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BIOQUÍMICA

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INVESTIGAÇÃO DOS EFEITOS DE OLIGÔMEROS β-AMILOIDE NO METABOLISMO ENERGÉTICO CEREBRAL E POTENCIAL EFEITO NEUROPROTETOR DA GUANOSINA EM CAMUNDONGOS.

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

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Porto Alegre, Setembro de 2020

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A minha mãe, que não teve oportunidade de estudar, mas plantou em seus filhos o desejo incessante de estudar e de mudar nossas vidas.

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The quality of strength lined with tenderness is an unbeatable combination, as are intelligence and necessity when unblunted by formal education.
(Maya Angelou, I Know Why the Caged Bird Sings)

RESUMO

A Doença de Alzheimer (DA) tem grande impacto na qualidade de vida das pessoas com a doença e na vida dos familiares por ser uma das doenças neurodegenerativas mais prevalentes, de curso crônico e com prejuízo cognitivo progressivo. Ainda não há tratamento capaz de impedir ou reverter a progressão da DA e a severidade dos sintomas vai aumentando com o tempo, causando importante perda cognitiva, funcional e comportamental nas fases mais avançadas. Nesses estágios mais críticos da doença, há grande necessidade de auxílio de outras pessoas para as atividades da vida diária, maior risco de acidentes e maior frequência de internações hospitalares.

Os oligômeros β-amiloide (AβOs) têm grande potencial tóxico e estão envolvidos no dano e morte neuronal em fase que precede a formação das placas amiloide, uma vez que os oligômeros se difundem facilmente pelo cérebro e são capazes de se ligar a receptores e a membranas lipídicas alterando suas funções. Além disso, estudos vem indicando que ocorre falha energética no cérebro de pessoas com DA e essa também pode ser uma das vias pelas quais a doença se inicia e progride. Nesse contexto, ocorre produção ineficiente de ATP e alterações mitocondriais que culminam em um ciclo de deterioração celular: aumento da fosforilação oxidativa para suprir a necessidade energética, com consequente formação de mais espécies reativas, e dano a moléculas e estruturas celulares que agravam intensamente a capacidade de produzir ATP. Por esse ciclo, ocorre disfunção sináptica, redução das espinhas dendríticas e morte neuronal.

Neste trabalho, utilizamos um modelo de DA, induzido com AβOs, e avaliamos a memória de curto prazo. Como estratégia terapêutica, utilizamos a Guanosina, um nucleosídeo purinérgico que interage com o sistema glutamatérgico, evitando a excitotoxicidade presente em situações patológicas. A hipótese deste estudo é verificar: i) a contribuição da administração in vivo (via injeção ICV) de oligômeros beta-amiloide nas alterações energéticas, por meio de avaliação mitocondrial e ii) se a administração in vivo (via gavagem) da Guanosina tem efeito neuroprotetor nesse contexto.

Os resultados mostram que os AβOs causam déficit de memória de curto prazo na tarefa de reconhecimento de objetos após 24 horas da administração e a GUO recupera o déficit causado pelos AβOs. Em 48h, os AβOs reduziram a captação e oxidação de glutamato em fatias de hipocampo, e reduziram as defesas antioxidantes, sem aumentar o dano oxidativo. Não houve alterações significativas de expressão de genes e de conteúdo de proteínas relacionadas a metabolismo bioenergético cerebral, dinâmica mitocondrial, e membrana sináptica. A GUO recuperou a captação de glutamato, sem alterar a oxidação e apresentou proteção antioxidante.

Na avaliação das funções mitocondriais na pré-sinapse (utilizando sinaptossoma e/ou mitocôndria sinaptossomal isolada), os AβOs reduziram a capacidade respiratória reserva e os níveis de ATP, aumentaram a produção de peróxido de hidrogênio, desregularam o tamponamento de Ca^{2+} e modificaram a morfologia mitocondrial. Enquanto isso, a GUO recuperou a homeostase do Ca^{2+} e reduziu a proporção de mitocôndrias danificadas. Assim, compreendemos que a GUO apresenta efeito protetor no metabolismo do hipocampo e na atividade mitocondrial, impedindo a deterioração induzida pelos AβOs em funções essenciais para o funcionamento da pré-sinapse hipocampal, incluindo processos de aprendizado e memória.

Palavras-chave: Doença de Alzheimer. Oligômeros β-amiloide. Mitocôndria pré-sináptica. Guanosina. Neuroproteção.

ABSTRACT

Alzheimer's disease (AD) has a significant impact on people's quality of life of with the disease and on the lives of family members as it is one of the most prevalent neurodegenerative diseases, with a chronic course and with progressive cognitive impairment. There is still no treatment able to prevent or reverse the AD's progression, and the severity of symptoms increases over time, causing a critical cognitive, functional, and behavioral loss in the most advanced stages. In these more critical phases of the disease, there is an increased demand for help from others for the daily living activities, increased risk of accidents, and higher frequency of hospital admissions.

Amyloid-β oligomers (AβOs) have high toxic potential and are involved in neuronal damage and death in the phase that precedes the formation of amyloid plaques since oligomers easily diffuse through the brain and can bind to receptors and lipid membranes, changing their functions. Also, studies have indicated that energy failure occurs in the brain of people with AD and this can also be one of the mechanisms through which the disease starts and progresses. In this context, there is inefficient production of ATP and mitochondrial changes that culminate in a cycle of cellular deterioration: increased phosphorylative activity to supply the energy need, with the consequent formation of more reactive species, and damage to molecules and cellular structures that aggravate more intensely the ability to produce ATP. Through this cycle, synaptic dysfunction, reduction of dendritic spines and neuronal death occur.

In this work, we used an AD model, induced with AβOs, and evaluated short-term memory. As a therapeutic strategy, we used Guanosine, a purinergic nucleoside that interacts with the glutamatergic system, avoiding the excitotoxicity present in pathological situations. The hypothesis in this study is to verify: i) the contribution of in vivo administration of β-amyloid oligomers (via ICV injection) in energy changes, through mitochondrial evaluation and ii) if the in vivo administration of Guanosine (via gavage) has a neuroprotective effect in this context. The results show that AβOs cause short-term memory deficits in the object recognition task in 24 h, and GUO recovers the deficit caused by AβOs. After 48 h AβOs reduced glutamate uptake and oxidation in hippocampus slices, and reduced antioxidant defenses, without increasing oxidative damage. There were no significant changes in gene expression, protein content to brain bioenergetic metabolism, mitochondrial dynamics, and synaptic membrane. GUO recovered glutamate uptake without changing oxidation and provided antioxidant protection.

In the assessment of mitochondrial functions in the pre-synapse (using synaptosome preparation or isolated mitochondria from synaptosome), AβOs reduced the spare respiratory capacity and ATP levels, increased hydrogen peroxide production, deregulated Ca^{2+} buffering, and led to changes in mitochondrial morphology. Meanwhile, GUO recovered Ca^{2+} homeostasis and reduced the ratio of damaged mitochondria. Thus, we understand that GUO has a protective effect on the hippocampus metabolism and mitochondrial activity, preventing the deterioration of essential functions for the functioning of the hippocampal pre-synapse, including learning and memory processes.

