURETTI, E.; DINCER, O.; PRATICÒ, D. Glycogen synthase kinase-3 signaling in Alzheimer's disease. **Biochimica et Biophysica Acta - Molecular Cell Research**, v. 1867, n. 5, p. 118664, 2020.

LAURIJSSENS, B.; AUJARD, F.; RAHMAN, A. Animal models of Alzheimer's disease and drug development. **Drug Discovery Today: Technologies**, v. 10, n. 3, p. e319–e327, 2013.

LEE, J. C.; KIM, S. J.; HONG, S.; KIM, Y. Diagnosis of Alzheimer's disease utilizing amyloid and tau as fluid biomarkers. **Experimental & Molecular Medicine**, v. 51, n. 5, p. 1-10, 2019.

LEE, Y. B.; LEE, H. J.; SOHN, H. S. Soy isoflavones and cognitive function. **The Journal of Nutritional Biochemistry**, v. 16, n. 11, p. 641–649, 2005.

LEONG, Y. Q.; NG, K. Y.; CHYE, S. M.; LING, A. P. K.; KOH, R. Y. Mechanisms of action of amyloid-beta and its precursor protein in neuronal cell death. **Metabolic Brain Disease**, v. 35, n. 1, p. 11-30, 2020.

LEROY, K.; YILMAZ, Z.; BRION, J. P. Increased level of active GSK-3β in Alzheimer's disease and accumulation in argyrophilic grains and in neurones at different stages of neurofibrillary degeneration. **Neuropathology and Applied Neurobiology**, v. 33, n. 1, p. 43–55, 2007.

LEUZY, A.; HEURLING, K.; ASHTON, N. J.; SCHÖLL, M.; ZIMMER, E. R. In vivo Detection of Alzheimer's Disease. **Yale Journal of Biology and Medicine**, v. 91, n. 3, p. 291-300, 2018.

LEWIS, J. *et al.* Enhanced Neurofibrillary Degeneration in Transgenic Mice Expressing Mutant Tau and APP. **Science**, v. 293, n. 5534, p. 1487–1491, 2001.

LIM, W. L. F.; MARTINS, I. J.; MARTINS, R. N. The Involvement of Lipids in Alzheimer's Disease. **Journal of Genetics and Genomics**, v. 41, n. 5, p. 261–274, 2014.

LIU, F.; GRUNDKE-IQBAL, I.; IQBAL, K.; GONG, C. X. Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation. **European Journal of Neuroscience**, v. 22, n. 8, p. 1942–1950, 2005.

LLORENS-MARTÍN, M.; JURADO, J.; HERNÁNDEZ, F.; ÁVILA, J. GSK-3β, a pivotal kinase in Alzheimer disease. **Frontiers in Molecular Neuroscience**, v. 7, p. 46, 2014.

LONG, J. M.; HOLTZMAN, D. M. Alzheimer Disease: An Update on Pathobiology and Treatment Strategies. **Cell**, v. 179, n. 2, p. 312-339, 2019.

LOPES, F. M. *et al.* Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. **Brain Research**, v. 1337, p. 85–94, 2010.

LU, C.; WANG, Y.; XU, T.; LI, Q.; WANG, D.; ZHANG, L.; FAN, B.; WANG, F.; LIU, X. Genistein Ameliorates Scopolamine-Induced Amnesia in Mice Through the Regulation of the Cholinergic Neurotransmission, Antioxidant System and the ERK/CREB/BDNF Signaling. **Frontiers in Pharmacology**, v. 9, p. 1153, 2018.

MA, W.; YUAN, L.; YU, H.; DING, B.; XI, Y.; FENG, J.; XIAO, R. Genistein as a neuroprotective antioxidant attenuates redox imbalance induced by β-amyloid peptides 25–35 in PC12 cells. **International Journal of Developmental Neuroscience**, v. 28, n. 4, p. 289–295, 2010.

MAGI, S.; CASTALDO, P.; MACRÌ, M. L.; MAIOLINO, M.; MATTEUCCI, A.; BASTIOLI, G.; GRATTERI, S.; AMOROSO, S.; LARICCIA, V. Intracellular Calcium Dysregulation: Implications for Alzheimer's Disease. **BioMed Research International**, v. 2016, p. 6701324, 2016.

MAGISTRETTI, P. J.; GEISLER, F. H.; SCHNEIDER, J. S.; LI, P. A.; FIUMELLI, H.; SIPIONE, S. Gangliosides: Treatment avenues in neurodegenerative disease. **Frontiers in Neurology**, v. 10, p. 859, 2019.

