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Efeitos farmacológicos de 6-((4-fluorofenil) selanil)-9*H*-purina, uma promissora alternativa terapêutica em modelos experimentais de doença de Alzheimer e suas comorbidades

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Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Bioprospecção do Centro de Ciências Químicas, Farmacêuticas e de Alimentos da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutora em Ciências com ênfase em Bioquímica e Bioprospecção.

Orientadora: Profª. Drª. Cristiane Luchese
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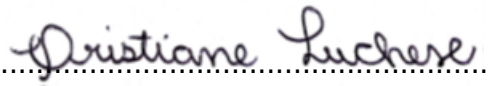

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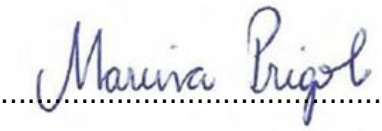

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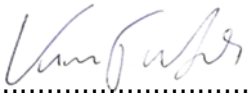

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Prof. Dr. Vinicius Farias Campos, Doutor em Ciências Biológicas (Biotecnologia) pela Universidade Federal de Pelotas, UFPEL, Brasil.

Dedico este trabalho a minha mãe e ao meu avô.

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Perseguir, sem cessar, uma meta: este é o segredo do sucesso
(ANNA PAVLOVA)

Resumo

PINZ, Mikaela Peglow. **Efeitos farmacológicos de 6-((4-fluorofenil) selanil)-9*H*-purina, uma promissora alternativa terapêutica em modelos experimentais de doença de Alzheimer e suas comorbidades.** Orientadora: Cristiane Luchese. 2021. 179 f. Tese (Doutorado em Ciências com ênfase em Bioquímica e Bioprospecção) - Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Pelotas, 2021.

A doença de Alzheimer (DA) é uma desordem neurodegenerativa gradual e debilitante, associada a diversas comorbidades, entre estas, ansiedade e dor. Estudos experimentais têm buscado alternativas terapêuticas que possam atuar nos múltiplos aspectos desta patologia complexa, dentre estes, os compostos derivados de selênio, que apresentam importante destaque nesta busca. Assim, o objetivo do presente estudo foi investigar o possível efeito farmacológico do 6-((4-fluorofenil) selanil)-9*H*-purina (FSP) em modelos experimentais de DA em camundongos machos *Swiss*. Primeiramente foi investigado a afinidade de ligação do FSP com a enzima acetilcolinesterase (AChE) por análises de *docking* molecular e observou-se que o FSP interagiu com os resíduos do sítio ativo da AChE. O primeiro modelo de DA estudado foi induzido por estreptozotocina (STZ). Os camundongos foram tratados com FSP (1 mg/kg, intragastricamente (i.g.)), 30 minutos antes da indução com STZ (2µl de uma solução 2,5 mg/ml, intracerebroventricular (i.c.v.)). O tratamento com o composto foi realizado diariamente até o 10º dia do protocolo experimental. A ansiedade foi avaliada no teste do labirinto em cruz elevado e o comprometimento da memória foi avaliado nos testes labirinto em Y, reconhecimento de objetos (RO) e esquiva inibitória. O FSP mitigou a indução de ansiedade e o prejuízo da memória causados pela STZ. Posteriormente o sistema colinérgico e a enzima Na^+/K^+-ATPase foram investigados . O FSP protegeu contra a disfunção do sistema colinérgico e no comprometimento da Na^+/K^+-ATPase. O FSP (300 mg/kg, i.g.) não modificou os parâmetros toxicológicos avaliados. A injeção do peptídeo beta amiloide (βA) (fragmento 25-35) foi utilizada como o segundo modelo de DA. Os camundongos foram tratados com o FSP (1 mg/kg, i.g.), 30 minutos antes da indução com βA (3 nmol/3 µl /por local, i.c.v.). O tratamento com o composto foi realizado diariamente até o 15º dia do protocolo experimental. Neste estudo, o efeito do FSP foi investigado

contra o comprometimento da memória e a sensibilidade a nocicepção induzidos pelo βA, que foram avaliados na tarefa de RO e no teste do Von-Frey (TVF), respectivamente. O FSP atenuou o comprometimento da memória e a hipernocicepção induzidos pelo βA. Observou-se um processo neuroinflamatório no córtex cerebral e hipocampo dos camundongos que foram induzidos pelo βA, com um aumento na expressão do fator nuclear-κB (NF-κB) e de citocinas pró- inflamatórias. O FSP diminuiu a expressão de NF-κB no hipocampo, e reduziu as citocinas pró-inflamatórias no córtex cerebral e hipocampo. Além disso, o βA aumentou os níveis de oxidantes e a peroxidação lipídica no córtex cerebral e hipocampo, bem como diminuiu a expressão da heme oxigenase-1 (HO-1) e peroxiredoxina-1 (Prx1) no hipocampo. O FSP protegeu contra o dano oxidativo, diminuindo os níveis de oxidantes, a peroxidação lipídica e aumentando as expressões de HO-1 e Prx1 no hipocampo de camundongos. Com isso, preveniu a ativação do fator nuclear 2 relacionado ao eritroide 2 no hipocampo do grupo βA. Em conclusão, o FSP apresenta-se como uma alternativa terapêutica para a DA, uma vez que é capaz de atuar em múltiplos alvos envolvidos nesta patologia.

Palavras-chave: doença de Alzheimer; organoselênio; purina; neuroinflamação; sistema colinérgico; estresse oxidativo.

Abstract

PINZ, Mikaela Peglow. **Pharmacological effects of 6-((4-fluorophenyl) selanyl)-9*H*-purine, a promising therapeutic alternative in experimental models of Alzheimer's disease and its comorbidities.** Advisor: Cristiane Luchese. 2021. 179 p. Thesis (Doctorate in Science with an emphasis on Biochemistry and Bioprospecting) - Center for Chemical, Pharmaceutical and Food Sciences, Federal University of Pelotas, Pelotas, 2021.

Alzheimer's disease (AD) is a gradual and debilitating neurodegenerative disorder associated with several comorbidities, including anxiety and pain. Experimental studies have been looking for therapeutic alternatives that can act on the multiple aspects of this complex pathology, among them, the compounds derived from selenium, which are important in this search. Thus, the aim of the present study was to investigate the possible pharmacological effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) in experimental AD models in male Swiss mice. First, the binding affinity of FSP with the enzyme acetylcholinesterase (AChE) was investigated by molecular docking analysis and it was observed that FSP interacted with residues of the active site of AChE. The first AD model studied was induced by streptozotocin (STZ). Mice were treated with FSP (1 mg/kg, intragastrically (i.g.)) 30 minutes before induction with STZ (2μl of 2.5 mg/ml solution, intracerebroventricular (i.c.v.)). The treatment with the compound was carried out daily until the 10th day of the experimental protocol. Anxiety was assessed in the elevated plus-maze test and memory impairment was assessed in the Y-maze, object recognition (OR) and inhibitory avoidance tests. FSP mitigated the anxiety induction and memory impairment caused by STZ. Subsequently, the cholinergic system and Na+/K+-ATPase enzyme were investigated. FSP protected against cholinergic system dysfunction and Na^+/K^+-ATPase impairment. FSP (300 mg/kg, i.g.) did not modify the evaluated toxicological parameters. Injection of beta amyloid peptide (Aβ) (fragment 25-35) was used as the second AD model. Mice were treated with FSP (1 mg/kg, i.g.), 30 minutes before induction with Aβ (3 nmol/3 μl /per site, i.c.v.). Treatment with the compound was carried out daily until the 15th day of the experimental protocol. In this study, the effect of FSP was investigated against Aβ-induced impairment of memory and hypernociception, which were evaluated in the OR

task and in the Von-Frey test (VFT), respectively. FSP attenuated Aβ-induced impairment of memory and nociception sensitivity. A neuroinflammatory process was observed in the cerebral cortex and hippocampus of mice that were induced by Aβ, with an increase in expression of nuclear factor-κB (NF-κB) and cytokines. FSP decreased the expression of NF-κB in the hippocampus, and reduced pro-inflammatory cytokines in the cerebral cortex and hippocampus. Furthermore, βA increased the levels of oxidants and lipid peroxidation in the cerebral cortex and hippocampus, as well as decreased the expression of heme oxygenase-1 (HO-1) and peroxiredoxin-1 (Prx1) in the hippocampus. FSP protected against oxidative damage, decreasing oxidant levels, lipid peroxidation and increasing HO-1 and Prx1 expressions in the mouse hippocampus. Thus, it prevented the activation of nuclear factor erythroid 2-related factor in the Aβ-induced mouse hippocampus. In conclusion, FSP presents itself as a therapeutic alternative for AD, as it is capable of acting on multiple targets involved in this pathology.

Keywords: Alzheimer's disease; organoselenium; purine; neuroinflammation; cholinergic system; oxidative stress.

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ACh	acetilcolina
AChE	acetilcolinesterase
AINE	anti-inflamatório não esteroidal
βA	peptídeo beta amilóide
DNA	anti-inflamatório não esteroidal
ChAT	colina acetiltransferase
CAS	*catalytic anionic site*
CAT	catalase
CO	monóxido de carbono
DA	doença de Alzheimer
DAE	doença de Alzheimer do tipo esporádica
DNA	ácido desoxirribonucléico
EROs	espécies reativas de oxigênio
FDA	Food and Drug Administration
GR	glutationa redutase
GPx	glutationa peroxidases
GSH	glutationa reduzida
GSSG	glutationa oxidada
H_2O_2	peróxido de hidrogênio
HO-1	heme oxigenasse-1
i.c.v.	intracerebroventricular
IFN-γ	interferon-gama
IL-1β	interleucina- 1 beta
K^+	potássio
MDA	malondialdeído
Na^+	Sódio
NADPH	fosfato de dinucleotídeo de adenina e nicotinamida
NF-κB	fator nuclear- kappa B
$NO^{\bullet}$	óxido nítrico
$O2^{\bullet-}$	radical ânion superóxido
PAS	*peripheral anionic site*
(PhSe)2	disseleneto de difenila
Prxs	peroxirredoxinas
PSEN-1	presenilina-1
αPPAs	proteína amilóide solúvel neurotrófica e neuroprotetora
PKC	proteína quinase C
PPA	proteína precursora amilóide
SNC	sistema nervoso central
STZ	estreptozotocina
TCA	ciclo do ácido tri carboxílico
TNF-α	fator de necrose tumoral-alfa

Sumário

1. INTRODUÇÃO

Ao longo do envelhecimento ocorre o declínio das funções fisiológicas e o aumento do risco de desenvolvimento de desordens neurodegenerativas, como a doença de Alzheimer (DA) (Weissman *et al.*, 2007). A fisiopatologia desta doença ainda não está totalmente esclarecida, mas sabe-se que é uma doença multifatorial (Avramovich, Amit e Youdim, 2002). A DA é caracterizada principalmente por alterações bioquímicas como a formação de placas senis contendo o peptídeo beta amilóide (βA) e emaranhados neurofibrilares intracelulares que tem em sua composição a proteína *tau* hiperfosforilada (Martins *et al.*, 2018). No entanto, a patologia da DA inclui não apenas estas características, mas também a neuroinflamação, a resistência à insulina, o estresse oxidativo e as anormalidades cerebrovasculares. Essas características patológicas apresentam interações recíprocas e complexas com a disfunção colinérgica (Hampel *et al.*, 2018) (Figura 1).

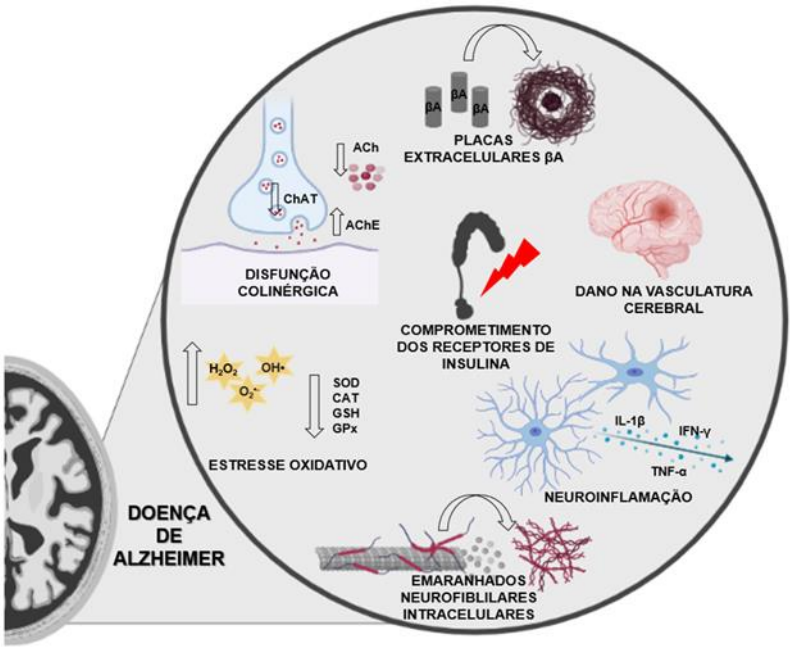

Figura 1. Principais características presentes na Doença de Alzheimer**.**

Fonte: Imagem do autor, 2021.

Sabe-se que a disfunção do sistema colinérgico é uma das características mais marcantes da DA. Esta é caracterizada por uma diminuição nos níveis do neurotransmissor acetilcolina (ACh) no cérebro de pacientes portadores desta doença. No sistema colinérgico, a colina acetiltransferase (ChAT) é uma enzima responsável pela síntese de ACh nos neurônios colinérgicos e tem sido considerada o indicador mais específico para monitorar o estado funcional desses neurônios e encontra-se reduzida na DA (Oda, 1999). Além disso, a acetilcolinesterase (AChE) finaliza a transmissão colinérgica hidrolisando a ACh. Um aumento na atividade da AChE tem sido associado à perda da função cerebral e, portanto, à perda de habilidades intelectuais. De fato, os inibidores da AChE são os tratamentos mais aceitos para os sintomas da DA, promovendo a restauração nos níveis do neurotransmissor ACh (Mokrani *et al.*, 2019).

Durante o curso clínico da doença, sintomas neuropsiquiátricos como irritabilidade, depressão, agitação e ansiedade (Masters, Morris e Roe, 2015) estão associados ao comprometimento cognitivo e a perda de memória. Uma outra condição que agrava ainda mais a qualidade de vida destes pacientes é a presença da dor. O sintoma neuropsiquiátrico de agitação pode ser ocasionado pela dor, muitas vezes subdiagnosticada e não tratada de forma adequada (Husebo *et al.*, 2011; Sampson *et al.*, 2015). A prescrição inadequada de medicamentos para o tratamento da dor em pacientes com a DA corrobora com a redução de mobilidade, fraqueza muscular e quedas, que consequentemente tem um grande impacto negativo na qualidade de vida destes pacientes.

Sendo assim, o desenvolvimento de terapias que possam atuar modulando simultaneamente diferentes alvos ou mecanismos envolvidos na cascata neurodegenerativa da DA é uma estratégia apropriada para obter eficácia no tratamento desta doença (León, Garcia e Marco-Contelles, 2013). A experiência clínica tem demonstrado que os fármacos disponíveis para a DA podem ser apenas paliativos, pois não abordam a etiologia complexa da doença, além de apresentarem efeitos adversos significativos (Ali *et al.*, 2015; Casey, Antimisiaris e O'Brien, 2010).

Com base na relevância da DA e no interesse em alcançar uma eficácia terapêutica para esta doença, o nosso grupo de pesquisa tem se dedicado ao estudo de compostos contendo selênio. Um destes compostos é o 6-((4-fluorofenil) selanil)-

9*H*-purina (FSP), um derivado de purina contendo selênio, que demonstrou efeito em aumentar as fases de consolidação e recuperação da memória, bem como inibir a atividade da AChE no córtex cerebral de camundongos (Duarte *et al.*, 2017). De fato, além do grupo organoselênio, o FSP é um derivado de purina que é um constituinte de muitos compostos heterocíclicos bioativos com diversas atividades biológicas relevantes (Hayallah, Talhouni e Abdel Alim, 2012; Jordheim *et al.*, 2013; Kinali-Demirci, Idil e Dişli, 2015).

Tendo em vista que (i) até o momento não existe uma terapia capaz de impedir a progressão da DA, (ii) os fármacos existentes para esta doença apresentam uma série de efeitos adversos significativos, e (iii) o FSP ter apresentado ação anticolinesterásica e na melhora da memória, propriedades estas relatadas na literatura, tem-se o interesse em aprofundar os estudos dos efeitos farmacológicos do FSP em modelos de DA. Acredita-se que o FSP, além de desempenhar uma ação anticolinesterásica, poderia também apresentar efeitos como antioxidante e anti-inflamatório devido a sua natureza química, um composto derivado de purina contendo selênio. Desta forma, o FSP poderia atenuar os danos de memória, aprendizado e as comorbidades em modelos de DA, tornando-se uma possível alternativa terapêutica multialvo para a DA, uma condição multifatorial.

2. REFERENCIAL TEÓRICO

2.1. DOENÇA DE ALZHEIMER

A DA foi identificada pela primeira vez em 1906 por Alois Alzheimer, um neurologista e psiquiatra alemão (Alzheimer's Association, 2010). Esta doença é a principal causa de demência em idosos e resulta em falha sináptica e destruição neuronal em regiões cerebrais essenciais para a memória e outros domínios cognitivos (Villemagne *et al.*, 2017). Apesar de os resultados epidemiológicos da DA serem reduzidos no Brasil, estima-se que um milhão de pessoas são acometidas pela demência no país (Ferri, 2012). Um estudo demonstrou que o Brasil é o segundo país em prevalência de demência no mundo (Nichols *et al.*, 2019), afetando cerca de 50 milhões de pessoas em todo mundo (Brookmeyer *et al.*, 2018; Prince *et al.*, 2015).

A DA é uma desordem multifatorial, uma vez que seu desenvolvimento envolve tanto fatores ambientais quanto genéticos. Dentre os principais fatores de risco relacionados à patologia encontram-se a idade avançada, o sexo feminino, a presença de injúria cerebral prévia e a expressão do alelo ε4 da apolipoproteína E (Dorszewska *et al.*, 2016). São descritas duas formas de DA: a DA do tipo familiar (~5% dos casos), na qual ocorrem alterações no gene da proteína precursora amiloide (PPA) ou das enzimas secretásicas (como a presenilina (PSEN)-1); e a DA do tipo esporádica (DAE) (~95% dos casos), em que apresenta múltiplos fatores genéticos e não genéticos, no qual o quadro demencial manifesta-se de forma mais tardia (Dorszewska *et al.*, 2016).

Os maiores marcadores da DA são a presença depósitos extracelulares de βA e a presença de emaranhados neurofiblilares, estes por sua vez, ocasionam disfunção neuronal, morte celular, perda das conexões sinápticas, principalmente devido a presença da inflamação e do estresse oxidativo (Zhao e Zhao, 2013). Além disso, a DA pode ser dividida em fases distintas sendo estas a fase bioquímica, a fase celular e a fase sintomática.

A fase bioquímica compreende o processamento anormal da PPA, a agregação anormal de βA e hiperfosforilação da proteína *tau* (Walker e Jucker, 2015). A formação de placas βA é um processo que envolve diversas etapas. Inicia-se pela clivagem da PPA transmembrana através das enzimas γ-secretase e β-secretase, seguido de formação de monômeros βA, estes monômeros formam várias estruturas,

como aglomerados desdobrados, oligômeros βA, fibrilas maiores e placas amiloides. Uma vez que tais agregados βA apresentam proximidade com as superfícies celulares, podem interagir fortemente com a membrana (Figura 2) (Drolle *et al.*, 2014).

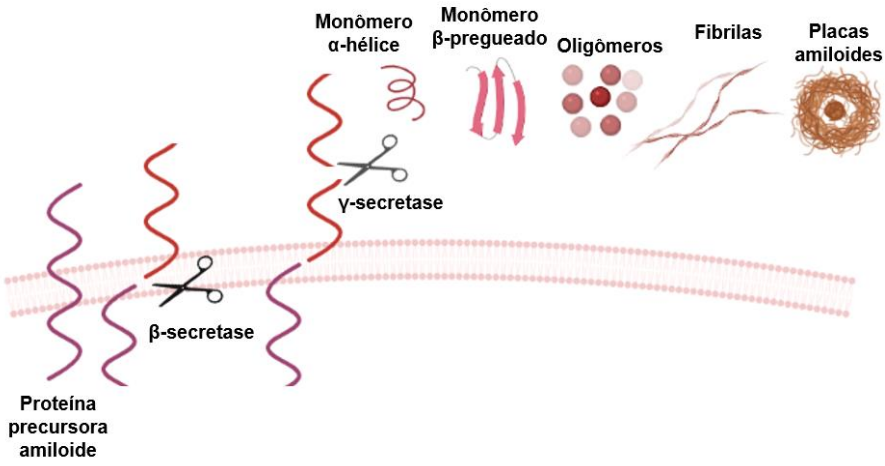

Figura 2. Formação das placas amiloides: a proteína precursora amiloide (PPA) é clivada pelas enzimas secretásicas, formando os monômeros βA, que se agregam gerando os oligômeros βA, fibrilas maiores e por fim as placas amiloides.

Fonte: Imagem do autor, 2021.

Ao longo dos anos, a visão prevalecente da patogênese da DA tem sido de que as mudanças no acúmulo de βA aceleram o processo da doença e iniciam uma cascata deletéria envolvendo a proteína *tau* (Busche e Hyman, 2020). Na DA ocorre uma hiperfosforilação irregular da proteína *tau* (responsáveis pela sustentabilidade dos microtúbulos neuronais presentes no axônio), resultando em despolimerização dos microtúbulos neuronais, formando assim oligômeros *tau* citoplasmáticos insolúveis, que se acumulam para formar filamentos helicoidais emparelhados, que se agregam e, por fim, levam à formação de emaranhados neurofibrilares (Mamun *et al.*, 2020) (Figura 3).

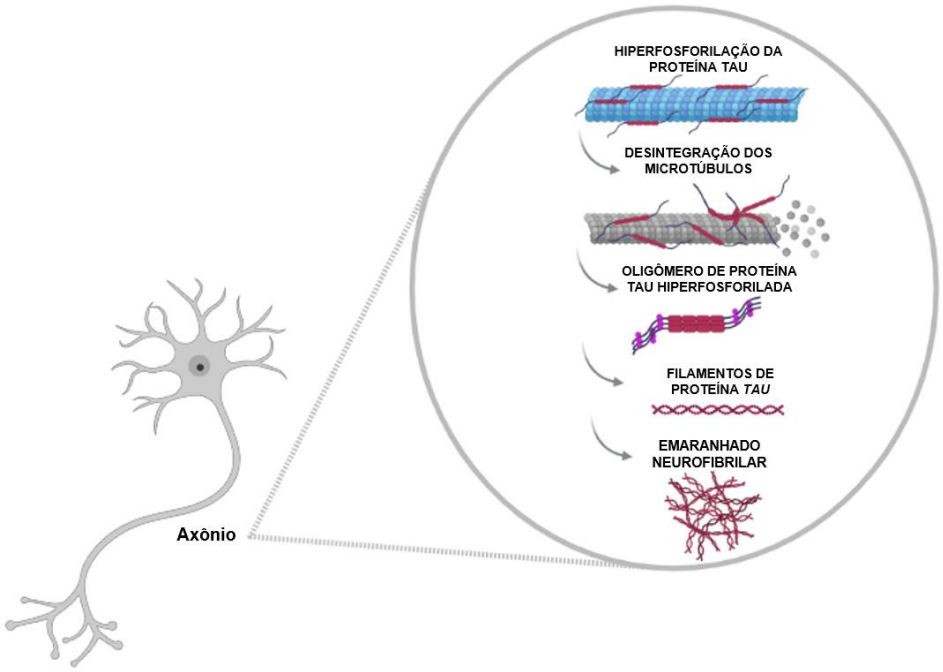

Figura 3. Formação dos emaranhados neurofibrilares intracelulares**:** a proteína *tau* hiperfosforilada resulta em desintegração dos microtúbulos neuronais e forma oligômeros *tau* citoplasmáticos insolúveis, que formam filamentos helicoidais emparelhados, estes filamentos agregam-se e, geram os emaranhados neurofibrilares.

Fonte: Imagem do autor, 2021.

No entanto, evidências atuais sugerem que ambas as disfunções têm efeitos sinérgicos (Busche e Hyman, 2020). Estas desordens são responsáveis por ocasionar o comprometimento na funcionalidade de diversas proteínas, interferir na estrutura da membrana celular, comprometer a sinalização e outras funções cerebrais (Palop e Mucke, 2010). Entretanto, existem reguladores chave, que são capazes de controlar as alterações ocasionas na fase bioquímica da DA. Dentre estes destaca-se o processo de autofagia que é responsável para degradação e reciclagem de componentes do citosol e organelas celulares danificadas (Nixon, 2013). Desta maneira, os portadores da DA podem sobreviver a este estresse por muitos anos. As respostas desencadeadas nesta fase são fisiológicas e podem ser autônomas celulares ou não celulares. Ainda, podem ocorrer respostas bioquímicas, que mantém a homeostase na rede proteostática (Labbadia e Morimoto, 2015), ou então, pode ser funcional, envolvendo vários mecanismos de plasticidade sináptica. A resposta inflamatória inicial pode contribuir para sustentar esta homeostase.

Acredita-se que a transição de uma resposta celular autônoma reversível para uma autônoma irreversível e crônica não seja mais dependente dos estressores iniciais, o βA e a proteína *tau*, e representa uma fase crítica no processo da doença (Braak e Braak, 1996). De Strooper e Karran (2016) realizaram um estudo demonstrando fortes evidências que apoiam uma fase celular longa e complexa, que consiste em um envolvimento e respostas de células como os astrócitos e a microglia, bem como uma ligação com a vasculatura na DA. Considera-se que esta fase ocorre após a fase bioquímica e antecede a fase clínica, onde surgem os sintomas da doença.

A homeostase da vasculatura cerebral é imprescindível, uma vez que alterações precoces na barreira sangue-cérebro poderiam exercer um papel principal no início da DA. Esta barreira consiste em células endoteliais firmemente conectadas e células astrogliais. A barreira endotelial não é permeável, contudo, apresenta proteínas de transporte especializadas que são responsáveis pela retirada do βA e da proteína *tau* da circulação. Já foi relatado que o comprometimento na funcionalidade dessas proteínas culmina em prejuízo no comportamento animal (Zhao *et al.*, 2015). Defeitos vasculares levam ao acúmulo do βA, contudo, pode ser que o acúmulo deste peptídeo possa ocasionar também o comprometimento da vasculatura cerebral. Sendo assim, esse ciclo destrutivo é uma parte importante da ação e reação celular subjacente à esta fase na DA (de Strooper e Karran, 2016).

A fase sintomática da DA ocorre cerca de 20 anos após o surgimento dos primeiros processos patológicos (de Strooper e Karran, 2016). Esta fase compreende os sintomas como afasia (a perda parcial ou total da capacidade de compreender ou expressar a linguagem escrita ou falada), amnésia, agnosia (perda da capacidade de identificar objetos), delírios, irritabilidade, labilidade, agressão, alterações do apetite e do sono, euforia, agitação, alucinações, ansiedade e depressão (Kamada *et al.*, 2018).

Um dos maiores problemas desta patologia é que não existe uma terapia capaz de curar ou impedir a progressão da DA. Além do mais, à medida que o dano cerebral progride, ocorre uma redução na eficácia da ação farmacológica dos medicamentos (inibidores das colinesterases) utilizados para o tratamento sintomático desta doença. Esta redução pode ocorrer até um ponto crítico onde estes medicamentos não exercem mais nenhum efeito na DA. Além do mais, cabe destacar que, além de

atuarem por tempo limitado, estes apresentam ações que variam de pessoa para pessoa (Mehta, Adem e Sabbagh, 2012; Mushtaq *et al.*, 2014). Os medicamentos inibidores da enzima AChE aprovados pela *Food and Drug Administration* (FDA) para o tratamento da DA são a donepezila, a galantamina e a rivastigmina.

Esses medicamentos apresentam efeitos benéficos modestos e possuem efeitos adversos como náusea, diarreia, distúrbio gastrintestinal e problemas com questões de biodisponibilidade (Ali *et al.*, 2015; Casey, Antimisiaris e O'Brien, 2010). Inclusive, o inibior da AChE, tacrina foi removido do mercado por apresentar hepatotoxicidade. Desta forma, os principais desafios em relação à DA incluem a falta de biomarcadores confiáveis para seu diagnóstico precoce, bem como a falta de estratégias eficazes de prevenção e tratamento (Mangialasche *et al.*, 2010; Wang *et al.*, 2018).

2.1.1. Doença de Alzheimer e ansiedade

Durante o curso clínico da demência, além do comprometimento cognitivo e da perda de memória, existe um desafio muito complexo apresentado pelos sintomas neuropsiquiátricos. Estes sintomas são caracterizados por irritabilidade, depressão, agitação, ansiedade, sintomas psicóticos e alterações motoras (Masters, Morris e Roe, 2015). A ansiedade é caracterizada por uma emoção básica que resulta em uma resposta adaptativa ao estresse ou a situações estressantes, facilitando a sobrevivência aos riscos potenciais (Lang, Bradley e Cuthbert, 1998). Contudo, quando essa resposta torna-se excessivamente frequente pode alcançar um grau patológico, qualificando-se como um distúrbio psiquiátrico (Fuchs e Flügge, 2006). Os transtornos de ansiedade estão entre as desordens mentais mais prevalentes no mundo (Bandelow e Michaelis, 2015).

Além do mais, de acordo com Johansson e colaboradores (2019) a ansiedade pode ser considerada como uma manifestação clínica precoce da DA. Existem algumas teorias sobre como a ansiedade poderia estar relacionada com a DA. No entanto, ainda ocorrem debates sobre a natureza da associação entre ansiedade e DA (Santabárbara *et al.*, 2019). O papel do sistema colinérgico na modulação do comportamento de ansiedade já foi relatado na literatura (Anagnostaras *et al.*, 1999; Klinkenberg e Blokland, 2010). Foi reportado que, a perda da função colinérgica do hipocampo, em um modelo animal de escopolamina, prejudica o processamento de

estímulos ameaçadores, resultando em um aumento no comportamento ansioso em ratos (Smythe *et al.*, 1998). Vale ressaltar que, o comprometimento da função colinérgica é uma das principais alterações presentes na DA. Uma outra hipótese é que, no início da DA, a ansiedade pode se desenvolver devido à interrupção das funções límbicas (Eysenck *et al.*, 2007).

Um estudo apontou que a ansiedade está significativamente associada a um risco aumentado de demência em um intervalo de tempo de pelo menos 10 anos (Gimson *et al.*, 2018). Portanto, a ansiedade pode também ser um fator de risco para o desenvolvimento de demência. Os níveis elevados de cortisol associados a transtornos de ansiedade (Mathew, Price e Charney, 2008) podem ocasionar o processo patológico da DA, como a atrofia hipocampal, a formação de βA e o acúmulo de proteína *tau* (Gulpers *et al.*, 2016). Além disso, os benzodiazepínicos que são medicamentos amplamente utilizados no tratamento da ansiedade apresentam-se como um possível fator de risco para o desenvolvimento da DA (Gage *et al.*, 2014). Esses diferentes mecanismos poderiam reforçar uns aos outros em ciclos de *feedback*. Sendo assim, novas intervenções terapêuticas direcionadas para a ansiedade em pacientes com a DA poderiam ser uma potencial forma de desaceleração do declínio cognitivo.

2.1.2. Doença de Alzheimer e dor

A agitação, um outro sintoma clínico neuropsiquiátrico presente em portadores da DA, pode ser causada por quadros de dor subdiagnosticada e não tratada nestes pacientes. Na verdade, estudos tem mostrado que a dor tem sido diagnosticada em pacientes com a DA (Husebo *et al.*, 2011; Sampson *et al.*, 2015). De fato, os mecanismos imunológicos e inflamatórios no sistema nervoso central (SNC) desempenham um papel fundamental em quadros de dor neuropática.

A dor neuropática é caracterizada por ocorrer em áreas ou órgãos envolvidos em lesões ou doenças neurológicas (Backonja, 2003). Este tipo de dor pode ser ocasionado por uma consequência direta de dano ou por uma disfunção dos axônios ou corpo dos neurônios que acarrete em interrupção da bainha de mielina, tanto no sistema nervoso periférico quanto no SNC (Backonja, 2003). Mudanças na função química e na estrutura neuronal estão diretamente envolvidas na alteração da sensibilidade, característica típica da dor neuropática.

Essas alterações têm sido estudadas de forma mais abrangente em neurônios periféricos e espinhais, mas modificações semelhantes ocorrem no cérebro, principalmente na região do córtex cerebral (Woolf e Mannion, 1999). Deste modo, a neuropatia é frequentemente desenvolvida por componentes mediados pelo SNC, com influência sobre a experiência cognitiva da dor, e que está relacionada com mudanças de humor (Ignatowski *et al.*, 1999).

Além disso, muitas destas alterações mencionadas anteriormente podem ser ocasionadas na DA. As placas contendo o βA e os emaranhados neurofibrilares intracelulares são acompanhadas por neuroinflamação e foram detectadas em regiões envolvidas no processo da dor (medula espinhal e o tálamo) (Aman *et al.*, 2016; Rüb *et al.*, 2002; Schmidt *et al.*, 2001) (Figura 4).

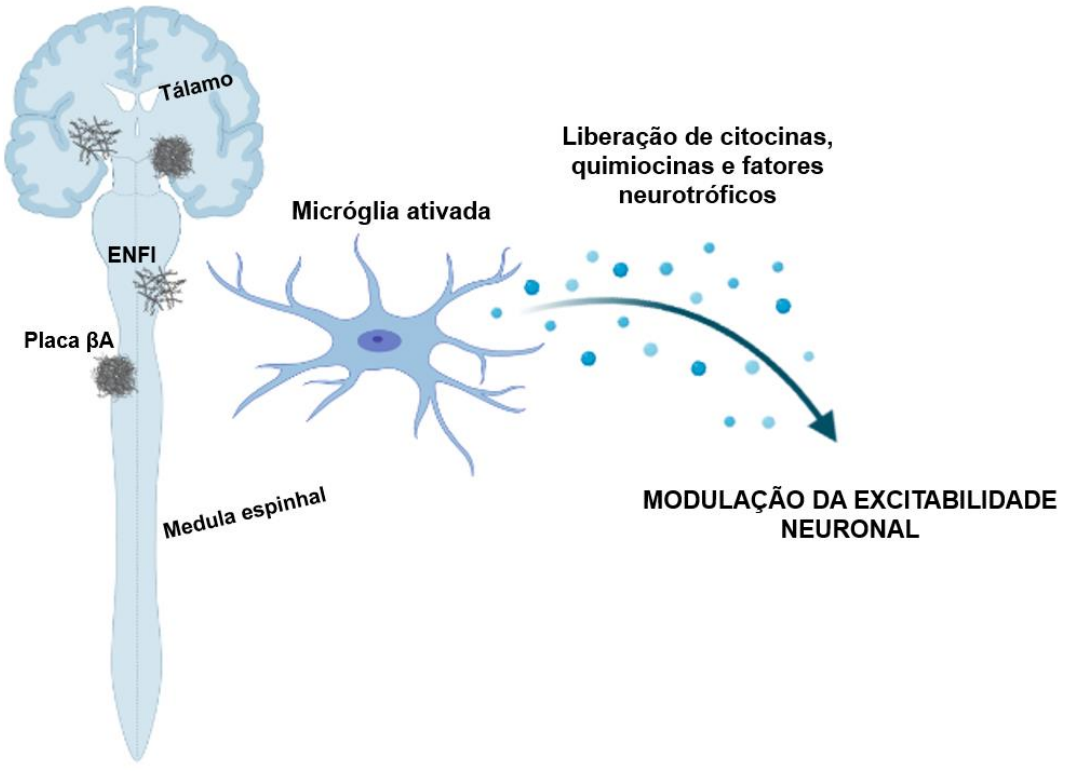

Figura 4. Processos patológicos da doença de Alzheimer que podem estar envolvidos na dor. Deposição de placas contendo o peptídeo beta amiloide (βA) e os emaranhados neurofibrilares intracelulares (ENFI) na medula espinhal e tálamo.

Fonte: Imagem do autor, 2021.