Keywords: Alzheimer's disease. Amyloid-β oligomers. Presynaptic mitochondria. Guanosine. Neuroprotection.

LISTA DE ABREVIATURAS

AMPA α-amino-3-hidróxi-metil-5-isoxazol- 4-propiônico (*α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid*)

APP proteína precursora amiloide (*amyloid precursor protein*)

ATP adenosina trifosfato

Aβ β- amiloide

AβOs oligômeros β- amiloide

BHE barreira hematoencefálica

CCCP desacoplador carbonilcianida-3-clorofenilhidrazona (*carbonyl cyanide m-chlorophenylhydrazone*)

DA Doença de *Alzheimer*

GSH glutationa

GSK-3 glicogênio sintase cinase 3 (*glycogen synthase kinase 3*)

ICV intracerebroventricular

LCR líquido cefalorraquidiano

LTD depressão de longa duração (*long term depression*).

LTP potenciação de longa duração (*long term potentiation*)

MCU canal uniporter mitocondrial (*mitochondrial calcium uniporter*)

mPTP poro de transição de permeabilidade mitocondrial (*mitochondrial permeability transition pore*)

NMDA receptor de glutamato N-metil D-Aspartato (*N-methyl-D-aspartate*)

OXPHOS fosforilação oxidativa mitocondrial (*oxidative phosphorylation*)

p-Tau proteína tau fosforilada (*hyperphosphorylated tau*)

RCR índice de controle respiratório (*respiratory control ratio*)

ROS espécies reativas de oxigênio (*reactive oxygen species*)

SNAP-25 *synaptosome-associated protein of relative molecular mass 25K*

SNARE *soluble NSF-attachment protein (SNAP) receptors*

SNC sistema nervoso central

SRC capacidade respiratória reserva (*spare respiratory capacity*)

TA temperatura ambiente

AV amor verdadeiro

SUMÁRIO

APRESENTAÇÃO

Esta tese está organizada em três capítulos, sendo cada um constituído dos seguintes itens:

Parte I Introdução e Objetivos

Parte II Resultados apresentados na forma de artigo científico publicado

Parte III Discussão, conclusão e referências bibliográficas citadas na Introdução e Discussão.

Em anexo, constam artigos publicados durante o doutorado, não relacionados a essa tese.

O trabalho dessa tese foi integralmente desenvolvido na Universidade Federal do Rio Grande do Sul

PARTE I

1. INTRODUÇÃO

1.1.DOENÇA DE ALZHEIMER

A Doença de Alzheimer (DA) é a principal causa de demência entre as doenças neurodegenerativas, cuja prevalência vem aumentando devido ao aumento da expectativa de vida (ALZHEIMER'S ASSOCIATION, 2020). Em 2019, a projeção de expectativa de vida no Brasil chegou a 75 anos. Em 2050, a projeção mundial esperada é de 77 anos, com a população maior que 65 anos correspondendo a 16% do total populacional. Esse avanço na expectativa de vida se deve à redução de morbidade e mortalidade populacional por, entre outros fatores, aprimoramentos em cuidados e tratamentos para doenças crônicas não transmissíveis e doenças infecciosas (World Population Prospects 2019: Highlights, 2019).

Considerando que o principal fator de risco para o desenvolvimento da DA seja a idade, esse aumento da expectativa de vida carrega de forma inerente o risco para o desenvolvimento da doença, cuja incidência atinge 5,6 % dos idosos entre 65 a 69 anos e 33,8 % daqueles com 90 anos ou mais (TOM; HUBBARD; CRANE; HANEUSE *et al.*, 2015). A prevalência mundial de pessoas vivendo com DA era de 43,8 milhões em 2016, com crescimento de 11% em relação a 1990 (COLLABORATORS, 2019). No Brasil, 1,6 milhões de pessoas apresentavam DA ou outras demências no ano de 2016, com incidência de 55 mil casos por ano (Gutierrez et al, 2014). Dessa forma, a prevalência da DA vem se tornando uma grande preocupação, uma vez que ainda não há tratamento capaz de curar ou impedir sua progressão (MASTERS; BATEMAN; BLENNOW; ROWE *et al.*, 2015).

A DA acomete principalmente pessoas idosas, com 65 anos ou mais, apresentando evolução progressiva, caracterizada histopatologicamente pelo acúmulo de peptídeo β-

amiloide, emaranhados neurofibrilares formados a partir da fosforilação da proteína Tau (p-Tau), além de morte de células neurais e atrofia de estruturas cerebrais (DETURE; DICKSON, 2019; SCHELTENS; BLENNOW; BRETELER; DE STROOPER *et al.*, 2016). Clinicamente, a doença é marcada pela perda de memória, prejuízo cognitivo, além de transtornos comportamentais e de humor (ALZHEIMER'S ASSOCIATION, 2020; CUMMINGS, 2004).

Devido à progressão da doença, o paciente gradativamente perde sua capacidade cognitiva e autonomia em atividades da vida diária, tornando-se dependente dos familiares ou cuidadores para a maioria das atividades básicas, inclusive de autocuidado (ALZHEIMER'S ASSOCIATION, 2020; CASTRO; DILLON; MACHNICKI; ALLEGRI, 2010). Além disso, pacientes com DA apresentam maior taxa de complicações de outras morbidades (cardiovasculares, pulmonares, infecções) assim como maior risco de acicentes e, por isso, têm maior frequência de admissão hospitalar, maior tempo de internação e maior risco de morte em comparação a pacientes com outras doenças (GOLÜKE; VAN DE VORST; VAARTJES; GEERLINGS *et al.*, 2019; TOLPPANEN; TAIPALE; PURMONEN; KOPONEN *et al.*, 2015) Dessa forma, a DA traz uma grande sobrecarga emocional, psicológica e física para os cuidadores, e elevado custo econômico para o sistema de saúde.

Com relação à fisiopatologia dessa doença, muitos fatores relacionados ao desenvolvimento e à progressão da doença foram caracterizados. Na primeira descrição clínica e correlação com os achados histopatológicos, Alois Alzheimer em 1907 observou uma paciente de 50 anos previamente diagnosticada com paranoia apresentando piora progressiva de alterações cognitivas e comportamentais (HIPPIUS; NEUNDÖRFER, 2003). Nas análises morfológica e histológica *post mortem* de fatias do cérebro dessa paciente, foram identificadas placas e agregados neurofibrilares. Dessa forma, Alzheimer chegou à proposição de uma nova doença com características específicas, diferente dos demais transtornos psiquiátricos

(HIPPIUS; NEUNDÖRFER, 2003).