MAGRANÉ, J.; ROSEN, K. M.; SMITH, R. C.; WALSH, K.; GOURAS, G. K.; QUERFURTH, H. W. Intraneuronal β-Amyloid Expression Downregulates the Akt Survival Pathway and Blunts the Stress Response. **The Journal of Neuroscience**, v. 25, n. 47, p. 10960–10969, 2005.

MAJD, S.; POWER, J. H.; GRANTHAM, H. J. M. Neuronal response in Alzheimer's and Parkinson's disease: the effect of toxic proteins on intracellular pathways. **BMC Neuroscience**, v. 16, p. 69, 2015.

MANNING, B. D.; TOKER, A. AKT/PKB Signaling: Navigating the Network. **Cell**, v. 169, n. 3, p. 381–405, 2017.

MARTIN, L.; LATYPOVA, X.; WILSON, C. M.; MAGNAUDEIX, A.; PERRIN, M. L.; TERRO, F. Tau protein phosphatases in Alzheimer's disease: The leading role of PP2A. **Ageing Research Reviews**, v. 12, n. 1, p. 39–49, 2013a.

MARTIN, L.; LATYPOVA, X.; WILSON, C. M.; MAGNAUDEIX, A.; PERRIN, M. L.; YARDIN, C.; TERRO, F. Tau protein kinases: Involvement in Alzheimer's disease. **Ageing Research Reviews**, v. 12, n. 1, p. 289–309, 2013b.

MASTERS, C. L.; BATEMAN, R.; BLENNOW, K.; ROWE, C. C.; SPERLING, R. A.; CUMMINGS, J. L. Alzheimer's disease. **Nature Reviews Disease Primers**, v. 1, p. 15056, 2015.

MEDINA, M.; AVILA, J. Understanding the relationship between GSK-3 and Alzheimer's disease: A focus on how GSK-3 can modulate synaptic plasticity processes. **Expert Review of Neurotherapeutics**, v. 13, n. 5, p. 495–503, 2013.

MEHAN, S.; MEENA, H.; SHARMA, D.; SANKHLA, R. JNK: A stress-activated protein kinase therapeutic strategies and involvement in Alzheimer's and various neurodegenerative abnormalities. **Journal of Molecular Neuroscience**, v. 43, n. 3, p. 376-390, 2011.

MESSINA, M. Soy foods, isoflavones, and the health of postmenopausal women. **The American Journal of Clinical Nutrition**, v. 100, Suppl 1, p. 423S-430S, 2014.

MESSINA, M.; NAGATA, C.; WU, A. H. Estimated Asian Adult Soy Protein and Isoflavone Intakes. **Nutrition and Cancer**, v. 55, n. 1, p. 1–12, 2006.

MINTER, M. R.; TAYLOR, J. M.; CRACK, P. J. The contribution of neuroinflammation to amyloid toxicity in Alzheimer's disease. **Journal of Neurochemistry**, v. 136, n. 3, p. 457–474, 2016.

MIRANDA, M.; MORICI, J. F.; ZANONI, M. B.; BEKINSCHTEIN, P. Brain-Derived Neurotrophic Factor: A Key Molecule for Memory in the Healthy and the Pathological Brain. **Frontiers in Cellular Neuroscience**, v. 13, p. 363, 2019.

MOOSAVI, F.; HOSSEINI, R.; SASO, L.; FIRUZI, O. Modulation of neurotrophic signaling pathways by polyphenols. **Drug Design, Development and Therapy**, v. 10, p. 23–42, 2015.

MORISHIMA, Y.; GOTOH, Y.; ZIEG, J.; BARRETT, T.; TAKANO, H.; FLAVELL, R.; DAVIS, R. J.; SHIRASAKI, Y.; GREENBERG, M. E. β-Amyloid Induces Neuronal Apoptosis Via a Mechanism that Involves the c-Jun N-Terminal Kinase Pathway and the Induction of Fas Ligand. **Journal of Neuroscience**, v. 21, n. 19, p. 7551–7560, 2001.

MUCKE, L.; SELKOE, D. J. Neurotoxicity of amyloid β-protein: synaptic and network dysfunction. **Cold Spring Harbor Perspectives in Medicine**, v. 2, n. 7, p. a006338, 2012.

NARISAWA-SAITO, M.; WAKABAYASHI, K.; TSUJI, S.; TAKAHASHI, H.; NAWA, H. Regional specificity of alterations in NGF, BDNF and NT-3 levels in Alzheimer's disease. **NeuroReport**, v. 7, n. 18, p. 2925–2928, 1996.

NASERI, N. N.; WANG, H.; GUO, J.; SHARMA, M.; LUO, W. The complexity of tau in Alzheimer's disease. **Neuroscience Letters**, v. 705, p. 183–194, 2019.