Embora não exista um total esclarecimento dos mecanismos subjacentes da dor na DA (Ballard *et al.*, 2009; Corbett *et al.*, 2012), acredita-se que a ativação da microglia e consequente liberação de moléculas bioativas, tais como as citocinas, as quimiocinas e os fatores neurotróficos, podem modular a excitabilidade de neurônios e desenvolver a dor nestes pacientes (Keller *et al.*, 2007; Scholz e Woolf, 2007). Estima-se que a prevalência de dor em pacientes com DA é de 50-80%, porém a avaliação e o tratamento adequado são frequentemente difíceis e isso tem um grande impacto negativo na qualidade de vida destes pacientes (Corbett *et al.*, 2012; Husebo *et al.*, 2011). Foi demonstrado que 60,51% de portadores de demência que fazem o uso de inibidores da AChE e de memantina foram tratados com analgésicos (Scuteri *et al.*, 2018). Adicionalmente, já foi reportado que há um aumento na prescrição e/ou uso de paracetamol (anti-inflamatório não esteroidal (AINE)) em indivíduos com comprometimento cognitivo, em comparação com indivíduos cognitivamente intactos (Sandvik *et al.*, 2016). Portanto, uma melhor compreensão dos mecanismos fisiopatológicos subjacentes as alterações na transmissão sensorial e uma terapia adequada são essenciais para melhorar a manejo clínico da dor em pacientes com DA.

2.2. ASPECTOS ENVOLVIDOS NA DOENÇA DE ALZHEIMER

2.2.1. Hipótese colinérgica

Os eventos que ocorrem na DA acarretam em alterações na neurotransmissão. A ACh é um neurotransmissor que está envolvido no aprendizado e na memória (Schliebs e Arendt, 2011). A progressão dos sintomas da doença está correlacionada a mudanças nas estruturas que envolvem as sinapses colinérgicas em determinadas regiões do cérebro, como o córtex e o hipocampo, que apresentam neurotransmissão colinérgica diminuída (Silva *et al.*, 2006).

Ainda que muitos sistemas neurotransmissores estão alterados na DA, o sistema colinérgico parece ser o mais comprometido. Este é o primeiro sistema envolvido com a disfunção cognitiva e o prejuízo de memória no envelhecimento normal e na DA. Desta maneira, com a estratégia de aumentar os níveis de ACh na fenda sináptica, medicamentos capazes de inibir a hidrólise da ACh foram desenvolvidos (Weinreb *et al.*, 2016). O defeito na neurotransmissão colinérgica (hipótese colinérgica) tem sido amplamente estudado na DA, bem como os distúrbios

de terminais pré-sinápticos colinérgicos decorrentes dos núcleos do prosencéfalo basal (núcleo basal de Meynert e núcleo da banda diagonal) (Felten, O'Banion e Maida, 2015).

O núcleo basal de Meynert é uma região cerebral envolvida com a produção da enzima ChAT. Neste sentido, a atrofia deste núcleo está relacionada com a deficiência da ACh na DA. Esta enzima é responsável por catalisar a reação de síntese do neurotransmissor ACh a partir da colina e da acetil-coenzima A. Depois de sintetizada, a ACh é liberada na fenda sináptica, onde será ligada a dois tipos de receptores, os muscarínicos e os nicotínicos. A ACh restante é degradada pela enzima AChE, na fenda sináptica, em acetato e colina (Martocchia e Falaschi, 2008) (Figura 5).

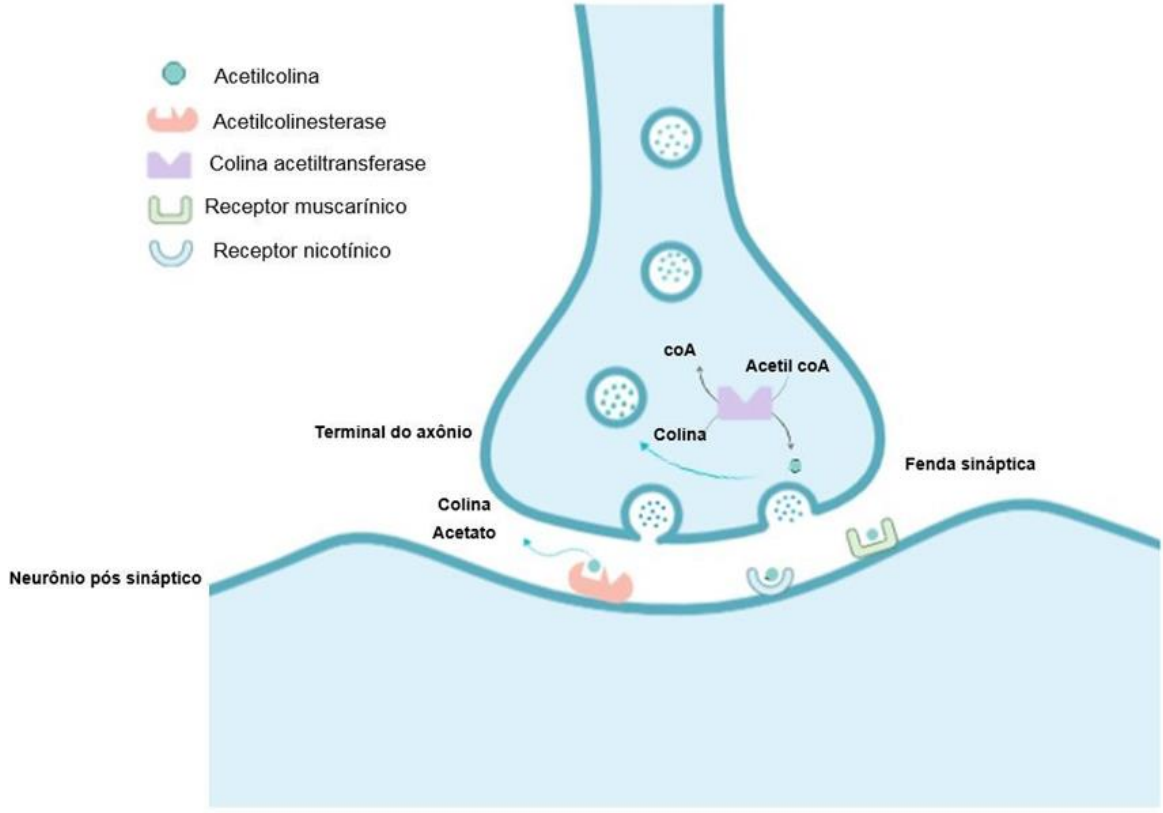

Figura 5. Sistema colinérgico. O neurotransmissor acetilcolina (ACh) é sintetizado pela colina acetiltransferase a partir dos substratos colina e acetilcoenzima A, posteriormente é armazenado em vesículas sinápticas no neurônio pré-sináptico. Em determinadas condições estas vesículas se rompem e liberam a ACh na fenda sináptica para atuarem em seus receptores (muscarínicos e nicotínicos) presentes no neurônio pós-sináptico. Na fenda sináptica a acetilcolinesterase (AChE) é responsável pela hidrólise da ACh em colina e acetato.

Fonte: Imagem do autor, 2020.

A hipótese colinérgica se baseia em diversas alterações nas quais ocorrem: (i) degeneração das projeções neuronais, (ii) perda dos terminais colinérgicos do córtex cerebral, (iii) redução na atividade da ChAT e consequentemente na síntese de ACh, (iv) redução da recaptação de colina, (v) aumento na atividade da AChE, (vi) redução no número de receptores muscarínicos, e (vii) redução do número de receptores nicotínicos (Gualtieri *et al.*, 1995).

Alterações neuroquímicas no sistema colinérgico têm sido observadas em cérebros de pacientes com a DA e esta disfunção colinérgica está envolvida em alterações de memória, aprendizagem, atenção e outros processos cognitivos comuns afetados nesses pacientes (Cummings e Back, 1998). Adicionalmente, já foi demonstrado que camundongos transgênicos que superexpressam os genes da proteína presenilina (envolvida com a porção catalítica da γ-secretase) e PPA, apresentam distrofia colinérgica e redução na atividade da enzima ChAT no córtex cerebral e no hipocampo, resultando na diminuição da função colinérgica (Zaborszky, Pol e Gyengesi, 2012). Por outro lado, Nitsch e colaboradores (1992) e Mori e colaboradores (1995) demonstraram que a estimulação dos receptores colinérgicos, tanto pelos agonistas muscarínicos quanto pelo tratamento com inibidores da colinesterase, deslocou o processamento PPA em direção a vias não amiloidogênicas. Também foi demonstrado que os déficits colinérgicos no cérebro de ratos podem interagir com mecanismos pró-inflamatórios agudos para produzir ou exacerbar o comprometimento cognitivo (Field, Gossen e Cunningham, 2012). Essas observações ilustram as interações altamente complexas que provavelmente existem entre a desenervação colinérgica e outras características patológicas da DA.

2.2.2. Enzima Acetilcolinesterase

Um dos principais focos da hipótese colinérgica é a enzima AChE. Esta enzima pertence à classe das serino-hidrolases e pode se apresentar na forma solúvel ou ligada à membrana. A forma solúvel está localizada nas terminações nervosas colinérgicas, onde é responsável por regular a concentração do neurotransmissor ACh livre. A forma ligada à membrana ocorre em locais como o eritrócito (Rang *et al.*, 2004). A primeira estrutura tridimensional (3D) da AChE foi determinada, em 1991 por Sussman e colaboradores, descrita como *Torpedo califórnica*.

A principal função da AChE é catalisar de maneira rápida e eficiente a hidrólise do neurotransmissor ACh. Esta hidrólise desativa o neurotransmissor após sua ligação ao seus respectivos receptores (muscarínicos ou nicotínicos), de maneira que impede seu acúmulo nas sinapses e evita a produção de estímulos contínuos por interações repetitivas (Gringauz, 1997). No sistema nervoso periférico esta enzima é responsável pelo controle dos batimentos cardíacos, pelos impulsos nervosos, pela contração de músculos lisos, dilatação de vasos sanguíneos, entre outros. Em contrapartida, no SNC a AChE modula o controle motor, a memória e a cognição. Esta enzima possui a capacidade de catalisar até $6x10^5$ moléculas de ACh por molécula de enzima a cada minuto (Petronilho, Pinto e Villar, 2011).

A AChE é uma proteína de aproximadamente 320.000 Da, apresentando duas cadeias polipeptídicas alfa (α) e beta (β). Ela expressa três regiões significativas na extensão catalítica: o sítio catalítico CAS (*catalytic anionic site*), o sítio periférico PAS (*peripheral anionic site*) e uma fenda hidrofóbica que liga o PAS ao CAS (Figura 6).

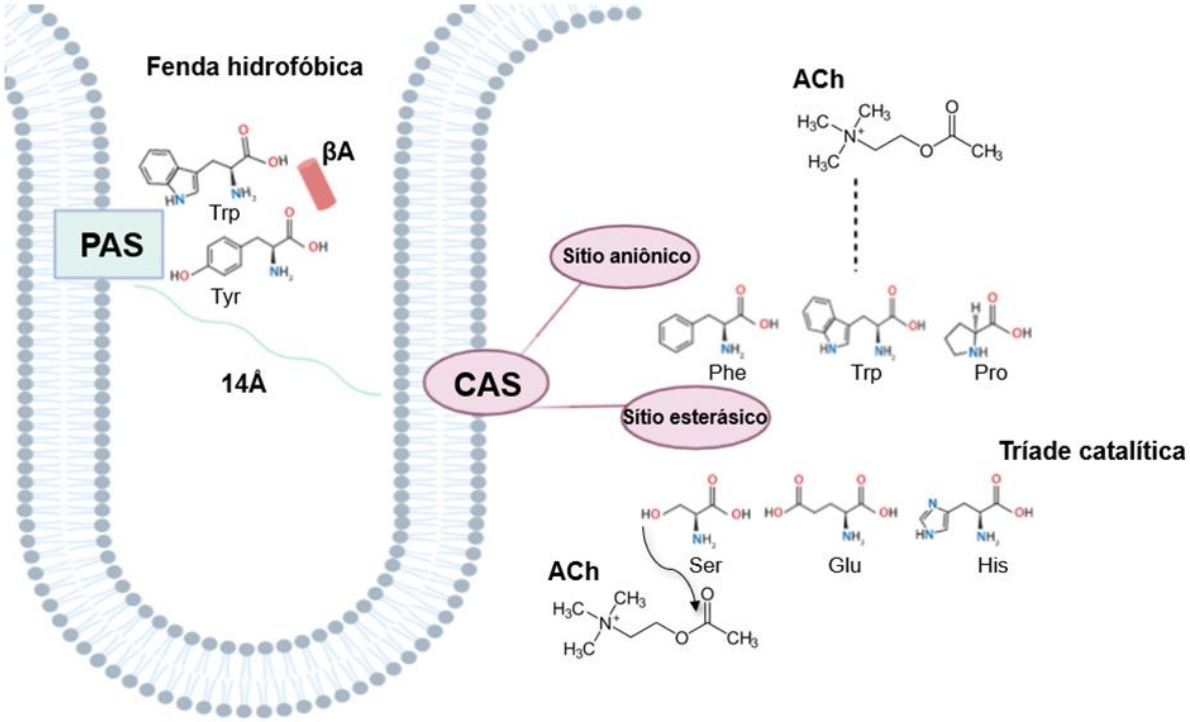

Figura 6. Extensão catalítica da enzima Acetilcolinesterase (AChE). Abreviações: βA (peptídeo beta amilóide), catalytic anionic site (CAS), Fenilalanina (Phe), Glutamato (Glu), Histidina (His), peripheral anionic site (PAS), Prolina (Pro), Triptofano (Trp), Serina (Ser).

Fonte: Imagem do autor, 2021.

O CAS é dividido em dois subsítios, um esterásico, encarregado pela hidrólise do grupo éster do substrato ACh, envolvendo os resíduos da tríade catalítica (serina, histidina e glutamato), e outro pertencente à interação com a região de colina, com a prevalência de aminoácidos hidrofóbicos, como o triptofano (Johnson e Moore, 2006). O PAS, encontrado na entrada da fenda catalítica, distante cerca de 14Å do CAS, contém o triptofano como o resíduo responsável pela função de aderência (Silva, 2002) (Figura 6).

O mecanismo pelo qual a AChE hidrolisa o neurotransmissor ACh envolve o ataque nucleofílico da serina ao carbono carbonílico da ACh, formando um intermediário tetraédrico, estabilizado por ligações hidrogênio, o que produz colina livre e serina acetilada. Por fim, a hidrólise da serina acetilada regenera o sítio catalítico da enzima (Silva, 2002) (Figura 7).

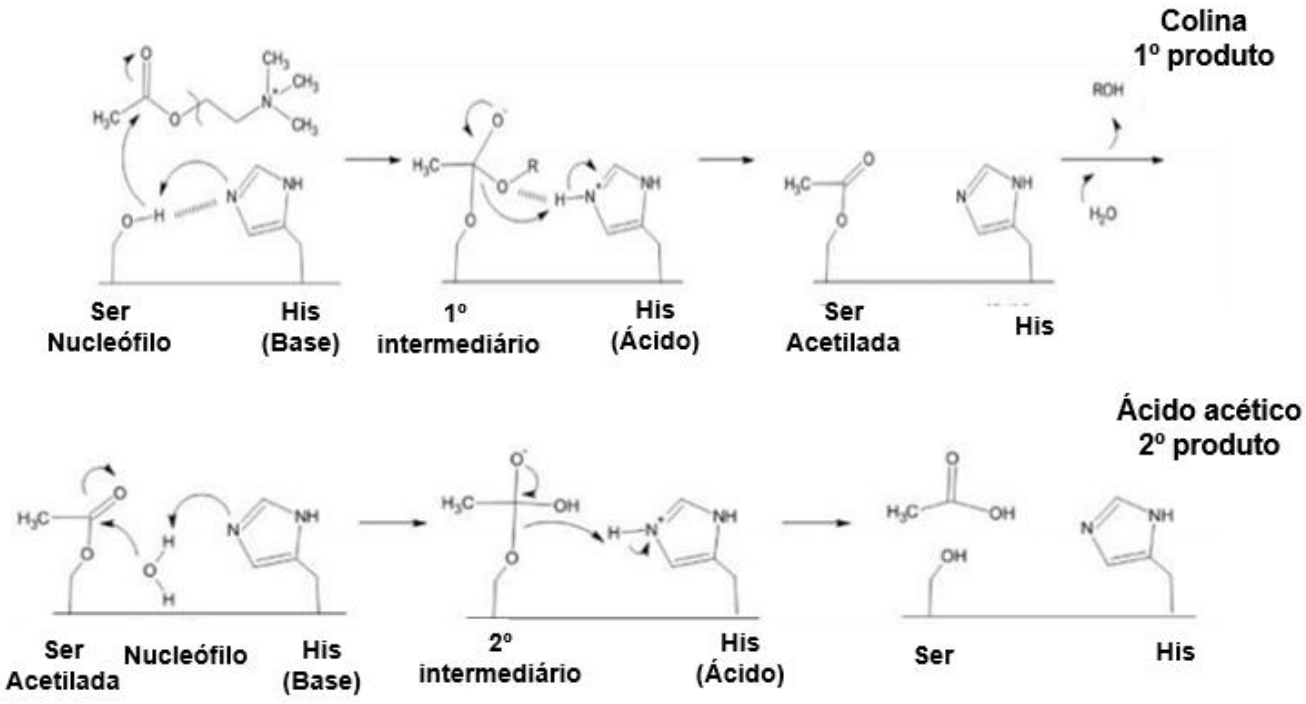

Figura 7. Mecanismo de hidrólise do neurotransmissor acetilcolina (ACh). Abreviações: Histidina (His), Serina (Ser).

Fonte: Adaptado de Riov e Jaffe, 1973, p. 526.

O PAS é um local envolvido com a modulação alostérica da catálise no CAS e é alvo de diversos agentes anticolinesterásicos. Este sítio também está relacionado com várias funções não clássicas da enzima, como por exemplo o depósito de

peptídeo βA, a adesão celular e o crescimento em expansão dos dendrímeros (Cheung *et al.*, 2012; Johnson e Moore, 2006; Muñoz-Torrero e Camps, 2006; Silva, da *et al.*, 2006). Estudos *in vitro* demonstram que a AChE é capaz de induzir a agregação de βA e formar complexos enzima-βA estáveis e fortemente ligados que são mais tóxicos do que o agregado de βA (Hou *et al.*, 2014; Luo *et al.*, 2011). Estes achados sugerem que a AChE pode participar do processo de formação de placas amiloidogênicas, fornecendo um núcleo heterogêneo durante a formação e crescimento das fibrilas amiloides (Muñoz e Inestrosa, 1999).

Estes complexos estáveis de βA-AChE são aptos a modificar as propriedades bioquímicas da enzima e ocasionar um aumento da neurotoxicidade das fibrilas de βA. Estes resultados demonstraram que a enzima está estritamente relacionada ao processo neurodegenerativo na DA. Desta maneira, os inibidores da AChE capazes de atuar neste sítio periférico da enzima poderiam exibir um relevante efeito neuroprotetor, podendo representar uma maneira de retardar o processo degenerativo na DA (Camps *et al.*, 2010; Muñoz-Torrero e Camps, 2006).

Adicionalmente, os efeitos destes inibidores na enzima podem estar relacionados com o processo de geração dos componentes não amiloidogênicos dos PPA (Muñoz-Torrero e Camps, 2006). A ativação dos receptores muscarínicos M1, por consequência da inibição da AChE, seguido da ativação da via da proteína quinase C (PKC), podem estimular a secreção da proteína amiloide solúvel neurotrófica e neuroprotetora (αPPAs), resultando na redução da liberação de βA. Esta circunstância indica que os inibidores da AChE podem prevenir a formação das placas amiloides e possibilitar o processamento normal da PPA (Muñoz-Torrero e Camps, 2006). A galantamina (inibidor da AChE), demonstrou uma capacidade em aumentar a captação microglial de βA (Shadfar *et al.*, 2015; Takata *et al.*, 2010). Com base nisso, pesquisas que visam estudar a DA e/ou desenvolver possíveis alternativas terapêuticas, buscam avaliar a atividade da enzima AChE (Jha, Panchal e Shah, 2018; Thomé *et al.*, 2018).

2.2.3. Neuroinflamação

As respostas neuroinflamatórias são altamente responsáveis pela patogênese da DA. Porém, a neuroinflamação desencadeia uma resposta imune inata responsável por apresentar efeitos benéficos ocasionados pelas células que degradam o βA

acumulado (Ahmad, Fatima e Mondal, 2019) (Figura 8). O papel da microglia e de maneira mais geral, o papel da inflamação estão se tornando processos cada vez mais investigados na patogênese da DA (Heneka *et al.*, 2005). Estudos histopatológicos mostram um aumento no número de células astrogliais e microgliais ativadas ao redor dos depósitos do βA (Wyss-coray, 2006).

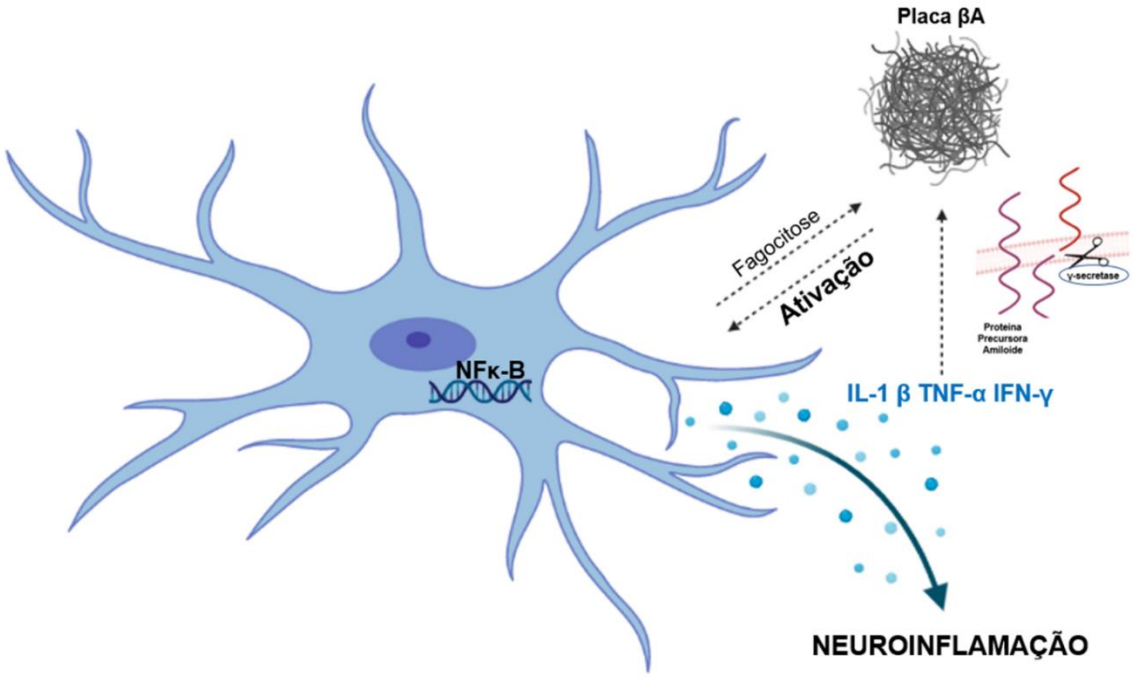

Figura 8. Processo neuroinflamatório na doença de Alzheimer (DA). A microglia realiza o processo de fagocitose para remoção dos peptídeos beta amiloides (βA) acumulados. Contudo, pode promover um aumento na produção de citocinas pró-inflamatórias quando há um desequilíbrio e é estimulada pelo βA. Abreviações: fator nuclear- kappa B (NFκB), fator de necrose tumoral-alfa (TNF-α), interferon-gama (IFN-γ), interleucina- 1 beta (IL-1β).

Fonte: Imagem do autor, 2021.

Um estudo mais recente sugere que a infusão crônica de oligômeros βA42 induz a neuroinflamação e ativa a microglia em hipocampos de ratos, levando ao declínio da memória espacial, como visto na DA (Fekete *et al.*, 2019). A neuroinflamação pode estar presente antes do surgimento de comprometimentos cognitivos graves ou demência nos pacientes com DA (Cuello, 2017). Desta forma, a neuroinflamação precoce pode ser um promissor alvo terapêutico à medida que seguem as buscas por um diagnóstico definitivo dos estágios pré-clínicos da DA (Cuello, 2017; Elfakhri *et al.*, 2019).

O βA quando está presente e ligado aos receptores de superfície da microglia pode ocasionar a ativação de fatores de transcrição como o fator nuclear kappa B (NF-

κB), levando a um aumento na produção de citocinas e resultando em um estado de inflamação crônica (Calsolaro e Edison, 2016) (Figura 8). Sabe-se que o NF-κB encontra-se envolvido em doenças neurodegenerativas como a DA (O'Neill e Kaltschmidt, 1997). De fato, este é um dos fatores de transcrição mais bem caracterizados, responsável por regular a expressão de muitos genes envolvidos com a codificação de proteínas encontradas nos processos inflamatórios (Li e Verma, 2002). Portanto, uma potencial abordagem terapêutica poderia envolver a modulação da ativação do NF-κB induzida pela sinalização de βA.

Além do mais, as citocinas pró-inflamatórias têm sido associadas à progressão da DA (Wang *et al.*, 2015). Foi demonstrado que o fator de necrose tumoral-alfa (TNF-α) pode potencializar a resposta astroglial, impulsionando o processo neuroinflamatório (Hensley, 2010). O TNF-α, juntamente com a interleucina-1beta (IL-1β) e o interferon-gama (IFN-γ) podem induzir a clivagem da PPA pela γ-secretase, através da ativação da via da proteína quinase ativada por mitógeno (Liao *et al.*, 2004) (Figura 8).

Neste sentido, a neuroinflamação demonstra um papel chave na patologia da DA, e alternativas terapêuticas com atividade anti-inflamatória, poderiam diminuir a progressão da DA. Já foi constatado que o uso a longo prazo de anti-inflamatórios demonstrou suprimir a progressão e o início da DA. Estudos epidemiológicos sugerem uma correlação inversa entre o uso de AINEs e a prevalência de DA (Wyss-coray, 2006), além de apresentarem um efeito protetor nestes pacientes (Calsolaro e Edison, 2016; Shadfar *et al.*, 2015). O ibuprofeno, um AINE, reduziu a deposição de βA e a hiperfosforilação da proteína *tau* no hipocampo de camundongos em um modelo de DA (Choi *et al.*, 2013).

O neurotransmissor ACh, amplamente conhecido por suas funções exercidas na memória e aprendizado, tem sido associado a doenças inflamatórias. Um estudo desenvolvido por Lehner e colaboradores (2019) propôs uma via anti-inflamatória colinérgica como um alvo viável no tratamento de doenças neurológicas. Foi observado uma redução dos níveis séricos de TNF-α através da estimulação seletiva do receptor muscarínico M1 de ACh. Apesar da ação a nível de sistema periférico este dado é de grande relevância uma vez que, várias anormalidades sistêmicas foram encontradas em pacientes com DA, inclusive inflamação (Wang *et al.*, 2017). De fora

complementar, a ativação do receptor nicotínico α7 está associada com vias anti-inflamatórias através da regulação negativa de NF-κB (Kalkman e Feuerbach, 2016).

Assim sendo, a busca por terapias com ação anti-inflamatória poderia contribuir para o controle da progressão da DA. A redução de processos inflamatórios, bem como, a inibição da resposta de células como a microglia e os astrócitos, poderiam favorecer uma proteção contra a morte celular ocasionada na DA (Morales *et al.*, 2014).

2.2.4. Estresse oxidativo e defesas antioxidantes

O estresse oxidativo é um distúrbio metabólico no qual moléculas instáveis, denominadas espécies reativas, ocasionam danos às células através de reações de oxido-redução com moléculas orgânicas (fosfolipídios, proteínas e ácido desoxirribonucléico (DNA)) (Figura 9). Esses danos podem levar à perda da função celular, causando disfunções importantes nos sistemas orgânicos (Campos e Leme, 2018). O estresse oxidativo, é um dos principais mecanismos envolvidos na etiologia da DAE (Popa-wagner *et al.*, 2013). O alto nível de espécies reativas de oxigênio (EROs) é uma das características típicas nos cérebros com DA (Gackowski *et al.*, 2008).Tais espécies tem sido associadas como contribuintes da ativação da microglia, atuando como moléculas de sinalização pró- inflamatórias (Taetzsch *et al.*, 2015), e podendo ocasionar uma superprodução das moléculas pró-inflamatórias (Bagyinszky *et al.*, 2017). Contudo, os mecanismos por trás da progressão da doença e como o estresse oxidativo inicia a ativação da cascata sensível ao processo redox na morte neuronal ainda não estão totalmente esclarecidos (Popa-wagner *et al.*, 2013).

O cérebro é um órgão suscetível a danos oxidativos principalmente devido à alta utilização de oxigênio, a grande quantidade de lipídios insaturados e a presença de metais de transição (Figura 9). Além disso, sabe-se que o cérebro é deficiente em defesas antioxidantes (Uttara *et al.*, 2009). A peroxidação lipídica consiste na degradação oxidativa dos lipídios ocasionada pelas espécies reativas (Ayala, Muñoz e Argüelles, 2014). O malondialdeído (MDA) é um composto orgânico gerado a partir da peroxidação lipídica e é considerado como um biomarcador para o estresse oxidativo (Tsikas, 2017).

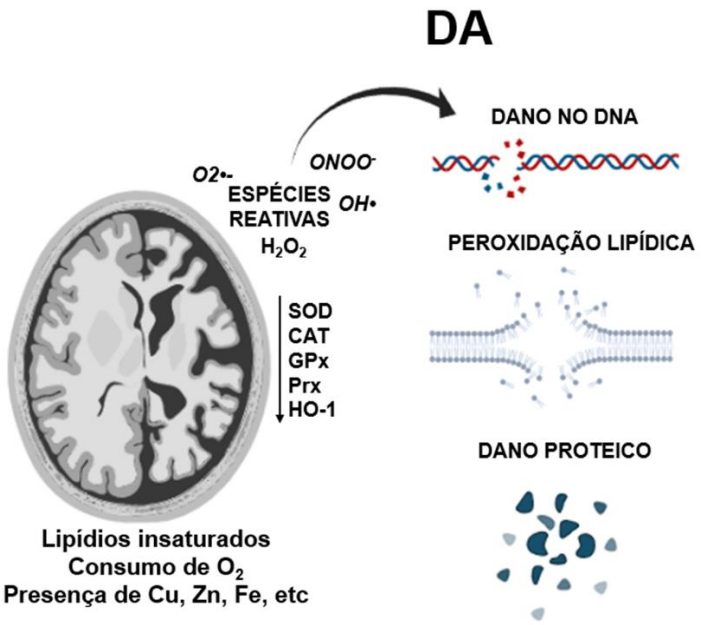

Figura 9. Causas e consequências do dano oxidativo na doença de Alzheimer (DA). O cérebro apresenta um alto consumo de oxigênio (O_2), grande quantidade de lipídios insaturados, presença de metais de transição e defesas antioxidantes reduzidas. Estes são fatores que corroboram para o surgimento do dano oxidativo na doença de Alzheimer. Abreviações: ácido desoxirribonucléico (DNA), catalase (CAT), cobre (Cu), ferro (Fe), glutationa peroxidase (GPx), heme oxigenase (HO)-1, peróxido de hidrogênio (H_2O_2), peroxinitrito ($O2^{\bullet-}$), peroxirredoxina (Prx), radical ânion superóxido ($O2^{\bullet-}$), radical hidroxil ($OH^{\bullet}$), superóxido dismutase (SOD), Zinco (Zn).

Fonte: Imagem do autor, 2021.

O radical ânion superóxido ($O_2^{\bullet-}$) é o precursor da maioria das EROs e mediador de reações em cadeia do estresse oxidativo. Apesar disso, os sistemas biológicos exibem uma resposta endógena para proteger as células contra danos oxidativos através do sistema de defesa antioxidante (Poljsak, Šuput e Milisav, 2013). O $O_2^{\bullet-}$ pode ser dismutado à peróxido de hidrogênio (H_2O_2) pela enzima superóxido dismutase (SOD) (Mn-SOD na matriz mitocondrial ou Cu/Zn-SOD no citosol) (Mailloux, Mcbride e Harper, 2013). Diversos estudos realizados sobre o envelhecimento e a disfunção relacionada à idade mostraram um aumento nos níveis de H_2O_2 no músculo de humanos e de camundongos (Capel *et al.*, 2004, 2005; Jackson, Ryan e Alway, 2011). Inclusive, níveis aumentados de H_2O_2 mitocondriais foram observados em cérebros de pacientes com a DA em modelos animais de DA (Du *et al.*, 2008; Manczak *et al.*, 2006).

Contudo, algumas enzimas antioxidantes são capazes de detoxificar o H_2O_2, dentre elas a peroxirredoxina (Prx), glutationa peroxidase (GPx) e catalase (CAT). A

Prx é oxidada pelo H_2O_2 em um sítio ativo de cisteína e, em seguida, é reduzida em um processo reacional que involve a tioredoxina, a tioredoxina redutase e o fosfato de dinucleotídeo de adenina e nicotinamida (NADPH). No processo de redução de H_2O_2 pela GPx ocorre a oxidação da glutationa reduzida (GSH) formando a glutationa oxidada (GSSG), que por sua vez, pode ser reduzida pela glutationa redutase (GR), às custas de NADPH (Sena e Chandel, 2013). Desta maneira, o GSH e indiretamente o NADPH são moléculas indispensáveis aos processos enzimáticos responsáveis pelo equilíbrio redox.

Embora o GSH seja utilizado como um cofator importante na função das enzimas antioxidantes, este é também um antioxidante intracelular essencial, fundamental para as defesas cerebrais frente ao estresse oxidativo. De fato, níveis aumentados ou diminuídos de GSH são considerados marcadores de estresse oxidativo (Karaman, Budak e Çiftci, 2018; Owen e Butterfield, 2010; Schulz *et al.*, 2000). O GSH representa 90% dos tiois não proteicos (NPSH) celulares, importantes para determinar o estado redox celular (Prakash *et al.*, 2009).

A função das peroxidases (CAT, GPx e Prx) depende da rapidez com que elas reagem com o H_2O_2, da concentração de H_2O_2 e da abundância destas enzimas *in vivo*. A Prx apresenta uma alta constante de taxa (k) e alta abundância e, portanto, acredita-se que é responsável pela eliminação de níveis nanomolares de H_2O_2 associados à sinalização celular. A GPx tem k semelhante, mas é menos abundante e, portanto, provavelmente é importante apenas em concentrações intracelulares mais altas de H_2O_2, quando a GPx pode começar a competir com a Prx pelo substrato H_2O_2 (Winterbourn e Hampton, 2008). Portanto, é possível que a Prx seja importante para desativar a sinalização de EROs. A CAT tem uma afinidade ainda menor com o H_2O_2, e o cérebro possui menores níveis desta enzima em relação a Prx e a GPx. A regulação dos níveis de atividade e expressão dessas defesas antioxidantes ocorre por uma variedade de mecanismos e funções, em parte para gerenciar os níveis de EROs (Sena e Chandel, 2013).

O estresse oxidativo em pacientes com a DA pode ocorrer em resposta a fatores genéticos, mutações na linha germinativa (como nos genes da APP, Presenilin-1 e Presenilin-2), fatores ambientais, fatores associados ao estilo de vida (como fumar), e certas doenças (como diabetes mellitus, lesão cerebral, doença

vascular hipertensiva e hipercolesterolemia) (Castellani *et al.*, 2009). Um estudo de revisão demonstrou o envolvimento do estresse oxidativo induzido pelo oligômero βA42 tanto na patogênese como na progressão da DA, e isto prejudica o metabolismo da glicose, levando à disfunção sináptica e eventual morte neuronal (Butterfield e Halliwell, 2019). Sabe-se que o βA é capaz de induzir a formação de peroxinitrito, um agente de nitração, e isso ocorre pela reação do óxido nítrico ($NO^{\bullet}$) e do $O_2^{\bullet-}$ (Coma *et al.*, 2005). Guix e colaboradores (2009) demonstraram que o dano nitro-oxidativo induzido pelo βA promove a nitrotirosinação da enzima glicolítica triosefosfato isomerase em células de neuroblastoma humano. A nitrotirosinação desta enzima promove uma diminuição no fluxo glicolítico. Além do mais, a nitro-triosefosfato isomerase liga os monômeros de proteína *tau* e induz a agregação desta proteína para formar filamentos helicoidais emparelhados, uma característica intracelular dos cérebros de pacientes com a DA.