Os avanços no estudo da DA permitiram identificar os mecanismos fisiopatológicos que ocorrem antes e durante o desenvolvimento das manifestações clínicas da doença, inclusive o aumento dos níveis de AβOs previamente à formação das placas, as regiões cerebrais primordialmente afetadas, a alteração dos genes cujo aumento ou redução da expressão estão relacionados à forma familiar de DA e sua evolução rápida, a disfunção mitocondrial associada à falha sináptica que resulta em déficit de memória, as alterações cognitivas e comportamentais da doença (FERREIRA; VIEIRA; DE FELICE, 2007; HU; TAN; TAN; YU, 2016; MASTERS; BATEMAN; BLENNOW; ROWE *et al.*, 2015). Contudo, em meio a muitos avanços sobre a compreensão da doença, ainda não foi possível alcançar um tratamento capaz de curar a doença ou impedir sua progressão. Os medicamentos atualmente disponíveis, como inibidores da colinesterase (donepezila, rivastigmina, galantamina) e antagonista não competitivo de receptor NMDA (memantina), melhoram alguns aspectos cognitivos da doença, contudo não impedem sua evolução e prognóstico. Dessa forma, a busca de alvos terapêuticos na investigação dos mecanismos de ação da DA e de fármacos capazes de atravessar a barreira hematoencefálica (BHE) vêm sendo tema relevante de pesquisa.

1.2.O PAPEL DOS OLIGÔMEROS β-AMILOIDE NA DA

1.2.1. Toxicidade dos AβOS

Classicamente, a DA é caracterizada por dois marcadores histopatológicos: os emaranhados neurofibrilares e as placas senis (WINBLAD; AMOUYEL; ANDRIEU; BALLARD *et al.*, 2016). Os emaranhados neurofibrilares são agregados intracelulares da proteína Tau – proteína responsável pela estabilização dos microtúbulos – que é hiperfosforilada por proteínas cinases, como a GSK 3 (glicogênio sintase cinase 3), induzindo

a mudança de conformação e desacoplamento dos microtúbulos (MEDINA; HERNANDEZ; AVILA, 2016). Dessa forma, além da toxicidade provocada pela p-Tau, as funções relacionadas ao citoesqueleto, transporte celular e estabilização de membranas são prejudicadas, contribuindo para a disfunção sináptica e mitocondrial presentes na DA (AVILA; DE BARREDA; PALLAS-BAZARRA; HERNANDEZ, 2013).

As placas senis, por sua vez, são formadas por fibrilas de β- amiloide (βA), as quais são oriundas do processamento da proteína precursora do amiloide (APP), e restos celulares (BALLARD; GAUTHIER; CORBETT; BRAYNE *et al.*, 2011). Esse processamento da proteína APP ocorre pela via amiloidogênica, permanecendo na forma oligomérica, solúvel, e agregando-se em oligômeros com maior massa molecular, mudando de conformação até chegar à forma fibrilar, insolúvel (HAMLEY, 2012). Por muito tempo, o dano cognitivo da DA foi atribuído ao efeito tóxico das placas amiloide (FERREIRA; LOURENCO; OLIVEIRA; DE FELICE, 2015). Contudo, os estudos vêm indicando que os níveis de AβOs solúveis é que se correlacionam melhor com a progressão da doença e os sintomas do que a presença de placas, sendo que os de baixa massa molecular são mais tóxicos do que aqueles com maior massa molecular (FIGUEIREDO; CLARKE; LEDO; RIBEIRO *et al.*, 2013; FONTANA; ZIMMER; ROCHA; GOSMANN *et al.*, 2020; HAMLEY, 2012), sendo o $A\beta_{1-42}$ um dos oligômeros mais produzidos na DA e que apresenta maior toxicidade (NASLUND; HAROUTUNIAN; MOHS; DAVIS *et al.*, 2000).

A explicação para esse efeito é que os oligômeros têm baixa massa molecular, são solúveis e difundem-se com facilidade pelo o cérebro, dessa forma atingindo mais terminações sinápticas e, além disso, apresentam afinidade por regiões sinápticas e maior potencial neurotóxico do que as placas (HAMLEY, 2012; YU; EDALJI; HARLAN; HOLZMAN *et al.*, 2009). Já as fibrilas, formadas no final no processo de agregação da proteína β-amiloide, tem

maior massa molecular, são insolúveis, com pouca capacidade de difusão, tendendo a agrupar-se no local em que estão, formando, junto com restos celulares, as placas senis, as quais exercem menor efeito tóxico comparadas aos oligômeros (AHMED; DAVIS; AUCOIN; SATO *et al.*, 2010; FERREIRA; LOURENCO; OLIVEIRA; DE FELICE, 2015; HAMLEY, 2012).

Fisiologicamente, a proteína APP é ancorada na membrana plasmática, apresentando um terminal N (extracelular) e um terminal C (citoplasmático), sendo processada em dois sítios específicos pelas enzimas α- e γ-secretase, resultando em um fragmento APPα e outro P3, e liberando o terminal C (ZHANG; CHEN; LEE, 2019). O fragmento extracelular APPsα apresenta funções relacionadas à neuroproteção e sobrevivência à apoptose. Contudo, quando a APP é clivada pela β-secretase, em vez da α-secretase, em sítio distinto dessa última, ocorre a formação dos fragmentos APPsβ e Aβ, sendo esse último com tamanho entre 39 a 43 aminoácidos (GALZITSKAYA; GALUSHKO; SELIVANOVA, 2018). Os fragmentos Aβ agrupam-se, formando oligômeros solúveis (AβOS), forma em que apresentam elevada neurotoxicidade e causam dano neural (FERREIRA; LOURENCO; OLIVEIRA; DE FELICE, 2015; SIVANESAN; TAN; RAJADAS, 2013; ZHAO; LONG; MU; CHEW, 2012).

Estudos *post-mortem* têm verificado maior comprometimento do hipocampo na DA em comparação a outras estruturas, sendo seguido pelo córtex cerebral (HALLIDAY, 2017). Uma vez que o hipocampo é uma das principais estruturas envolvidas em processos de aprendizagem e memória (FELD; BORN, 2020), o dano causado pelos AβOs tem importante repercussão clínica (JACK; WISTE; VEMURI; WEIGAND *et al.*, 2010). Em alguns casos a atrofia do hipocampo ocorre de maneira isolada, e a severidade clínica da doença se correlaciona melhor com a perda neuronal hipocampal em relação a outros achados patológicos (HALLIDAY, 2017). Além disso, o envolvimento do hipocampo na forma clássica da DA se correlaciona com quadro clínico mais severo e progressão mais rápida da doença, enquanto as formas de DA que

poupam o hipocampo apresentam quadro clínico mais leve e com evolução mais lenta (FERREIRA; VERHAGEN; HERNÁNDEZ-CABRERA; CAVALLIN *et al.*, 2017; JACK; WISTE; VEMURI; WEIGAND *et al.*, 2010). Deve-se ressaltar, contudo, que a quantidade de AβOs isoladamente não apresenta correlação com piora clínica, e sim quando associada à perda neuronal hipocampal (JACK; WISTE; VEMURI; WEIGAND *et al.*, 2010).