ODDO, S.; CACCAMO, A.; KITAZAWA, M.; TSENG, B. P.; LAFERLA, F. M. Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. **Neurobiology of Aging**, v. 24, n. 8, p. 1063-1070, 2003a.

ODDO, S.; CACCAMO, A.; SHEPHERD, J. D.; MURPHY, M. P.; GOLDE, T. E.; KAYED, R.; METHERATE, R.; MATTSON, M. P.; AKBARI, Y.; LAFERLA, F. M. Triple-transgenic model of Alzheimer's Disease with plaques and tangles: Intracellular Aβ and synaptic dysfunction. **Neuron**, v. 39, n. 3, p. 409–421, 2003b.

ORGANIZAÇÃO MUNDIAL DA SAÚDE - OMS. Disponível em: https://www.who.int/. Acesso em: 15 mai. 2020.

PALMANO, K.; ROWAN, A.; GUILLERMO, R.; GUAN, J.; MCJARROW, P. The role of gangliosides in neurodevelopment. **Nutrients**, v. 7, n. 5, p. 3891–3913, 2015.

PENG, S.; WUU, J.; MUFSON, E. J.; FAHNESTOCK, M. Precursor form of brain-derived neurotrophic factor and mature brain-derived neurotrophic factor are decreased in the pre-clinical stages of Alzheimer's disease. **Journal of Neurochemistry**, v. 93, n. 6, p. 1412–1421, 2005.

PENG, S.; GARZON, D. J.; MARCHESE, M.; KLEIN, W.; GINSBERG, S. D.; FRANCIS, B. M.; MOUNT, H. T. J.; MUFSON, E. J.; SALEHI, A.; FAHNESTOCK, M. Decreased brain-derived neurotrophic factor depends on amyloid aggregation state in transgenic mouse models of Alzheimer's disease. **Journal of Neuroscience**, v. 29, n. 29, p. 9321–9329, 2009.

PERKIN, A. G.; NEWBURY, F. G. LXXIX. - The colouring matters contained in dyer's broom (Genista tinctoria) and heather (Calluna vulgaris). **Journal of the Chemical Society, Transactions**, v. 75, p. 830-839, 1899.

PERLUIGI, M.; BARONE, E.; DI DOMENICO, F.; BUTTERFIELD, D. A. Aberrant protein phosphorylation in Alzheimer disease brain disturbs pro-survival and cell death pathways. **Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease**, v. 1862, n. 10, p. 1871–1882, 2016.

PERUSINI, G. Über klinisch und histologisch eigenartige psychische Erkrankungen des späteren Lebensalters. *In:* NISSL, F.; ALZHEIMER, A. **Histologische und Histopathologische Arbeiten**. Jena: Verlag G. Fischer, 1909. p. 297-351.

PHILLIPS, H. S.; HAINS, J. M.; ARMANINI, M.; LARAMEE, G. R.; JOHNSON, S. A.; WINSLOW, J. W. BDNF mRNA is decreased in the hippocampus of individuals

with Alzheimer's disease. **Neuron**, v. 7, n. 5, p. 695–702, 1991.

PICONE, P.; NUZZO, D.; GIACOMAZZA, D.; DI CARLO, M.. β-Amyloid Peptide: the Cell Compartment Multi-faceted Interaction in Alzheimer's Disease. **Neurotoxicity Research**, v. 37, n. 2, p. 250–263, 2020.

PIERZYNOWSKA, K.; PODLACHA, M.; GAFFKE, L.; MAJKUTEWICZ, I.; MANTEJ, J.; WĘGRZYN, A.; OSIADŁY, M.; MYŚLIŃSKA, D.; WĘGRZYN, G. Autophagy-dependent mechanism of genistein-mediated elimination of behavioral and biochemical defects in the rat model of sporadic Alzheimer's disease. **Neuropharmacology**, v. 148, p. 332–346, 2019.

PINHO, B. R.; FERRERES, F.; VALENTÃO, P.; ANDRADE, P. B. Nature as a source of metabolites with cholinesterase-inhibitory activity: an approach to Alzheimer's disease treatment. **Journal of Pharmacy and Pharmacology**, v. 65, n. 12, p. 1681–1700, 2013.

POLKOWSKI, K.; MAZUREK, A. Biological Properties of Genistein. A Review of in Vitro and in Vivo Data. **Acta Poloniae Pharmaceutica - Drug Research**, v. 57, n. 2, p. 135–155, 2000.

POOLER, A. M.; NOBLE, W.; HANGER, D. P. A role for tau at the synapse in Alzheimer's disease pathogenesis. **Neuropharmacology**, v. 76, p. 1–8, 2014.