Ceylan e colaboradores (2019) demonstraram que a administração de ferro a longo prazo ocasionou efeitos deletérios no córtex de ratos, causando prejuízos no metabolismo antioxidante e alterações na expressão de genes relacionados à patologia da DA. Outro fator capaz de contribuir com o estresse oxidativo é o acúmulo descontrolado do biometal zinco, através da citotoxicidade mediada por βA. Este dano ocorre uma vez que, a resposta imunológica/ inflamatória às placas de βA não solúveis envolvem a interrupção da homeostase deste biometal (Pal, Badyal e Vasishta, 2013).

A heme oxigenasse (HO)-1 possui a capacidade de atuar como uma enzima catalisadora sobre o grupo heme, transformando-o em três produtos: monóxido de carbono (CO), biliverdina, que é rapidamente convertida em bilirrubina por meio da ação da biliverdina redutase, e ferro livre, que promove a indução de ferritina, responsável pelo armazenamento e detoxificação do ferro. Estas moléculas formadas desempenham papeis como antioxidantes e anti-inflamatórios (Barone *et al.*, 2011). A HO-1 é rapidamente induzida por exposição a metais pesados, H_2O_2, citocinas pró-inflamatórias e pela DA (Keyse e Tyrrell, 1989). De fato, foi demonstrado que há um aumento na expressão da HO-1 após a injeção intracerebroventricular (i.c.v.) de estreptozotocina (STZ) em um modelo experimental de DA em ratos, e com o passar do tempo houve uma redução na expressão desta enzima, indicando um dano na

atividade da HO-1 com a progressão dos danos ocasionados na DA (Bhardwaj *et al.*, 2016).

Como o dano oxidativo é um fator crítico presente na patologia e na progressão da DA (Butterfield e Boyd-kimball, 2018), estudos que colaborem para uma melhor compreensão dos mecanismos antioxidantes e uma abordagem terapêutica que tem como foco a diminuição deste dano na DA são de importante relevância.

2.2.5. Na^+/K^+-ATPase

A Na^+/K^+-ATPase é uma enzima transmembrana envolvida no estabelecimento do gradiente de íons sódio (Na^+) e potássio (K^+) (Figura 10).

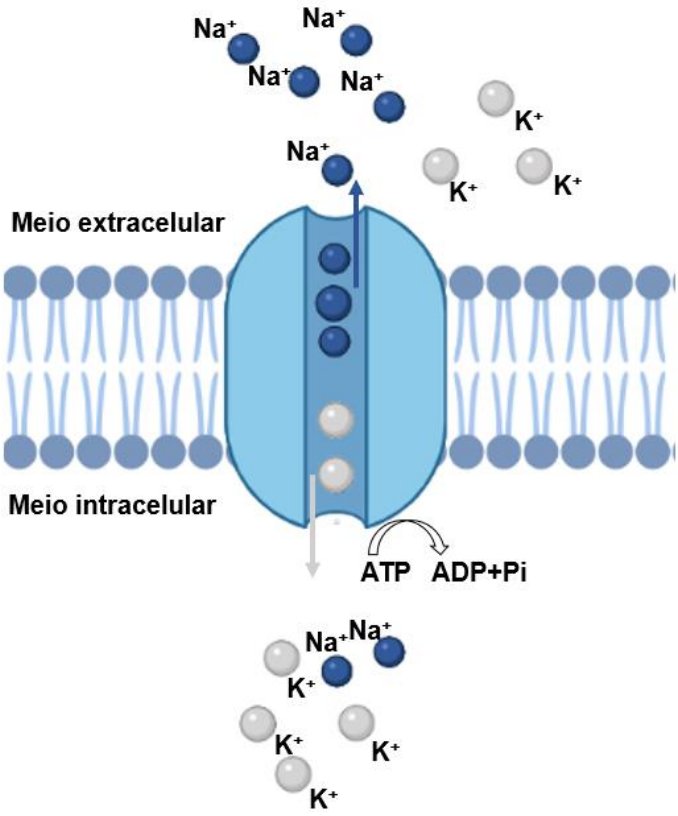

Figura 10. Extrusão de 3 Na^+ da célula enquanto 2 K^+ entram na célula devido a ativação da enzima Na^+/ K^+- ATPase. Resultando em uma concentração intracelular aumentada de K^+ e uma concentração intracelular reduzida de Na^+ em relação ao fluido intersticial.

Fonte: Imagem do autor, 2021.

Nas células neuronais, esta enzima, além de desempenhar a função do gradiente iônico, está envolvida na geração do potencial de ação, fornecendo as

propriedades eletrônicas dos neurônios (Zhang *et al.*, 2013). A diminuição da atividade/níveis de Na^+/K^+-ATPase pode interferir nas propriedades eletrônicas normais dos dendritos e contribuir para a distrofia neurítica. Também, pode levar à interrupção do processamento normal de informações e à disfunção da memória (Dickey *et al.*, 2005). Além disso, esta importante enzima é um dos alvos do estresse oxidativo neuronal (Kurella, Tyulina e Boldyrev, 1997).

Já foi verificado em modelos animais transgênicos de DA que o aumento do estresse oxidativo induz uma diminuição na Na^+/K^+-ATPase, gerando comprometimento da atividade cerebral na memória e na cognição, antes da formação de lesões patológicas (Fu *et al.*, 2009). Assim, o βA pode induzir um dano oxidativo às proteínas da membrana neuronal, que, por sua vez, ocasiona um prejuízo da homeostase iônica e uma degeneração celular (Mark *et al.*, 1997). Além disso, Lakunina e colaboradores (2017) relataram que o βA inibe diretamente a atividade Na^+/K^+-ATPase. Alguns estudos mostraram que substâncias como a rivastigmina e a vitamina E, que são capazes de restaurar ou aumentar a atividade da Na^+/K^+-ATPase reduzida pelo βA, bem como o estresse colinérgico e oxidativo, podem exercer efeitos protetores contra a DA (Carageorgiou *et al.*, 2008; Ishrat *et al.*, 2008; Zhang *et al.*, 2013).

Adicionalmente, existe uma relação não completamente esclarecida entre a Na^+/K^+-ATPase e o sistema colinérgico (Zhang *et al.*, 2013). No entanto, já foi relatado que a estimulação da Na^+/K^+-ATPase ativa os receptores colinérgicos nicotínicos e muscarínicos, e a inibição da atividade dessa enzima causa um efeito oposto (Pivovarov, Calahorro e Walker, 2019), demonstrando desta forma, a importância desta enzima como alvo terapêutico e de estudo na DA.

2.3. MODELOS ANIMAIS PARA O ESTUDO DA DOENÇA DE ALZHEIMER

O estudo através de modelos experimentais é uma ferramenta útil não só para a seleção de novos alvos farmacológicos, como também para o mapeamento da fisiopatologia envolvida na DA, uma patologia que até o presente momento não é completamente elucidada. Desta forma, modelos experimentais que mimetizam e transpõe os sintomas de pacientes com a DA para animais são de grande relevância.

O βA é um dos principais agentes neurotóxicos envolvidos na DA, e nas últimas décadas muitas estratégias foram desenvolvidas para compreender como essa

proteína afeta o SNC (Esquerda-Canals *et al.*, 2017; Newman, Musgrave e Lardelli, 2007). Existem algumas formas diferentes do βA, com comprimentos variados de 39 a 43 aminoácidos (Kang *et al.*, 1987). Entre eles o peptídeo de 40 resíduos de aminoácidos (βA(1-40)) e o peptídeo de 42 aminoácidos (βA(1-42)), que são as formas mais comuns (Suzuki *et al.*, 1994). Ambas as formas estão presentes nas placas amiloides (Saido *et al.*, 1995). O βA(1-40) é o mais abundante, mas, o βA(1-42) é muito mais amiloidogênico, o qual oligomeriza e forma fibrilas com mais facilidade (Harper e Lansbury, 1997).

Diante disso, a injeção intracerebral do βA em roedores é amplamente utilizada entre os modelos para o estudo da DA (Spiazzi *et al.*, 2015; Ianiski *et al.*, 2012; Pinz *et al.*, 2018; Shin *et al.*, 2020; Wu *et al.*, 2018) e pode representar tanto os efeitos dos peptídeos solúveis e oligômeros, quanto das placas amiloides. O fragmento βA (25-35) também é utilizado em modelos animais, e é originado do peptídeo βA (1-42) (Kubo *et al.*, 2002). O fragmento βA (25-35) apresenta uma toxicidade de forma mais rápida e causa mais dano oxidativo que o peptídeo de origem (βA (1-42)) e isso parece ser devido a metionina, que desempenha papel crucial na toxicidade de ambos os fragmentos, o fato de a metionina estar na posição C-terminal no fragmento βA (25-35), ocasiona estes efeitos mais exacerbados (Varadarajan *et al.*, 2001). Tendo em vista estas características, o fragmento βA (25-35) tem sido utilizado em diversos laboratórios como indutor experimental da DA.

Estes modelos de injeção intracerebral do βA permitem avaliar vários pontos da cascata patológica e são ferramentas inestimáveis para identificar diversas alterações envolvidas na DA. Dentre estas alterações pode-se destacar o dano oxidativo (Ianiski *et al.*, 2012; Pinz *et al.*, 2018; Varadarajan *et al.*, 2001), a neuroinflamação (Spiazzi *et al.*, 2015), citotoxicidade (Pinton, Souza, *et al.*, 2013) e a alteração no sistema colinérgico com o aumento na atividade da enzima AChE (He *et al.*, 2018; Pinz *et al.*, 2018).

Além do mais, os modelos animais que apresentam alterações genéticas com o intuito de aumentar os níveis de expressão do βA em camundongos também são amplamente utilizados (Esquerda-Canals *et al.*, 2017; Fu *et al.*, 2009; Newman, Musgrave e Lardelli, 2007). Tais modelos fornecem informações valiosas sobre os mecanismos moleculares subjacentes ao declínio da memória e da cascata amiloide.

Contudo, devido à manipulação de genes especificamente relacionados com o aumento do βA (PPA, PSEN-1 ou PSEN-2), esses modelos se assemelham à forma familiar da doença, que representa apenas ~5% dos casos de DA relatados (Esquerda-Canals *et al.*, 2017).

Neste sentido, modelos animais que representam a DAE são de grande relevância. Cabe destacar que, as causas exatas da DAE ainda são desconhecidas. Apesar disso, sabe-se que um fator de risco substancial para o desenvolvimento da DA é a resistência à insulina e o estresse oxidativo (Butterfield e Halliwell, 2019). O metabolismo da glicose em cérebros com DA é significativamente prejudicado, sendo que este órgão apresenta uma alta demanda de energia e depende fortemente da produção eficiente de trifosfato de adenosina (ATP) por meio da glicólise, do ciclo do ácido tri carboxílico (TCA) e da fosforilação oxidativa (Butterfield e Halliwell, 2019).

A STZ, uma glucosamina-nitrosoureia, substância derivada de bactérias do solo e amplamente utilizada como uma toxina diabetogênica (Figura 11), tem sido empregada como um modelo não transgênico representativo de DAE (Grieb, 2016).

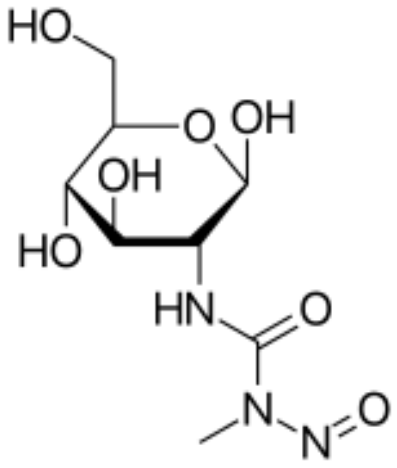

Figura 11. Estrutura química da estreptozotocina (STZ).
Fonte: Imagem do autor, 2021.

Este modelo foi proposto inicialmente por Lannert e Hoyer (1998), através da aplicação i.c.v. de STZ em ratos. Foi identificado que a STZ ocasionava déficits na aprendizagem, memória e comportamento cognitivo, acompanhado por déficit de energia cerebral permanente e contínuo em ratos (Lannert e Hoyer, 1998). Um dos mecanismos de ação pelo qual a STZ causa estes prejuízos é a dessensibilização dos

receptores neuronais de insulina e a redução na atividade das enzimas da via glicolítica, tais como a hexoquinase e a fosfofrutoquinase no córtex cerebral e hipocampo, provocando assim, um redução no metabolismo da energia cerebral (Plaschke e Hoyer, 1993).

Desde então, muitos estudos foram desenvolvidos baseados neste modelo e diversas características observadas na DA podem ser identificadas, tais como: déficit cognitivo (Fronza *et al.*, 2019), resistência à insulina no cérebro (Kamat, Kalani, Rai, Tota, *et al.*, 2016), déficit colinérgico (Salkovic-Petrisic *et al.*, 2013), aumento de βA e fosforilação da proteína *tau* (Ravelli *et al.*, 2017), neuroinflamação (Rai *et al.*, 2013) e estresse oxidativo (Martini *et al.*, 2019). Com isso, esse modelo caracteriza-se como adequado para *screening* de novos agentes com função terapêutica para a DA (Pinton, Sampaio, *et al.*, 2013; Thomé *et al.*, 2018).

2.4. BUSCA POR ALTERNATIVAS TERAPÊUTICAS PARA O TRATAMENTO DA DOENÇA DE ALZHEIMER

Apesar do paradigma tradicional de descoberta de medicamentos utilizado na indústria farmacêutica ser “um alvo, um medicamento, uma doença”, uma abordagem mais promissora para o tratamento da DA consiste em projetar os medicamentos ligantes direcionados para múltiplos alvos (Panek *et al.*, 2017). Neste sentido, duas ou mais porções farmacofóricas podem gerar moléculas híbridas, e com isso essas moléculas são capazes de interagir com múltiplos alvos (Prati *et al.*, 2018). Além disso, a união de duas porções farmacoforicamente ativas pode ser capaz de potencializar as atividades biológicas dos precursores individuais.

O desenvolvimento de moléculas que podem interagir com mais de um alvo é de grande relevância, uma vez que a DA apresenta aspectos multi-patológicos, dificultando e, muitas vezes, impossibilitando a cura. O progresso na pesquisa de novos medicamentos candidatos ao tratamento da DA também depende da inovação e do aprendizado na exploração de novos alvos patológicos. Assim como em outras doenças crônicas, uma fase longa de estudos precedeu períodos cujos sucessos incrementais sequenciais levaram a um progresso significativo em terapias eficientes (Cummings *et al.*, 2019). As indústrias farmacêuticas e diversas pesquisas já realizaram alguns estudos para encontrar medicamentos capazes de tratar a DA.

Grande parte destes medicamentos apresentaram potencial, inclusive entraram nos ensaios clínicos de fase II e fase III. Dentre eles encontram-se o solanezumab, (Salloway *et al.*, 2014) , o gantenerumab (Ostrowitzki *et al.*, 2017) e o idalopirdina (Atri *et al.*, 2018). Contudo, os resultados posteriores foram quase sempre decepcionantes, especialmente nos ensaios clínicos de tratamento tardio para o metabolismo amiloide.

Entretanto, os resultados de um estudo de fase III de um oligossacarídeo extraído de algas marrons, o GV-971, foram bastante promissores. O GV-971 apresentou significância estatística e clínica em termos de melhoria da função cognitiva dos pacientes com DA. Os mecanismos potenciais do GV-971 incluem a inibição do acúmulo do βA, a regulação do desequilíbrio da flora intestinal e a redução da neuroinflamação (Cummings *et al.*, 2019; Wang *et al.*, 2019). Este fornece um mecanismo de ação inovador e multidirecionado, demonstrando assim, que o desenvolvimento deste tipo de medicamentos para a terapia da DA é uma ferramenta bastante promissora.

Recentemente, o FDA aprovou o uso do Aduhelm (aducanumabe) por meio da aprovação acelerada, que corresponde a liberação de um medicamento para uma doença grave ou com risco de vida que pode fornecer benefício terapêutico significativo sobre os tratamentos existentes. Contudo ainda pode permanecer alguma incerteza sobre o benefício clínico do medicamento. Desde 2003, o Aduhelm é a primeira terapia aprovada para a DA. Ademais, o Aduhelm é o primeiro tratamento direcionado à fisiopatologia subjacente da DA, a presença de placas βA no cérebro. Os ensaios clínicos demonstraram uma redução nestas placas βA. Os dados mostram que no primeiro ensaio clínico de fase III, o Aduhelm demonstrou redução no declínio cognitivo. O segundo ensaio clínico de fase III não atingiu o *endpoint* primário que observou-se esta redução no declínio cognitivo. Entretanto, todos os estudos em que foi avaliado, o Aduhelm reduziu de forma consistente e muito convincente o nível de placas amiloides no cérebro de uma forma dependente da dose e do tempo (Cavazzoni, 2021).

2.4.1. Compostos orgânicos de selênio e doença de Alzheimer

Os compostos orgânicos de selênio tem se tornado cada vez mais interessantes na pesquisa de novas alternativas terapêuticas para o tratamento da

DA. Inclusive, alguns destes compostos já demonstraram ações multialvo tais como, o 7-cloro-4-(fenilselanil)quinolina (4-PSQ) (Figura 12) e o Ebselen (Figura 12) (Barth *et al.*, 2019; Martini *et al.*, 2019; Pinz *et al.*, 2018). Em parte, estas atividades podem ser atribuídas ao selênio, que é um elemento traço importante e desempenha um papel crítico em vários processos redox e metabólicos, e tem sido sugerida a sua utilização na prevenção do início e na progressão da DA (Loef *et al.*, 2011).

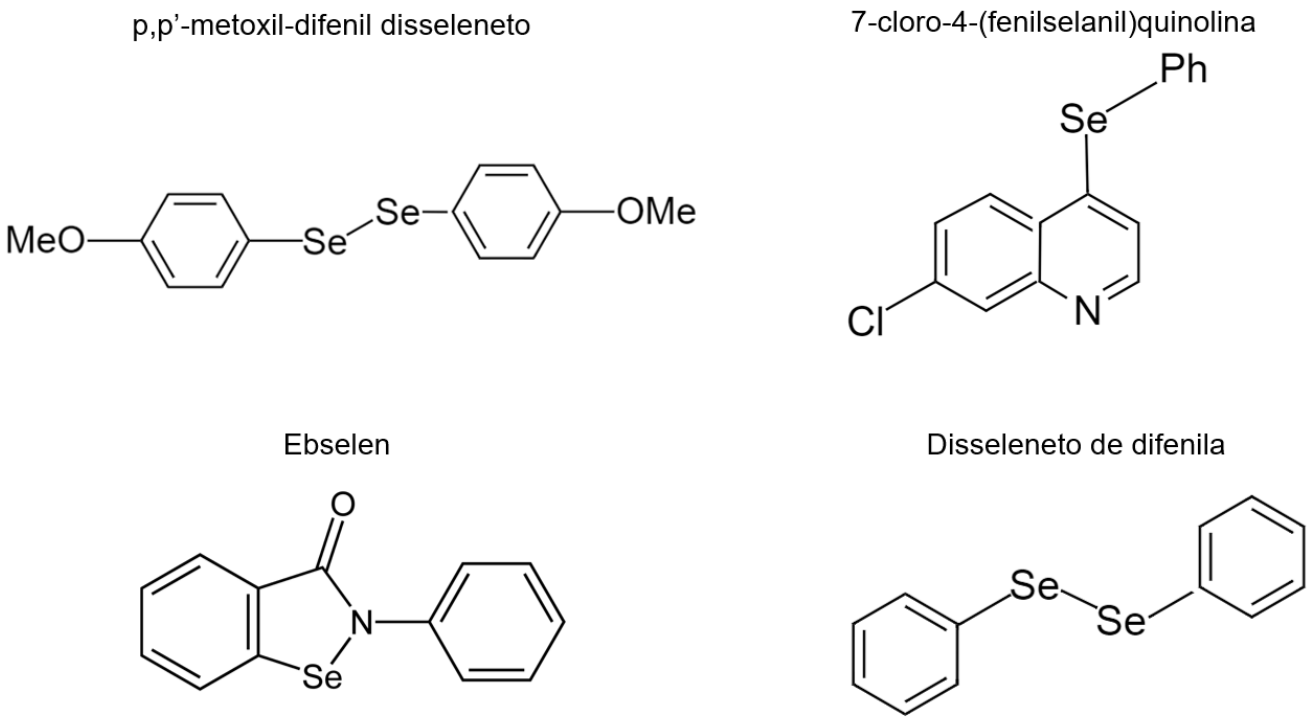

Figura 12. Estrutura química dos compostos orgânicos de selênio.
Fonte: Imagem do autor, 2021.

A selenocisteína é a principal forma biológica de selênio em humanos, e está presente nas selenoproteínas, podendo ser adquirida pela dieta, ou através de subprodutos do metabolismo de selênio, ou ainda do subproduto da degradação de selenoproteínas (Shetty e Copeland, 2018). Apesar da menor concentração de selênio no cérebro, este é altamente estável (Seale *et al.*, 2018). Diante das várias doenças atribuídas ao status alterado de selênio estão as doenças neurodegenerativas e, especificamente, a DA. Atualmente existe uma possível conexão entre a deficiência de selênio e a DA, que está atraindo considerável atenção (Varikasuvu *et al.*, 2018). Inclusive a selenometionina, a forma química predominante de selênio ingerido por humanos, retardou o declínio da função cognitiva, reduziu a hiperfosforilação da

proteín *tau* e déficits sinápticos em camundongos (Song *et al.*, 2014). Este elemento está presente como resíduo de selenocisteína no sítio ativo de enzimas como a GPx e tiorredoxina redutase, enzimas que desempenham um papel essencial no equilíbrio redox do organismo, atuando como importantes antioxidantes (Holmgren, 1985; Ursini *et al.*, 1999).

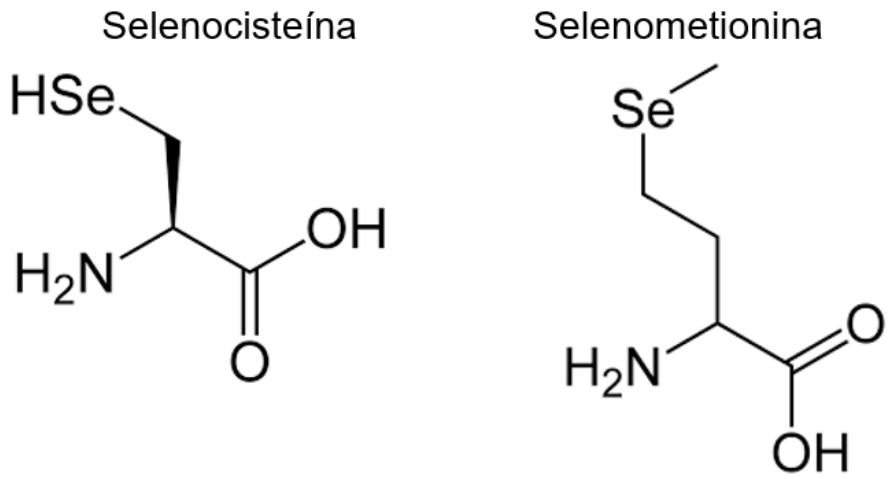

Figura 13. Estrutura química da selenocisteína e da selenometionina.
Fonte: Imagem do autor, 2021.

Alguns compostos orgânicos de selênio, dentre eles o disseleneto de difenila [$(PhSe)_2$] (Figura 12), apresentam atividade mimética da enzima GPx e, portanto, protegem contra condições relacionadas ao estresse oxidativo (Barbosa *et al.*, 2017). Durante os últimos anos diferentes compostos orgânicos de selênio se tornaram alvos de estudos que investigam o potencial farmacológico destes compostos (Lopes *et al.*, 2019; Pinz *et al.*, 2016; Reis *et al.*, 2019; Wilhelm *et al.*, 2017). O 4-PSQ tem sido um composto amplamente estudado por nosso grupo de pesquisa e já apresentou ações anticolinestarásica, antioxidantes e efeito protetor contra o comprometimento da aprendizagem e memória em um modelo de DA em camundongos (Pinz *et al.*, 2018). Cabe destacar que Vogt e colaboradores (2018) evidenciaram que o grupamento contendo selênio é fundamental para a atividade antioxidante do 4-PSQ. Além disso, o 4-PSQ também foi capaz de restaurar o comprometimento de memória causado pelo envelhecimento em ratos através da modulação da plasticidade sináptica, do sistema colinérgico e dos níveis de colesterol (Barth *et al.*, 2019), bem como o estresse oxidativo periférico em ratos envelhecidos (Luchese *et al.*, 2020).

Além do mais, outro composto orgânico de selênio, o p,p'-metoxil-difenil disseleneto (Figura 12) também apresenta atividades promissoras para o tratamento da DA. Este composto exerceu efeitos neuroprotetores *in vitro* (em neurônios corticais) e *in vivo* (em ratos e camundongos) melhorando o déficit cognitivo e de memória em diferentes modelos animais (STZ e βA, i.c.v.) de DA. Tais ações do p,p'-metoxil-difenil disseleneto foram relacionadas com a sua atividade antiapoptótica, a preservação de dendritos e sinapses, bem como a inibição da ativação da microglia e astrogliose (Pinton *et al.*, 2013a; Pinton *et al.*, 2013b).

De modo geral, outros trabalhos também tem dedicado estudar os compostos orgânicos de selênio e destacaram suas promissoras atividades no tratamento e/ou prevenção da DA (Thomé *et al.*, 2018; Xie *et al.*, 2017; Yan *et al.*, 2019). Diante do que foi exposto, compostos derivados de selênio tem se tornado importantes alvos de estudo e podem representar uma boa estratégia terapêutica para o tratamento/prevenção de desordens neurodegenerativas como a DA.

2.4.2. Compostos orgânicos derivados de purinas

Os compostos orgânicos derivados de purina representam outra classe de relevância bastante evidenciada. As purinas apresentam uma gama de propriedades biológicas, a sua estrutura consiste em uma pirimidina fundida com um anel imidazol (Figura 14) e são os heterocíclicos de nitrogênio amplamente distribuídos na natureza (Rosemeyer, 2004) e explorados para o desenvolvimento de moléculas bioativas.

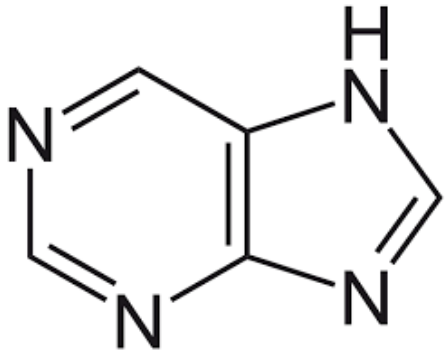

Figura 14. Estrutura química de purina.
Fonte: Imagem do autor, 2021.

As purinas apresentam diversas propriedades conhecidas como anticonvulsivante (Skerritt, Davies e Johnston, 1982), antibacteriana (Hirokawa *et al.*,

2009), antimalárica (Mallari, Guiguemde e Guy, 2009) e anti-inflamatória (Wang *et al.*, 2010). Vários medicamentos à base de purina foram aprovados e estão sendo usados para o tratamento de diversas patologias como a síndrome da imunodeficiência adquirida, herpes, artrite reumatoide grave e leucemia linfoide aguda. Dentre estes medicamentos encontram-se a didanosina, azatioprina, aciclovir e 6-tioguanina (Figura 15).

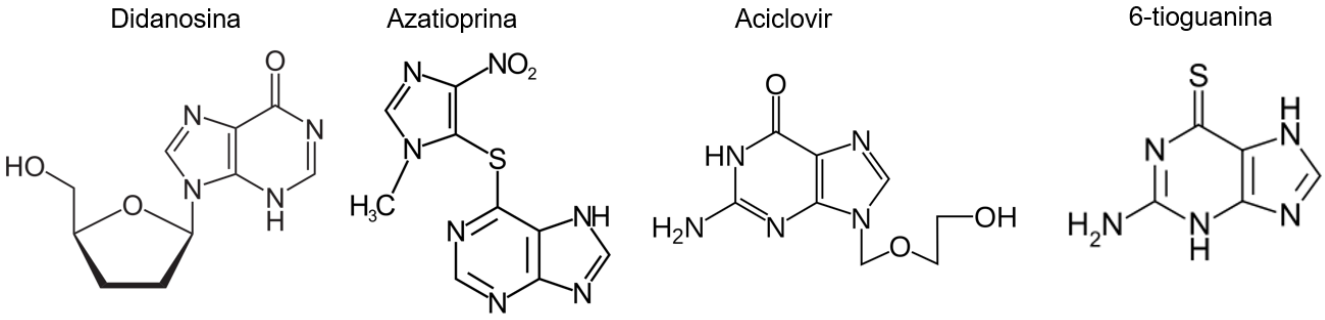

Figura 15. Estrutura química dos fármacos derivados de purina.
Fonte: Imagem do autor, 2021.

Entre outros compostos derivados de purina, os mais estudados para os distúrbios que acometem a memória e o aprendizado são as metilxantinas (Figura 16). Estas atuam como antagonistas de receptores purinérgicos do tipo P1, que também são conhecidos como receptores de adenosina (Martínez-pinilla, Oñatibia-astibia e Franco, 2015). A cafeína, é uma metilxantina bastante conhecida, e tem demonstrado ação neuroprotetora em diferentes doenças neurodegenerativas (Oñatibia-Astibia, Franco e Martínez-Pinilla, 2017). Apesar de os subtipos de receptores de adenosina e as vias intracelulares exatas relacionadas com as ações da cafeína não estejam claramente definidas, sabe-se que o antagonismo dos receptores de adenosina do subtipo A2 de neurônios nos estágios iniciais da DA evitam a perda sináptica do hipocampo e melhoram os déficits de memória (Canas *et al.*, 2009; Silva *et al.*, 2016).

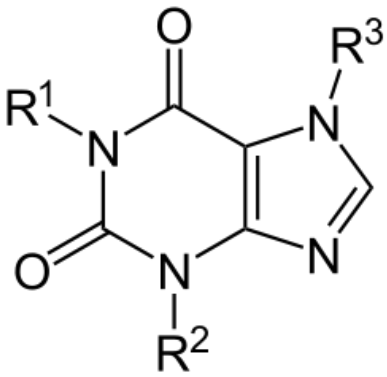

Figura 16. Estrutura química das metilxantinas.
Fonte: Imagem do autor, 2021.

Em um estudo realizado por Leite e colaboradores (2011) a cafeína demonstrou uma capacidade em reverter o comprometimento da memória associado à idade em ratos, reduzindo parcialmente do estresse oxidativo. Dall'Igna e colaboradores (2007) evidenciaram que a cafeína impede a neurotoxicidade induzida pelo βA em culturas primárias de neurônios cerebelares de ratos com o envolvimento do receptor de adenosina A2.

Recentemente, Teixeira e colaboradores (2020) demonstraram o efeito benéfico da inosina (Figura 17), um nucleosídeo purínico de ocorrência natural, que evitou déficits de memória e apresentou uma interação com múltiplos alvos envolvidos com funções cognitivas (enzimas colinérgicas, atividades de bomba iônica e estado redox) em um modelo experimental de DA, reforçando desta forma, o importante papel desta classe de compostos no auxílio da busca por um tratamento para a DA .

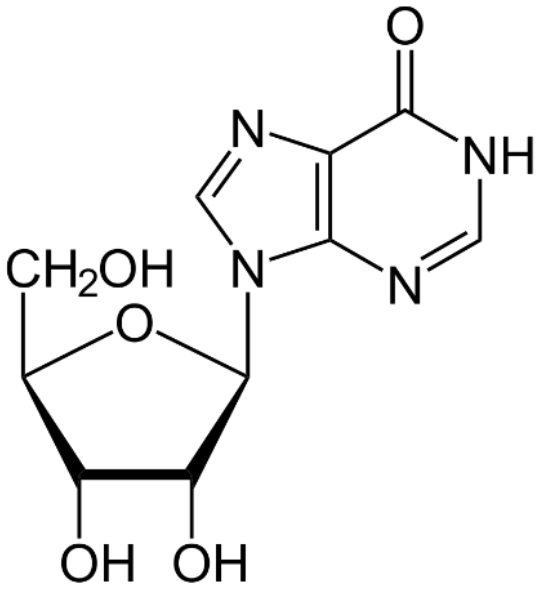

Figura 17. Estrutura química da inosina.

Fonte: Imagem do autor, 2021.

2.4.3. 6-((4-fluorofenil) selanil)-9*H*-purina (FSP)

Tendo em vista as propriedades biológicas relevantes dos compostos orgânicos de selênio e o fato de os compostos à base de purina poderem interagir com uma grande variedade de alvos biológicos envolvidos em várias doenças, o nosso grupo de pesquisa buscou sintetizar uma molécula combinando estes dois núcleos farmacológicos (Figura 18).

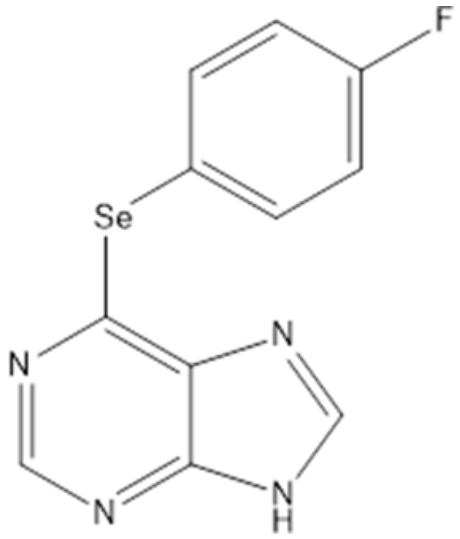

Figura 18. Estrutura química do 6-((4-fluorofenil) selanil)-9*H*-purina (FSP).

Fonte: Imagem do autor, 2021.

O interesse na combinação de selênio e purina intensificou-se já que estes compostos apresentam atividades farmacológicas de grande relevância para o tratamento da DA. O FSP foi sintetizado com bons rendimentos através de um método simples. Além disso, este composto inibiu *in vitro* a atividade da enzima AChE no córtex cerebral de camundongos (Duarte *et al.*, 2017). Outro achado relevante foi evidenciado através do aprimoramento das fases de consolidação e recuperação da memória em camundongos, e a inibição da atividade da AChE *ex vivo* no córtex cerebral destes camundongos (Duarte *et al.*, 2017). Diante deste contexto, o FSP tem despertado um grande interesse pelo nosso grupo de pesquisa, que visa elucidar outras propriedades farmacológicas deste composto em modelos animais de DA. Dessa forma, este composto pode ser considerado um promissor agente no desenvolvimento de um fármaco para o tratamento da DA.

3. OBJETIVOS

3.1. OBJETIVO GERAL

Investigar os possíveis efeitos protetores do FSP em dois modelos experimentais de DA em camundongos, bem como investigar os possíveis mecanismos pelos quais o composto está agindo.

3.2. OBJETIVOS ESPECÍFICOS

a) Realizar análises *in silico* do composto FSP para investigar a sua interação com a enzima AChE;

b) Investigar o efeito do composto FSP em um modelo de DA induzida por STZ em camundongos:

- Avaliar, o efeito terapêutico do FSP contra o prejuízo na memória e aprendizado ;
- Verificar efeito tipo-ansiolítico do FSP;
- Avaliar a atividade do FSP em modular a via colinérgica nas estruturas cerebrais dos animais;
- Investigar o efeito protetor do FSP na modulação da bomba iônica Na^+K^+-ATPase no córtex cerebral e hipocampo de camundongos;
- Mensurar a expressão das proteínas quinase B (Akt) nas estruturas cerebrais dos camundongos;

c) Investigar o possível potencial toxicológico agudo e sub-crônico do composto FSP em camundongos;

d) Estudar o efeito do FSP no modelo de DA induzida pelo βA (25-35) em camundongos:

- Investigar se a indução com o βA (25-35) pode ocasionar uma hiperalgesia mecânica nos animais;
- Verificar o efeito protetor deste derivado de purina contendo selênio no comprometimento de memória e aprendizado;
- Investigar um possível efeito anti-hiperalgésico do FSP;
- Estudar o envolvimento do estresse oxidativo induzido por βA e a possível ação do FSP contra o dano oxidativo;

- Determinar a expressão relativa de ácido ribonucleico mensageiro (RNAm) da AChE nas estruturas cerebrais de camundongos;
- Investigar o possível efeito anti-inflamatório do FSP no córtex cerebral e hipocampo de camundongos.

4. CAPÍTULOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigo e manuscrito. As seções materiais e métodos, resultados, discussão e referências encontram-se no artigo e no manuscrito, representando a íntegra deste estudo. O artigo está estruturado conforme a revista onde foi publicado e o manuscrito está estruturado de acordo com revista onde foi submetido.

4.1. ARTIGO

Effect of a purine derivative containing selenium to improve memory decline and anxiety through modulation of the cholinergic system and Na+/K+-ATPase in an Alzheimer's disease model

Artigo publicado na revista *Metabolic Brain Disease*

Metabolic Brain Disease
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ORIGINAL ARTICLE

Effect of a purine derivative containing selenium to improve memory decline and anxiety through modulation of the cholinergic system and Na^+/K^+-ATPase in an Alzheimer's disease model

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Abstract
Alzheimer's disease (AD) is a worldwide problem, and there are currently no treatments that can stop this disease. To investigate the binding affinity of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) with acetylcholinesterase (AChE), to verify the effects of FSP in an AD model in mice and to evaluate the toxicological potential of this compound in mice. The binding affinity of FSP with AChE was investigated by molecular docking analyses. The AD model was induced by streptozotocin (STZ) in Swiss mice after FSP treatment (1 mg/kg, intragastrically (i.g.)), 1st-10th day of the experimental protocol. Anxiety was evaluated in an elevated plus maze test, and memory impairment was evaluated in the Y-maze, object recognition and step-down inhibitory avoidance tasks. The cholinergic system was investigated based on by looking at expression and activity of AChE and expression of choline acetyltransferase (ChAT). We evaluated expression and activity of Na^+/K^+-ATPase. For toxicological analysis, animals received FSP (300 mg/kg, i.g.) and aspartate aminotransferase, alanine aminotransferase activities were determined in plasma and δ-aminolevulinate dehydratase activity in brain and liver. FSP interacts with residues of the AChE active site. FSP mitigated the induction of anxiety and memory impairment caused by STZ. FSP protected cholinergic system dysfunction and reduction of activity and expression of Na^+/K^+-ATPase. FSP did not modify toxicological parameters evaluated and did not cause the death of mice. FSP protected against anxiety, learning and memory impairment with involvement of the cholinergic system and Na^+/K^+-ATPase in these actions.

Keywords Alzheimer's disease · Anxiety · Cholinergic system · Na^+/K^+-ATPase · Organoselenium · Molecular docking

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder, affecting about 50 million people worldwide (Brookmeyer et al. 2018). The neuropathological hallmarks of AD are constituted by cerebral beta-amyloid (Aβ) deposition, aggregated tau protein, neuronal loss and cognitive decline (Jack et al. 2018). Neuropsychiatric symptoms (NPS), including anxiety, have been linked to these pathological changes. Moreover, associations between apathy and anxiety with cerebral Aβ accumulation and longitudinal cognitive decline point to these NPS as early clinical manifestations of AD (Johansson et al. 2019). Sporadic AD (SAD) comprise most cases of this disease and multiple genetic and nongenetic factors contribute to susceptibility to this disorder (Dorszewska et al. 2016).

Glucose metabolism and insulin signaling disorders (Rani et al. 2016), as well as a reduction in acetylcholine (ACh) synthesis (Gibson and Blass 1976) are characteristic of SAD. In the cholinergic system, choline acetyltransferase (ChAT) is a synthesizing enzyme of ACh (Oda 1999). Furthermore, acetylcholinesterase (AChE) hydrolyzes ACh and an increase in AChE activity has been associated with loss of brain function, and thus a loss of intellectual abilities (Mokrani et al. 2019).

Additionally, Na^+/K^+-ATPase is an important enzyme that controls intracellular ion homeostasis and maintains the resting membrane potential and excitable properties of neurons (Takeuchi et al. 2008). The decrease in activity/levels of Na^+/K^+-ATPase can disrupt normal information processing and lead to memory dysfunction (Dickey et al. 2005). Studies showed that modifications in AChE and Na^+/K^+-ATPase in cerebral synaptosomes may compromise synaptic activities (Abdalla et al. 2013; Lakunina et al. 2017) and therefore, cause behavioral changes.

The main challenges regarding AD include the lack an early diagnosis and the lack of effective preventive and treatments strategies (Mangialasche et al. 2010; Wang et al. 2018). AChE inhibitors drugs for the AD treatment have demonstrated modest benefits with some limitations, including side effects (Ali et al. 2015).

The trace element Selenium (Se) is essential to human health. Possible role of Se in the prevention and treatment of AD, either alone or in combination with other elements, has been demonstrated (Agnieszka et al. 2016). The beneficial properties of this trace element are attributed in part to its ability to be incorporated in various proteins. Se is important to reduce oxidative stress and to control abnormal molecular processes in AD (Agnieszka et al. 2016; Varikasuvu et al. 2018).

In this sense, the organoselenium compounds have gained great attention from de scientific community. Several Se compounds, such as, Ebselen (Martini et al., 2019), 7-chloro-4-(phenylselanyl) quinoline (Pinz et al. 2018), selenothymidine (Thomé et al. 2018), selenepezil (Yan et al. 2019) and others, have shown promising therapeutic potential against AD. Recently, Martini et al. (2019) presented the beneficial effect of Ebselen,a multifunctional selenoorganic compound, to reverse memory impairment, hippocampal oxidative stress and apoptosis in a mouse model of SAD induced by streptozotocin (STZ).

Based on the relevance of AD and the need to search for better therapeutic alternatives for AD, our research group have a continued interest in the search promising an alternative for the treatment of AD. We showed that 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP), a purine derivative with an organoselenium group is a promising therapeutic agent for the treatment of AD. This compound was synthesized in good yields using a simple method. Additionally, we demonstrated the inhibitory effect of FSP on in vitro AChE activity in the cerebral cortex of mice. Another relevant finding was that FSP had an effect in enhancing the consolidation and retrieval memory phases, and also inhibited ex vivo AChE activity in the cerebral cortex of mice (Duarte et al. 2017). In this context, this compound is a promising agent to be tested for the prevention of memory loss in an AD model in mice.

In view of this, firstly we seek to elucidate how FSP blunted the increase of AChE through an in silico study. Furthermore, another purpose of this study was to examine the effect of FSP on memory impairment and anxiety, as well as the mechanisms involved in its pharmacological actions in an AD model in mice.

Materials and methods

Molecular docking studies

Molecular docking analyses were performed in order to further our knowledge about the direct interaction affinity of FSP with the therapeutic target AChE. To elucidate the importance of the selenium atom in the observed properties of FSP, we carry out an isosteric replacement of the Selenium (Se) atom for Sulfur (S). Firstly, 2D structures of FSP, FSP analog with S (S-FSP), ACh and donepezil were drawn with Chemdraw, converted to 3D using the Avogadro 0.9.4 software and their geometry was optimized following the MMFF94 method (Hanwell et al. 2012). The Auto Dock Tools 1.5.4 program was used to make all rotatable bonds of ligands rotate freely and the protein receptors were considered rigid (Morris et al. 2009).

The 3D X-ray crystal structures of AChE complex with an inhibitor were retrieved from the Protein Data Bank (human PDB ID: 4EY7) and prepared using the Auto Dock Tools 1.5.4. software. The protein preparation consisted of fixing structures, deleting molecules, ions, and water, fixing hetero groups and finally optimizing the fixed structure using

Gasteiger charges with 500 steps of minimization. CHIMERA 1.5.3 software was used previously to remove ligands in the 3D structure (Pettersen et al. 2004).

Molecular docking was conducted using the Autodock vina (version 1.1.1) software with a grid box centered on all atom structures, allowing the program to search for additional sites of probable interactions (Trott and Olson 2010). The protein−ligand interactions were analyzed by Discovery studio visualizer 2016.

Before performing docking studies, the Autodock vina program and our docking configuration were validated by redocking the donepezil with co-crystal protein structure 4EY7. The accuracy of the docking software was measured using RMSD between the redocked and co-crystal ligand. The docking poses were selected based on docking score.

Chemicals

STZ was obtained from Sigma (St. Louis, MO) and it was dissolved in sterile filtered water. FSP (Fig. 1) was prepared and characterized in our laboratory using the method previously described by Duarte et al. (2017). Analysis of the ^{1}H nuclear magnetic resonance spectroscopy (NMR) and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of FSP (99.9%) was determined by gas chromatography–mass spectrometry (GC/MS). FSP was dissolved in canola oil. All the other chemicals used in this experiment were of the highest purity and obtained from standard commercial suppliers.

Animals

Male adult Swiss mice (25–35 g) from the Federal University of Pelotas, Brazil were used in this study. The animals were maintained at a constant temperature (23 ± 1 °C), on a 12 h dark/light cycle (with lights on at 6:00 a.m.), with free access to food and water. Animal care and all experimental procedures were conducted in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publications no. 80–23, revised in 1996) (National Research Council 1996) and in accordance with the Committee on Care and Use of Experimental Animal Resources, Federal University of Pelotas, Brazil (CEEA 1974/2016). Every effort was made to minimize the number of animals used and their discomfort. The size of the group used for each experiment was based on studies that used protocols similar to those proposed here.

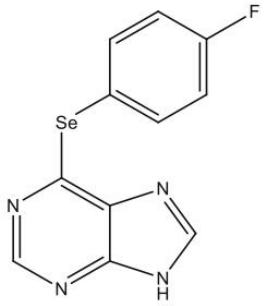

Fig. 1 Chemical structure of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP)

Experimental protocol

Mice were randomly divided into 4 experimental groups (7 animals/group) (Table 1). Thirty minutes before initiating induction, the mice belonging to sham and STZ groups received the canola oil (vehicle, 10 ml/kg); animals of the FSP and STZ + FSP groups received the compound (1 mg/kg) intragastrically (i.g.) via gavage. After treatments, mice belonging to the STZ and STZ + FSP groups were induced with STZ (2 μl of 2.5 mg/ml solution, intracerebroventricular (i.c.v.)) (Haley and Mccormick 1957). The i.c.v. infusion of STZ (glucosamine–nitrosurea compound) chronically decreases the cerebral glucose uptake. This infusion mimics AD in mice (Grieb 2016; Martini et al. 2019). Sham and FSP groups received saline (vehicle) (2 μl, i.c.v.). The i.c.v. infusion of STZ or saline was administered using a microsyringe with a 28-gauge, 3.0 mm long stainless-steel needle (Hamilton) according to a previous report (Haley and Mccormick 1957). On the third day of experimental protocol, the same induction i.c.v. was repeated. All animals were anesthetized with isoflurane before i.c.v. injection. Mice were treated with FSP or canola oil every day, until the tenth day of the experimental protocol. On the seventh day of experimental protocol, behavioral tests were initiated. All observations were performed by an observer blinded to the study plan. On the eleventh day, mice were anaesthetized by inhalation of isoflurane for blood collection by cardiac puncture. Later,

Table 1 Experimental groups

Group	Treatment
Sham	Saline (i.c.v.)+Canola oil (i.g)
FSP	Saline (i.c.v.)+FSP (i.g)
STZ	STZ (i.c.v.)+Canola oil (i.g)
STZ+FSP	STZ (i.c.v.)+FSP (i.g)

Animals were treated intragastrically (i.g.) with 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) or canola oil 30 min before intracerebroventricular (i.c.v.) streptozotocin (STZ)) or i.c.v. saline infusion. On day three of the experimental protocol, the i.c.v. induction. With STZ or saline was repeated. The i.g. treatments were performed every day, until the tenth day of the experimental protocol. The number of mice tested was 7 each group

mice were euthanized by inhalation of isoflurane and liver and brain were removed for ex vivo experiments. The experimental protocol is demonstrated in Fig. 2.

Behavioral tests

Open field test (OFT)

The OFT evaluated the spontaneous locomotor and exploratory behaviors of mice (Walsh and Cummins 1976). The place for the OFT was made of plywood and surrounded by 30 cm-high walls. The floor of the open-field (40 cm long × 40 cm wide) was divided into 9 squares (3 rows of 3). Each animal was placed at the center of the open field and observed for 4 min to record the locomotor (number of segments crossed with the four paws) and exploratory (number of rearings on the hind limbs) activities. This test was carried out to identify locomotors disabilities, which might influence other tasks. The OFT test was performed on the seventh day of experimental protocol (Fig. 2).

Elevated plus maze test (EPMT)

The EPMT is widely validated to measure anxiety in rodents (Pellow et al. 1985). The apparatus consists of two opposed open arms (16 × 5 cm) and two opposed closed arms (16 × 5 × 10 cm) mounted at an angle of 90°, all facing a central platform (5 × 5 cm) elevated 50 cm from the floor. Animals were evaluated on the seventh day of the experimental protocol (Fig. 2). Each animal was placed individually at the center of the apparatus facing one of the open arms. The frequency of entries into either open or closed arms, the time spent in each type of arm and number of dives were measured for 5 min.

Y-maze task

The Y-maze task was performed as described by Sarter et al. (1988) and it was used as a measure of working memory. The Y-maze apparatus consisted of a three-arm horizontal maze (40 cm long and 3 cm wide with walls 12 cm high) in which the three arms at 120° angles to each other, radiated out from a central point. The Y-maze task was performed on the seventh day of experimental protocol (Fig. 2). Mice were initially placed within one arm (A), and the arm entry sequence (e.g. ABCCAB, where letters indicate arm codes) and the number of arm entries were recorded manually for each mouse over an 8 min period. Alternation was determined from successive entries into the three arms on overlapping triplet sets in which three different arms are entered. An actual alternation was defined as entries into all three arms consecutively (i.e. ABC, CAB or BCA but not BAB). An entry was defined as placing all four paws within the boundaries of the arm.

Object recognition task (ORT)

ORT was used to assess the short-term (STM) and long-term (LTM) memories of mice and was used according to Stangherlin et al. (2009). ORT was performed in an open-field apparatus. On the day of the task (eighth day of experimental protocol) (Fig. 2) each animal was submitted to a

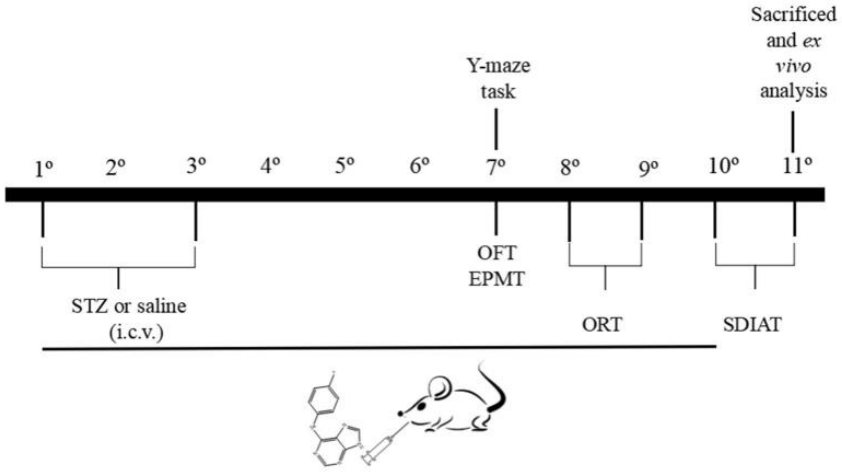

Fig. 2 Scheme of experimental protocol. Thirty minutes after intragastric (i.g.) treatments, mice received streptozotocin (STZ) or vehicle (saline) intracerebroventricularly (i.c.v.). On day three of the experimental protocol the i.c.v. injections were repeated. The i.g. treatments were performed every day, until the tenth day of the experimental protocol. Behavioral tasks started on the seventh day of the experimental protocol. On the seventh day open-field test (OFT), elevated plus maze test (EPMT) and Y-maze task were performed. On the eighth and ninth days the object recognition (ORT) task was performed. On the tenth and eleventh days the step-down inhibitory avoidance task (SDIAT) was performed. On the eleventh day, after the SDIAT test, the mice were sacrificed

habituation session in the absence of objects for 5 min. Subsequently, four objects were used: A1, A2, B and C. Each object had the following color pattern: blue, red and yellow. All objects were made of plastic, measuring 10 × 10 cm (length x height). During the training, on the eighth day of the experimental protocol, the animals were placed in the arena containing two identical objects (objects A1 and A2) for 5 min. Exploration was defined when the animal directed its nose within 2 cm of the object while looking, sniffing, or touching it. The STM of mice was evaluated 1.5 h after training in the presence of a familiar object (A1) and a new object (B), and the total time spent in exploring each object was determined during 5 min to measure the learning and recognition memory. The LTM was performed 24 h after training, on the ninth day of the experimental protocol, where mice were placed to explore a familiar object (A1) and a new object (C) for 5 min and the total time spent in exploring each object was determined.

In order to analyze the cognitive performance, the exploratory preference was calculated and data were expressed as percentage as follows: Training = (A2 / (A1 + A2)) × 100; STM = (B / (A1 + B)) × 100; LTM = (C / (A1 + C)) × 100).

Step-down inhibitory avoidance task (SDIAT)

SDIAT was performed to evaluate aversive and non-spatial LTM (Sakaguchi et al. 2006). During the training session (on the tenth day of the experimental protocol) (Fig. 2), each mouse was placed on the platform. When it stepped down and placed its four paws on the grid floor, an electric shock (0.5 mA) was delivered for 2 s. Twenty-four hours after the training session, the mice were tested under the same conditions without electric shock. Each mouse was placed again on the platform, and the transfer latency time (i.e., time taken to step down from the platform) (seconds) was measured as in the training session. The maximum transfer latency time (seconds) was 300 s.

Neurochemical analysis

Sample preparation

The cerebral cortices and hippocampus ($n = 6$–7 for each experimental group) were separated in order to submit each sample to all neurochemical determinations. The cerebral structures (cerebral cortices and hippocampus) were washed with cold saline solution (0.9%). The samples were homogenized in 0.25 M sucrose buffer (1:10, *w/v*) and 50 mM Tris-HCl, (1:10, w/v), to determine AChE and Na^+/K^+-ATPase activities, respectively. Thus, they were centrifuged at 900 *x*g for 10 min at 4 °C, to obtain supernatant fraction (S1). Additionally, cerebral cortices and hippocampus were separated for the RNA extraction and determination of AChE, ChAT, Na^+/K^+-ATPase, protein kinase B (Akt) 1, Akt2 and Akt3 expression. For this, samples were immediately processed and adequately stored (−80 °C) until the expression was evaluated.

AChE activity

The AChE enzymatic assay was performed according to the method of Ellman et al. (1961), with some modifications, using acetylthiocholine as substrate. The method is based on the formation of the yellow anion, 5-thio-2-nitro-benzoic acid, measured by absorbance at 412 nm during a 2 min incubation. Results were expressed as μmol/(acetylthiocholine) AcSCh/h/mg protein.

Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity was estimated by the method of Fiske and Subbarow (1925). The S1 (3 pools (2 hippocampi of mice/pool) was utilized; to obtain the appropriate volume of S1) it was mixed with 3 mM MgCl, 125 mM $NaCl_2$, 20 mM KCl and 50 mM Tris–HCl, pH 7.4. After 10 min pre-incubation at 37 °C in the presence of 0.1 mM ouabain, which specifically inhibits the Na^+/K^+-ATPase activity, the reaction was initiated by addition of ATP (3.0 mM) and terminated after 30 min of incubation by addition of trichloroacetic acid (TCA) solution (10%) with 10 mM $HgCl_2$. The samples were then centrifuged, an aliquot was removed, and color reagent added (ammonium molybdate 2%). Na^+/K^+-ATPase activity was calculated from the difference between amounts of inorganic phosphate found after incubation in the absence and presence of ouabain. The color reaction was assayed spectrophotometrically at 650 nm. Enzyme activity was expressed as nmol phosphate inorganic (Pi)/ min/mg protein.

RNA extraction and relative expression of AChE, ChAT, Na^+/K^+-ATPase, Akt1, Akt2 Akt3 by real-time PCR

Total mRNA was extracted from thawed samples of cerebral cortices and hippocampus ($n = 6$ for each experimental group) weighing between 50 and 70 mg using TRIzol reagent (Invitrogen™, Carlsbad, USA) followed by DNase treatment with DNase I Amplification Grade (Invitrogen™, Carlsbad, USA) in order to ensure minimum DNA contamination of the samples. The total RNA isolated was quantified and its purity (260/280 and 260/230 ratios) was examined by NanoVue spectrophotometer (GE, Fairfield, CT, USA). The cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription kit (AppliedBiosystems™, UK) according to the manufacturer's protocol. For reverse transcription, 2 μg of total RNA were used in a reaction volume of 20 μl. The amplification was performed with GoTaq® qPCR Master Mix (Promega, Madison, WI) using the Agilent Mx3005P qPCR

System (Agilent Technologies Inc., Santa Clara, CA) and the sequence of primers used **is** indicated in Table 2. The qPCR conditions were as follows: 10 min at 95 °C to activate the hot-start Taq polymerase, followed by 35 cycles of denaturation for 15 s at 95 °C, primer annealing for 60 s at 60 °C, and extension for 30 s at 72 °C (fluorescence signals were detected at the end of every cycle). Baseline and threshold values were automatically set by the Stratagene MxPro software. The number of PCR cycles required to reach the fluorescence threshold in each sample was defined as the Ct value, and each sample was analyzed in duplicate to obtain an average Ct. The 2-ΔΔCT method was used to normalize the fold change in gene expressions (Livak and Schmittgen 2001), using Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene.

Plasma glucose levels

Plasma glucose levels were determined to further confirm that 2 μl of 2.5 mg/ml of STZ is a subdiabetogenic dose. The samples were separated by centrifugation at 2400 *x*g for 15 min and glucose levels were determined by an enzymatic colorimetric method using a commercial kit (Bioclin, Brazil). Glucose levels were expressed as mg/dl.

Toxicological analysis

Oxidative parameters in liver

Samples of liver were collected to determine: δ-aminolevulinate dehydratase (δ-ALA-D) activity, thiobarbituric acid reactive species (TBARS) content, non-protein thiol (NPSH) levels. The metalloenzyme δ-ALA-D that contains the sulfhydryl group is responsible for catalyzing the synthesis of porphobilinogen and it is proposed as a marker of organochalcogen toxicity (Nogueira and Rocha 2011). TBARS content was used as a marker of lipid peroxidation (Ohkawa et al. 1979). NPSH levels were used as a marker of oxidative damage (Ellman 1959). These dosages were determined for the evaluation of toxicological analysis in the liver of mice. The samples were homogenized in 50 mM Tris/HCl pH 7.5, (1:10, *w*/*v*) and centrifuged at 900 *x*g for 10 min at 4 °C to yield S1. δ-ALA-D activity was assayed by the method of Sassa (1982). An aliquot of S1 was pre-incubated for 10 min at 37 °C. The enzymatic reaction was initiated by adding the substrate (δ-ALA) to a final concentration of 2.2 mM in a medium containing 45 mM phosphate buffer, pH 6.8 and incubated for 1 h at 37 °C. The incubation was stopped by adding 10% TCA solution with 10 mM $HgCl_2$. The reaction product porphobilinogen (PBG) was measured at 555 nm using modified Ehrlich's reagent. The values were expressed as nmol PBG/mg protein/h.

TBARS levels were determined as described by Ohkawa et al. (1979). An aliquot of S1 was added to the reaction mixture containing: thiobarbituric acid (0.8%), sodium dodecyl sulfate (8.1%), and acetic acid (pH 3.4) and incubated at 95 °C for 2 h. The absorbance was measured at 532 nm in a spectrophotometer. Results were reported as nmol malondialdehyde (MDA)/mg protein.

NPSH levels were determined as described by Ellman (1959). An aliquot of S1 was mixed (1:1) with 10% TCA and centrifuged at 900 *x*g for 10 min. Afterwards, the protein pellet was discarded and free -SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer, pH 7.4, and 10 mM 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). The color reaction was measured at 412 nm. NPSH levels were expressed as mmol NPSH/g tissue.

Table 2 Primers used for quantitative real-time polymerase chain reaction. The forward and reverse primer sequences used to amplify each target gene as well as the the GAPDH endogenous control are listed

Primer Name	Sequence	Reference
AChE Forward	5' TTAGGGCTGGGATATAATACGAC 3′	(Silverman et al. 2014)
AChE Reverse	5' GCCCCTAGTGGGAGGAAGT 3′	
Akt1 Forward	5' TCGTGTGGCAGGATGTGTAT 3′	(Brand et al. 2015)
Akt1 Reverse	5' ACCTGGTGTCAGTCTCAGAGG 3′	
Akt2 Forward	5' CAGCTGGGAGACCCAAGA 3′	
Akt2 Reverse	5' CACACGCTGTCACCTAGCTT 3′	
Akt3 Forward	5' TGGACCACTGTTATAGAGAGAACATTT 3′	
Akt3 Reverse	5' TGGATAGCTTCCGTCCACTC 3′	
Na^+/K^+ATPase Forward	5' TTTCAGAACGCCTACCTAGAGC 3′	(Wang et al. 2015)
Na^+/K^+ATPase Reverse	5' TGGAGATAAGACCCACGAAGC 3′	
ChAT Forward	5' CTGTGCCCCCTTCTAGAGC 3′	(Gu et al. 2015)
ChAT Reverse	5' CAAGGTTGGTGTCCCTGG 3'	
GAPDH Forward	5' TGCGACTTCAACAGCAACTC 3'	(Bruckert et al. 2016)
GAPDH Reverse	5' ATGTAGGCAATGAGGTCCAC 3′	

Abbreviations: Acetylcholinesterase (AChE), Acetyltransferase (ChAT), protein kinase B (Akt), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Acute toxicity

To examine the potential acute toxicity caused by FSP, two independent groups of three mice/each were used. One group received a single oral dose of FSP (300 mg/kg i.g.) or canola oil (vehicle, 10 ml/kg i.g.). After administration, animals were observed for up to 72 h to determine the lethal potential of this compound. After this time of exposure, mice were anesthetized for a heart puncture through which blood was collected in tubes containing heparin. Plasma was obtained by centrifugation at 900× g for 10 min and used for biochemical assays, which were performed with commercial test kits. Alanine (ALT) and aspartate aminotransferase (AST) activities were used as biochemical markers for early acute hepatic damage and they were determined by the colorimetric method (Reitman and Frankel 1957). The values were expressed as U/l. Renal function was analyzed by determining plasma urea levels (MacKay and MacKay 1927). The values were expressed as mg/dl. Later, mice were euthanized, and liver and brain samples were also collected to determine the δ-ALA-D activity which was assayed by the method of Sassa (1982) described above. The inhibition of this enzyme has been proposed as a marker of organoselenium compound toxicity (Nogueira and Rocha 2011).

Protein determination

The protein concentration was measured by the Bradford method (1976), using bovine serum albumin as the standard.

Statistical analysis

The data are expressed as mean ± standard error of the mean (S.E.M.). Data were analyzed by Graphpad Prism® 5 and the normality of data was evaluated by the D'Agostino and Pearson omnibus normality test. Statistical analysis was performed using one-way ANOVA followed by the Newman-Keuls test for behavioral tests. In the quantitative real-time polymerase chain reaction and ex vivo analyses, the statistical significance of differences between groups was determined by one-way ANOVA followed by Tukey's multiple comparison test. Statistical analysis of acute toxicity data was performed using a non-paired t-test. Values of $p < 0.05$ were considered statistically significant.

Results

FSP has a strong binding affinity with AChE in molecular docking analyses

The pharmacological target of this study was to investigate how FSP acts as an AChE inhibitor, which is considered a potential treatment for AD, mainly due to its ability to reduce ACh degradation. Molecular docking analyses revealed a strong binding affinity of FSP, ACh and positive control donepezil on AChE of −9.0 kcal/mol, −5.9 kcal/mol and −8.2 kcal/mol respectively (Table 3). The best position of FSP interacts mainly with residues of active sites, through hydrogen-bonds with Trp86 and Gly122, pi-stacked hydrophobic interaction with Phe338 and additional van der Waals intermolecular attractions with His447 and Gly121 (Fig. 3a). FSP also interacts with a peripheral anionic site (PAS) through Pi-Stacked interaction with Tyr124. Besides, our results showed that S-FSP presented a considerably lower affinity with AChE, of −6.8 kcal/mol. S-FSP interacts with AChE through three hydrogen bonds with the residues His381, Gln527 and Ala258, making also an additional halogen interaction with Arg525 which comprises the center of the four-helix bundle (Fig. 3c). When we superimposed the both ligands in AChE tridimensional structure is clearly visible the different binding modes of both compounds, as can be seen in Fig. 3d.

FSP and STZ did not cause any significant change in locomotor activity

The one-way ANOVA followed by Newman-Keul's test demonstrated that treatments did not cause any significant change in the number of crossings (ANOVA: $F_{3,\ 24} = 0.6310$, $p > 0.05$) or rearings (ANOVA: $F_{3,\ 24} = 0.6109$, $p > 0.05$) (Fig. 4a and Fig. 4b).

FSP protects against anxiety induced by i.c.v. injections of STZ

STZ administration promoted a significant decrease in the number of dives (around 75%), in the percentage of open arms entries (around 47%) and in the percentage of time spent in the open arms (around 45%) when compared with the sham group. FSP (1 mg/kg (i.g.)/day) administration significantly prevented the decrease in the number of dives and the percentage of open arms entries, when compared with the STZ group (ANOVA: $F_{3,\ 24} = 14.0$ $p < 0.0001$ for number of dives) (Fig.

Table 3 Results of molecular docking analyses

Molecules	Docking score on AChE enzyme (kcal/mol)
FSP	−9.0
ACh	−5.9
Donepezil	−8.2

Abbreviation: 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP), Acetylcholine (ACh), Acetylcholinesterase (AChE)

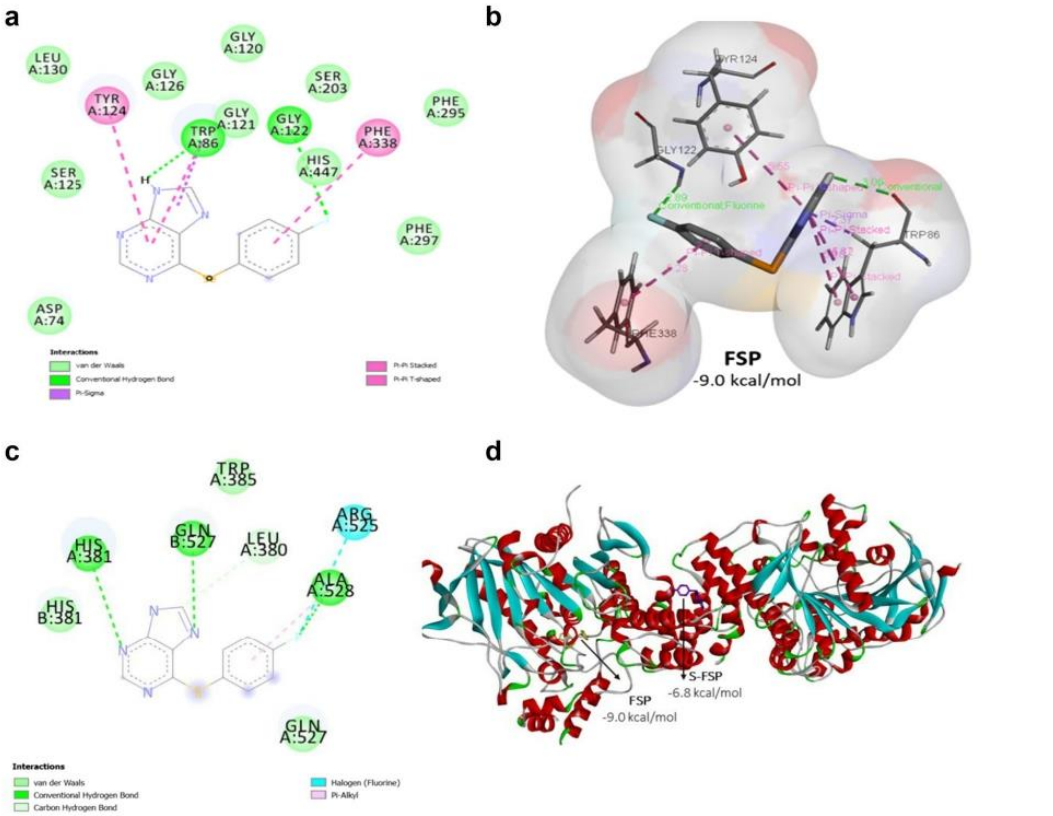

Fig. 3 Binding mode and protein-ligand interactions of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) on the acetylcholinesterase (AChE) enzyme in (**a**) 2D and (**b**) 3D conformation. Binding mode of (**c**) FSP analog with sulfur (S) (S-FSP) and (**d**) superimposition of FSP and S-FSP on AChE enzyme

4c), (ANOVA: $F_{3,\ 24} = 7.798$, $p < 0.001$ for percentage of open arms entries) (Fig. 4d). However, FSP did not protect against the decrease in the percentage of time spent in the open arms when compared with the STZ group (ANOVA: $F_{3,\ 24} = 4.988$, $p < 0.01$) (Fig. 4e).

FSP protects against cognitive and memory impairment induced by i.c.v. injections of STZ

As expected, the one-way ANOVA followed by Newman-Keul's test demonstrated that STZ reduced (around 26%) the spontaneous alternation behavior, when compared with the sham group. It is noteworthy that FSP (1 mg/kg (i.g.)/day) prevented the reduction of spontaneous alternation behavior, when compared with the STZ group (ANOVA: $F_{3,\ 24} = 7.540$, $p < 0.001$) (Fig. 4f). The treatments did not cause any significant change in the number of arm entries (ANOVA $F_{3,\ 24} = 2.980$, $p > 0.05$) (data not shown) in the Y-maze task.

ORT was used to access STM 1.5 h after the presentation of identical objects (training phase). The one-way ANOVA followed by Newman-Keul's test revealed that there was no difference in the exploratory preference of objects among groups in the training phase (ANOVA: $F_{3,24} = 0.8111$, $p > 0.05$) (data not shown). On the other hand, STZ induced cognitive deficits (Fig. 4g) demonstrated by a significant reduction (around 20%) in the exploratory preference of the new object in the STM, when compared with the sham group. Treatment with FSP was able to prevent this reduction, when compared with the STZ group (ANOVA: $F_{3,24} = 5.666$, $p < 0.01$). The LTM was valued 24 h after the training phase. The one-way ANOVA followed by the Newman-Keul's test revealed that STZ decreased (around 22%) the exploratory preference of the new object, when compared with the sham group. Remarkably, FSP treatment prevented this reduction, when compared with the STZ group (ANOVA: $F_{3,24} = 7.397$, $p < 0.01$) (Fig. 4h).