1.3. PRÉ-SINAPSE: FATORES ENVOLVIDOS NA MEMÓRIA E APRENDIZADO

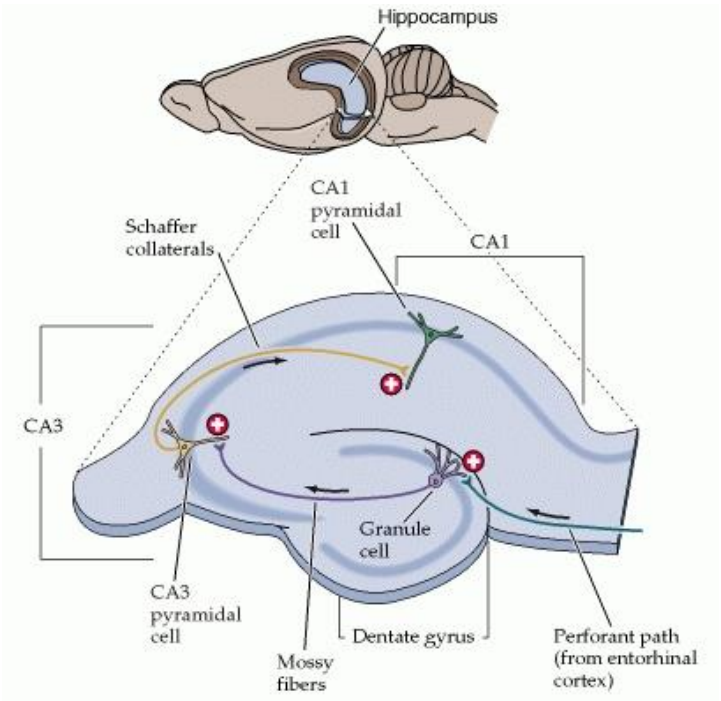
1.3.1. Mecanismos sinápticos de formação de memória

Assim como em outros circuitos cerebrais, no hipocampo as LTPs (potenciações de longo termo) e as LTDs (depressões de longo termo) precisam estar em equilíbrio e são eventos fundamentais na modulação de memórias e na plasticidade sináptica (BLISS; COLLINGRIDGE, 2019; CITRI; MALENKA, 2008; FELD; BORN, 2020; SÜDHOF, 2018). Embora os estudos tenham observado maior frequência de LTPs na pós-sinapse, mediadas pelos receptores NMDA, elas ocorrem também na pré-sinapse, sendo esse processo mediado principalmente por canais de Ca^{2+} voltagem dependente, modulando a conexão sináptica e a liberação de neurotransmissores (CITRI; MALENKA, 2008; LU; HAWKINS, 2006; NINAN; ARANCIO, 2004).

Como exemplo clássico, as fibras musgosas (mossy fibers), que estão envolvidas na via indireta de conexão do córtex entorrinal com a região CA3 do hipocampo e as fibras colaterais de Schaffer, que conectam os neurônios piramidais da região CA3 com os neurônios piramidais da região CA1, representadas na figura 1, apresentam importante mecanismo de regulação da LTP na pré-sinapse, assegurando a estabilidade e robustez sináptica para a manutenção do estímulo neuronal (CITRI; MALENKA, 2008; LU; HAWKINS, 2006; SUDHOF, 2004). Essa LTP pré-sináptica ocorre mediante a entrada de Ca^{2+} e liberação de neurotransmissores na fenda

sináptica, principalmente o glutamato, o qual se liga a receptores, sobretudo AMPA e NMDA, modulando a presença desses receptores na densidade sináptica e reforçando a estabilidade sináptica (BLISS; COLLINGRIDGE, 2019; CITRI; MALENKA, 2008). Nesses processos são formadas as espinhas dendríticas, estruturas com características bioquímicas e morfológicas reconhecidas nas regiões de alta densidade sináptica e de modulação de memórias (KASAI; FUKUDA; WATANABE; HAYASHI-TAKAGI *et al.*, 2010; YANG; PAN; GAN, 2009).

Figura 1. Fibras da via indireta no hipocampo



Respresentação dos mechanismos de regulação da LTP na pré-sinapse do hipocampo, que ocorre principalmente pelas fibras musgosas e fibras colaterais de Schaffer. Fonte: Brain Stuff (https://brainstuff.org/blog/how-does-ltp-work-at-the-hippocampal-synapse

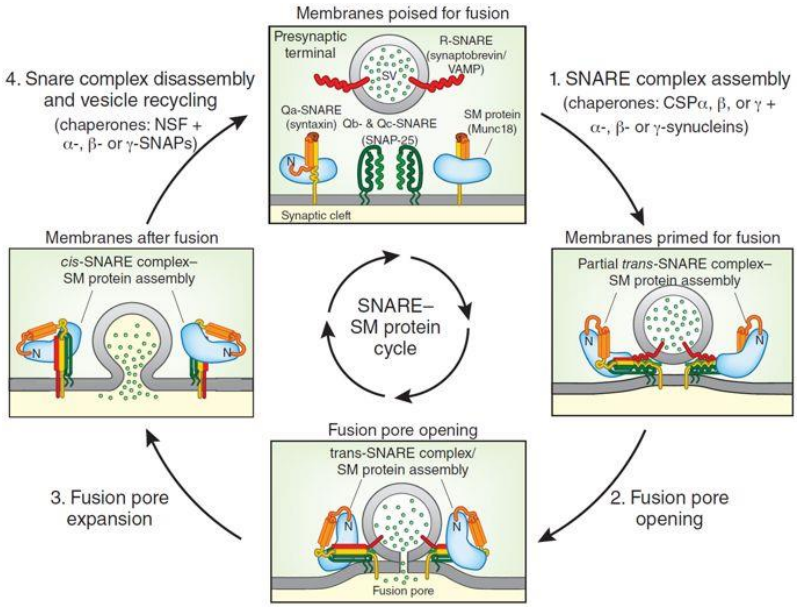
1.3.2. Papel do Ca^{2+} na neurotransmissão

O Ca^{2+} é um íon essencial para que ocorra a neurotransmissão (KATZ; MILEDI,

1967b). Estudos com sinapse de axônios gigantes evidenciaram que os íons Na^+ e K^+ são importantes para a propagação do potencial de ação, para a manutenção e restabelecimento do potencial de membrana, mas não são suficientes para a liberação de neurotransmissores, uma vez que o uso de inibidores dos canais de Na^+ e K^+ não impediram o processo de liberação dessas moléculas (BLOEDEL; GAGE; LLINÁS; QUASTEL, 1966; KATZ; MILEDI, 1967a; b). Esses experimentos observaram que a entrada de Ca^{2+} na pré-sinapse pelos canais de Ca^{2+} voltagem-dependentes é a condição necessária para desencadear a liberação de neurotransmissores na fenda sináptica pelo ancoramento e fusão das vesículas sinápticas na membrana pré-sináptica (KATZ; MILEDI, 1967b; SUDHOF, 2004).

Na pré-sinapse, conforme representado da figura 2, o Ca^{2+} liga-se a proteínas de ancoramento presentes na membrana pré-sináptica e nas vesículas, ativando outras proteínas e complexos, como o SNARE-SM, que compreende a sinaptotagmina, sintaxina, sinaptobrevina, SNAP-25, entre outras (DOLPHIN; LEE, 2020; SUDHOF, 2004; SÜDHOF, 2013). A ativação dessas proteínas pelo Ca^{2+} permite a fusão das membranas e formação de poro para a liberação dos neurotransmissores (CITRI; MALENKA, 2008; DOLPHIN; LEE, 2020; SÜDHOF, 2013).

Figura 2. Modelo de ciclo de fusão das vesículas sinápticas mediado pelo complexo SNARE-SM.