POON, W. W.; CARLOS, A. J.; AGUILAR, B. L.; BERCHTOLD, N. C.; KAWANO, C. K.; ZOGRABYAN, V.; YAOPRUKE, T.; SHELANSKI, M.; COTMAN, C. W. β-Amyloid (Aβ) oligomers impair brain-derived neurotrophic factor retrograde trafficking by down-regulating ubiquitin C-terminal hydrolase, UCH-L1. **Journal of Biological Chemistry**, v. 288, n. 23, p. 16937–16948, 2013.

PUGAZHENTHI, S.; WANG, M.; PHAM, S.; SZE, C. I.; ECKMAN, C. B. Downregulation of CREB expression in Alzheimer's brain and in Aβ-treated rat hippocampal neurons. **Molecular Neurodegeneration**, v. 6, p. 60, 2011.

QUERFURTH, H. W.; LAFERLA, F. M. Alzheimer's Disease. **New England Journal of Medicine**, v. 362, n. 4, p. 329–344, 2010.

RAI, S. N.; DILNASHIN, H.; BIRLA, H.; SINGH, S. S.; ZAHRA, W.; RATHORE, A. S.; SINGH, B. K.; SINGH, S. P. The Role of PI3K/Akt and ERK in Neurodegenerative Disorders. **Neurotoxicity Research**, v. 35, n. 3, p. 775-795, 2019.

RAMSER, E. M.; GAN, K. J.; DECKER, H.; FAN, E. Y.; SUZUKI, M. M.; FERREIRA, S. T.; SILVERMAN, M. A. Amyloid-β oligomers induce tau-independent disruption of BDNF axonal transport via calcineurin activation in cultured hippocampal neurons. **Molecular Biology of the Cell**, v. 24, n. 16, p. 2494–2505, 2013.

REN, B.; LIU, Y.; ZHANG, Y.; CAI, Y.; GONG, X.; CHANG, Y.; XU, L.; ZHENG, J. Genistein: A Dual Inhibitor of Both Amyloid β and Human Islet Amylin Peptides. **ACS Chemical Neuroscience**, v. 9, n. 5, p. 1215–1224, 2018.

RIEDEL, B. C.; THOMPSON, P. M.; BRINTON, R. D. Age, APOE and sex: Triad of risk of Alzheimer's disease. **The Journal of Steroid Biochemistry and Molecular Biology**, v. 160, p. 134–147, 2016.

RUSSO, M.; RUSSO, G. L.; DAGLIA, M.; KASI, P. D.; RAVI, S.; NABAVI, S. F.; NABAVI, S. M. Understanding genistein in cancer: The "good" and the "bad" effects: A review. **Food Chemistry**, v. 196, p. 589-600, 2016.

SANTOS, A. L.; PRETA, G. Lipids in the cell: organisation regulates function. **Cellular and Molecular Life Sciences**, v. 75, n. 11, p. 1909–1927, 2018.

SASTRY, P. S. Lipids of nervous tissue: Composition and metabolism. **Progress in Lipid Research**, v. 24, n. 2, p. 69-176, 1985.

SCHELTENS, P.; BLENNOW, K.; BRETELER, M. M. B.; STROOPER, B.; FRISONI, G. B.; SALLOWAY, S.; VAN DER FLIER, W. M.. Alzheimer's disease. **The Lancet**, v. 388, n. 10043, p. 505–517, 2016.

SCHILLING, L. P.; ZIMMER, E. R.; SHIN, M.; LEUZY, A.; PASCOAL, T. A.; BENEDET, A. L.; BORELLI, W. V.; PALMINI, A.; GAUTHIER, S.; ROSA-NETO, P. Imaging Alzheimer's disease pathophysiology with PET. **Dementia & Neuropsychologia**, v. 10, n. 2, p. 79-90, 2016.

SELKOE, D. J.; HARDY, J. The amyloid hypothesis of Alzheimer's disease at 25 years. **EMBO Molecular Medicine**, v. 8, n. 6, p. 595–608, 2016.

SEZGIN, E.; LEVENTAL, I.; MAYOR, S.; EGGELING, C. The mystery of membrane organization: composition, regulation and roles of lipid rafts. **Nature Reviews Molecular Cell Biology**, v. 18, n. 6, p. 361–374, 2017.

SHOJI, M.; IWAKAMI, N.; TAKEUCHI, S.; WARAGAI, M.; SUZUKI, M.;

KANAZAWA, I.; LIPPA, C. F.; ONO, S.; OKAZAWA, H. JNK activation is associated with intracellular β-amyloid accumulation. **Molecular Brain Research**, v. 85, n. 1–2, p. 221–233, 2000.

SI, H.; LIU, D. Phytochemical Genistein in the Regulation of Vascular Function: New Insights. **Current Medicinal Chemistry**, v. 14, n. 24, p. 2581–2589, 2007.