The SDIA task is substantially used to evaluate learning and aversive memory. In the training phase of SDIA, there was no difference in the transfer latency time among groups (ANOVA: $F_{3,24} = 1.047$, $p > 0.05$) (Fig. 4i). In the test phase, STZ decreased the transfer latency time (around 62%), when

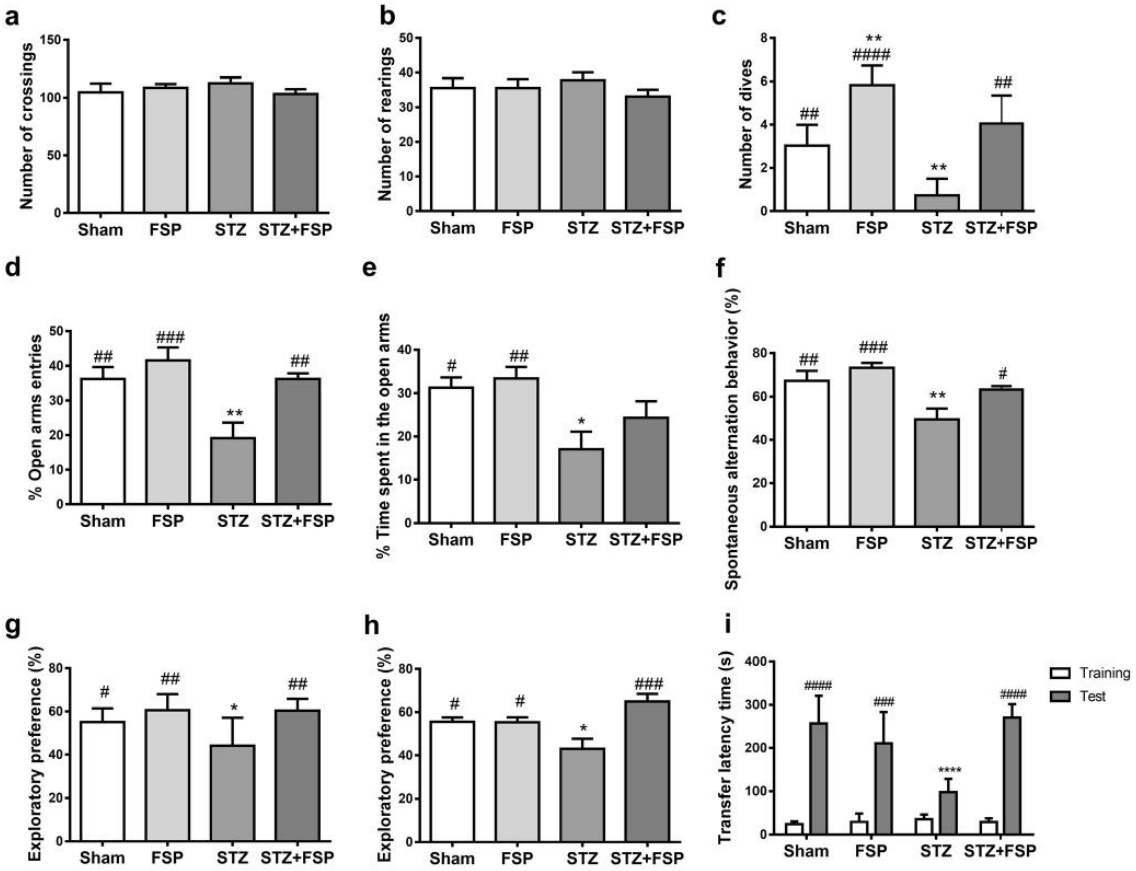

Fig. 4 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) (1 mg/kg intragastric (i.g.)/day) and/or streptozotocin (STZ) on behavioral tests of mice. Number of crossings (**a**) and rearings (**b**) in the open field test (OFT); number of dives (**c**), % open arms entries (**d**) and % time spent in the open arms (**e**) in the elevated plus maze task (EPMT); spontaneous alternation behavior (**f**) in the Y-maze task; first trial (percentage of time spent exploring the novel object, test carried out 1.5 h after training) (**g**) and second trial (percentage of time spent exploring the novel object, test carried out 24 h after training) (**h**) in the object recognition task (ORT); training and test (latency (s) to fall from the platform) (**i**) in the step-down inhibitory avoidance task (SDIAT). Data are reported as mean ± standard error of the mean (SEM) of seven animals per group (one-way analysis of variance/ Newman-Keul's test). (*) $p < 0.05$, (**) $p < 0.01$, (****) $p < 0.0001$ as compared with the sham group. (#) $p < 0.05$, (##), $p < 0.01$, (###), $p < 0.001$, (####), $p < 0.0001$ as compared with the STZ group

compared with the sham group. As depicted in Fig. 4I, FSP significantly prevented this reduction (ANOVA: $F_{3,24} = 15.11$, $p < 0.0001$), when compared with the STZ group.

FSP protects against cholinergic system dysfunction induced by i.c.v. injections of STZ

STZ administration promoted a significant increase in AChE activity in the cerebral cortices (around 47%) and hippocampus (around 40%) of mice, when compared with the sham group (Fig. 5a and Fig. 5b). FSP (1 mg/kg (i.g.)/day) treatment significantly prevented the increase of AChE activity caused by STZ in the hippocampus (ANOVA: $F_{3,20} = 9.395$, $p < 0.001$), but not in the cerebral cortices (ANOVA: $F_{3,24} = 3.252$, $p < 0.05$).

The one-way ANOVA followed by Tukey's test showed that STZ was able to increase the relative expression of mRNA AChE in hippocampi (around 50%), when compared with the sham group. The treatment with FSP reduced the STZ-induced relative expression of mRNA AChE in the hippocampi (ANOVA: $F_{3,24} = 7.45$, $p < 0.01$) (Fig. 5d). On the other hand, no difference was observed in the relative expression of mRNA AChE in the cerebral cortices after the treatments (ANOVA: $F_{3,24} = 1.294$, $p > 0.05$) (Fig. 5c). Additionally, Fig. 5f shows that STZ decreased the levels of relative expression of mRNA ChAT (54%), when compared with the sham group in the hippocampus of mice. However, it was not observed in the cerebral cortices of mice (Fig. 5e). FSP treatment significantly prevented the decrease of levels of

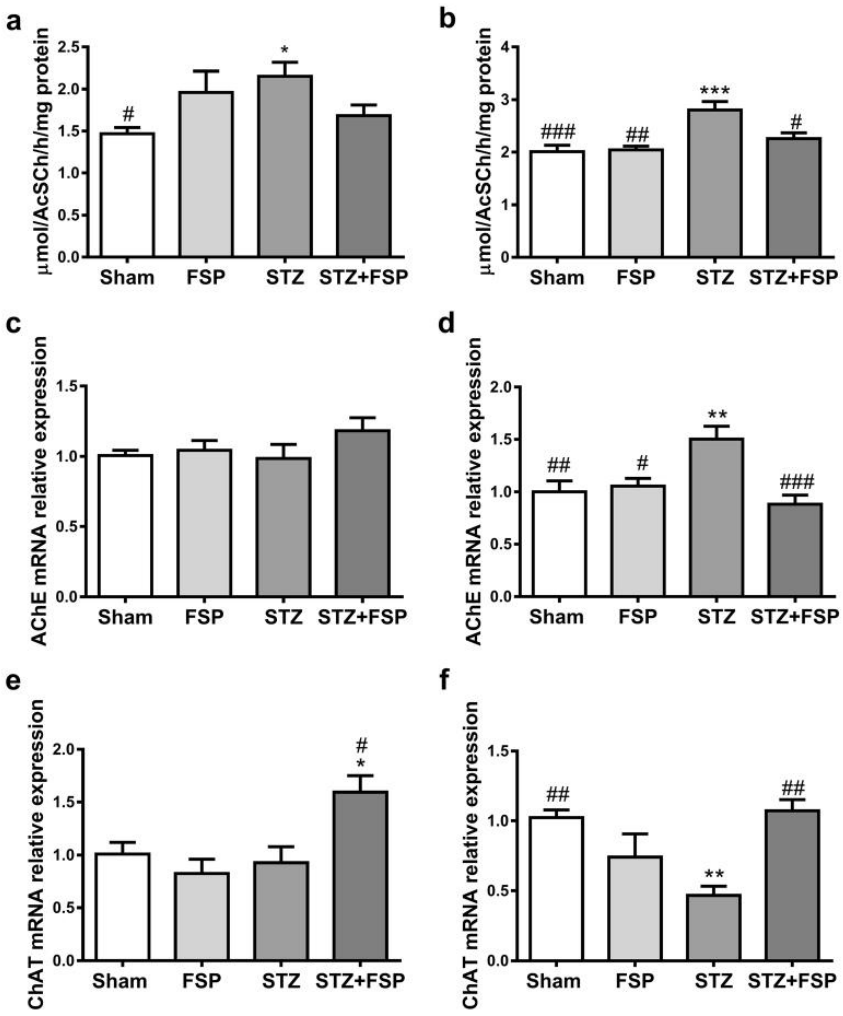

Fig. 5 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) (1 mg/kg intragastric (i.g.)/day) and/or streptozotocin (STZ) on cholinergic system. Acetylcholinesterase (AChE) activity in cerebral cortex (**a**) and hippocampus (**b**) of mice. Relative expression of mRNA AChE on cerebral cortex (**c**) and hippocampus (**d**) of mice. Relative expression of mRNA choline acetyltransferase (ChAT) on cerebral cortex (**e**) and hippocampus (**f**) of mice. Data are reported as mean ± standard error of the mean (SEM) of six-seven animals per group. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ as compared with the sham group. (#) $p < 0.05$, (##) $p < 0.01$, (###) $p < 0.001$ as compared with the STZ group (one-way analysis of Tukey's test)

relative expression of mRNA ChAT caused by STZ in the hippocampus of mice (ANOVA: $F_{3,24} = 7.57$, $p < 0.001$). The FSP + STZ group presented an increase in the levels of relative expression of mRNA ChAT when compared with the sham and STZ groups in the cerebral cortices (ANOVA: $F_{3,24} = 6.04$, $p < 0.01$) of mice.

FSP acts as neuroprotective modulator through the Na^+/K^+-ATPase enzyme

Figure 6b shows that STZ inhibited the Na^+/K^+-ATPase activity in the hippocampus (around 96%) of mice, when compared with the sham group. FSP (1 mg/kg (i.g.)/day) treatment significantly prevented the inhibition of hippocampus Na^+/K^+-ATPase activity induced by STZ (ANOVA: $F_{3,8} = 21.46$, $p < 0.001$). The results observed in Fig. 6a demonstrated that no alteration was observed in cerebral cortices Na^+/K^+-ATPase activity of mice (ANOVA: $F_{3,24} = 0.8083$, $p > 0.05$). Additionally, STZ decreased the levels of relative expression of mRNA Na^+/K^+-ATPase in the cerebral cortices (around 78%) (Fig. 6c) and hippocampus (around 73%) (Fig. 6d) of mice, when compared with the sham group. Treatment with FSP was able to prevent this decrease in the cerebral cortices (ANOVA: $F_{3,24} = 24.52$, $p < 0.0001$) and hippocampus (ANOVA: $F_{3,24} = 12.48$, $p < 0.0001$) caused by STZ.

Akt1, Akt2 and Akt3 signaling is not involved in FSP pharmacological action

The levels of relative expression of mRNA Akt1 (ANOVA: $F_{3,24} = 0.7914$, $p > 0.05$), Akt2 (ANOVA: $F_{3,24} = 1.707$, $p >$

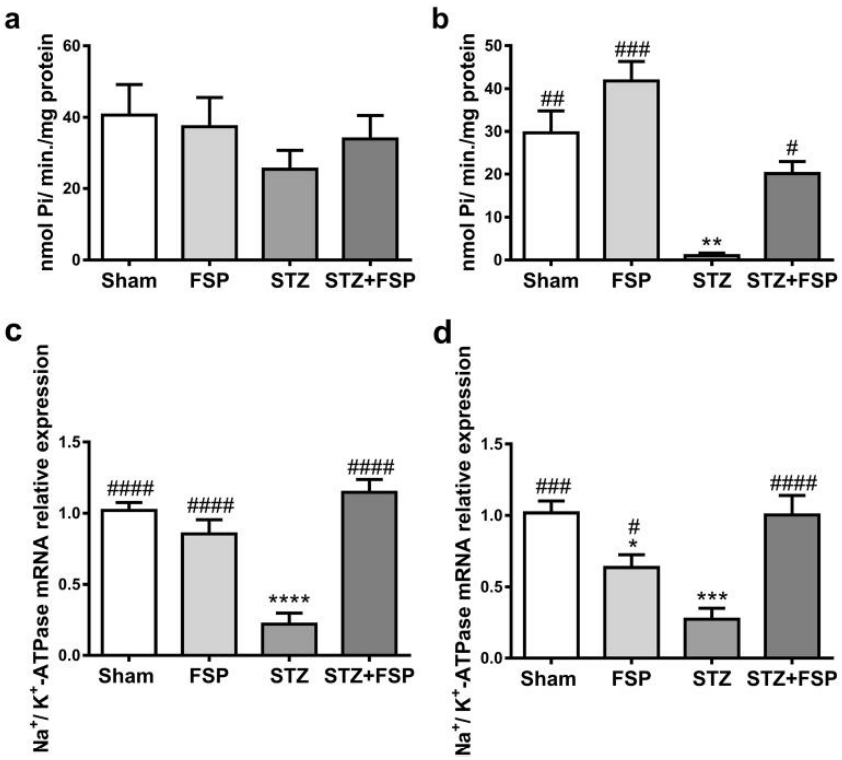

Fig. 6 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) (1 mg/kg intragastric (i.g.)/day) and/or streptozotocin (STZ) on Na^+/K^+-ATPase enzyme. Na^+/K^+-ATPase activity in cerebral cortex (**a**) and hippocampus (**b**) of mice. Relative expression of mRNA Na^+/K^+-ATPase in cerebral cortex (**c**) and hippocampus (**d**) of mice. Data are reported as mean ± standard error of the mean (SEM) of six- seven animals per group. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$, as compared with the sham group. (#) $p < 0.05$, (##) $p < 0.01$, (###) $p < 0.001$, (####) $p < 0.0001$ as compared with the STZ group (one-way analysis of Tukey's test)

0.05) and Akt3 (ANOVA: $F_{3,24} = 1.279$, $p > 0.05$) in cerebral cortices and Akt1 (ANOVA: $F_{3,24} = 1.859$, $p > 0.05$), Akt2 (ANOVA: $F_{3,24} = 2.515$, $p > 0.05$) and Akt3 (ANOVA: $F_{3,24} = 0.9730$, $p > 0.05$) in the hippocampus of mice were not altered by treatments (Fig. 7).

FSP did not induce oxidative damage in liver

Several undesirable side effects, such as hepatotoxicity, are observed in AD. The δ-ALA-D activity (ANOVA: $F_{3,24} = 0.3311$ $p > 0.05$) (Fig. 8a), TBARS (ANOVA: $F_{3,24} = 1.299$ $p > 0.05$) (Fig. 8b) and NPSH levels (ANOVA: $F_{3,24} = 0{,}7983 p > 0.05$) (Fig. 8c) in the liver of mice were not altered by treatments.

FSP did not induce acute toxicity

A single oral administration of FSP (300 mg/kg) did not cause the death of exposed mice. In Table 4, the administration of FSP did not alter any biomarker of hepatic (ALT ($df = 4$; $t = 1.821$ $p > 0.05$) and AST ($df = 4$; $t = 0.2683$ $p > 0.05$) activities) and renal (levels of urea ($df = 4$; $t = 2.157$, $p > 0.05$)) damage, as well as δ-ALA-D activity in the liver and brain of mice ($df = 4$; $t = 0.6393$, $p > 0.05$ for liver, $df = 4$; $t = 1.096$ $p > 0.05$ for brain), when compared to the control group.

Discussion

In this study we showed, for the first time, interaction of FSP with crucial residues for AChE inhibition. Moreover, this study provided evidence that FSP protected against anxiety behavior, as well as learning and memory impairment induced by STZ, which was accompanied by the modulation of the cholinergic system (AChE and ChAT) and Na^+/K^+-ATPase in the cerebral cortex and hippocampi of mice. Furthermore, no changes were observed in plasma markers of kidney and liver damage, in addition to oxidative markers in these tissues.

Previously, we demonstrated that FSP, inhibited AChE activity (Duarte et al. 2017). These results motivated us to understand how the FSP interacts with the AChE enzyme. Thus, we performed a molecular docking analysis to elucidate this question. Importantly, FSP interacts with crucial residues for AChE inhibition, such as His447, part of a catalytic triad. The initial step of both deacylation or acylation reactions, catalyzed by AChE in ACh hydrolysis, is firstly facilitated by a simultaneous proton transfer from Ser203 to His447. Furthermore, FSP can interact with Trp86 and Phe338, which are residues belonging to an anionic catalytic subsite and responsible for binding the quaternary trimethylammonium choline moiety of the substrate. Additional interactions with Gly122 and Gly121 were also observed. These residues are

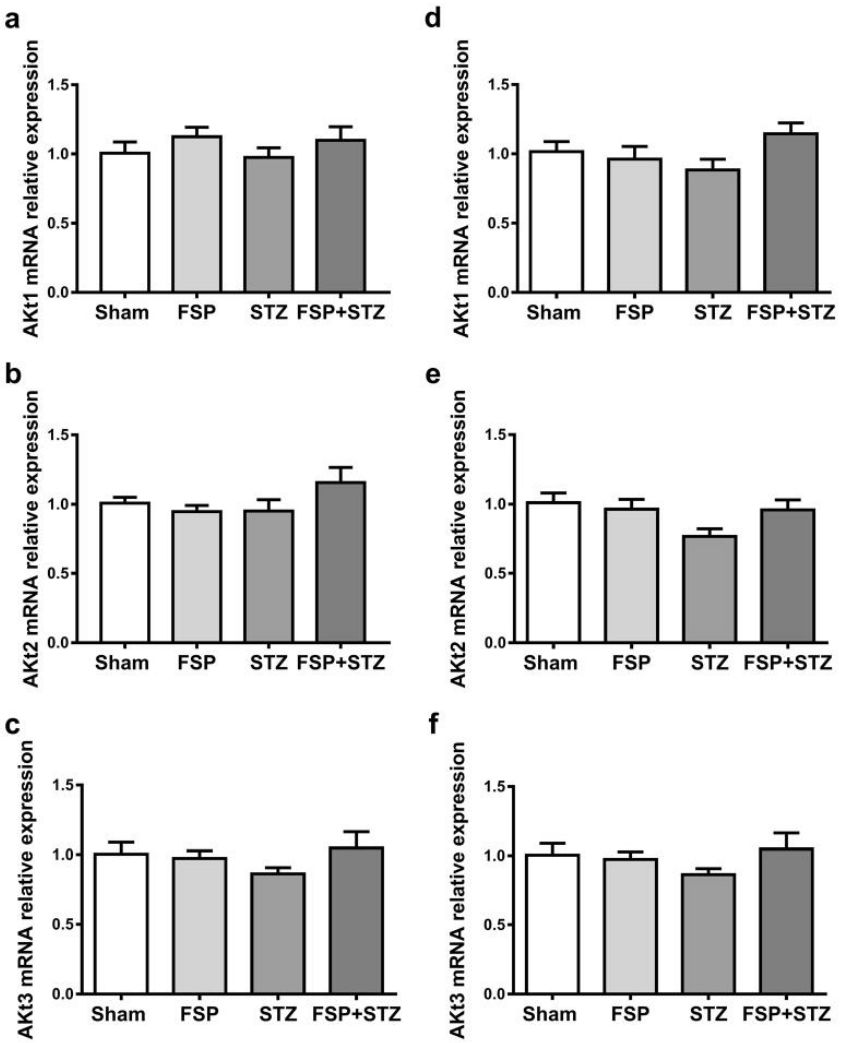

Fig. 7 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) (1 mg/kg intragastric (i.g.)/day) and/or streptozotocin (STZ) on relative expression of mRNA protein kinase B (Akt)1 (**a**), Akt2 (**b**) Akt3 (**c**) on cerebral cortex and Akt1 (**d**), Akt2 (**e**), Akt3 (7f) on hippocampus of mice. Data are reported as mean ± standard error of the mean (SEM) of six animals per group (one-way analysis of variance/ Tukey's test)

part of an oxyanion hole, which provides hydrogen bond donors that stabilize the tetrahedral transition state of the substrate (Zhang et al. 2002; Johnson and Moore 2006; Wiesner et al. 2007; Jang et al. 2018). Regarding the described data, these findings can suggest that FSP inhibitory AChE activity might be attributed, at least in part, to a competitive action mechanism.

We hypothesized that FSP acts impeding the ACh binding, through the combination of both superior molecular affinity and the interaction with residues responsible for stabilizing the ACh structure on the AChE. Studies have demonstrated that AChE interacts with Aβ through PAS (Tyr72, Tyr124, Trp286 and Tyr341) and promotes amyloid fibril formation, increasing its deposition and toxicity (De Ferrari et al. 2001; Gupta and Mohan 2014; Hou et al. 2014). Interestingly, FSP interacts with the motif Tyr124, which flanks the indole of Trp286 on the bottom groove of PAS. This evidence might suggest the dual inhibition activity of FSP on both the catalytic active site (CAS) and PAS of AChE enzyme. Additionally, we demonstrated the importance of the Se atom for the affinity of FSP with AChE, S-FSP presented a considerably lower affinity with this enzyme. Besides Se and S are two chalcogens, they differ from each other in some properties. The Se atom is bigger than S, having almost the double of the atomic weight. This could influence in the tridimensional structure of FSP and also the fitting in the structural cavities of the enzyme. Furthermore, Se atom is also more electronegative than S, due the nuclear shielding effect, which directly contributes to the residue's interactions and affinities, culminating in a different binding mode in AChE. These facts provide useful

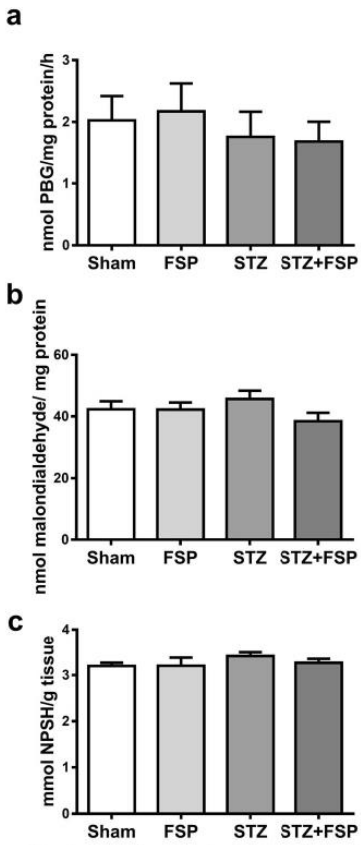

Fig. 8 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) (1 mg/kg intragastric (i.g.)/day) on δ-aminolevulinate dehydratase (δ-ALA-D) activity (**a**), thiobarbituric acid reactive species (TBARS) levels (**b**) and non-protein thiol (NPSH) levels (**c**) in liver of mice after streptozotocin (STZ) treatment. Data are reported as mean ± standard error of the mean (SEM) of seven animals per group (one-way analysis of variance/Tukey's test)

information regarding the importance of Se atom on the inhibitory activity of AChE exerted by FSP. Indeed, the beneficial effects of Se-derivates in preclinical studies involving AD are widely show (Pinz et al. 2018; Thomé et al. 2018; Martini et al., 2019; Yan et al. 2019).

In this sense, different online databases and in silico techniques can be useful to identify or design compounds against AD. These studies can effectively improve the success rate of the drug development process (Abeijon et al. 2017; Ambure and Roy 2017). Based on the promising result of the dual action mechanism of AChE inhibition in in silico analysis in this study and on the results that we observed previously (Duarte et al. 2017), we evaluated the effect of FSP in an AD model in mice.

We confirmed that STZ caused learning and memory impairment of animals, as noted in the different behavioral tests to assess different types of memory, without changing blood glucose. We demonstrated that STZ caused a loss of working memory, STM, LTM and aversive memory and non-spatial LTM. Importantly, the main finding of this study was that FSP protected against learning and memory impairment induced by STZ in mice, and that the beneficial effects were in all types of memory studied. These findings complement the results that we had already obtained (Duarte et al. 2017), demonstrating that, in the current study, FSP had an effect on acquisition memory.

Moreover, in this study, we observed that STZ caused an anxiety-like behavior in animals. Previous studies demonstrated that insulin/insulin receptors could be involved in neuropsychiatric disorders, including anxiety (Akasofu et al. 2008; Marks et al. 2009). Here, we believe that the impairment of insulin signaling caused by i.c.v. injection of STZ may interfere in the anxiety-like behavior. Another surprising result of the present study was that FSP presented, for the first time, an anxiolytic-like effect in an AD model in mice.

A main advantage of FSP over traditional AD treatments is the possibility of the administration of one drug for treatment of two disorders, AD and its comorbid, anxiety. This is important since it increases patients' adherence to treatments. In addition, this finding is very important since the treatment of patients with anxiety consists in the use of benzodiazepines. However, the administration of benzodiazepines has been shown to be a risk factor for AD and dementia (He et al. 2019). Here, our results suggest that anxiety is present in the early phase in a mouse AD model. In this sense, anxiety can be used as an early clinical manifestation of AD. This finding is highly relevant, given that it is believed that when treatment is started following the onset of cognitive symptoms it may be too late to alter disease progression (Mehta et al. 2017).

Table 4 Effect of a single administration of FSP (300 mg/kg) on acute toxicity in mice

		Groups	
		Control	FSP
AST (U/l)		115 ± 14	119 ± 7
ALT (U/l)	Plasma	52 ± 4	44 ± 2
Urea (mg/dl)		56 ± 5	40 ± 5
δ-ALA-D (nmol PBG/mg protein/h)	Brain	5.9 ± 1.0	5.1 ± 0.8
	Liver	54 ± 2	50 ± 3

Data are reported as mean ± standard error of the mean (SEM) of three animals per group. Statistical analysis was performed using a non-paired t test. *Abbreviations:* Aspartate (AST) and alanine (ALT) aminotrasferases, δ-aminolevulinate dehydratase (δ-ALA-D), 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP)

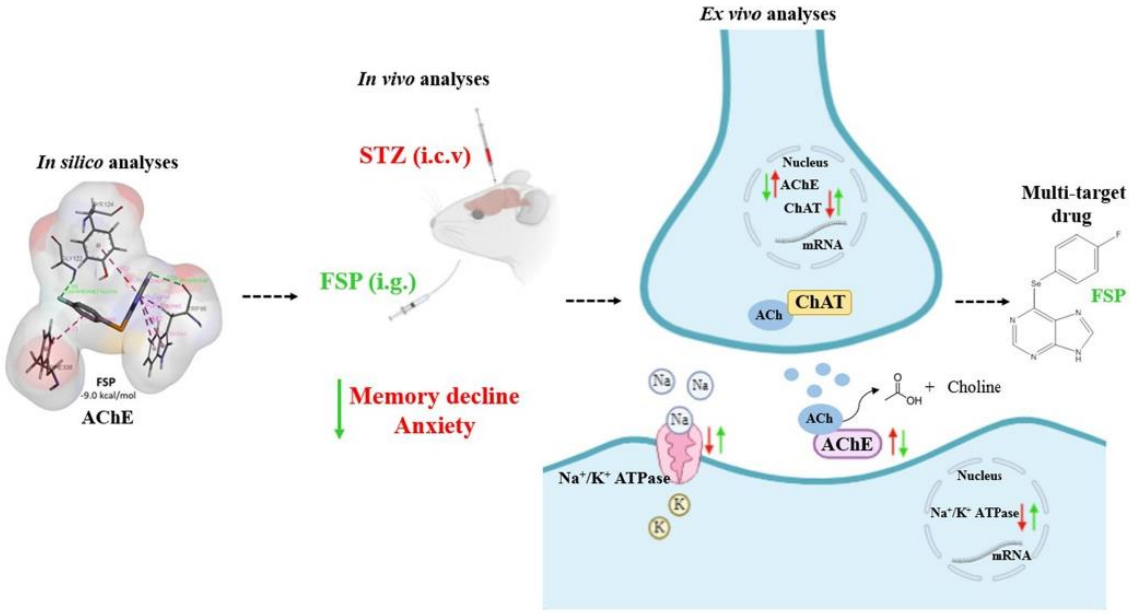

Fig. 9 Summary of action mechanism of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP). Our results have inferred that FSP is a multi-target compound. FSP interacts with residues of the acetylcholinesterase (AChE) active site as observed in in silico analyses. FSP mitigated the induction of anxiety and memory impairment caused by streptozotocin (STZ). FSP protected cholinergic system dysfunction and reduction of activity and expression of Na^+/K^+-ATPase. *Abbreviations:* acetylcholine (ACh); acetyltransferase (ChAT); intracerebroventricular (i.c.v.); intragastrically (i.g.); messenger ribonucleic acid (mRNA)

In order to clarify the mechanism by which FSP exerts an effect in preventing behavioral changes, mainly learning memory impairment, caused by STZ, we investigated the possible involvement of the cholinergic pathway. We showed that FSP modulated the cholinergic system, since the compound protected against the increase in the activity and gene expression of AChE, and also protected against the decrease in the ChAT gene expression caused by STZ in cerebral structures of mice. These results probably indicate that the modulation of AChE and ChAT by FSP promotes an increase in ACh levels in the synaptic cleft of neurons, contributing to the improvement in memory and learning caused by this compound.

Complementarily, we investigated the Na^+/K^+-ATPase enzyme aiming to expand the action mechanisms involved in effects already demonstrated by the FSP. In the present study, we observed that Na^+/K^+-ATPase showed an inhibition in the activity (hippocampi) and a reduction in the levels of gene expression (cerebral cortex and hippocampi) of mice exposed to STZ. Indeed, the oligomers of Aβ inhibit the Na^+/K^+-ATPase at the synapse (Ding et al. 2019) and a previous report by Ravelli et al. (2017) demonstrated that the i.c.v. injection of STZ induces accumulation of Aβ. The ionic imbalance caused by the dysfunction in the enzymatic activity of Na^+/K^+-ATPase may interfere in its expression. According to Nørgaard et al. (1981) and Vér et al. (1997) the ionic status may also play a role in the altered expression of Na^+/K^+-ATPase isoforms. The protective effect of FSP was further confirmed through prevention in the enzyme activity and gene expression of Na^+/K^+-ATPase. Moreover, Zhang et al. (2013) revealed that stimulators of Na^+/K^+-ATPase activity could be used as neuromodulator agents against AD. Additionally, it has already been demonstrated that Na^+/K^+-ATPase modulates anxiety-like behavior, and spatial learning and memory in mice (Moseley et al. 2007).

Thus, we believe that the mechanism by which the FSP exerts an effect in preventing behavioral changes caused by STZ is related to improvement of the cholinergic deficit and Na^+/K^+-ATPase modulation. These findings agree with a previous report, where rivastigmine (a drug used to treat AD) decreases AChE activity and increases Na^+/K^+-ATPase activity of aged rat brain and improves cognitive performance (Carageorgiou et al. 2008). It is known that, AChE interacts with Aβ through PAS and promotes amyloid fibril formation, increasing its deposition and toxicity (De Ferrari et al. 2001; Gupta and Mohan 2014; Hou et al. 2014). Here, in an in silico analysis, we observed that FSP interacts with the PAS residue. In this sense, this site of AChE will be less available to bind. This may contribute to a reduction of Aβ deposition and

consecutive increase of Na^+/K^+-ATPase activity, since Aβ directly decreases Na^+/K^+-ATPase activity (Lakunina et al. 2017).

Based on the results obtained we carried out Akt 1, Akt 2, and Akt 3 expression in cerebral cortex and hippocampi of mice. The reduction in Akts levels favors the deposition and aggregation of Aβ through the glycogen synthase kinase 3 pathway (Phiel et al. 2003). Chen et al. (2012) showed that the i.c.v. injection of STZ decreased levels of gene expression Akts in cerebral cortex and hippocampi in mice. However, in the present study, we did not observe statistically significant changes in levels of gene expression of Akts. Notably, in the hippocampus of mice exposed to STZ we observed a tendency to reduce the levels of gene expression Akts when compared to the sham group. In this sense, we cannot rule out, the involvement of this pathway in the events that corroborate the damage caused by STZ. Further studies are required to evaluate the Akts levels through other methodologies.

Taken together, our results evidenced that FSP is an efficient new candidate and multi-target drug for the clinical symptoms (memory and learning) and comorbidity (anxiety) of AD. In spite of the traditional drug discovery paradigm, "one-target, one-drug, one disease" used in the pharmaceutical industry, a more promising approach consists of designing the multi-target directed ligand drugs (Panek et al. 2017). In this context, two or more pharmacophoric portions can generate hybrid molecules which are therefore able to interact with multiple targets (Prati et al. 2016).

Concerning signs of hepatic and renal toxicity, FSP did not change plasma biochemical parameters and oxidative markers in these tissues. In addition, the absence of an unspecific behavior of FSP was proved in the OFT, since no animal showed reduced locomotion when compared to the control group.

Conclusions

In conclusion, the present study demonstrates that FSP presented dual inhibition activity of AChE, both CAS and PAS and Se atom is important for this affinity. Moreover, FSP was effective in protecting against learning and memory impairment in a mouse model of AD, and also had an effect in protecting against the anxiety-like type behavior of animals. The modulation of the cerebral cholinergic pathway and Na^+/K^+-ATPase contributes to the beneficial effects of FSP on memory and anxiety (Fig. 9). Despite that, the study has some limitations, as example, to evaluate the enzymes through more refined techniques. Thus, future studies are required to understand the complete mechanisms exerted by FSP and its physiological effects in other AD models.

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Author contributions MPP: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft. CL and EAW: Conceptualization, Verification, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. LS, DA and VFC: Methodology, Formal analysis, Funding acquisition. AGV, KCR, ASR, EBB and LFBD: Methodology. MGF: Methodology, Formal analysis, Writing Original Draft. WBD: Methodology, Formal analysis.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval Animal care and all experimental procedures were conducted in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publications no. 80–23, revised in 1996) (National Research Council 1996) and in accordance with the Committee on Care and Use of Experimental Animal Resources, Federal University of Pelotas, Brazil (CEEA 1974/2016). All efforts were made to minimize the number of animals used and their suffering.

Conflict of interest The authors declare that they have no conflict of interest.

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4.2. MANUSCRITO

A purine derivative containing organoselenium group protects against memory impairment, sensitivity to nociception, oxidative damage and neuroinflammation in a mouse model of Alzheimer's disease

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A purine derivative containing an organoselenium group protects against memory impairment, sensitivity to nociception, oxidative damage and neuroinflammation in a mouse model of Alzheimer's disease

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Abstract

In the present study, the effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) was tested against memory impairment and sensitivity to nociception induced by intracerebroventricular injection of amyloid-beta peptide (Aβ) (25-35 fragment), 3 nmol/3 μl/per site in mice. Memory impairment was determined by the object recognition task (ORT) and nociception by the Von-Frey test (VFT). Aβ caused neuroinflammation with upregulation of nuclear factor-κB (NF-κB) and the proinflammatory cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) in cerebral cortex and hippocampus. Additionally, Aβ increased oxidant levels and lipid peroxidation in cerebral cortex and hippocampus, but decreased heme oxygenase-1 (HO-1) and peroxiredoxin-1 (Prdx1) expression in the hippocampus. Anti-neuroinflammatory effects of FSP were demonstrated by a decrease in the expression of NF-κB in the hippocampus, as well as a decrease in proinflammatory cytokines in both the hippocampus and cerebral cortex FSP protected against oxidative stress by decreasing oxidant levels, lipid peroxidation and by increasing HO-1 and Prdx1 expressions in the hippocampus of mice. Moreover, FSP prevented the activation of nuclear factor erythroid 2-related factor 2 (Nrf-2) in the hippocampus of mice induced by Aβ. In conclusion, treatment with FSP attenuated memory impairment, nociception sensitivity by decreasing oxidative stress and neuroinflammation in a mouse model of Alzheimer's disease.

Keywords: Alzheimer's disease; Pain; Neuroinflammation; Oxidative stress; Organoselenium; Purine

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and is officially listed as the sixth leading cause of death in the United States. AD has a physical, psychological, social, and economic impact on patients and society at large [1, 2]. It is a multifactorial condition, given that amyloid deposition, *tau* protein hyperphosphorylation, cholinergic dysfunction, oxidative stress, neuroinflammation, among other factors, constitute the pathological hallmarks of this disease [3]. Moreover, purinergic signaling is involved in pathological processes of AD [4].

In addition, chronic pain is often present in AD patients (around 46% of patients) [5], and although this correlation exists, there is no clear mechanistic link. When in pain, a person with dementia may respond with agitation, aggression and resistance to care [6]. This understanding is important for the development of appropriate treatments and to improve the patients' quality of life.

Regarding AD therapy, it is known that acetylcholinesterase (AChE) inhibitors are the most popular treatments for AD symptoms. However, it should be noted that these inhibitors act for limited periods and present a risk of side effects. In this context, there is a continued search for new treatments for this condition. Over the last decade, more than 50 candidates have successfully passed phase II clinical trials, but none has passed phase III [7]. Recently, the FDA approved Aduhelm (aducanumab) using the accelerated approval pathway. Aduhelm is the first treatment directed at the presence of amyloid beta plaques in the brain. However, there remains some uncertainty about the drug's clinical benefit [8]. Therefore, the discovery of drugs to treat or prevent AD progression consists in developing new compounds or in the repositioning of known drugs that could act on multiple molecular targets involved in the pathogenesis of the disease.

In this sense, our research group has a continued interest in seeking promising alternative compounds for the treatment or prevention of AD progression [9–14]. Organoselenium compounds have proved to be protective in AD models with a putative application to counteract disease progression [15–18].

In addition to organoselenium compounds, purine derivatives prevent memory deficits and promote cognitive functions by modulating the cholinergic system, activity of ion pumps, and redox status in an experimental model of AD [19]. Purine derivatives also possess anticonvulsant [20], analgesic [21], antioxidant [22] and anti-inflammatory effects [22] and drugs affecting purinergic signaling are used in the treatment of central nervous system (CNS) disorders[23].