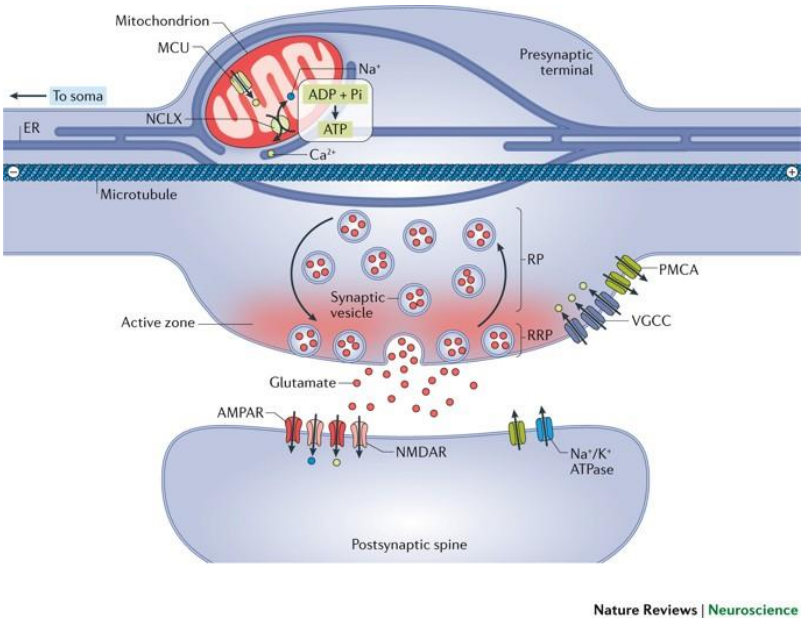
Funções do Ca^{2+} na pré-sinapse, participando do processo de neutrotransmissão e do ciclo das vesículas ao mediar o ancoramento das vesículas às proteínas da membrana pré-sinaptica. Fonte: (SÜDHOF, 2013).

1.3.3. Função mitocondrial na região pré-sináptica

As regiões sinápticas, caracterizadas pela elevada demanda energética necessária para a manutenção do processo de neurotransmissão, possuem elevada concentração de mitocôndrias, as quais desempenham funções essenciais na plasticidade sináptica (MARLAND; HASEL; BONNYCASTLE; COUSIN, 2016; MÜLLER; AHUMADA-CASTRO; SANHUEZA; GONZALEZ-BILLAULT *et al.*, 2018; NICHOLLS; BRAND; GERENCSER, 2015; VOS; LAUWERS; VERSTREKEN, 2010). O ATP liberado pelas mitocôndrias pré-sinápticas é fundamental para a manutenção do ciclo das vesículas, liberação de neurotransmissores, manutenção do potencial de membrana, tamponamento de espécies reativas de O_2, entre outras funções (MARLAND; HASEL; BONNYCASTLE; COUSIN, 2016; NICHOLLS; BRAND; GERENCSER, 2015; VOS; LAUWERS; VERSTREKEN, 2010). Dentro da célula, ocorrem sinalizações oriundas das regiões de maior necessidade energética para a migração de mitocôndrias por transporte pelo citoesqueleto como forma de

regular a distribuição das mitocondrias dentro da célula, conforme ilustrado na figura 3 (VOS; LAUWERS; VERSTREKEN, 2010).

Figura 3. Esquema de transporte das mitocôndrias para a região sináptica



O transporte das mitocôndrias ocorre pelo citoesqueleto para regiões de alta demanda energética.

Fonte: (DEVINE; KITTLER, 2018)

A mitocôndria pré-sináptica também participa da regulação dos níveis de Ca^{2+} no citosol da pré-sinapse, apresentando grande capacidade de tamponamento de Ca^{2+}, que ocorre mediante a captação desse íon pelos transportadores uniporter de cálcio (MCU) (MARLAND; HASEL; BONNYCASTLE; COUSIN, 2016; NICHOLLS; BRAND; GERENCSER, 2015). O tamponamento preciso do Ca^{2+} é uma das condições fundamentais para a ativação das proteínas relacionadas à exocitose das vesículas sinápticas e para a formação das LTPs (NICHOLLS; BRAND; GERENCSER, 2015; SUDHOF, 2004). Alguns estudos indicam que a mitocôndria tem maior participação no tamponamento de Ca^{2+} em condições de saturação da bomba

Na^+/Ca^{2+} e de concentração elevada de Ca^{2+} no citosol (KIM; KOROGOD; SCHNEGGENBURGER; HO *et al.*, 2005), enquanto outros defendem que o tamponamento de Ca^{2+} promovido pela mitocôndria agiliza o processo de recuperação da depressão sináptica, mantendo a neurotransmissão (BILLUPS; FORSYTHE, 2002; DEVINE; KITTLER, 2018).

Além disso, as concentrações intracelulares de Ca^{2+} modulam a formação de ATP, por provável mecanismo de indução do ciclo do ácido cítrico, aumentando a fosforilação oxidativa (OXPHOS) e os seus produtos (LLORENTE-FOLCH; RUEDA; PARDO; SZABADKAI *et al.*, 2015). Alguns mecanismos de regulação dos níveis de ATP pelo Ca^{2+} envolvem a ativação das enzimas desidrogenases que participam do ciclo de Krebs, ativação do complexo V, ativação de transportadores como o do aspartato/glutamato e do ATP-Mg/Pi (RUEDA; TRABA; AMIGO; LLORENTE-FOLCH *et al.*, 2015). Contudo, quando em altas concentrações de Ca^{2+} na mitocôndria ou rápido influxo de Ca^{2+} para a matriz mitocondrial, ocorre ativação e abertura do poro de permeabilidade transitória da mitocôndria, liberando o Ca^{2+} em excesso, porém prejudicando a função da F1F0 ATP sintase, uma vez que o poro está vinculado a esse complexo e muda a sua conformação (KWONG; MOLKENTIN, 2015; MNATSAKANYAN; BEUTNER; PORTER; ALAVIAN *et al.*, 2017)

1.4. DISFUNÇÃO SINÁPTICA NA DA

A disfunção sináptica provocada pela toxicidade dos AβOs causa redução da síntese de proteínas sinápticas levando à perda de espinhas dendríticas (SIVANESAN; TAN; RAJADAS, 2013). Além disso, os AβOs inibem as LTP e facilitam as LTD, resultando em prejuízo da comunicação interneuronal, da modulação de memórias e da plasticidade neuronal (LI; HONG; SHEPARDSON; WALSH *et al.*, 2009; LI; JIN; KOEGLSPERGER; SHEPARDSON *et al.*, 2011a; SIVANESAN; TAN; RAJADAS, 2013; TANAKA; SAKAGUCHI; HIRANO, 2019).

Embora os mecanismos que induzem essas alterações ainda não estejam completamente elucidados, sabe-se que eles envolvem a hiperestimulação de receptores glutamatérgicos e redução da captação de glutamato da fenda sináptica, levando à excitotoxicidade glutamatérgica e consequente grande influxo de Ca^{2+} para o citosol, que por sua vez ativa cascatas de apoptose (BIRNBAUM; BALI; RAJENDRAN; NITSCH *et al.*, 2015; LI; HONG; SHEPARDSON; WALSH *et al.*, 2009; LI; JIN; KOEGLSPERGER; SHEPARDSON *et al.*, 2011a; SIVANESAN; TAN; RAJADAS, 2013).