SILVA, M. V. F.; LOURES, C. M. G.; ALVES, L. C. V.; SOUZA, L. C.; BORGES, K. B. G.; CARVALHO, M. G. Alzheimer's disease: risk factors and potentially protective measures. **Journal of Biomedical Science**, v. 26, n. 1, p. 33, 2019.

SPAGNUOLO, C.; RUSSO, G. L.; ORHAN, I. E.; HABTEMARIAM, S.; DAGLIA, M.; SUREDA, A.; NABAVI, S. F.; DEVI, K. P.; LOIZZO, M. R.; TUNDIS, R.; NABAVI, S. M. Genistein and Cancer: Current Status, Challenges, and Future Directions. **Advances in Nutrition**, v. 6, n. 4, p. 408–419, 2015.

STEARDO, L.; BRONZUOLI, M. R.; IACOMINO, A.; ESPOSITO, G.; STEARDO, L.; SCUDERI, C. Does neuroinflammation turn on the flame in Alzheimer's disease? Focus on astrocytes. **Frontiers in Neuroscience**, v. 9, p. 259, 2015.

SUWANNA, N.; THANGNIPON, W.; SOI-AMPORNKUL, R. Neuroprotective effects of diarylpropionitrile against β-amyloid peptide-induced neurotoxicity in rat cultured cortical neurons. **Neuroscience Letters**, v. 578, p. 44–49, 2014.

SWAMINATHAN, A.; JICHA, G. A. Nutrition and prevention of Alzheimer's dementia. **Frontiers in Aging Neuroscience**, v. 6, p. 282, 2014.

SWEATT, J. D. Mitogen-activated protein kinases in synaptic plasticity and memory. **Current Opinion in Neurobiology**, v. 14, n. 3, p. 311–317, 2004.

TACKENBERG, C.; NITSCH, R. M. The secreted APP ectodomain sAPPα, but not sAPPβ, protects neurons against Aβ oligomer-induced dendritic spine loss and increased tau phosphorylation. **Molecular Brain**, v. 12, p. 27, 2019.

TAKEDA, A.; LOVEMAN, E.; CLEGG, A.; KIRBY, J.; PICOT, J.; PAYNE, E.; GREEN, C. A systematic review of the clinical effectiveness of donepezil, rivastigmine and galantamine on cognition, quality of life and adverse events in Alzheimer's disease. **International Journal of Geriatric Psychiatry**, v. 21, n. 1, p. 17–28, 2006.

TANILA, H. The role of BDNF in Alzheimer's disease. **Neurobiology of Disease**, v.

97, p. 114–118, 2017.

TAYLOR, C. K.; LEVY, R. M.; ELLIOTT, J. C.; BURNETT, B. P. The effect of genistein aglycone on cancer and cancer risk: A review of in vitro, preclinical, and clinical studies. **Nutrition Reviews**, v. 67, n. 7, p. 398-415, 2009.

TEJEDA, G. S.; DÍAZ-GUERRA, M. Integral Characterization of Defective BDNF/TrkB Signalling in Neurological and Psychiatric Disorders Leads the Way to New Therapies. **International Journal of Molecular Sciences**, v. 18, n. 2, p. 268, 2017.

THANGAVEL, P.; PUGA-OLGUÍN, A.; RODRÍGUEZ-LANDA, J. F.; ZEPEDA, R. C. Genistein as Potential Therapeutic Candidate for Menopausal Symptoms and Other Related Diseases. **Molecules**, v. 24, n. 21, p. 3892, 2019.

THORP, A. A.; SINN, N.; BUCKLEY, J. D.; COATES, A. M.; HOWE, P. R. C. Soya isoflavone supplementation enhances spatial working memory in men. **British Journal of Nutrition**, v. 102, n. 9, p. 1348–1354, 2009.

TONG, B. C. K.; WU, A. J.; LI, M.; CHEUNG, K. H. Calcium signaling in Alzheimer's disease & therapies. **Biochimica et Biophysica Acta - Molecular Cell Research**, v. 1865, n. 11, p. 1745-1760, 2018.

TONG, L.; BALAZS, R.; THORNTON, P. L.; COTMAN, C. W. β-amyloid peptide at sublethal concentrations downregulates brain-derived neurotrophic factor functions in cultured cortical neurons. **Journal of Neuroscience**, v. 24, n. 30, p. 6799–6809, 2004.

TOSELLI, M.; TOSETTI, P.; TAGLIETTI, V. Functional changes in sodium conductances in the human neuroblastoma cell line SH-SY5Y during in vitro differentiation. **Journal of Neurophysiology**, v. 76, n. 6, p. 3920–3927, 1996.