In this context, a purine derivative containing an organoselenium group is a promising compound as it combines the well-known applicability of the purine skeleton with that of the selenium proprieties. Based on this, we have studied the compound 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP). We have already demonstrated that FSP inhibits AChE activity from cerebral cortex *in vitro.* It enhances the consolidation and retrieval memory phases and inhibits the *ex vivo* AChE activity in mice cerebral cortex [12]. Moreover, when we performed a molecular docking study we observed that this compound interacts with residues in the AChE active site (Trp^{86}, Gly^{122}, Phe^{338} His^{447} and Gly^{121}) and in the peripheral anionic site (PAS) (Tyr^{124}), a site of Aβ deposition in AChE [24–26], reinforcing the anticholinesterase potential of FSP [27]. We also demonstrated that FSP was an efficient new candidate and multi-target drug for AD, considering that it protected against (i) learning and memory impairment, (ii) cholinergic system dysfunction in cerebral structures, and (iii) reduction of activity and expression of Na^+/K^+-ATPase in mice CNS. Concerning signs of toxicity, in preliminary tests, we found that FSP did not change plasma biochemical parameters and oxidative markers in mice tissue (brain and liver) [27].

Based on the above description and on our interest in seeking a more efficient and multifunctional alternative for the treatment of AD, a multifactorial condition, the aim of the present study was to investigate whether FSP ameliorates memory impairment, and decreases hypernociception, oxidative damage and neuroinflammation in a mouse model of AD induced by amyloid-beta peptide (Aβ) (25-35).

Materials and Methods

Chemicals

FSP (Fig. 1) was prepared and characterized in the Laboratory of Clean Organic Synthesis (LASOL), according to the method previously described by Duarte et al. [12]. FSP was dissolved in canola oil and administered to mice intragastrically (i.g.) at a dose of 1 mg/kg in a volume of 10 ml/kg via gavage. This dose was selected in accordance with a previous study [27]. The Aβ (fragment 25-35) was purchased from Sigma-Aldrich (St Louis, USA). It was dissolved in sterile filtered water, aggregated by incubation at 37°C for 4 days before use, and administered by intracerebroventricular (i.c.v.) injection at a dose of 3 nmol/3 μl/per site [10, 28, 29]. Despite the numerous similarities in the properties of Aβ (1-42) and Aβ (25-35), the shorter peptide is more rapidly toxic than the full-length peptide. Furthermore, Aβ (25-35) often causes more oxidative damage than Aβ (1-42) [30]. In this sense, the Aβ (fragment 25-35) has proved to adequately show many biochemical changes in animal models like those observed in AD patients [31]. All other analytical grade chemicals were obtained from standard commercial suppliers.

Animals

Male adult Swiss mice (25-35 g) from the Federal University of Pelotas, Brazil, were used in this study. The animals were maintained at a constant temperature (23 ± 1ºC), on a 12 h dark/light cycle (with lights on at 6:00 a.m.), with free access to food and water. Animal care

and all experimental procedures were conducted in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publications no. 80-23, revised in 1996) [32] and in accordance with the Committee on Care and Use of Experimental Animal Resources, Federal University of Pelotas, Brazil (CEEA 1974/2016). The number of animals and intensity of noxious stimuli used were the minimum needed to demonstrate the consistent effects of the treatments.

Experimental protocol

After three days of adaptation to colony room conditions, mice were randomly divided into 4 experimental groups:

Group 1 (sham): Saline + canola oil

Group 2 (FSP): Saline + FSP

Group 3 (Aβ): Aβ + canola oil

Group 4 (Aβ + FSP): Aβ + FSP

On day 1, mice were submitted to the Von-Frey test (VFT) to access the basal sensitivity of animals. On day 2, mice belonging to groups 1 and 3 received the canola oil (10 ml/kg, i.g.) and animals of groups 2 and 4 received the FSP compound (1 mg/kg, i.g.). Thirty minutes (min) later, mice belonging to groups 3 and 4 were induced with Aβ (3 nmol/3 μl/per site, i.c.v.) and those from the groups 1 and 2 received saline (3 μl/per site, i.c.v.). The i.c.v. infusion of Aβ or saline was administered using a microsyringe with a 28-gauge, 3.0 mm long stainless-steel needle (Hamilton), according to a previous report [29]. All animals were anesthetized with isoflurane before i.c.v. injection. Mice were treated with FSP or canola oil every day, until day 15 of the experimental protocol. On the 1st day of the experimental protocol, behavioral tests were initiated. An observer blinded to the study design performed all observations and these behavioral analyses were done in real-time by hand. On day 16, mice were euthanized by

inhalation of isoflurane and brains were removed for *ex vivo* experiments. The experimental protocol is demonstrated in Fig. 2.

Behavioral tests

Von-Frey test (VFT)

VFT was performed using a digital analgesimeter (Insight, Brazil). This test was used for the assessment of pain sensitivity [33] on days 1, 8 and 16 of the experimental protocol (Fig. 2). The digital analgesimeter was calibrated to record a maximum force of 150 g, maintaining an accuracy of 0.1 g to 80 g force. The contact between the pressure transducer and the hind limb was performed using a disposable tip made of polypropylene with a 0.5 mm diameter adapted to it. For this purpose, mice (8 animals per group) were placed individually in clear Plexiglas boxes, with twelve compartments, on elevated wire mesh platforms to allow access to the ventral surface of the right hind paw. Through the holes in the mesh the researcher applied linearly increasing pressure on the central region of the plantar hind limb until the paw withdrawal stimuli were reached. The paw withdrawal latency of mice was recorded in grams (g).

Rota Rod test (RRT)

A rota-rod apparatus (Insight, Brazil) was used for the assessment of motor coordination on day 8 of the experimental protocol (Fig. 2), as described previously [34] with some modifications. Mice (10 animals per group) were placed on the rod and then they ran while the rod was rolling. At the start, speed was set at 5 rpm, and then speed gradually increased from 6 to 30 rpm during 5 min., three times. The mice were tested similarly on the apparatus, 24 hours (h) after the training. An arbitrary time limit of 300 seconds (s) was set for the testing procedure. The latency time during which the mice remained on the accelerating rod was recorded in s.

Open field test (OFT)

OFT was carried out to identify locomotor disabilities, which might influence other tasks. OFT was performed on day 6 of the experimental protocol (Fig. 2). The apparatus was made of plywood and surrounded by 30 cm-high walls. The floor of the open-field (40 cm long × 40 cm wide) was divided into 9 squares (3 rows of 3). Each animal (10 animals per group) was placed at the center of the apparatus and observed for 4 min to record the number of segments crossed with the four paws and the number of rearings on the hindlimbs [35].

Object recognition task (ORT)

ORT was used to assess the short-term (STM) and long-term (LTM) memories of mice. ORT was performed in an open-field apparatus. On the day of the task (day 9 of the experimental protocol) (Fig. 2) each animal (10 animals per group) was submitted to a habituation session in the absence of objects for 5 min. Subsequently, four objects were used: A1, A2, B and C. Each object had the following color pattern: blue, red and yellow. All objects were of plastic material, measuring 10 x 10 cm (length x height). During the training, on day 10 of the experimental protocol, the animals were placed in the arena containing two identical objects (objects A1 and A2) for 5 min. Exploration was defined when the animal directed its nose within 2 cm of the object while looking, sniffing, or touching it. The STM of mice was evaluated 1.5 h after training in the presence of a familiar object (A1) and a new object (B), and the total time spent in exploring each object was determined during 5 min to measure the learning and recognition memory. The LTM was performed 24 h after training, on the ninth day of the experimental protocol, where mice were placed to explore a familiar object (A1) and a new object (C) for 5 min and the total time spent in exploring each object was determined. This test was performed according to Stangherlin et al. [36]. In order to analyze the cognitive performance, the exploratory preference was calculated and data were expressed as percentage as follows: Training = (A2 / (A1 + A2)) x 100; STM = (B / (A1 + B)) x 100; LTM = (C / (A1 + C)) x 100).

Ex vivo analyses

Tissue preparation

Cerebral cortices and hippocampi (6-9 samples per group) were homogenized in 50 mM Tris-HCl, pH 7.4 (1:10, w/v). Afterwards they were centrifuged at 900 *xg* for 10 min at 4°C to obtain a supernatant fraction (S1) to determine oxidant, thiobarbituric acid reactive substances (TBARS) and non-protein thiol (NPSH) levels.

Oxidant level quantification

The oxidant quantification was determined by a spectrofluorimetric method, using 2′,7′-dichlorofluorescein diacetate (DCHF-DA) assay [37]. The S1 was incubated with DCHF-DA (1 mM). The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) was measured for the detection of intracellular oxidants. The DCF fluorescence intensity emission was determined at 520 nm (with 480 nm excitation), 15 min after the addition of DCHF-DA to the medium. The results were expressed as arbitrary units of fluorescence (UF).

Thiobarbituric acid reactive substances (TBARS) level

Lipid peroxidation was evaluated by TBARS formation [38]. An aliquot of S1 was added to the reaction mixture containing thiobarbituric acid (0.8%), sodium dodecyl sulfate (8.1%), and acetic acid (pH 3.4), and incubated at 95°C for 2 h. The absorbance was measured at 532 nm. Results were expressed as nmol malondialdehyde (MDA) /mg protein.

Non protein thiol (NPSH) level

NPSH levels were determined as described by Ellman [39]. An aliquot of S1 was mixed (1:1) with 10% TCA and centrifuged at 900 *xg* for 10 min. Afterwards, the protein pellet was discarded and free -SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer (pH 7.4), and 10 mM 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). The color reaction was measured at 412 nm. NPSH levels were expressed as µmol NPSH/g tissue.

RNA extraction and gene expression by real-time PCR

Total mRNA was extracted from thawed samples of cerebral cortices and hippocampi (8 samples per group) using TRIzol reagent (Invitrogen™, Carlsbad, USA) followed by DNase treatment with DNase I Amplification Grade (Invitrogen™, Carlsbad, USA). The total RNA amount and purity (260/280 and 260/230 ratios) were examined by NanoVue spectrophotometer (GE, Fairfield, CT, USA). The cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription kit (AppliedBiosystems™, UK) according to the manufacturer's protocol. The amplification was done with GoTaq® qPCR Master Mix (Promega, Madison, WI) using the Agilent Mx3005P qPCR System (Agilent Technologies Inc., Santa Clara, CA) and the sequence of primers used is indicated in Table 1. The qPCR conditions were as follows: 10 min at 95°C, 15 s at 95°C, 60 s at 60°C, 30 s at 72°C (fluorescence signals were detected at the end of every cycle). Baseline and threshold values were automatically set by the Stratagene MxPro software. The 2-ΔΔCT method was used to normalize the fold change in gene expressions[40], using Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene of AChE, glutathione peroxidase (GPx), nuclear factor- κB (NF-κB), superoxide dismutase (SOD), interferon- γ (IFN-γ) and tumor necrosis factor-α (TNF-α).

Western-blot analysis

The samples of cerebral cortices and hippocampi (3-5 samples per group) were homogenized in lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1mM dithiothreitol (DTT), 2% IGEPAL® CA-630, commercial phosphatase and protein inhibitor cocktail (Sigma-Aldrich Company, St. Louis, Missouri, United States), centrifuged at 11,000 *xg* at 4 °C for 20 min to obtain the supernatant (enriched with cytoplasmic fraction). The pellet was homogenized in extraction buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 300 mM NaCl, 0.25 mM EDTA, 25 % glycerol, 8 M urea, 1 nM DTT, commercial phosphatase and protein inhibitor cocktail (Sigma-Aldrich Company, St. Louis, Missouri, United States), centrifuged at 20,000

xg at 4 °C for 5 min to obtain the supernatant (enriched with nuclear fraction). The samples were diluted to a final protein concentration of 50 µg/µl in 5× sample buffer, which contains 329 mM Tris-HCl (pH 6.8), 10% SDS, 0.01% bromophenol blue, 50% glycerol and 5% β-mercaptoethanol as reducing agent. The samples and pre-stained molecular weight standards (Bio-rad Laboratories, Hercules, CA, USA) were separated by 10% SDS-PAGE electrophoresis and transferred to PVDF membrane (Bio-rad Laboratories, Hercules, CA, USA) using the TransferBlot® Turbo™ Transfer System. After blocking with 5% bovine serum albumin or 5% skimmed milk solution, the blots were incubated overnight at 4°C with mouse anti-heme oxygenase1 (HO-1) (1:250; Abcam), rabbit anti-peroxiredoxin-1 (Prdx1) (1:1000; Cell Signaling), rabbit anti-NF-κB p65 (1:1000; Cell Signaling), and rabbit anti-nuclear factor erythroid 2-related factor 2 (Nrf2) (1:200; Abcam). After primary antibody incubation, membranes were washed and incubated with their respective secondary antibodies (1:10000) conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA) for 1 h at room temperature. For protein detection, we used an enhanced chemiluminescence (ECL) kit (Bio-rad Laboratories, Hercules, CA, USA). Optical density (O.D.) of the western blotting bands was quantified using Image J (NIH, Bethesda, MD, USA) software for Windows. Protein loading was normalized by Coomassie staining of the membrane.

Cytokines levels

Interleukin-1β (IL-1β), IFN-γ and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA) mouse kits in cerebral cortices and hippocampi (3-6 samples per group) homogenates as recommended by the manufacturer (R&D Systems, Minneapolis, USA).

Protein determination

The protein concentration was measured by the Bradford method [41], using bovine serum albumin as the standard.

Statistical analysis

The data are expressed as mean ± standard error of the mean (S.E.M.). Data were analyzed by Graphpad Prism® 5 and data normality was evaluated by the D'Agostino and Pearson omnibus normality test. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test and two-way ANOVA followed by Tukey's multiple comparison test for VFT. Values of $p < 0.05$ were considered statistically significant.

Results

Effects of FSP on behavioral evaluations

FSP and Aβ did not cause any significant change in locomotor and motor activities

The one-way ANOVA followed by Tukey's test revealed that treatments did not cause any significant change in the number of crossings (supplementary Fig. S1a) (ANOVA: $F_{3, 36} = 2.373$, $p = 0.08640$) or rearings (supplementary Fig. S1b) (ANOVA: $F_{3, 36} = 1.198$, $p = 0.3243$) in the OFT, or in the time of permanence of mice in the RRT (supplementary Fig. S1c) (ANOVA: $F_{3, 36} = 1.108$, $p = 0.3585$).

FSP protects against cognitive and memory impairment caused by i.c.v. injection of Aβ

The one-way ANOVA followed by Tukey's test revealed that there was no difference in the exploratory preference for objects among groups in the training phase (supplementary Fig. S2) (ANOVA: $F_{3,36} = 0.2194$, $p = 0.8823$). Aβ decreased the exploratory preference for the new object in STM (around 26%) (Fig. 3a) and LTM (around 28%) (Fig. 3b), when compared with the sham group. Treatment with FSP was able to attenuate these reductions in STM (Fig. 3a) (ANOVA: $F_{3,36} = 13.80$, $p < 0.0001$) and LTM (Fig. 3b) (ANOVA: $F_{3,36} = 13.53$, $p < 0.0001$). FSP alone did not change the exploratory preference in STM (Fig. 3a) and LTM (Fig. 3b).

FSP protects against increased nociception sensitivity induced by i.c.v. injection of Aβ

The two-way ANOVA followed by Tukey's test indicated a significant interaction of groups x days ($F_{6, 84} = 4.573$, $p = 0.0005$) in the VFT. Post-hoc comparisons demonstrated that on day 1 (basal sensitivity) no change was observed between groups in the VFT (Fig. 3c). Aβ increased the paw withdrawal stimuli on the 8th (around 16%) and 16th (around 21%) days, when compared with the sham group (Fig. 3c). FSP treatment was not able to attenuate the increase in the paw withdrawal stimuli on the 8th day, but it attenuated the alterations caused by Aβ on the 16th day, when compared with the Aβ group (Fig. 3c). FSP alone did not change the paw withdrawal stimuli in the VFT on any of the days analyzed (Fig. 3c).

Effects of FSP on ex vivo analyses

FSP modulate the NF-κB transcription factor

The one-way ANOVA followed by Tukey's test revealed no difference in the relative expression of NF-κB mRNA in the cerebral cortices of mice after the treatments (Fig. 4a) (ANOVA: $F_{3,28} = 0.7110$, $p = 0.5536$). On the other hand, Aβ increased (around 32%) the relative expression of NF-κB mRNA in the hippocampi of mice, when compared with the sham group (Fig. 4b). FSP treatment significantly prevented the increase in relative expression of NF-κB mRNA induced by Aβ in the hippocampi (Fig. 4b) (ANOVA: $F_{3,28} = 9.986$, $p = 0.0001$). FSP alone did not change the relative expression of NF-κB mRNA in cerebral cortex (Fig. 4a) or hippocampus (Fig. 4b).

Western-blot analysis demonstrated that Aβ increased the NF-κBp65 protein levels in the cerebral cortex (around 455%) (Fig. 4c and 4d) and hippocampus (around 295%) (Fig. 4e and 4f) when compared with the sham group. Treatment with FSP was able to prevent the increase in hippocampus (Fig 4e and 4f) (ANOVA: $F_{3,11} = 11.23$, $p = 0.0011$), but not in cerebral cortex (Fig 4c and 4d) (ANOVA: $F_{3,12} = 17.32$, $p = 0.0001$). FSP alone did not change the NF-κBp65 protein levels (Fig 4c and 4d for cerebral cortices; Fig 4e and 4f for hippocampi).

Proinflammatory cytokines modulation is involved in FSP pharmacological action

The one-way ANOVA followed by Tukey's test revealed that Aβ increased the relative expression of TNF-α mRNA in cerebral cortex (around 27%) (Fig. 5a) and hippocampus (around 41%) (Fig. 5b) when compared with the sham group. Treatment with FSP was able to inhibit this increase in both the cerebral cortex (Fig. 5a) (ANOVA: $F_{3,28} = 12.94$, $p > 0.0001$) and hippocampus (Fig. 5b) (ANOVA: $F_{3,28} = 26.67$, $p < 0.0001$). FSP alone reduced (around 24%) the relative expression of TNF-α mRNA in the hippocampus (Fig. 5b), but there were no changes in cerebral cortex (Fig. 5a).

Regarding TNF-α levels, Aβ induced a slight but not significant increase (12.26%) in cerebral cortex (ANOVA: $F_{3,12} = 0.6801$, $p = 0.5809$) (Fig.5c). In the hippocampus, FSP alone reduced (around 20%) TNF-α levels but no statistically significant change was observed in the other groups when compared to the sham group (ANOVA: $F_{3,15} = 6.001$, $p = 0.0068$) (Fig. 5d).

Relative expression of IFN-γ mRNA was increased after injection of Aβ into cerebral cortex (around 74%) (Fig. 5e) and hippocampus (around 50%) (Fig. 5f) when compared with the sham group. Treatment with FSP precluded it in the cerebral cortex (Fig. 5e) (ANOVA: $F_{3,28} = 14.85$, $p < 0.0001$), but not in the hippocampus (Fig. 5f) (ANOVA: $F_{3,28} = 6.704$, $p = 0.0015$). FSP alone increased IFN-γ mRNA in hippocampus (Fig. 5f).

Regarding IFN-γ levels, Aβ induced to a 10.61% no significant increase in the cerebral cortices (ANOVA: $F_{3,15} = 1.264$, $p = 0.3223$) (Fig.5g). However, Aβ significantly increased IFN-γ levels in the hippocampi (around 50%), when compared with the sham group (Fig. 5h). FSP treatment partially precluded the increase IFN-γ levels (Fig. 5h). FSP alone increased the IFN- γ levels in the hippocampi of mice (around 37%) (Fig. 5h) (ANOVA: $F_{3,14} = 19.84$, $p < 0.0001$). No changes were observed in IL-1β levels in either cerebral cortices (Fig. 5i) (ANOVA: $F_{3,12} = 0.5569$, $p = 0.6534$) or hippocampi (Fig. 5j) (ANOVA: $F_{3,12} = 2.487$, $p = 0.1103$).

FSP protects against oxidative damage induced by i.c.v. injection of Aβ

Aβ administration increased the production of oxidants in cerebral cortex (Fig. 6a) and hippocampus (Fig. 6b), around 39 and 38% respectively, whereas FSP treatment inhibited this increase in the hippocampus (Fig. 6a) (ANOVA: $F_{3,25} = 5.671$ $p = 0.0042$), but not in the cerebral cortex (Fig. 6b) (ANOVA: $F_{3,28} = 4.170$, $p = 0.0146$). FSP alone did not change the production of oxidants (Fig. 6a for cerebral cortices; Fig. 6b for hippocampi).

One-way ANOVA followed by Tukey's test showed that Aβ increased the TBARS levels in the cerebral cortex (around 36%) (Fig. 6c) and hippocampus (around 24%) (Fig. 6d) when compared with the sham group. Treatment with FSP inhibited this increase in the hippocampus (Fig. 6d) (ANOVA: $F_{3,31} = 5.761$ $p = 0.0030$), but not in the cerebral cortex (Fig. 6c) (ANOVA: $F_{3,29} = 5.860$, $p = 0.0030$). FSP alone did not change the TBARS levels (Fig. 6c for cerebral cortices; Fig. 6d for hippocampi).

FSP acts in Nrf2 transcription factor and antioxidant defenses modulation

Fig. 7a and Fig. 7b show that Aβ increased the Nrf2 protein levels in the hippocampus around 810 % when compared with the sham group and FSP treatment prevented this increase (Fig. 7a and 7b) (ANOVA: $F_{3,10} = 32.77$, $p < 0.0001$). FSP alone did not change the Nrf2 protein levels in the hippocampi of mice (Fig. 7a and 7b). No changes in Nrf2 were detected in cerebral cortices (data not show).

The one-way ANOVA followed by Tukey's test show that Aβ increased the relative expression of SOD mRNA (around 40%) (ANOVA: $F_{3,28} = 8.097$, $p = 0.0005$) (Fig. 7c) and GPx mRNA (around 21%) (ANOVA: $F_{3,28} = 8.798$, $p = 0.0003$) in hippocampi when compared with the sham group (Fig. 7c for SOD and Fig. 7d for GPx). Treatment with FSP was not able to prevent these changes. No change was observed in mice hippocampi by the treatment with FSP alone (Fig. 7c for SOD and Fig. 7d for GPx). In the cerebral cortices, treatments did not cause any significant changes in the relative expression of SOD mRNA (supplementary Fig.

S3b) (ANOVA: $F_{3,28} = 0.7763$, $p = 0.5171$) and GPx mRNA (supplementary Fig. S3c) (ANOVA: $F_{3,28} = 3.448$ $p = 0.0299$).

Additionally, Aβ decreased HO-1 (Fig. 7e and 7f) and Prdx1 (Fig. 7g and 7h) levels around 68 and 83% respectively, in the hippocampi. FSP treatment significantly prevented protein decrease (ANOVA: $F_{3,9} = 9.193$, $p = 0.0042$ for HO-1 levels Fig. 7e and Fig. 7f; ANOVA: $F_{3,13} = 15.54$ $p = 0.0001$ for Prdx1 levels, Fig. 7g and 7h). No change was observed from the treatment with FSP alone. No alteration was observed in HO-1 (ANOVA: $F_{3,9} = 1.374$, $p = 0.3119$) (supplementary Fig. S3d and S3e) and Prdx1 levels (ANOVA: $F_{3,14} = 3.190$, $p = 0.0567$) (supplementary Fig. S3f and. S3g) in cerebral cortices.

No difference was observed in the NPSH levels in the cerebral cortices of mice after the treatments (Supplementary Fig. S4a) (ANOVA: $F_{3,24} = 2.497$, $p = 0.0840$). The FSP+Aβ group presented a reduction of the NPSH levels in the hippocampi when compared with the Aβ group (Supplementary Fig. S4b) (ANOVA: $F_{3,24} = 3.536$ $p = 0.0298$).

FSP protects against the increase in the relative expression of mRNA AChE

One-way ANOVA followed by Newman-Keuls' test showed that Aβ increased the relative expression of AChE mRNA in the cerebral cortices (around 22%) (Fig. 8a) and hippocampi (around 23%) (Fig. 8b) when compared with the sham group. FSP reduced the relative expression of AChE mRNA induced by Aβ in the hippocampi (Fig. 8b) (ANOVA: $F_{3,28} = 17.76$, $p < 0.0001$), but had no effect on cerebral cortices (Fig. 8a) (ANOVA: $F_{3,28} = 5.036$, $p = 0.0065$). Additionally, FSP alone decreased (around 20%) the relative expression of AChE mRNA in the hippocampi (Fig. 8b) without affecting AChE mRNA in cerebral cortices (Fig. 8a).

Discussion

The present study demonstrated that Aβ (25-35) caused memory impairment accompanied by hypernociception in mice. In this study, we demonstrated, for the first time,

that FSP, a purine derivative with an organoselenium group, attenuated memory impairment and hypernociception in a mice model of AD. Furthermore, molecular and biochemical analyses provided evidence that FSP decreases the neuroinflammatory process and the oxidative stress caused by Aβ (25-35).

As inflammation and oxidative stress are thought to be a central phenomenon in the early pathogenesis of AD and in other conditions linked to AD, therapeutic alternatives that may increase antioxidant and/or anti-inflammatory defense have been recommended [42]. During the neuroinflammation process, NF-κB is involved in the inflammatory signaling pathway and in cytokine production by microglia [43]. In the current study, we found an increase in NF-κB expression in the hippocampus and cerebral cortex of mice exposed to Aβ (25-35). Indeed, the activation of the NF-κB transcription factor has been detected in the brains of AD patients [44]. FSP protected against this augmentation of the hippocampus, but not of the cerebral cortex. Accordingly, Pan et al. [45] reported that an organoselenium compound (Se-methylselenocysteine) had a potent anti-inflammatory effect via downregulation of NF-κB in lipopolysaccharide-activated macrophages.

The pro-inflammatory cytokines TNF-α, IFN-γ and IL-1β are reported to be augmented in AD brains [46]. We also investigated these cytokines to learn more about the mechanisms exercised by FSP. We observed that FSP protected against the increase in TNF-α and IFN-γ in both cerebral structures of mice exposed to Aβ, but FSP *per se* induced an increase in the IFN-γ levels in the hippocampus of mice. Here, we cannot explain the exact reason for this increase in IFN-γ and further studies need to be carried out for a better understanding of this effect. In parallel, a previous report showed that selenofuranoside compound *per se* caused an increase in interleukin 6 (IL-6) [47], which we similarly observed for IFN-γ levels. Moreover, no changes were observed in the IL-1β levels after treatments, which might be due to a different time course for expression of this cytokine. As observed for TNF-α and IFN-γ there was a time-

lapse in gene expression and protein secretion, since mRNA was altered, but no change was observed in the cytokine levels. Importantly, FSP reduced the neuroinflammation induced by Aβ.

There is a relationship between the production of oxidants and NF-κB in AD. In fact, Aβ induces oxidants generation, which, in turn, could activate NF-κB [44], whereas NF-κB can potentiate oxidant formation through upregulation of nitric oxide synthase [48], and a positive feedback occurs that fosters further neuronal damage. Increased oxidant production and oxidative damage have been implicated in the pathogenesis and progression of AD [42, 49].

Corroborating this information, our results demonstrate that Aβ increased oxidant levels and lipid peroxidation in cerebral cortex and hippocampus. Lipid peroxidation affects the integrity/functions of biological membranes such as neuron membranes [50] and it is frequently generated by excessive oxidant production in AD [51]. FSP treatment was able to reduce these increases in the hippocampi, but not in the cerebral cortices of mice induced by Aβ.

It was shown that FSP effectively reduced oxidative damage in this pathological model since it decreased oxidant content and TBARS levels. The exact antioxidant mechanism of the molecule has not been described, but it likely acts as other organoselenium compounds, such as selenofuranosides, diphenyl diselenide, 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) and Ebselen. Theoretically, FSP would form a selenolate (RSe^{-1}) in a thiol-rich environment and then react with oxidants. In the reaction with H_2O_2, it would form a selenic acid, which reacts with a thiol group yielding selenyl sulfide. [52, 53]. Additionally, we observed a reduction in NPSH levels in the hippocampus of mice treated with FSP plus Aβ. The consumption of NPSH may explain this theory.

In an attempt to understand how FSP modulates neuroinflammation and oxidative damage, Nrf2 transcription factor and antioxidant defenses were evaluated. Under oxidative conditions, Nrf2 translocates to the nucleus initiating the transcription of an ensemble of

defense proteins, such as γ-glutamylcysteine synthetase, GPx, SOD, HO-1 and Prdx1[54]. The decline of the Nrf2 function has been observed in brains of AD patients [55]. However, our study demonstrated that Aβ activated Nrf2 in mice hippocampus. This is in line with other studies, which demonstrated activation of Nrf2 in human brain and mouse models of AD [56, 57]. In fact, Nrf2 activity varies according to region and age [58]. Migration of Nrf2 might be an effort to limit the progression of AD. FSP treatment significantly prevented Nrf2 activation, which could characterize a lower insult in FSP treated mice, with a decreased trigger of protection mechanisms. The Nrf2 activation was accompanied by an increase in the relative expression of SOD mRNA and GPx mRNA. FSP did not prevent the increase of the relative expression of these enzymes. Controversially, mice induced with Aβ presented a reduction in the expression of HO-1 and Prdx1 in their hippocampus. HO-1 degrades pro-oxidant heme [59] and Prdx1 belongs to a family of cysteine peroxidases (peroxiredoxins) that are responsible for antioxidant defense [60]. A reduction in the expression of these enzymes can contribute to oxidative stress and inflammation. Further studies are required to better understand how the Aβ can reduce the expression of these enzymes. Meanwhile, these observations are in line with Mota et al. [57] who observed an increase in the levels of Nrf2 in nuclear fractions and a significant decrease in HO-1 mRNA and a trend for decreased Prdx-1 mRNA in brain samples of a 3xTg-AD mice model. Importantly, we demonstrated, for the first time, that FSP was effective in protecting against the decrease in the expression of HO-1 and Prdx1 caused by Aβ in the hippocampus of mice. FSP is likely a multi-target compound and could modulate the function of other transcription factors, such as the Forkhead O box, another canonical antioxidant pathway [61, 62]. We did not observe changes in the Nrf2 transcription factor and antioxidant defenses after treatments in the cerebral cortices of mice. This result may be due to regional variations observed in AD.

It has been reported that i.c.v. injection of Aβ causes memory impairment and a cognitive deficit [10, 28, 47]. In fact, FSP attenuated the loss of STM and LTM induced by Aβ (25-35) in mice, without changing locomotor, exploratory and motor activities. These discoveries reinforce our previous results, we have already demonstrated that FSP improved different types of memory (working memory, STM, LTM and aversive memory and non-spatial LTM) in a mice AD model induced by streptozotocin [27], and this indicates that FSP is a promising alternative therapy for AD. However, the effect of Aβ (25-35) on nociception, as far as we know, has not been explored. Although the correlation between pain and AD has been well described [5], a consolidated mechanistic link needs clarification. For this reason, we evaluated the mechanical hyperalgesia of animals. Our results showed that FSP attenuated the increased nociception caused by Aβ in mice.

Growing evidence suggests that patients with AD and/or pain hypersensitivity share some common pathologies such as activation of microglia in brain areas and increased neuroinflammation [63]. In fact, microglia activation is essential in pain pathophysiology [64]. Aβ aggregates can activate the microglia through specific receptors which, in turn, activate transcription factors inducing cytokine release and oxidant production [65]. The inflammatory and neuropathic pain-related symptoms can be reduced by selective microglial α7 nicotinic acetylcholine receptor (nAChR) agonists [66–68]. In our previously study, FSP improved cholinergic function through the inhibition of AChE and overexpression of choline acetyltransferase (ChAT) [27]. Additionally, here we showed that FSP protected against the increase in AChE expression caused by Aβ in the hippocampus. This would be a putative mechanism for anti-hypernociceptive effect of the compound. These results suggest that FSP can improve memory impairment and decrease nociception by reestablishing ACh levels, reducing neuroinflammation and oxidative damage.

Altogether, the results suggest that the FSP (i) decrease neuroinflammatory process, (ii) decrease oxidant levels and lipid peroxidation, and (iii) increase antioxidant defenses establishing a favorable condition that prevents behavioral alterations. Previously, we reported the protective effects of FSP by modulating cholinergic function and ion pump Na^+/K^+-ATPase activity [27]. Here, we found additional effects of FSP as an anti-inflammatory agent. In this sense, FSP can provide a multifunctional approach to AD, since this is a multifactorial condition.

Conclusion

In summary, the results of the present study demonstrated that FSP orchestrated an adaptive response to oxidative damage through an increase in Prdx1 and HO-1 expression, decrease in oxidant levels and lipid peroxidation and prevention of the inflammatory process. The protective effect of FSP promoted memory improvement and reduced hypernociception in Aβ treated mice. Although further investigations are necessary (to evaluate the FSP effects on other animal models of AD and in males and females), our findings shed new light on the mechanism underlying the protective effects of FSP, a new multi-targeted drug, as a promising therapeutic strategy for AD and its comorbidity, pain.

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Author contributions

MPP: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft. CL and EAW: Conceptualization, Verification, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. FCM: Resources, Writing - Review & Editing, Supervision, Funding acquisition. DA and VFC: Methodology, Formal analysis, Funding acquisition. RLO, CARF, GTV, HGO and LFBD: Methodology. BPS and WBD: Methodology, Formal analysis.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval

Animal care and all experimental procedures were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications no. 80–23, revised in 1996) (National Research Council 1996) and in accordance with the Committee on Care and Use of Experimental Animal Resources, Federal University of Pelotas, Brazil (CEEA 1974/2016). All efforts were made to minimize the number of animals used and their suffering.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table 1. Primers used for quantitative real-time polymerase chain reaction. The forward and reverse primer sequences used to amplify each target gene as well as the GAPDH endogenous control are listed.