Um dos mecanismos de sinaptotoxicidade provocado pelos AβOs inclui a disfunção mitocondrial (CAI; TAMMINENI, 2017), o que causa importante impacto na função sináptica, uma vez que essa região apresenta alta densidade de mitocôndrias e é extremamente dependente de suas atividades (THOMAS; KEINE; OKAYAMA; SATTERFIELD *et al.*, 2019). Os AβOs interferem no acoplamento mitocondrial, causando vazamento de prótons e perda do potencial de membrana mitocondrial, o que resulta em menor eficiência respiratória e energética associada à maior produção de espécies reativas de O_2 (BIRNBAUM; BALI; RAJENDRAN; NITSCH *et al.*, 2015; PAGANI; ECKERT, 2011; WANG; WANG; LI; PERRY *et al.*, 2014). Desse modo, o declínio na produção de ATP resulta em importante prejuízo das funções neuronais e vários estudos vêm indicando a disfunção mitocondrial causada pelos AβOs como importante fator causador da falha sináptica da DA e um dos eventos iniciais a ocorrerem no cérebro de pessoas com a doença (CADONIC; SABBIR; ALBENSI, 2016; CAI; TAMMINENI, 2017; HU; TAN; TAN; YU, 2016; ZHU; SMITH; PERRY; ALIEV, 2004).

Alguns estudos também já apontaram alterações na dinâmica mitocondrial em modelos animais de DA correlacionados a déficit de memória em tarefas comportamentais (DU, H.; GUO, L.; YAN, S.; SOSUNOV, A. A. *et al.*, 2010; WANG; GUO; LU; SUN *et al.*, 2016). Essas alterações estruturais impedem o transporte de mitocôndrias e inibem os processos de

fusão e fissão, que são necessários para o controle de qualidade mitocondrial e funcionamento eficiente (RUI; ZHENG, 2016; SHENG; CAI, 2012; WANG; GUO; LU; SUN *et al.*, 2016).

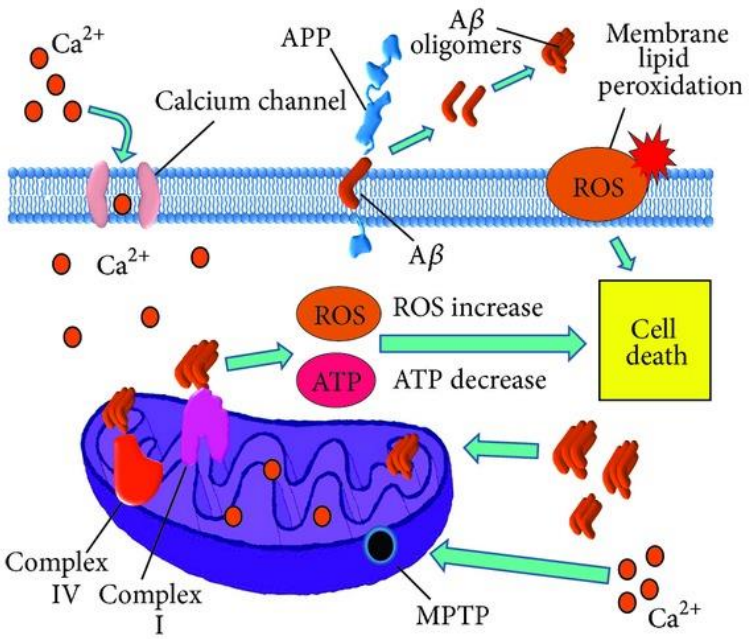
Como descrito anteriormente, a mitocôndria possui função importante no tamponamento e liberação do Ca^{2+}, associado aos processos de aprendizado e memória (ZÜNDORF; REISER, 2011). A modulação do Ca^{2+} é essencial para sinalização intracelular para ancoramento de vesículas na membrana pré-sináptica, liberação de neurotransmissores, reciclagem das vesículas e indução de LTP (SUDHOF, 2004). No entanto, quando ocorre dano à mitocôndria, impedindo a manutenção da homeostase do Ca^{2+}, as células neurais tornam-se mais suscetíveis à excitotoxicidade glutamatérgica e à disfunção sináptica, deteriorando as funções de aprendizado e memória (NICHOLLS, 2009a; ZÜNDORF; REISER, 2011).

Vários estudos apontam a desregulação da homeostase do Ca^{2+} sináptico como um dos fatores que contribuem para a progressão da DA, pois, além de estar diretamente envolvido na liberação de neurotransmissores e na modulação da formação de memórias, níveis elevados de Ca^{2+} no citosol estão associados à excitotoxicidade glutamatérgica e ativação de vias pró-apoptóticas (DEVINE; KITTLER, 2018; MÜLLER; AHUMADA-CASTRO; SANHUEZA; GONZALEZ-BILLAULT *et al.*, 2018). Modelos animais transgênicos de DA e induzidos por AβOs demonstraram aumento do Ca^{2+} citosólico e mitocondrial associados à morte neuronal, além do déficit no efluxo de Ca^{2+} aumentar a suscetibilidade mitocondrial para abertura do poro de transição de permeabilidade mitocondrial (mPTP) e perda do potencial de membrana, contribuindo para a disfunção sináptica e a progressão da doença (CALVO-RODRIGUEZ; HOU; SNYDER; KHARITONOVA *et al.*, 2020; JADIYA; KOLMETZKY; TOMAR; DI MECO *et al.*, 2019).

É importante frisar que a abertura do mPTP causada pelos AβOs leva à disfunção mitocondrial severa, edema de membranas mitocondriais e liberação do citocromo C e de outros

fatores pró-apoptóticos (DU; YAN, 2010; JADIYA; KOLMETZKY; TOMAR; DI MECO *et al.*, 2019; MÜLLER; AHUMADA-CASTRO; SANHUEZA; GONZALEZ-BILLAULT *et al.*, 2018). Também foi observado em análises *post-mortem* de cérebros humanos com DA que a expressão de genes relacionados ao influxo de Ca^{2+} estava reduzida, enquanto a de genes relacionados ao efluxo de Ca^{2+} estava aumentada, o que foi interpretado como um mecanismo para contrabalançar a sobrecarga de Ca^{2+} mitocondrial (CALVO-RODRIGUEZ; HOU; SNYDER; KHARITONOVA *et al.*, 2020).