TOSETTI, P.; TAGLIETTI, V.; TOSELLI, M. Functional Changes in Potassium Conductances of the Human Neuroblastoma Cell Line SH-SY5Y During In Vitro Differentiation. **Journal of Neurophysiology**, v. 79, n. 2, p. 648–658, 1998.

TU, S.; OKAMOTO, S.; LIPTON, S. A.; XU, H. Oligomeric Aβ-induced synaptic dysfunction in Alzheimer's disease. **Molecular Neurodegeneration**, v. 9, p. 48, 2014.

TULI, H. S.; TUORKEY, M. J.; THAKRAL, F.; SAK, K.; KUMAR, M.; SHARMA, A. K.; SHARMA, U.; JAIN, A.; AGGARWAL, V.; BISHAYEE, A. Molecular

mechanisms of action of genistein in cancer: Recent advances. **Frontiers in Pharmacology**, v. 10, p. 1336, 2019.

UDDIN, M. S.; KABIR, M. T. Emerging Signal Regulating Potential of Genistein Against Alzheimer's Disease: A Promising Molecule of Interest. **Frontiers in Cell and Developmental Biology**, v. 7, p. 197, 2019.

UDDIN, M. S.; RAHMAN, M. M.; JAKARIA, M.; RAHMAN, M. S.; HOSSAIN, M. S.; ISLAM, A.; AHMED, M.; MATHEW, B.; OMAR, U. M.; BARRETO, G. E.; ASHRAF, G. M. Estrogen Signaling in Alzheimer's Disease: Molecular Insights and Therapeutic Targets for Alzheimer's Dementia. **Molecular Neurobiology**, v. 57, n. 6, p. 2654-2670, 2020.

VALLÉS, S. L.; BORRÁS, C.; GAMBINI, J.; FURRIOL, J.; ORTEGA, A.; SASTRE, J.; PALLARDÓ, F. V.; VIÑA, J. Oestradiol or genistein rescues neurons from amyloid beta-induced cell death by inhibiting activation of p38. **Aging Cell**, v. 7, n. 1, p. 112–118, 2008.

VALLES, S. L.; DOLZ-GAITON, P.; GAMBINI, J.; BORRAS, C.; LLORET, A.; PALLARDO, F. V.; VIÑA, J. Estradiol or genistein prevent Alzheimer's disease-associated inflammation correlating with an increase PPARγ expression in cultured astrocytes. **Brain Research**, v. 1312, p. 138–144, 2010.

VAN DAM, D.; DE DEYN, P. P. Animal models in the drug discovery pipeline for Alzheimer's disease. **British Journal of Pharmacology**, v. 164, n. 4, p. 1285–1300, 2011.

VAN ECHTEN-DECKERT, G.; WALTER, J. Sphingolipids: Critical players in Alzheimer's disease. **Progress in Lipid Research**, v. 51, n. 4, p. 378–393, 2012.

VAN ERP-BAART, M. A. J.; BRANTS, H. A. M.; KIELY, M.; MULLIGAN, A.; TURRINI, A.; SERMONETA, C.; KILKKINEN, A.; VALSTA, L. M. Isoflavone intake in four different European countries: the VENUS approach. **British Journal of Nutrition**, v. 89, n. S1, p. S25–S30, 2003.

VAUZOUR, D. Dietary Polyphenols as Modulators of Brain Functions: Biological Actions and Molecular Mechanisms Underpinning Their Beneficial Effects. **Oxidative Medicine and Cellular Longevity**, v. 2012, p. 914273, 2012.

WANG, Y.; CAI, B.; SHAO, J.; WANG, T. T.; CAI, R. Z.; MA, C. J.; HAN, T.; DU, J. Genistein suppresses the mitochondrial apoptotic pathway in hippocampal neurons in

rats with Alzheimer's disease. **Neural Regeneration Research**, v. 11, n. 7, p. 1153–1158, 2016.

WANG, Y. X.; XIA, Z. H.; JIANG, X.; LI, L. X.; AN, D.; WANG, H. G.; HENG, B.; LIU, Y. Q. Genistein Inhibits Aβ25–35-Induced Neuronal Death with Changes in the Electrophysiological Properties of Voltage-Gated Sodium and Potassium Channels. **Cellular and Molecular Neurobiology**, v. 39, n. 6, p. 809–822, 2019.

WILKINS, H. M.; SWERDLOW, R. H. Amyloid precursor protein processing and bioenergetics. **Brain Research Bulletin**, v. 133, p. 71–79, 2017.

WILLIAMS, R. J.; SPENCER, J. P. E. Flavonoids, cognition, and dementia: Actions, mechanisms, and potential therapeutic utility for Alzheimer disease. **Free Radical Biology and Medicine**, v. 52, n. 1, p. 35-45, 2012.