Primer Name	Sequence	Reference
AChE Forward	5' TTAGGGCTGGGATATAATACGAC 3'	[69]
AChE Reverse	5' GCCCCTAGTGGGAGGAAGT 3'	
IFN-γ Forward	5' AGCGGCTGACTGAACTCAGATTGTAG 3'	[70]
IFN-γ Reverse	5' GTCACAGTTTTCAGCTGTATAGGG 3'	
GPx Forward	5' TGTGGAAATGGATGAAAGTCCAG 3'	[71]
GPx Reverse	5' CATGGGACCATAGCGCTTCAC 3'	
NF-κB Forward	5' AGAGAAGCACAGATACCACTAAG 3'	[72]
NF-κB Reverse	5' CAGCCTCATAGAAGCCATCC 3'	
SOD Forward	5' GGACCTCATTTTAATCCTCAC 3'	[73]
SOD Reverse	5' TGCCCAGGTCTCCAACATG 3'	
TNF-α Forward	5' CCCTCACACTCAGATCATCTTCT 3'	[74]
TNF-α Reverse	5' CTACGACGTGGGCTACAG 3'	
GAPDH Forward	5' TGCGACTTCAACAGCAACTC 3'	[75]
GAPDH Reverse	5' ATGTAGGCAATGAGGTCCAC 3'	

Abbreviations: Acetylcholinesterase (AChE), Interferon- γ (IFN-γ), glutathione peroxidase (GPx), nuclear factor- κB (NF-κB), superoxide dismutase (SOD), Tumor necrosis factor-α (TNF-α), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Legend of Figures

Fig. 1 Chemical structure of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP)

Fig. 2 Scheme of experimental protocol. On day 1, mice were submitted to the Von-Frey test (VFT). On day 2, thirty min after intragastric (i.g.) treatments, mice received beta amyloid (Aβ) or vehicle (saline), intracerebroventricularly (i.c.v.). The i.g. treatments were performed every day, until day 15 of the experimental protocol. The open-field test (OFT) was performed on day 6, the rota rod test (RRT) on days 7 and 8, VFT on day 8, the object recognition task (ORT) on days 9 and 10, and VFT on day 16. On day 16, after the VFT, the mice were sacrificed. *Abbreviation:* 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP)

Fig. 3 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) and/or beta amyloid (Aβ) on behavioral tests of mice. First trial (percentage of time spent exploring the novel object, test carried out 1.5 h after training) (3a) and second trial (percentage of time spent exploring the novel object, test carried out 24 h after training) (3b) of mice in the object recognition task (ORT). Paw withdrawal latency (grams) (3c) in the Von-Frey test (VFT). Data are reported as mean ± standard error of the mean (SEM) of eight-ten animals per group (one-way analysis of variance/ Tukey's test for ORT and two-way analysis of variance/Tukey's test for VFT). (**) $p < 0.01$, (****) $p < 0.0001$ as compared with the sham group. (#) $p < 0.05$, (##), $p < 0.01$, (###), $p < 0.001$, (####), $p < 0.0001$ as compared with the Aβ group. (&) $p < 0.0001$ as compared with the respective group on day 1

Fig. 4 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) on nuclear factor-κB (NF-κB). Relative expression of NF-κB mRNA on cerebral cortices (4a) and hippocampi (4b); NF-κBp65 levels (4c) and quantification of protein blots by densitometry (4d) on nuclear fraction of cerebral cortices and NF-κBp65 levels (4e) and quantification of protein blots by densitometry (4f) on nuclear fraction of hippocampi of mice after beta amyloid (Aβ) treatment. Data are reported as mean ± standard error of the mean (SEM) of three-eight animals per group (one-

way analysis of variance/Tukey's test). (**) $p < 0.01$, (***) $p < 0.001$ as compared with the sham group. (#), $p < 0.05$, (##), $p < 0.01$, (###), $p < 0.001$ as compared with the Aβ group

Fig. 5 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) on proinflammatory cytokines. Relative expression of tumor necrosis factor-α (TNF-α) mRNA in cerebral cortices (5a) and hippocampi (5b); TNF-α levels in cerebral cortices (5c) and hippocampi (5d); Relative expression of interferon-γ (IFN-γ) mRNA in cerebral cortices (5e) and hippocampi (5f); IFN-γ levels in cerebral cortices (5g) and hippocampi (5h); Interleukin-1β (IL-1β) levels in cerebral cortices (5i) and hippocampi (5j) of mice after beta amyloid (Aβ) treatment. Data are reported as mean ± standard error of the mean (SEM) of three-eight animals per group (one-way analysis of variance/Tukey's test). (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ (****) $p < 0.0001$ as compared with the sham group. (#), $p < 0.05$, (##), $p < 0.01$, (###), $p < 0.001$, (####), $p < 0.0001$ as compared with the Aβ group

Fig. 6 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) on oxidant levels in cerebral cortices (6a) and hippocampi (6b); thiobarbituric acid reactive substances (TBARS) levels in cerebral cortices (6c) and hippocampi (6d) of mice after beta amyloid (Aβ) treatment. Data are reported as mean ± standard error of the mean (SEM) of six-nine animals per group (one-way analysis of variance/Tukey's test). (*) $p < 0.05$ as compared with the sham group. (#), $p < 0.05$, (##) $p < 0.01$ as compared with the Aβ group

Fig. 7 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) on antioxidant defenses. Nuclear factor erythroid 2-related factor 2 (Nrf2) levels (7a) and quantification of protein blots by densitometry (7b) on nuclear fraction of hippocampi; Relative expression of superoxide dismutase (SOD) mRNA on hippocampi (7c); Relative expression of glutathione peroxidase (GPx) mRNA on hippocampi (7d); Heme oxygenase-1 (HO-1) content (7e) and quantification of protein blots by densitometry (7f) on cytoplasmic fraction of hippocampi; Peroxiredoxin1 (Prdx1) content (7g) and quantification of protein blots by densitometry (7h) on cytoplasmic

fraction of hippocampi of mice after beta amyloid (Aβ) injection. Data are reported as mean ± standard error of the mean (SEM) of three-eight animals per group (one-way analysis of variance/Tukey's test). (**) $p < 0.01$, (****) $p < 0.0001$ as compared with the sham group. (#) $p < 0.05$, (##) $p < 0.01$, (###) $p < 0.001$, (####) $p < 0.0001$ as compared with the Aβ group

Fig. 8 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) on relative expression of acetylcholinesterase (AChE) mRNA on cerebral cortices (8a) and hippocampi (8b) of mice after beta amyloid (Aβ) injection. Data are reported as mean ± standard error of the mean (SEM) of eight animals per group (one-way analysis of variance/Tukey's test). (*) $p < 0.05$ as compared with the sham group. (#) $p < 0.05$, (####) $p < 0.0001$ as compared with the Aβ group

Figures

Fig. 1

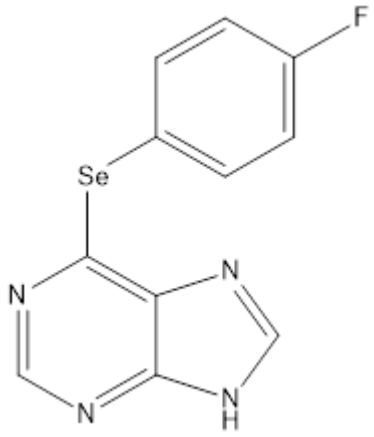


Fig. 2

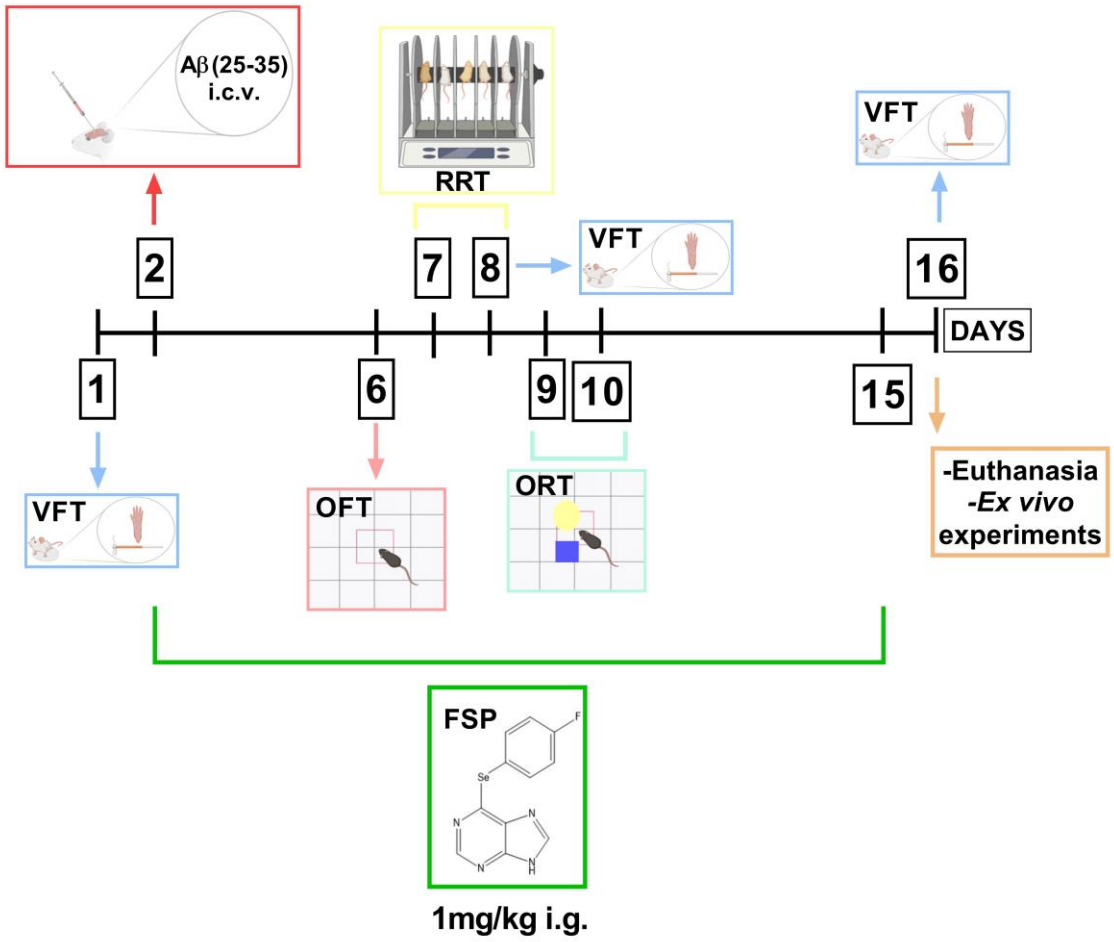

Aβ (25-35)
i.c.v.
RRT
VFT
VFT
2
7
8
16
DAYS
1
6
9
10
15
VFT
OFT
ORT
-Euthanasia
-Ex vivo
experiments
FSP
1mg/kg i.g.

Fig. 3

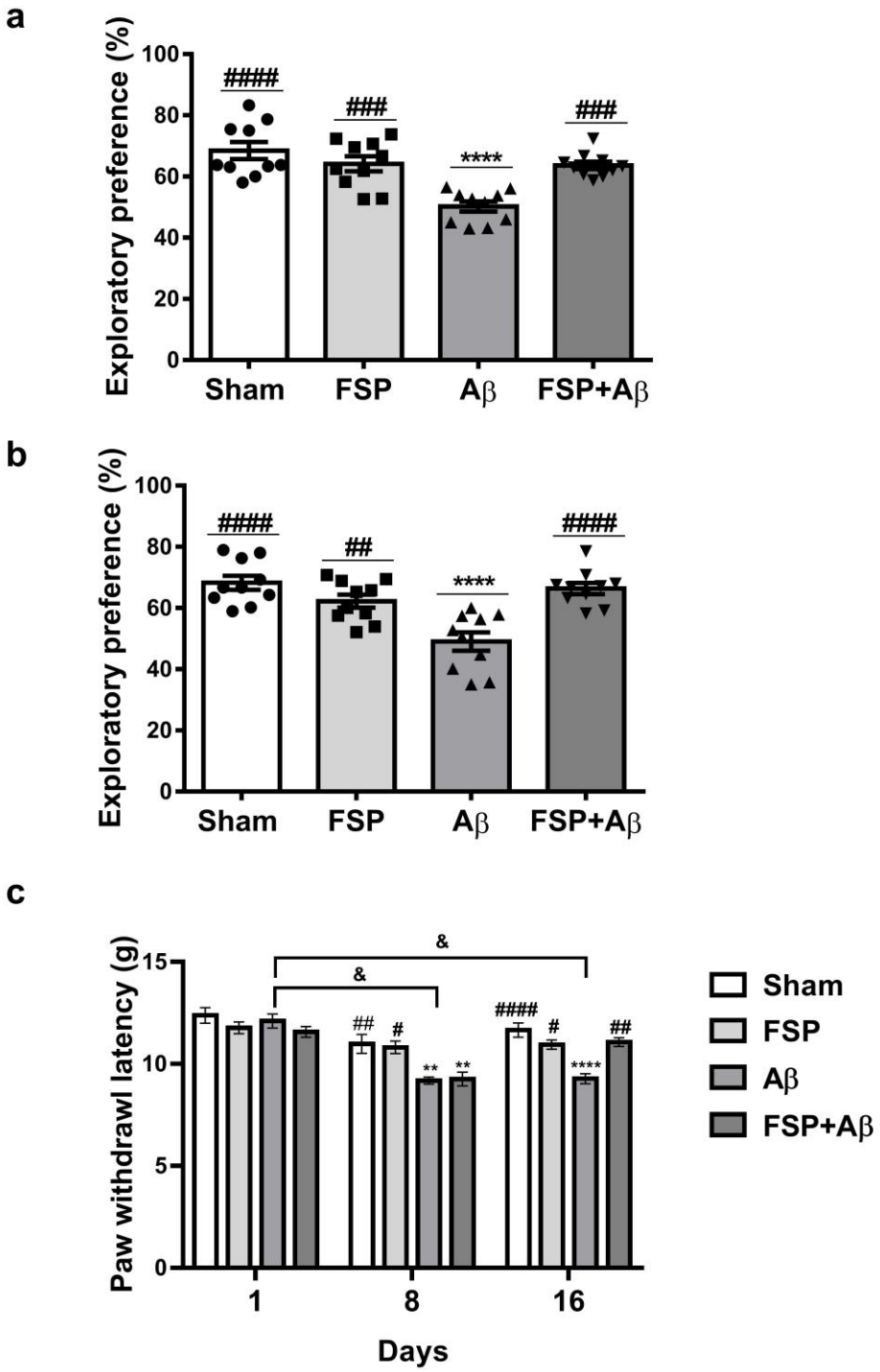

a
Exploratory preference (%)
100
80
60
40
20
0
####
###
****
###
Sham
FSP
Aβ
FSP+Aβ
b
Exploratory preference (%)
100
80
60
40
20
0
####
##
****
####
Sham
FSP
Aβ
FSP+Aβ
c
Paw withdrawl latency (g)
15
10
5
0
&
&
##
#
**
**
####
#
****
##
1
8
16
Days
Sham
FSP
Aβ
FSP+Aβ

Fig. 4

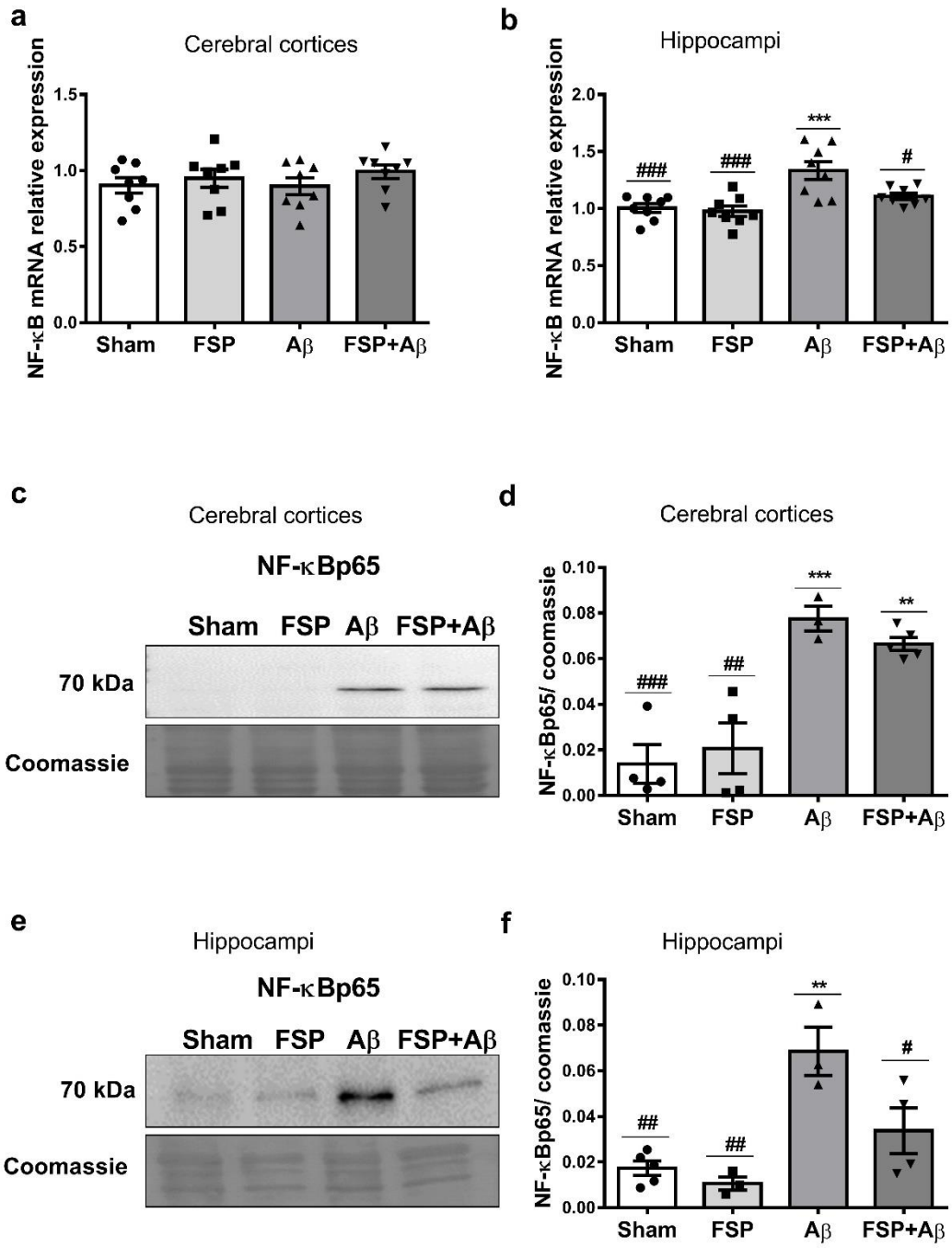

a
Cerebral cortices
NF-κB mRNA relative expression
1.5
1.0
0.5
0.0
Sham
FSP
Aβ
FSP+Aβ
b
Hippocampi
NF-κB mRNA relative expression
2.0
1.5
1.0
0.5
0.0
###
###
***
#
Sham
FSP
Aβ
FSP+Aβ
c
Cerebral cortices
NF-κBp65
Sham FSP Aβ FSP+Aβ
70 kDa
Coomassie
d
Cerebral cortices
NF-κBp65/ coomassie
0.10
0.08
0.06
0.04
0.02
0.00
###
##
***
**
Sham
FSP
Aβ
FSP+Aβ
e
Hippocampi
NF-κBp65
Sham FSP Aβ FSP+Aβ
70 kDa
Coomassie
f
Hippocampi
NF-κBp65/ coomassie
0.10
0.08
0.06
0.04
0.02
0.00
##
##
**
#
Sham
FSP
Aβ
FSP+Aβ

Fig. 5

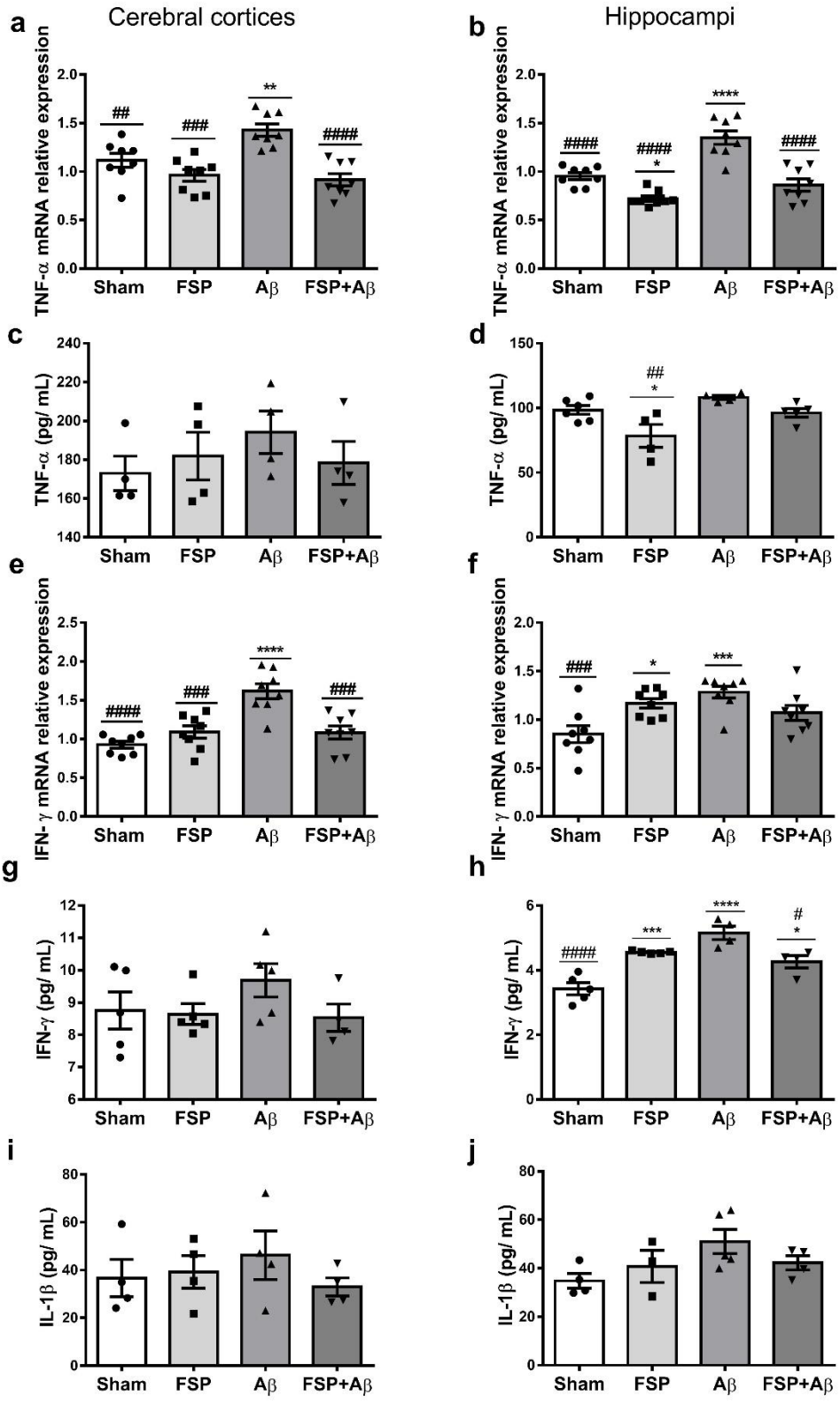

a
Cerebral cortices
b
Hippocampi
TNF-α mRNA relative expression
TNF-α (pg/ mL)
IFN-γ mRNA relative expression
IFN-γ (pg/ mL)
IL-1β (pg/ mL)
Sham
FSP
Aβ
FSP+Aβ
c
d
e
f
g
h
i
j

Fig. 6

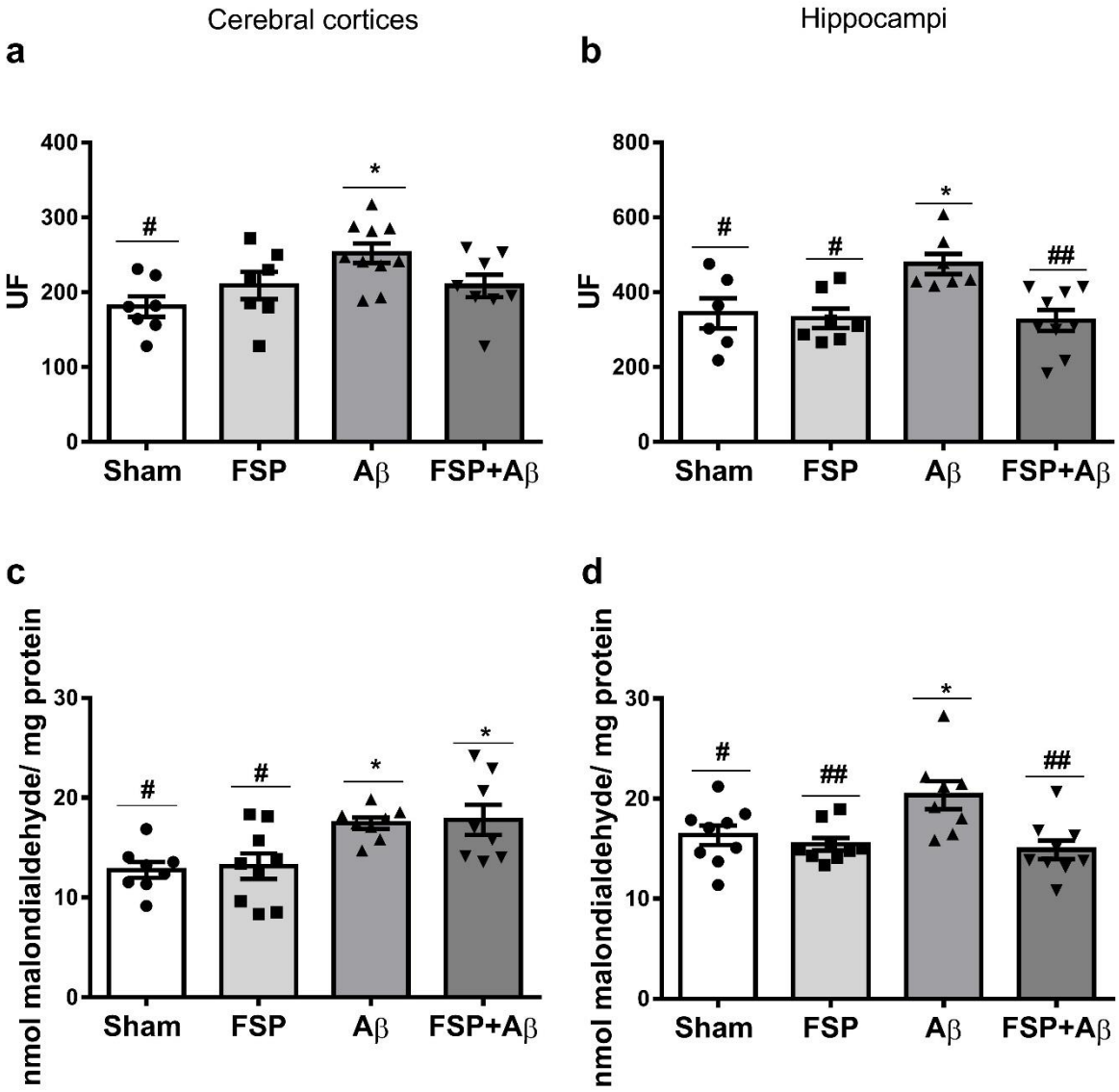
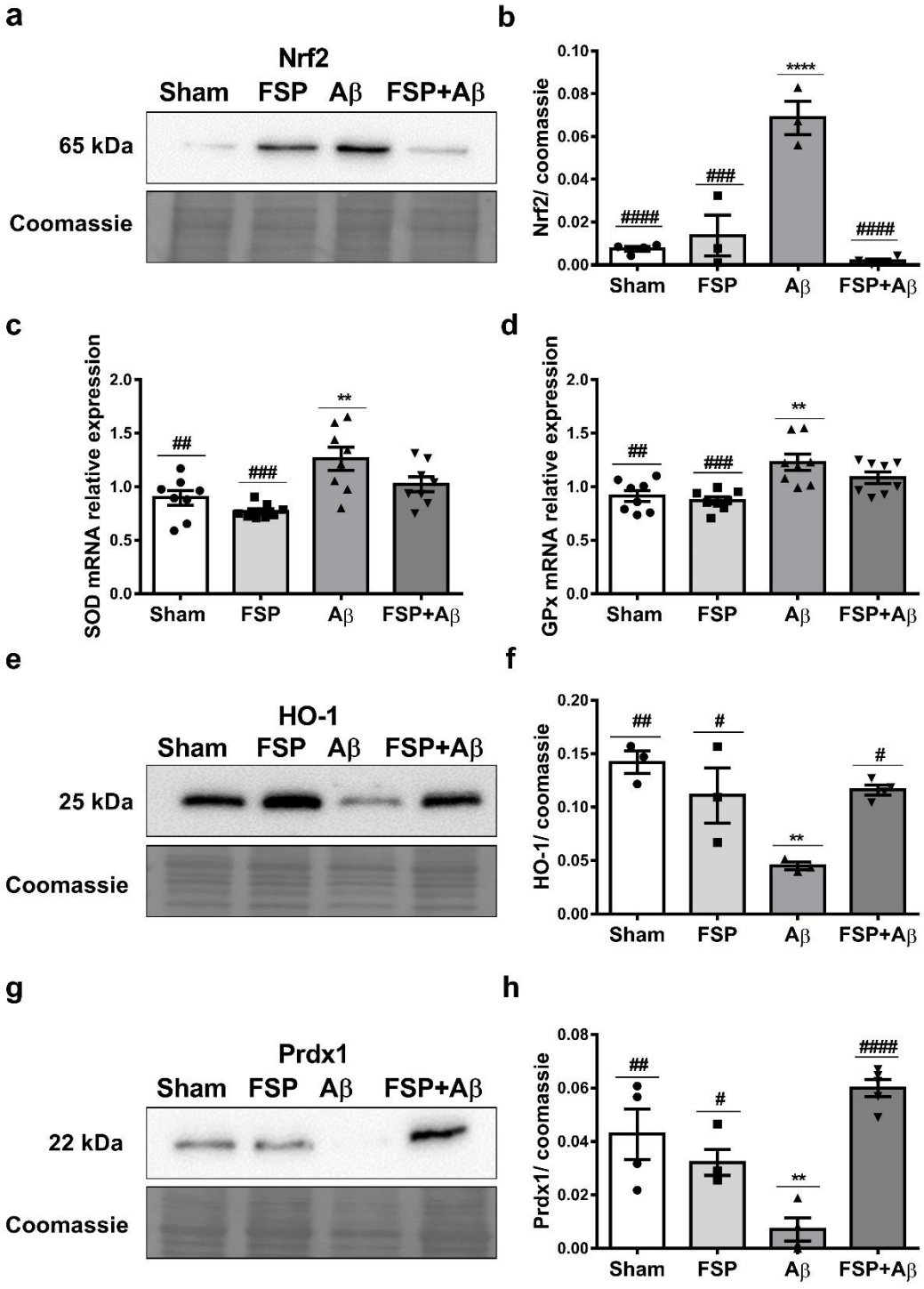

Cerebral cortices
Hippocampi
a
b
c
d
UF
nmol malondialdehyde/ mg protein
Sham
FSP
Aβ
FSP+Aβ
#
##
*

Fig. 7


a
Nrf2
Sham FSP Aβ FSP+Aβ
65 kDa
Coomassie
b
Nrf2/ coomassie
0.10
0.08
0.06
0.04
0.02
0.00
****
###
####
####
Sham FSP Aβ FSP+Aβ
c
SOD mRNA relative expression
2.0
1.5
1.0
0.5
0.0
##
###
**
Sham FSP Aβ FSP+Aβ
d
GPx mRNA relative expression
2.0
1.5
1.0
0.5
0.0
##
###
**
Sham FSP Aβ FSP+Aβ
e
HO-1
Sham FSP Aβ FSP+Aβ
25 kDa
Coomassie
f
HO-1/ coomassie
0.20
0.15
0.10
0.05
0.00
##
#
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#
Sham FSP Aβ FSP+Aβ
g
Prdx1
Sham FSP Aβ FSP+Aβ
22 kDa
Coomassie
h
Prdx1/ coomassie
0.08
0.06
0.04
0.02
0.00
##
#
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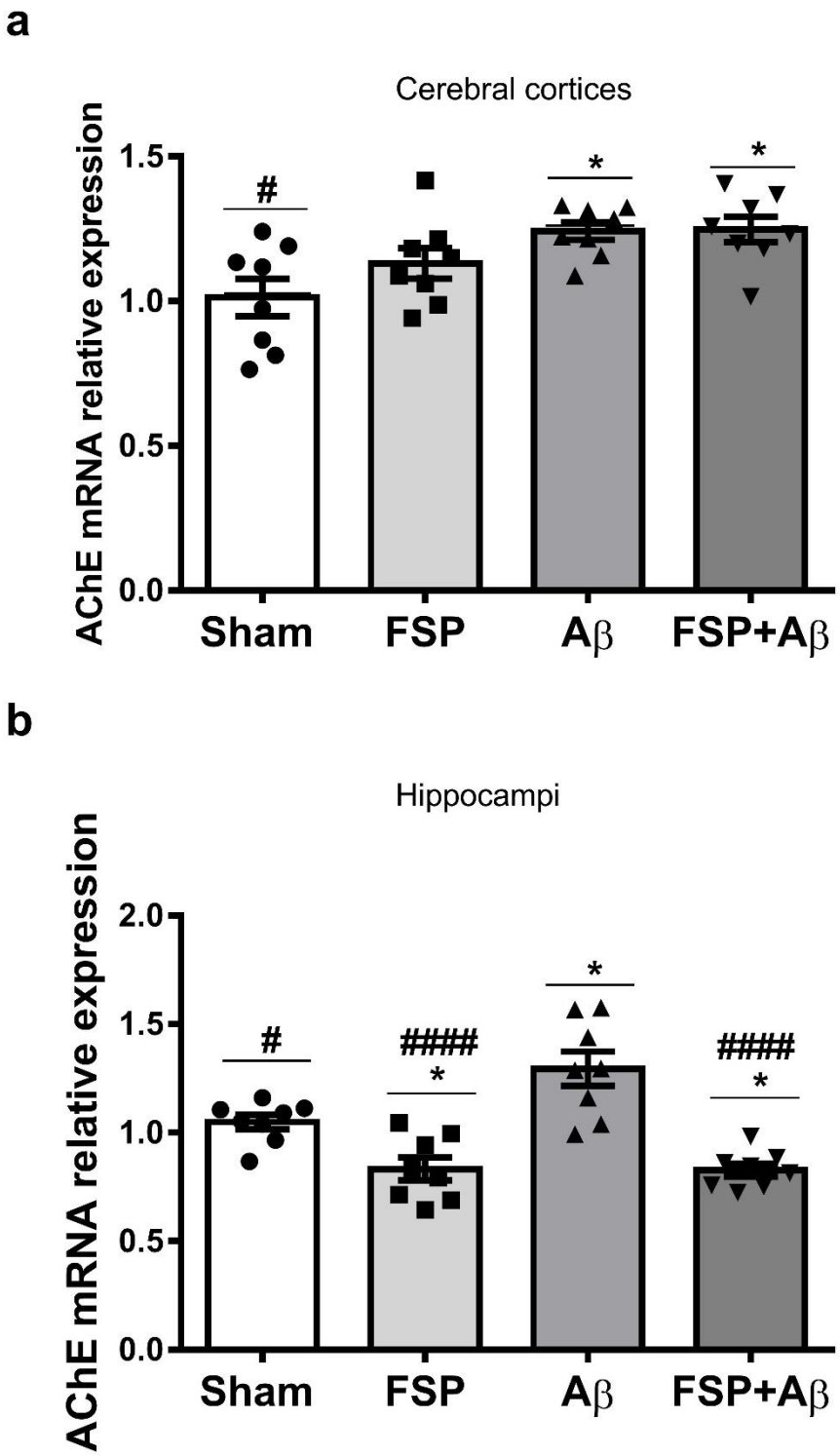
####
Sham FSP Aβ FSP+Aβ

Fig. 8


a
Cerebral cortices
AChE mRNA relative expression
1.5
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0.5
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Sham
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Aβ
FSP+Aβ
b
Hippocampi
AChE mRNA relative expression
2.0
1.5
1.0
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FSP+Aβ

Supplementary material

Fig. S1

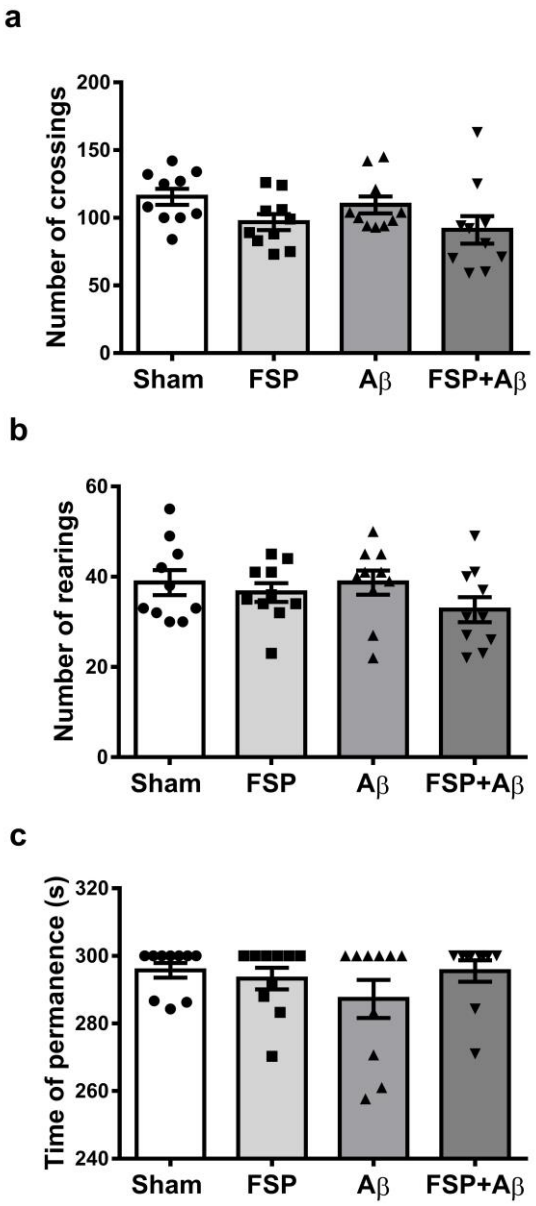

Fig. S1 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) and/or beta amyloid (Aβ) on locomotor, exploratory and motor activities of mice. Number of crossings (S1a) and rearings (S1b) in the open field test (OFT); time of permanence in seconds (s) (S1c) in the rota rod test (RRT). Data are reported as mean ± standard error of the mean (SEM) of ten animals per group (one-way analysis of Tukey's test).