Figura 4. Mecanismos de dano mitocondrial na DA



Esquema dos efeitos dos AβOs na mitocôndria, apresentando falha energética com baixa produção de ATP, redução do tamponamento de Ca^{2+}, aumento de espécies reativas de O_2 e dano oxidativo às membranas, resultando em morte celular. Fonte: (LUQUE-CONTRERAS; CARVAJAL; TORAL-RIOS; FRANCO-BOCANEGRA *et al.*, 2014)

1.5. EXCITOTOXICIDADE GLUTAMATÉRGICA NA DA

A excitotoxicidade glutamatérgica consiste na hiperestimulação de receptores glutamatérgicos devido ao aumento excessivo dos níveis de glutamato na fenda sináptica, causado pela liberação de glutamato pelas vesículas pré-sinápticas e/ou redução da captação de glutamato da fenda sináptica pelos transportadores astrocitários de glutamato (KABOGO; RAUW; AMRITRAJ; BAKER *et al.*, 2010; MAHMOUD; GHARAGOZLOO; SIMARD; GRIS, 2019). Assim como em outras doenças do SNC, a excitotoxicidade glutamatérgica é um dos fatores que contribuem para o processo de neurodegeneração na DA (KODIS; CHOI; SWANSON; FERREIRA *et al.*, 2018). Entre os efeitos da excitotoxicidade glutamatérgica, estão a disfunção mitocondrial, apontada por Schinder e colegas já em 1998 como um primeiros alvos da excitotoxicidade (SCHINDER; OLSON; SPITZER; MONTAL, 1996), com aumento da permeabilidade da membrana mitocondrial e consequente perda do potencial de membrana e redução da eficiência energética, conforme descrito anteriormente (NICHOLLS; BUDD, 2000). Além disso, o excesso de glutamato na fenda sináptica causa desregulação dos níveis de Ca^{2+} ao aumentar a entrada desse íon no citosol, aumenta a produção e liberação de espécies reativas de oxigênio e ativa cascatas de apoptose e morte celular (DONG; WANG; QIN, 2009; HYND; SCOTT; DODD, 2004).

Em modelo in vitro, a incubação com AβOs despolariza células neuronais e leva à liberação de glutamato em cerca de 10-30 minutos, com acúmulo na fenda sináptica em 1h e pico de glutamato extracelular em 4h, o qual foi relacionado à possível inibição dos transportadores astrocitários de glutamato (MORKUNIENE; CIZAS; JANKEVICIUTE; PETROLIS *et al.*, 2015). A cascata de excitotoxicidade desencadeada pelos AβOs é atribuída principalmente à hiperestimulação dos receptores NMDA (LI; JIN; KOEGLSPERGER; SHEPARDSON *et al.*, 2011a), pelo influxo excessivo e persistente de Ca^{2+} com consequente

inibição das LTP, indução de LTD, aumento de estresse oxidativo e perda neuronal (ALBERDI; SANCHEZ-GOMEZ; CAVALIERE; PEREZ-SAMARTIN *et al.*, 2010; DE FELICE; VELASCO; LAMBERT; VIOLA *et al.*, 2007; FERREIRA; BAJOUCO; MOTA; AUBERSON *et al.*, 2012; LI; HONG; SHEPARDSON; WALSH *et al.*, 2009; LI; JIN; KOEGLSPERGER; SHEPARDSON *et al.*, 2011b; MIGUEL-HIDALGO; ALVAREZ; CACABELOS; QUACK, 2002). Além disso, em pacientes com DA também foi observada redução dos receptores de NMDA e dos níveis de RNAm desse receptor e suas isoformas (GluN1, GluN2A, GluN2B), principalmente no hipocampo (GUO; ZHANG; ZENG; HUANG *et al.*, 2020; HYND; SCOTT; DODD, 2004; LI; JIN; KOEGLSPERGER; SHEPARDSON *et al.*, 2011b).

Uma vez que o glutamato é um dos principais neurotransmissores envolvidos na plasticidade sináptica e na modulação de memórias envolvendo os processos de LTP (BLISS; COLLINGRIDGE, 2019), a excitotoxicidade glutamatérgica desencadeada pelos AβOs têm importante impacto na plasticidade sináptica (DANYSZ; PARSONS, 2012; LI; JIN; KOEGLSPERGER; SHEPARDSON *et al.*, 2011b). O aumento dos níveis extracelulares de glutamato está relacionado à hiperativação dos receptores extra sinápticos NR2B (subunidade 2 NMDA), envolvidos na ativação das LTD e inibição das LTP, além de redução dos receptores NR1 (subunidade 1 NMDA) sinápticos, envolvidos na plasticidade neuronal (DANYSZ; PARSONS, 2012; LI; JIN; KOEGLSPERGER; SHEPARDSON *et al.*, 2011b).

1.6. GUANOSINA

A Guanosina (GUO) é um nucleosídeo derivado da guanina, uma purina endógena produzida pelo metabolismo dos metabólitos da guanina, pela ação da 5’-ecto-nucleotidase, sendo liberada por neurônios e células gliais (CIRUELA, 2013). Suas funções neuroprotetoras estão relacionadas à modulação do sistema glutamatérgico, estimulando a captação de

glutamato pelos astrócitos, principalmente pelo transportador astrocitário de glutamato GLT-1 (Glutamate transporter-1), com consequente redução dos níveis desse neurotransmissor na fenda sináptica, evitando a excitotoxicidade glutamatérgica e reduzindo a produção de ROS (CITTOLIN-SANTOS; DE ASSIS; GUAZZELLI; PANIZ *et al.*, 2017; HANSEL; RAMOS; DELGADO; SOUZA *et al.*, 2014). Além disso, os efeitos da GUO têm sido observados em funções tróficas, por estimular a neurogênese em zonas específicas de reparo celular (LANZNASTER; DAL-CIM; PIERMARTIRI; TASCA, 2016), na modulação de respostas inflamatórias (DAL-CIM; LUDKA; MARTINS; REGINATO *et al.*, 2013; HANSEL; RAMOS; DELGADO; SOUZA *et al.*, 2014), na prevenção da produção de espécies reativas em situações de privação de oxigênio e glicose (THOMAZ; DAL-CIM; MARTINS; CUNHA *et al.*, 2016) e na interação com o sistema adenosinérgico e glutamatérgico (ALMEIDA; COMASSETO; RAMOS; HANSEL *et al.*, 2017; DAL-CIM; POLUCENO; LANZNASTER; DE OLIVEIRA *et al.*, 2019; LANZNASTER; MASSARI; MARKOVÁ; ŠIMKOVÁ *et al.*, 2019; MASSARI; CONSTANTINO; MARQUES; BINDER *et al.*, 2020; SCHMIDT; LARA; DE FARIA MARASCHIN; DA SILVEIRA PERLA *et al.*, 2000).

Estudos do nosso grupo têm mostrado o efeito da GUO em modelos animais de isquemia focal permanente, com redução do volume de infarto, resultando em ganho de funcionalidade motora (HANSEL; TONON; GUELLA; PETTENUZZO *et al.*, 2015). Em modelo de ansiedade, a GUO inibiu a liberação excessiva de glutamato pelo terminal pré-sináptico, e teve efeito agonista em receptores adenosinérgicos A_1, reduzindo o comportamento ansioso dos animais (ALMEIDA; COMASSETO; RAMOS; HANSEL *et al.*, 2017). Em modelo de convulsão induzido por ácido quinolínico, a GUO teve efeito anticonvulsivante, reduzindo em até 80% dos episódios convulsivos (SCHMIDT; LARA; DE FARIA MARASCHIN; DA SILVEIRA PERLA *et al.*, 2000; SCHMIDT, 2010). Em modelo de encefalopatia hepática

induzido por ligamento do ducto biliar, a GUO modulou os parâmetros eletroencefalográficos, reduziu os níveis de glutamato no LCR e o dano oxidativo em estruturas cerebrais (PANIZ; CALCAGNOTTO; PANDOLFO; MACHADO *et al.*, 2014). Em encefalopatia hiperamonêmica, causada pela administração in vivo de acetato de amônio, a GUO reduziu a taxa de mortalidade e duração do coma em ratos (CITTOLIN-SANTOS; DE ASSIS; GUAZZELLI; PANIZ *et al.*, 2017).