WILLIAMSON, R.; SUTHERLAND, C. Neuronal Membranes are Key to the Pathogenesis of Alzheimer's Disease: the Role of Both Raft and Non-Raft Membrane Domains. **Current Alzheimer Research**, v. 8, n. 2, p. 213–221, 2011.

WONG, M. W.; BRAIDY, N.; POLJAK, A.; PICKFORD, R.; THAMBISETTY, M.; SACHDEV, P. S. Dysregulation of lipids in Alzheimer's disease and their role as potential biomarkers. **Alzheimer's & Dementia**, v. 13, n. 7, p. 810–827, 2017.

WU, M.; WANG, B.; FEI, J.; SANTANAM, N.; BLOUGH, E. R. Important roles of Akt/PKB signaling in the aging process. **Frontiers in Bioscience (Scholar edition)**, v. 2, p. 1169–1188, 2010.

XI, Y. D.; ZHANG, D. D.; DING, J.; YU, H. L.; YUAN, L. H.; MA, W. W.; HAN, J.; XIAO, R. Genistein Inhibits Aβ25–35-Induced Synaptic Toxicity and Regulates CaMKII/CREB Pathway in SH-SY5Y Cells. **Cellular and Molecular Neurobiology**, v. 36, n. 7, p. 1151–1159, 2016.

XICOY, H.; WIERINGA, B.; MARTENS, G. J. M. The SH-SY5Y cell line in Parkinson's disease research: a systematic review. **Molecular Neurodegeneration**, v. 12, n. 1, p. 10, 2017.

XU, H. N.; LI, L. X.; WANG, Y. X.; WANG, H. G.; AN, D.; HENG, B.; LIU, Y. Q. Genistein inhibits Aβ25-35-induced SH-SY5Y cell damage by modulating the expression of apoptosis-related proteins and Ca2+ influx through ionotropic glutamate receptors. **Phytotherapy Research**, v. 33, n. 2, p. 431–441, 2019.

YADAV, R. S.; TIWARI, N. K. Lipid Integration in Neurodegeneration: An Overview of Alzheimer's Disease. **Molecular Neurobiology**, v. 50, n. 1, p. 168–176, 2014.

YANG, Y.; LEE, M.; FAIRN, G. D. Phospholipid subcellular localization and dynamics. **Journal of Biological Chemistry**, v. 293, n. 17, p. 6230–6240, 2018.

YAO, M.; NGUYEN, T. V. V.; PIKE, C. J. β-Amyloid-induced neuronal apoptosis involves c-Jun N-terminal kinase-dependent downregulation of Bcl-w. **Journal of Neuroscience**, v. 25, n. 5, p. 1149–1158, 2005.

YARZA, R.; VELA, S.; SOLAS, M.; RAMIREZ, M. J. c-Jun N-terminal kinase (JNK) signaling as a therapeutic target for Alzheimer's disease. **Frontiers in Pharmacology**, v. 6, p. 321, 2016.

YE, S.; WANG, T. T.; CAI, B.; WANG, Y.; LI, J.; ZHAN, J. X.; SHEN, G. M. Genistein protects hippocampal neurons against injury by regulating calcium/calmodulin dependent protein kinase IV protein levels in Alzheimer's disease model rats. **Neural Regeneration Research**, v. 12, n. 9, p. 1479–1484, 2017.

YOU, F.; LI, Q.; JIN, G.; ZHENG, Y.; CHEN, J.; YANG, H. Genistein protects against Aβ25–35 induced apoptosis of PC12 cells through JNK signaling and modulation of Bcl-2 family messengers. **BMC Neuroscience**, v. 18, n. 1, p. 12, 2017.

YU, H. L.; LI, L.; ZHANG, X. H.; XIANG, L.; ZHANG, J.; FENG, J. F.; XIAO, R. Neuroprotective effects of genistein and folic acid on apoptosis of rat cultured cortical neurons induced by β-amyloid 31-35. **British Journal of Nutrition**, v. 102, n. 5, p. 655–662, 2009.

ZEKE, A.; MISHEVA, M.; REMÉNYI, A.; BOGOYEVITCH, M. A. JNK Signaling: Regulation and Functions Based on Complex Protein-Protein Partnerships. **Microbiology and Molecular Biology Reviews**, v. 80, n. 3, p. 793–835, 2016.

ZENG, H.; CHEN, Q.; ZHAO, B. Genistein ameliorates β-amyloid peptide (25–35)-induced hippocampal neuronal apoptosis. **Free Radical Biology and Medicine**, v. 36, n. 2, p. 180–188, 2004.

ZHENG, Z.; SABIRZHANOV, B.; KEIFER, J. Oligomeric amyloid-β inhibits the proteolytic conversion of brain-derived neurotrophic factor (BDNF), AMPA receptor trafficking, and classical conditioning. **Journal of Biological Chemistry**, v. 285, n. 45, p. 34708–34717, 2010.