Fig. S2

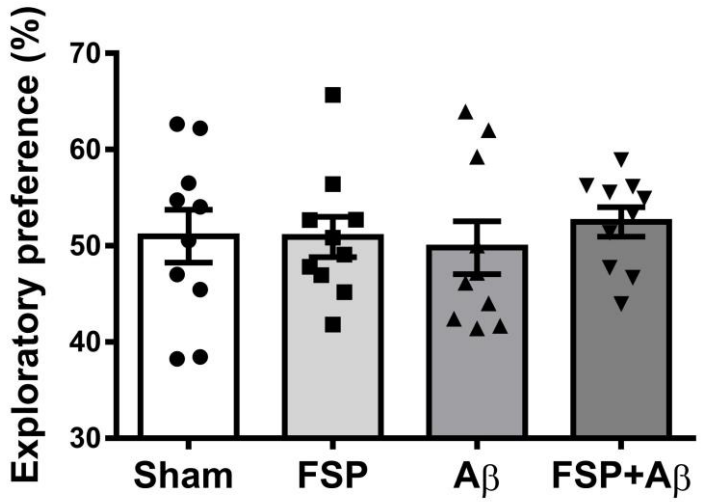

Fig. S2 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) and/or beta amyloid (Aβ) on exploratory preference (%) of mice in the training phase of the object recognition task (ORT). Data are reported as mean ± standard error of the mean (SEM) of ten animals per group (one-way analysis of Tukey's test).

Fig. S3

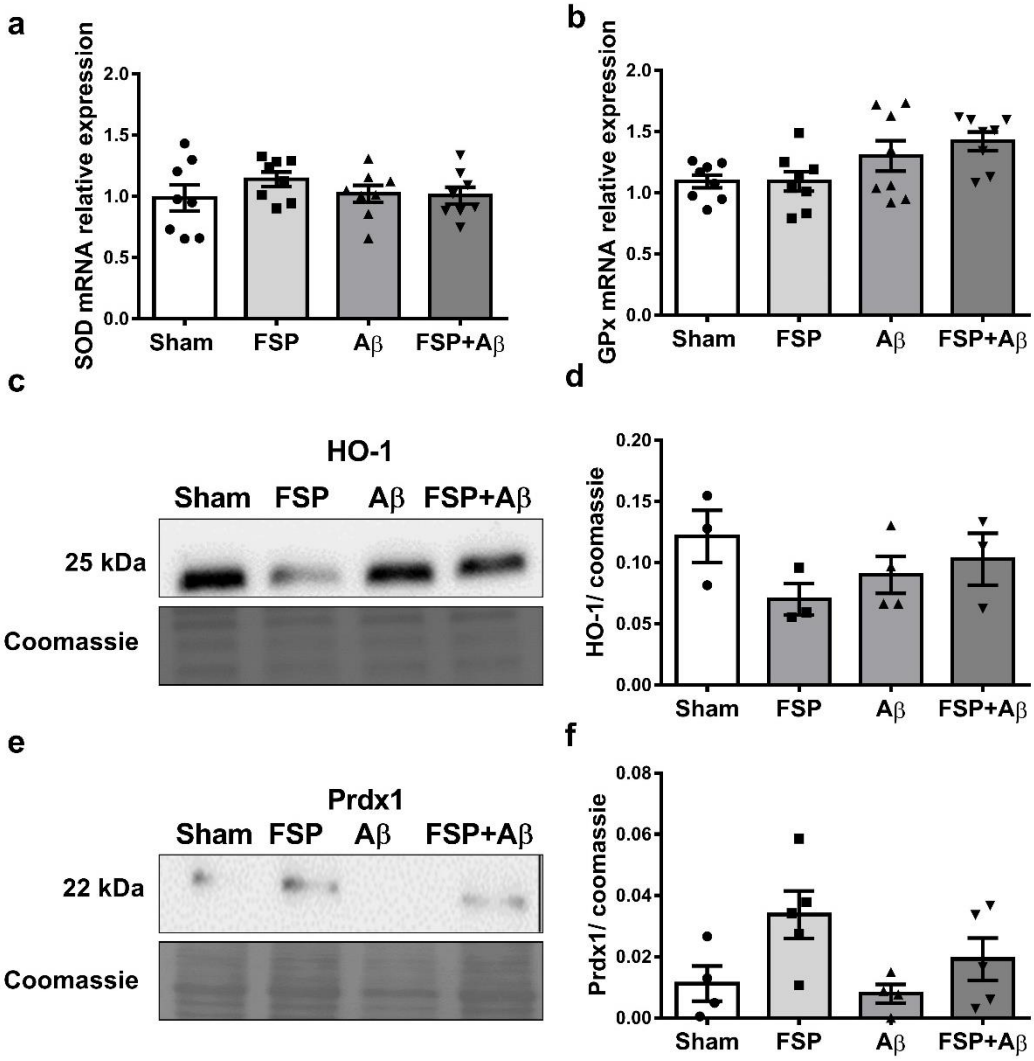

Fig. S3 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) on antioxidant defenses. Relative expression of mRNA superoxide dismutase (SOD) (S3a); Relative expression of mRNA (glutathione peroxidase) GPx (S3b); Heme oxygenase-1 (HO-1) protein content (S3c) and quantification of protein blots by densitometry (S3d) on cytoplasmic fraction; Peroxiredoxin-1 (Prdx1) protein content (S3e) and quantification of protein blots by densitometry (S3f) on cytoplasmic fraction of the cerebral cortices of mice after beta amyloid (Aβ) treatment. Data are reported as mean ± standard error of the mean (SEM) of three-eight animals per group (one-way analysis of variance/Tukey's test).

Fig. S4

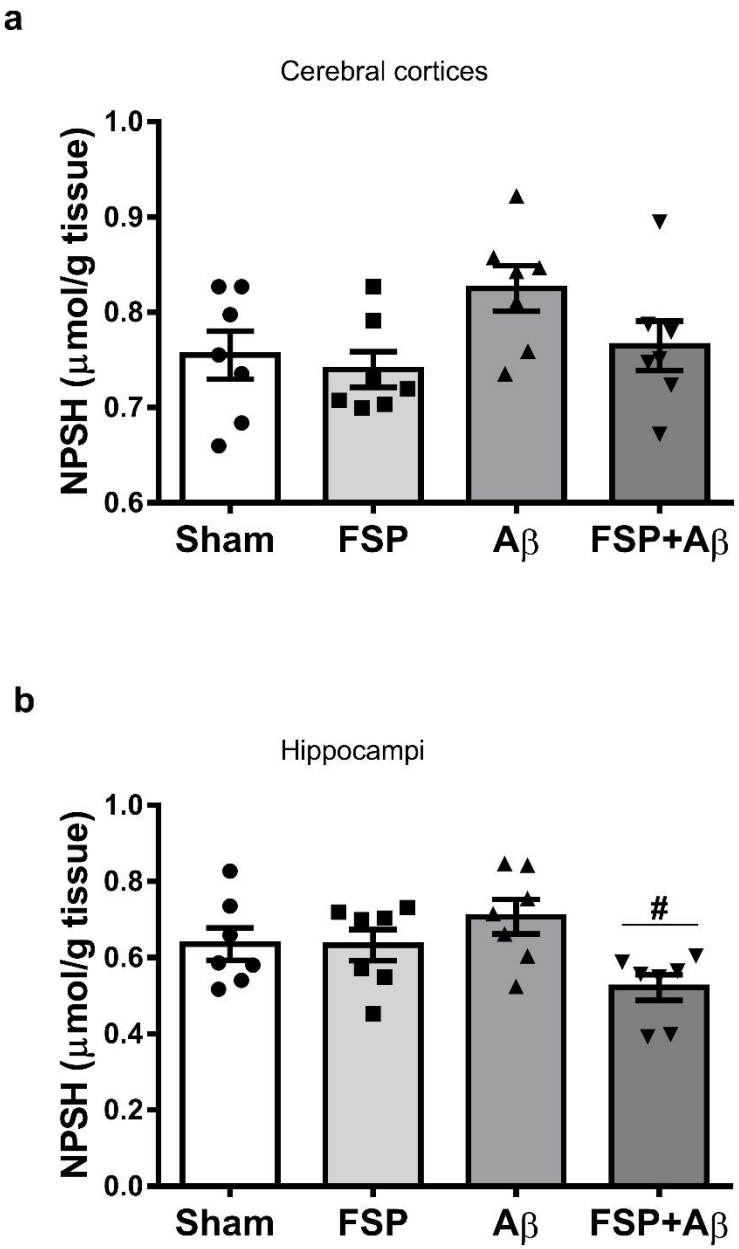

Fig. S4 Effect of 6-((4-fluorophenyl) selanyl)-9*H*-purine (FSP) on Non protein thiol (NPSH) levels in cerebral cortices (S4a) and hippocampi (S4b) of mice after beta amyloid (Aβ) treatment. Data are reported as mean ± standard error of the mean (SEM) of seven animals per group (one-way analysis of variance/Tukey's test). (#), $p < 0.05$ as compared with the Aβ group.

5. DISCUSSÃO

Atualmente, nenhum dos tratamentos farmacológicos disponíveis para a DA diminui ou interrompe o dano e a destruição dos neurônios que causam os sintomas e tornam esta doença fatal. Ao longo de décadas muitos esforços foram feitos para encontrar uma terapia mais eficaz para a DA, contudo os resultados observados nos testes clínicos normalmente são desanimadores (Bachurin, Bovina e Ustyugov, 2017). Outro agravante é que não existem tratamentos específicos aprovados pela FDA para os sintomas comportamentais e psiquiátricos que podem se desenvolver nos estágios moderados e graves da DA (Alzheimer's association report, 2020). Além disso, os medicamentos utilizados, como por exemplo alguns antipsicóticos, estão associados a um risco aumentado de acidente vascular cerebral e morte em indivíduos com demência (Ralph e Espinet, 2018).

Diante disso, pesquisas que buscam por alternativas terapêuticas para o tratamento da DA e suas comorbidades são essenciais. Baseado nisso, em um estudo anterior desenvolvido por um grupo parceiro e pelo nosso grupo de pesquisa, realizou-se a síntese de uma classe de compostos derivados de purina contendo um grupo organosselênio (6-arilselanilpurinas). O FSP foi o melhor composto dentre esta classe em inibir a atividade da enzima AChE *in vitro.* Outro achado relevante foi que o FSP teve efeito em melhorar as fases de consolidação e recuperação da memória, e também inibiu a atividade da AChE *ex vivo* no córtex cerebral de camundongos. (Duarte *et al.*, 2017). Nesse sentido, a proposta desta tese foi avaliar o possível efeito protetor do FSP na disfunção cognitiva, declínio de memória, ansiedade e aumento de sensibilidade à nocicepção em modelos de DA em camundongos.

Inicialmente, por meio de um estudo *in sílico* procurou-se compreender de que maneira o composto exercia sua ação anticolinesterásica. Verificou-se que o FSP interagiu com resíduos cruciais para a inibição da AChE, como os resíduos do sítio ativo (Trp86, Gly122, Phe338 His447 e Gly121) e também interagiu com os resíduos do sítio periférico, o PAS (Tyr124), um sítio de deposição de βA na AChE (Ferrari, De *et al.*, 2001; Gupta e Mohan, 2014; Hou *et al.*, 2014), reforçando o potencial anticolinesterásico do FSP (**Artigo**). Além do mais, este trabalho reforçou a importância do átomo de selênio para as ações terapêuticas deste composto. No presente estudo foi demonstrado que, quando o átomo de selênio foi substituído por

um átomo de enxofre, que também é um calcogênio mas com algumas propriedades diferentes, ocorreu uma modificação e redução da afinidade de ligação do composto pela AChE (**Artigo**). Com isso, pode-se evidenciar que o grupo orgânico de selênio na estrutura do composto é essencial para as sua ação.

No **Artigo** também foi verificado que os camundongos do grupo STZ apresentaram déficit na memória de trabalho, na memória aversiva e não espacial de longo prazo e memórias de curto e longo prazo. De fato, é bem descrito que a STZ mimetiza a DA em camundongos (Fronza *et al.*, 2019; Grieb, 2016; Martini *et al.*, 2019). O composto FSP foi eficaz em proteger todos os tipos de memória avaliados. Estes resultados estão de acordo com os encontrados na literatura, que reportam o efeito farmacológico de compostos orgânicos de selênio em modelos de DA induzida por STZ (Martini *et al.*, 2019; Thomé *et al.*, 2018).

Outro achado de grande relevância observado neste trabalho foi que a STZ causou um comportamento semelhante à ansiedade nos animais, conforme observado também por Pinton e colaboradores (2011). Estes resultados sugerem que a ansiedade está presente em uma fase inicial da doença. Além disso, um estudo de Johansson e colaboradores (2019) provou que existem associações entre a apatia e a ansiedade com o acúmulo de βA cerebral e declínio cognitivo longitudinal, e esses sintomas neuropsiquiátricos podem ser considerados como manifestações clínicas iniciais de DA. Nesse sentido, a ansiedade poderia ser utilizada como uma manifestação clínica precoce presente em estágios pré-clínicos ou prodrômicos da DA (Carrillo *et al.*, 2013). Surpreendentemente, o FSP apresentou, pela primeira vez, um efeito do tipo-ansiolítico em um modelo de DA em camundongos.

A fim de elucidar quais os mecanismos de ação estão envolvidos nos efeitos exercidos pelo FSP, alguns dos principais alvos que são comprometidos nesta patologia foram estudados. Primeiramente, investigou-se o envolvimento da via colinérgica, e identificou-se que o FSP atenuou o aumento na atividade da enzima AChE, bem como reduziu os níveis de RNAm desta enzima. De fato, a AChE é o principal alvo terapêutico utilizado atualmente para o tratamento da DA. Ademais, o FSP já havia demostrado ação anticolinesterásica *in vitro* e *ex vivo* (Duarte *et al.*, 2017), sendo assim, o presente estudo reforçou esta ação do FSP em um modelo de DA. Além disso, o FSP proporcionou um aumento nos níveis de RNAm da enzima ChAT (responsável pela síntese de ACh (Oda, 1999)), que foi reduzida após a injeção

de STZ. Com esses achados, pode-se inferir que o FSP estaria promovendo um aumento nos níveis do neurotransmissor ACh, e desta maneira contribuindo para a melhora da memória e do aprendizado.

Adicionalmente, os resultados do presente estudo revelaram que camundongos expostos a STZ obtiveram uma redução nos níveis de expressão gênica e na atividade da Na^+/K^+-ATPase. Esta é uma importante enzima transmembrana responsável pela homeostase iônica celular (Takeuchi *et al.*, 2008). Inclusive, Moseley e colaboradores (2007) revelaram que a Na^+/K^+-ATPase modula o comportamento semelhante à ansiedade, a memória e aprendizagem espacial em ratos. Sendo assim, o prejuízo causado pela STZ na Na^+/K^+-ATPase pode ser mais um dos fatores que contribuíram para as alterações comportamentais observadas. O efeito protetor do FSP foi mais uma vez confirmado, por meio da prevenção dos danos na atividade enzimática e expressão gênica de Na^+/K^+-ATPase.

Anteriormente foi reportado que alterações nas enzimas AChE e Na^+/K^+-ATPase resultam em comprometimento de memória (Pacheco *et al.*, 2018; Zhang *et al.*, 2013). Sendo assim, o efeito do FSP em modular estas importantes enzimas provavelmente possibilitou uma melhora no comprometimento da memória e ansiedade dos animais.

Além disso, investigou-se as proteínas Akts. Estas proteínas são uma família de serina/treonina quinases que consistem em três isoformas: Akt1, Akt2 e Akt3, que exibem diferentes padrões de expressão dependendo da região do cérebro e do tipo de célula envolvidos (Levenga *et al.*, 2017). A Akt regula a sobrevivência celular, a proliferação, a organização do citoesqueleto, o metabolismo celular, o tráfego de vesículas e o transporte de glicose (Noguchi e Suizu, 2012). A redução nos níveis de Akts favorece a deposição e agregação de βA através da via da glicogênio sintase quinase 3 (Phiel *et al.*, 2003). No presente estudo, não foram observadas alterações estatisticamente significativas nos níveis de expressão gênica das Akts após as injeções de STZ. Apesar disso, pode-se notar uma tendência na redução dos níveis de expressão gênica das isoformas no hipocampo dos animais induzidos com STZ. Nesse sentido, não pode-se descartar o envolvimento dessa via nos eventos que corroboram com os danos causados pela STZ.

Como trata-se de um novo composto com atividades biológicas promissoras também realizou-se uma avaliação preliminar de toxicidade (**Artigo**). Com relação aos sinais de toxicidade hepática e renal, o FSP não alterou os parâmetros bioquímicos do plasma e os marcadores oxidativos hepáticos. Os tratamentos disponíveis atualmente para a DA podem causar hepatotoxicidade (Marucci *et al.*, 2020). Este é um problema que quando pensa-se em pacientes idosos é bastante grave, pois muitas vezes a função hepática já está comprometida (Mitchell e Hilmer, 2010). Além do mais, normalmente ocorre a polimedicação, corroborando ainda mais para o dano hepático. Sendo assim, o FSP poderia ser uma alternativa terpêutica que forneceria uma maior segurança em relação a estes aspectos.

Estudos *in vivo* e *in vitro* reportam que a AChE, considerada um componente de placa senil, pode promover a formação de fibrila amiloide e formar complexos βA-AChE altamente tóxicos, que apresentam um efeito neurotóxico maior do que o ocasionado pelo peptídeo βA sozinho (Inestrosa, Urra e Colombres, 2004). Como verificou-se no **Artigo,** por meio de um estudo *in sílico,* o FSP interagiu com os aminoácidos presentes na região PAS da enzima AChE, onde o βA se deposita, e com isso buscou-se expandir a investigação dos efeitos farmacológicos do FSP em um modelo de DA induzido pelo βA em camundongos. Acredita-se que, se o FSP se ligar a esta região poderia promover um efeito protetor por reduzir os danos causados pelo βA. De fato, verificou-se no **Manuscrito** que o FSP atenuou a perda de memória de curto prazo e memória de longo prazo induzidas pelo βA (25-35) em camundongos. Essas descobertas reforçam os resultados anteriores encontrados no **Artigo** e indicam que o FSP é uma alternativa terapêutica promissora para a DA.

É bem descrita a correlação entre dor e DA pois cerca de 46% dos pacientes com DA apresentam algum tipo de dor crônica (Kooten *et al.*, 2016), entretanto um vínculo mecanístico consolidado precisa ser esclarecido. Crescentes evidências demonstram que pacientes com DA e/ou hipersensibilidade à dor compartilham algumas patologias em comum, como a ativação da microglia em áreas do cérebro e aumento da neuroinflamação (Salter e Stevens, 2017). Contudo, o efeito do βA (fragmento 25-35) na hipernocicepção, até onde se sabe, não foi explorado. Neste trabalho (**Manuscrito**), observou-se que os animais que foram induzidos com o βA apresentaram um aumento da nocicepção, reforçando uma importante relação da DA

com a dor. A DA abrange principalmente a população idosa nas quais, normalmente, a dor pode ser ocasionada por outros fatores como por exemplo, diabetes, artrite e fibromialgia (Bicket e Mao, 2015). Neste sentido, o achado do presente estudo é de suma importância, pois revela que os prejuízos ocasionados pela patologia amiloide podem desencadear quadros de dor. Isto implica diretamente na importância de um manejo clínico mais adequado para o tratamento da dor em pacientes com DA. Quando sente dor, uma pessoa com demência pode responder com agitação, agressão e resistência ao cuidado (Sampson *et al.*, 2015). É importante ressaltar que o presente estudo demonstrou que o FSP apresentou efeito em reduzir a hipernocicepção de camundongos no modelo de DA induzido pelo βA. Este é um resultado relevante pois aumenta a adesão dos pacientes ao tratamento, proporcionando a possibilidade de administração de um medicamento para o tratamento da DA e sua comorbidade, a dor.

A neuroinflamação pode estar diretamente associada com as alterações comportamentais observadas nos pacientes com DA e inclusive é um dos principais fatores patológicos desta doença (Calsolaro e Edison, 2016; Cao *et al.*, 2019). Portanto, no presente estudo, primeiramente procurou-se investigar o mecanismo de ação do FSP (**Manuscrito**) observando o seu efeito sob o principal fator de transcrição responsável pela produção de mediadores inflamatórios, o NFκB (Frakes *et al.*, 2014). Foi verificado um aumento na expressão de NF-κB no hipocampo e córtex cerebral de camundongos expostos ao βA (25-35), corroborando com o que é observado em pacientes com DA (Snow e Albensi, 2016). Compostos orgânicos derivados de selênio são conhecidos por apresentarem efeito anti-inflamatório (Casaril *et al.*, 2017; Pinz *et al.*, 2016). O FSP protegeu contra o aumento da expressão de NF-κB no hipocampo dos camundongos. Além disso, este efeito foi acompanhado por uma redução na expressão das citocinas TNF-α e IFN-γ em camundongos expostos ao βA.

Ainda, os sintomas inflamatórios e neuropáticos relacionados à dor podem ser reduzidos por agonistas seletivos do receptor nicotínico α7 de ACh presente na microglia (Abbas *et al.*, 2019; Loram *et al.*, 2012; Rowley *et al.*, 2010). Sendo assim, a melhora na função colinérgica por meio da inibição da atividade e redução da expressão gênica da AChE, bem como um aumento na expressão da ChAT pelo FSP observado no **Artigo,** poderia ser um mecanismo putativo para o efeito anti-

hipernociceptivo do composto. No **Manuscrito** reforçamos estes efeitos, o FSP protegeu contra o aumento na expressão da AChE causado por βA no hipocampo dos camundongos. Esses resultados sugerem que o FSP pode melhorar o comprometimento da memória e diminuir a nocicepção ao restabelecer os níveis de ACh. Adicionalmente, pode-se averiguar que o FSP reduziu por si só a expressão de RNAm da AChE e a expressão de RNAm e níveis proteicos do TNF- α no hipocampo dos camundongos. Baseado nisso, pode-se inferir que a ação anticolinesterásica do FSP é importante para o seu efeito anti-inflamatório.

O aumento na produção de oxidantes e o dano oxidativo têm sido implicados na patogênese e progressão da DA (Butterfield e Halliwell, 2019; Martins *et al.*, 2018). Adicionalmente, existe uma relação entre a produção de oxidantes e o NF-κB na DA (Snow e Albensi, 2016). Neste contexto, no **Manuscrito** foi observado que o βA aumentou os níveis de oxidantes e a peroxidação lipídica no córtex cerebral e no hipocampo dos camundongos. O tratamento com FSP foi capaz de reduzir o aumento desses parâmetros nos hipocampos, mas não nos córtices cerebrais dos camundongos do grupo βA. Este efeito protetor do FSP poderia explicar, ao menos em parte, o resultado obtido no **Artigo,** onde ocorreu um aumento na atividade da enzima Na^+/K^+-ATPase no hipocampo dos animais que receberam o tratamento com o FSP. É bem descrito que a Na^+/K^+-ATPase, pode ter a sua atividade comprometida em casos de dano oxidativo (Kurella, Tyulina e Boldyrev, 1997).

O mecanismo antioxidante exato do FSP ainda permanece elusivo, mas é possível que seu mecanismo de ação ocorra de modo semelhante a outros compostos de orgânicos de selênio, como selenofuranosídeos, $[(PhSe)_2]$, 4-PSQ e Ebselen. Teoricamente, o FSP formaria um selenolato (RSe^{-1}) em um ambiente rico em tiol e, então, reagiria com oxidantes. (Hassan, Ibrahim e Rocha, 2009; Vogt *et al.*, 2018). De fato, observa-se uma redução nos níveis de NPSH no hipocampo de camundongos tratados com FSP e induzidos com βA. O consumo de NPSH corroborou a hipótese descrita acima. Contudo, com base nos dados obtidos na presente tese, não podemos afirmar se o FSP exerce um efeito antioxidante direto, ou se de alguma maneira modula as defesas antioxidantes e assim contribui para uma redução do dano oxidativo observado anteriormente.

Na tentativa de entender como o FSP modula a neuroinflamação e o dano oxidativo e para ampliar os mecanismos de ação do composto, o fator de transcrição Nrf2 e as defesas antioxidantes foram avaliados. Em condições oxidativas, o Nrf2 se transloca para o núcleo iniciando a transcrição de um conjunto de proteínas de defesa, como a γ-glutamilcisteína sintetase, GPx, SOD, HO-1 e Prx1 (Uruno, Yagishita e Yamamoto, 2015). O nosso estudo demonstrou que o βA ativou o Nrf2 no hipocampo de camundongos. Isso está de acordo com outros estudos, que demonstraram a ativação de Nrf2 no cérebro humano e em modelos experimentais de DA (Lastres-Becker *et al.*, 2014; Mota *et al.*, 2015).

A migração do Nrf2 para o núcleo pode ser um esforço para limitar a progressão da DA. O tratamento com FSP preveniu significativamente a ativação do Nrf2, isso poderia ser explicado pelo fato de os camundongos que foram tratados com FSP apresentam um insulto menor, proporcionando uma diminuição do gatilho dos mecanismos de proteção. A ativação de Nrf2 foi acompanhada por um aumento na expressão relativa de RNAm de SOD e GPx. Controversamente, os animais do grupo βA apresentaram uma redução na expressão de HO-1 e Prx1 nos hipocampos. Uma redução na expressão dessas enzimas pode contribuir para o estresse oxidativo e a neuroinflamação. Estudos futuros são necessários para entender melhor como o βA pode reduzir a expressão dessas enzimas. Contudo, vale ressaltar que, essas observações estão de acordo com Mota e colaboradores (2015) que verificaram um aumento nos níveis de Nrf2 em frações nucleares, uma diminuição significativa no RNAm de HO-1 e uma tendência para diminuição do RNAm de Prx-1 no cérebro de camundongos em um modelo transgênico de DA. Além disso, é importante destacar que no presente estudo demonstrou-se, pela primeira vez, que o FSP foi eficaz na proteção contra a diminuição na expressão de HO-1 e Prx1 causada pelo βA no hipocampo de camundongos. De fato, como a inflamação e o estresse oxidativo são considerados um fenômeno central na patogênese inicial da DA e em outras condições associadas à DA, alternativas terapêuticas que podem aumentar a defesa antioxidante e/ou anti-inflamatória têm sido recomendadas (Martins *et al.*, 2018).

Em conjunto, os resultados sugerem que o FSP (i) modula a função colinérgica, (ii) aumenta a atividade e expressão gênica da bomba de íons Na^+/K^+-ATPase, (iii) aumenta as defesas antioxidantes, (iv) diminui os níveis de oxidantes e peroxidação

lipídica e (v) diminui o processo neuroinflamatório, estabelecendo uma condição favorável que previne as alterações comportamentais. Além do mais, cabe ressaltar que o FSP não apresentou indícios de toxicidade nos testes preliminares. Nesse sentido, o FSP pode ser uma abordagem multifuncional para a DA, uma condição multifatorial. Uma das grandes vantagens do FSP em relação os tratamentos tradicionais para DA é a possibilidade de administração de um medicamento para o tratamento da DA e suas comorbidades, a ansiedade e a dor. Isso é importante, pois aumenta a adesão dos pacientes ao tratamento, levando-se em consideração que esta possibilidade facilita o manejo clínico dos portadores da DA.

6. CONCLUSÃO

Como conclusão, os resultados da presente tese demonstraram que o FSP orquestrou uma resposta protetora atuando por diferentes mecanismos (Figura 19), tais como disfunção colinérgica, dano oxidativo, desequilíbrio iônico e neuroinflamação.

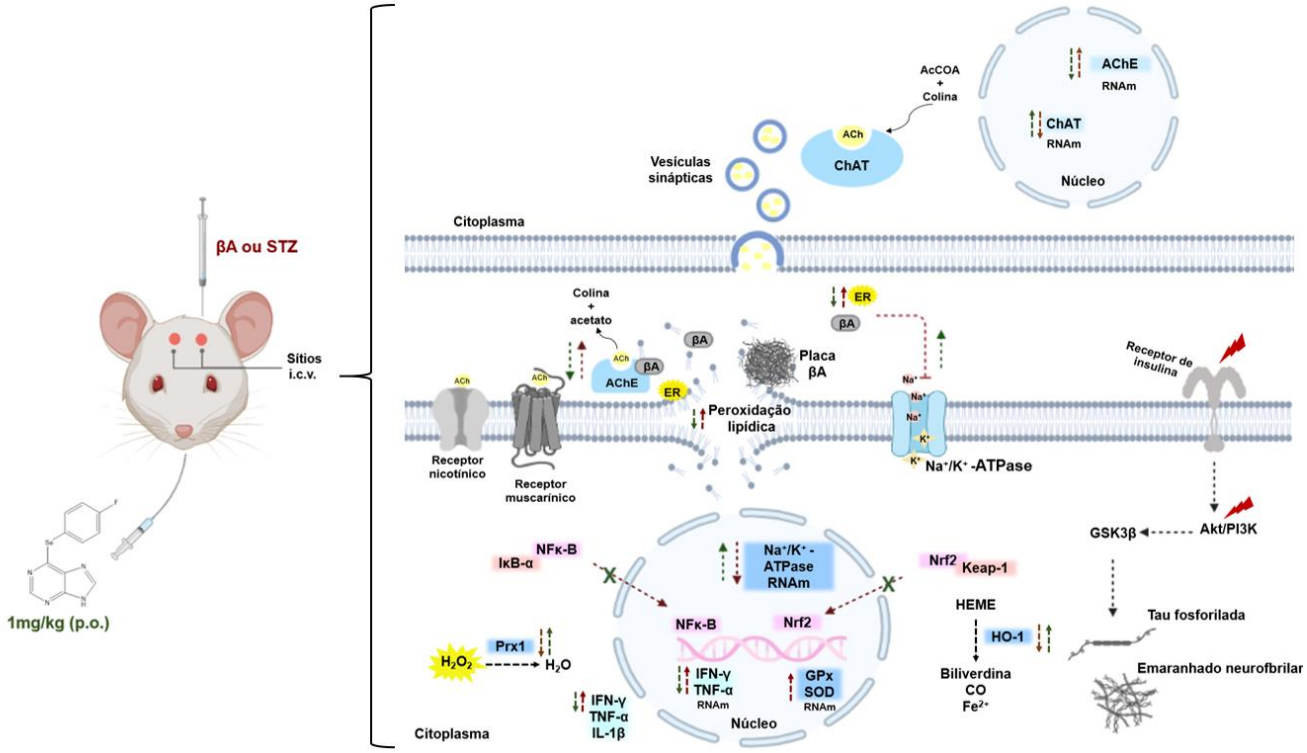

Figura 19. Resumo gráfico dos eventos ocasionados pelas induções com estreptozotocina (STZ) e/ou peptídeo beta amilóide (βA), e dos mecanismos de ação do 6-((4-fluorofenil) selanil)-9*H*-purina (FSP) em camunongos. Abreviações: Acetilcolina (ACh), Acetilcoenzima A (AcCOA), Acetilcolinesterase (AChE), ácido ribonucleico mensageiro (RNAm), colina acetiltransferase (ChAT), espécie reativa (ER), ferro (Fe^{2+}), glutationa peroxidase (GPx), glicogênio sintase quinase 3 beta (GSK3β), heme oxigenase (HO)-1, interleucina-1 beta (IL-1β), interferon- gama (IFN-γ), intracerebroventricular (i.c.v.), fator de necrose tumoral α (TNF-α), fator nuclear kappa B (NFκB*),* fosfoinositídeo 3-quinase (PI3K), Keap-1 *(Kelch-like ECH-associated protein 1*), monóxido de carbono (CO), IκB- α (*nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha)*, *per* oral (p.o.), peróxido de hidrogênio (H_2O_2), peroxirredoxina (Prx)1, Potássio (K^+), proteína quinase B (Akt),Sódio (Na^+), superóxido dismutase (SOD).

Fonte: Imagem do autor, 2021.

Estas ações do composto FSP puderam promover uma melhora na memória, no déficit cognitivo, na ansiedade, bem como, uma redução na hipernocicepção em modelos experimentais de DA em camundongos. De modo geral, embora mais investigações sejam necessárias, os resultados indicam que o FSP apresenta um grande potencial para atuar como uma estratégia terapêutica para a DA, e com

algumas vantagens em relação aos medicamentos atuais. Dentre estas, encontra-se a possibilidade de redução dos medicamentos administrados, uma vez que, o FSP apresentou efeitos em atenuar também as comorbidades (dor e ansiedade), favorecendo assim uma maior segurança para estes pacientes. Além disso, o FSP é capaz de atuar em diversos alvos patológicos da DA, uma característica que não é encontrada nos medicamentos usuais.

7. PERSPECTIVAS

As próximas etapas deste trabalho serão avaliar um possível efeito anti-amiloidogênico do FSP, investigar as possíveis ações do FSP na modulação da ativação da micróglia, bem como, expandir a compreensão dos mecanismos de dor na DA. E possibilitar o estudo das ações do FSP em testes pré-clínicos.

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ANEXOS

ANEXO A

Comprovante de submissão do manuscrito "A purine derivative containing organoselenium group protects against memory impairment, sensitivity to nociception, oxidative damage and neuroinflammation in a mouse model of Alzheimer's disease"

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Responder a: Molecular Neurobiology <jerseyanne.madrid@springernature.com>
Para: Mikaela Peglow Pinz <mikaelappinz@gmail.com>

Re: "A purine derivative containing organoselenium group protects against memory impairment, sensitivity to nociception, oxidative damage and neuroinflammation in a mouse model of Alzheimer's disease"
Full author list: Mikaela Peglow Pinz; Renata Leivas de Oliveira; Caren Aline Ramson da Fonseca; Guilherme Teixeira Voss; Beatriz Pereira da Silva; Luis Fernando Barbosa Duarte; William Borges Domingues; Hadassa Gabriela Ortiz; Flavia Carla Meotti; Diego Alves; Vinicius Farias Campos; Ethel Antunes Wilhelm; Cristiane Luchese

Dear MSc Mikaela Pinz,

We have received the submission entitled: "A purine derivative containing organoselenium group protects against memory impairment, sensitivity to nociception, oxidative damage and neuroinflammation in a mouse model of Alzheimer's disease" for possible publication in Molecular Neurobiology, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. Dr Cristiane Luchese who will be able to track the status of the paper through his/her login.

If you have any objections, please contact the editorial office as soon as possible. If we do not hear back from you, we will assume you agree with your co-authorship.

Thank you very much.

With kind regards,

Springer Journals Editorial Office
Molecular Neurobiology

ANEXO B

Carta de aprovação dos protocolos experimentais pelo Cômite de Ética em Experimentação Animal da Universidade Federal de Pelotas

Pelotas, 19 de junho de 2017

De: M.V. Dra. Anelize de Oliveira Campello Felix

Presidente da Comissão de Ética em Experimentação Animal (CEEA)

Para: Profa. Cristiane Luchese

Centro de Ciências Químicas, Farmacêuticas e de Alimentos

Senhora Professora:

Atendendo à sua solicitação, alteramos o título do projeto intitulado **Avaliação do efeito de compostos orgânicos sintéticos na memória e no déficit cognitivo em camundongos"** cadastrado na CEEA sob o número **1974-2016** para **"Avaliação da atividade anticolinesterásica de compostos derivados de purinas e quinolinas contendo selênio: implicações na memória e no déficit cognitivo em camundongos"**, permanecendo com mesmo número de cadastro e com as mesmas informações, que seguem:

Finalidade	(X) Pesquisa ( ) Ensino
Vigência da autorização	17/05/2016 a 01/04/2019
Espécie/linhagem/raça	*Mus musculus*/Swiss
N° de animais	2474
Idade	60 dias
Sexo	Machos
Origem	Biotério Central - UFPel

Solicitamos, após tomar ciência do parecer, reenviar o processo à *CEEA*.

M.V. Dra. Anelize de Oliveira Campello Felix

Presidente da CEEA

Ciente em: ____/____/2017

Assinatura do Professor Responsável: _____

ANEXO C

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Licensed Content Author	Mikaela Peglow Pinz et al
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Title	Efeitos farmacológicos de 6-((4-fluorofenil) selanil)-9H-purina, uma promissora alternativa terapêutica para a doença de Alzheimer e suas comorbidades em modelos experimentais
Institution name	Universidade Federal de Pelotas
Expected presentation date	Jul 2021
Requestor Location	Mr. Mikaela Pinz Rua gramado, n 320 Cristal, Rio grande do sul 96195-000 Brazil Attn: Mr. Mikaela Pinz
Total	0.00 USD

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