ZHU, D.; BUNGART, B. L.; YANG, X.; ZHUMADILOV, Z.; LEE, J. C. M.; ASKAROVA, S. Role of membrane biophysics in Alzheimer's-related cell pathways. **Frontiers in Neuroscience**, v. 9, p. 186, 2015.

ZHU, X.; RAINA, A. K.; ROTTKAMP, C. A.; ALIEV, G.; PERRY, G.; BOUX, H.; SMITH, M. A. Activation and redistribution of c-Jun N-terminal kinase/stress activated protein kinase in degenerating neurons in Alzheimer's disease. **Journal of Neurochemistry**, v. 76, n. 2, p. 435–441, 2001.

8. ANEXOS

8.1. Prêmios recebidos durante a realização do doutorado

- **2018 -** SBBq Award for best poster presented during the 47th Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology (item 8.2.1 abaixo).

- **2019 -** Prêmio de melhor apresentação oral na VI Mostra da Bioquímica, Departamento de Bioquímica e Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da UFRGS (item 8.2.3 abaixo).

8.2. Trabalhos apresentados em congressos durante o doutorado

8.2.1. **PETRY, F. S.**; COELHO, B. P.; GAELZER, M. M.; KREUTZ, F.; GUMA, F. C. R.; SALBEGO, C. G.; TRINDADE, V. M. T. Genistein prevents $A\beta_{25-35}$-induced toxicity in SH-SY5Y cells via Akt/GSK-3β signaling pathway. In: 47a Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2018, Joinville, SC. Livro de Resumos, 2018.

8.2.2. **PETRY, F. S.**; HOPPE, J. B.; KLEIN, C. P.; SANTOS, B. G.; HOZER, R. M.; SALBEGO, C. G.; TRINDADE, V. M. T. Neuroprotective Effect of Genistein Against Amyloid-β-induced Cognitive Impairment in Rats. In: 48a Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2019, Águas de Lindoia, SP. Livro de Resumos, 2019.

8.2.3. **PETRY, F. S.**; HOPPE, JULIANA B.; KLEIN, C. P.; SANTOS, B. G.; HOZER, R. M.; BIFI, F.; MATTE, C.; SALBEGO, CHRISTIANNE G.; TRINDADE, V. M. T. Efeito neuroprotetor da genisteína em modelo *in vivo* de toxicidade do peptídeo beta-amiloide. In: VI Mostra da Bioquímica, Porto Alegre, 2019.

8.2.4. **PETRY, F. S.**; HOPPE, J. B.; KLEIN, C. P.; SANTOS, B. G.; HOZER, R. M.; BIFI, F.; SALBEGO, C. G.; TRINDADE, V. M. T. A administração de genisteína protege contra o déficit cognitivo e a sinaptotoxicidade em modelo *in*

vivo de toxicidade do peptídeo beta-amiloide. In: 39ª Semana Científica do HCPA, 2019, Porto Alegre. Anais da 39ª Semana Científica do HCPA, 2019.

8.2.5. **PETRY, F. S.**; COELHO, B. P.; GAELZER, M. M.; KREUTZ, F.; GUMA, F. C. R.; SALBEGO, C. G.; TRINDADE, V. M. T. Efeito neuroprotetor da genisteína sobre a toxicidade induzida pelo peptídeo beta-amiloide em modelo *in vitro*: mecanismos relacionados à prevenção da hiperfosforilação da proteína Tau. In: 39ª Semana Científica do HCPA, 2019, Porto Alegre. Anais da 39ª Semana Científica do HCPA, 2019.

8.3. Trabalhos publicados em coautoria durante o doutorado

8.3.1. SCHMITZ, F.; PIEROZAN, P.; BIASIBETTI-BRENDLER, H.; FERREIRA, F. S.; **PETRY, F. S.**; TRINDADE, V. M. T.; PESSOA-PUREUR, R.; WYSE, A. T. S. Methylphenidate disrupts cytoskeletal homeostasis and reduces membrane-associated lipid content in juvenile rat hippocampus. **Metabolic Brain Disease**, v. 33, p. 693-704, 2018.

8.3.2. COELHO, B. P.; GAELZER, M. M.; **PETRY, F. S.**; HOPPE, J. B.; TRINDADE, V. M. T.; SALBEGO, C. G.; GUMA, F. T. C. R. Dual Effect of Doxazosin: Anticancer Activity on SH-SY5Y Neuroblastoma Cells and Neuroprotection on an In Vitro Model of Alzheimer's Disease. **Neuroscience**, v. 404, p. 314-325, 2019.