A GUO também já foi testada em um modelo de DA (LANZNASTER; MACK; COELHO; GANZELLA *et al.*, 2017), mostrando que a administração por 14 dias foi capaz de recuperar a memória de curto prazo prejudicada pelos AβOs, além de modular a captação de glutamato em fatias de hipocampo (LANZNASTER; DAL-CIM; PIERMARTIRI; TASCA, 2016; LANZNASTER; MACK; COELHO; GANZELLA *et al.*, 2017). Em resumo, esses estudos indicam o potencial efeito neuroprotetor da GUO, trazendo a perspectiva de uma nova droga de ação no SNC.

2. OBJETIVO GERAL:

Avaliação dos efeitos de Oligômeros β-amiloide no metabolismo energético cerebral e do potencial terapêutico da guanosina em camundongos.

2.1. Objetivos específicos:

- Avaliação do efeito dos AβOs e da GUO no comportamento dos animais por meio das tarefas de reconhecimento de objeto;
- Avaliar a duração do efeito dos AβOs em tarefas comportamentais;
- Avaliar os efeitos dos AβOs e da GUO no metabolismo do hipocampo, mediante avaliação do sistema glutamatérgico, estresse oxidativo, conteúdo de proteínas e expressão gênica.
- Verificar os efeitos dos AβOs e da GUO em parâmetros mitocondriais da pré-sinapse.

PARTE II

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Guanosine Neuroprotection of Presynaptic Mitochondrial Calcium Homeostasis in a Mouse Study with Amyloid-β Oligomers

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Abstract

Amyloid-β oligomers (AβOs) toxicity causes mitochondrial dysfunction, leading to synaptic failure in Alzheimer's disease (AD). Considering presynaptic high energy demand and tight Ca^{2+} regulation, impairment of mitochondrial function can lead to deteriorated neural activity and cell death. In this study, an AD mouse model induced by ICV (intracerebroventricular) injection of AβOs was used to investigate the toxicity of AβOs on presynaptic function. As a therapeutic approach, GUO (guanosine) was given by oral route to evaluate the neuroprotective effects on this AD model. Following 24 h and 48 h from the model induction, behavioral tasks and biochemical analyses were performed, respectively. AβOs impaired object recognition (OR) short-term memory and reduced glutamate uptake and oxidation in the hippocampus. Moreover, AβOs decreased spare respiratory capacity, reduced ATP levels, impaired Ca^{2+} handling, and caused mitochondrial swelling in hippocampal synaptosomes. Guanosine crossed the BBB, recovered OR short-term memory, reestablished glutamate uptake, recovered mitochondrial Ca^{2+} homeostasis, and partially prevented mitochondrial swelling. Therefore, this endogenous purine presented a neuroprotective effect on presynaptic mitochondria and should be considered for further studies in AD models.

Keywords Presynaptic mitochondria · Guanosine · Amyloid-β oligomers · Alzheimer's disease · Neuroprotection

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease leading to memory loss, cognitive disability, and mood disorders that generate high costs for health systems [1, 2]. These aspects, combined with the increasing global longevity along with the rising prevalence of AD, are an alarming cause of concern for health policymakers. Also, the difficult clinical diagnosis in the initial phase and the lack of treatment options are reasons pressing the need for developing a treatment for this disease [2].

There is a close association between AD development and amyloid-β protein toxicity, mainly from soluble amyloid-β oligomers (AβOs) [3], which is related to their high affinity for synapses [4]. The AβOs toxicity results in glutamatergic excitotoxicity, dendritic spine damage, mitochondrial failure, impaired Ca^{2+} signaling, and oxidative injuries [4, 5]. Glutamatergic excitotoxicity, considered a triggering factor for neural death in AD [6], is characterized by glutamate accumulation in the synaptic cleft resulted from increased glutamate release from the presynaptic terminal [7] and/or reduced astrocytic glutamate uptake [8], besides the subsequent overactivation of glutamate receptors in synaptic terminals [9]. The neuronal exposure to high glutamate levels is implicated in cytosolic Ca^{2+} overload, mitochondrial impairment, ROS (reactive oxygen species) damage, energetic failure, and cell death [5, 10].

The synaptic mitochondrial damage caused by AβOs leads to membrane potential depolarization and dysregulated Ca^{2+} signaling, impairing ATP production and causing severe energetic insufficiency in the synapse, a region of high energy demand [11, 12]. In physiological conditions, mechanisms involving the high

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mitochondrial Ca^{2+} storage capacity and affinity for buffering Ca^{2+} are responsible for maintaining low intracellular free Ca^{2+} levels under strict regulation, without disturbing mitochondrial respiratory activity or ATP synthesis [13]. However, in conditions of cytosolic Ca^{2+} overload, as in AD, the mitochondrial buffering set point is overwhelmed, impairing the synaptic mechanisms involved in learning and memory and even releasing apoptotic signals [14]. In line with these mechanisms of disease, investigators have raised questions about the lack of medications that are able to cross the BBB and target mitochondria for AD therapy since the initial alterations caused by this disease are related to mitochondrial dysfunction [15].

Considering a neuroprotective therapy, guanosine (GUO), an endogenous guanine-based purine, has shown protective effects with in vivo and in vitro models of brain diseases, including ischemic stroke, AD, hepatic encephalopathy, and seizures [16–20]. In these studies, GUO modulated several neurochemical processes and behavioral parameters, reducing inflammation, oxidative stress, and glutamate excitotoxicity [21].

In the present study, a mouse model of AD was induced by in vivo injection of AβO, hypothesizing AβOs as the pivotal promoters of mitochondrial dysfunction leading to OR memory impairment to test GUO administration as a neuroprotective strategy. Since the hippocampus is one of the most affected brain regions in AD, with reduction of synaptic proteins, neuronal loss, and volume atrophy correlated with cognitive disability [22], this structure was selected for biochemical experiments.

First, the effect of GUO on in vivo synaptotoxicity was evaluated in animal behavioral performance 24 h after the injection of AβOs. The biochemical analyses were performed 48 h after the injection of AβOs using the whole hippocampus for evaluation of glutamate metabolism, oxidative damage and antioxidant molecules, protein content, and gene expression related to the synaptic function. These experiments were followed by the assessment of presynaptic mitochondrial function using synaptosomes prepared from the hippocampus. These assays mostly focused on respiratory chain efficacy, energy supply, and Ca^{2+} regulation. Thus, this work highlights the AβO's damaging mechanisms in an early phase of the disease and the presynaptic functions that are overwhelmed by this insult, thus reinforcing the role of mitochondria as notable mediators of AβO synaptotoxicity. Importantly, this model of GUO neuroprotection involves mitochondrial presynaptic Ca^{2+} regulation and brings new perspectives for the evaluation of this drug in other AD models.

Results

Time Curve of AβOS Effect on Short-Term Memory

Mice were evaluated at 24 h and 7, 14, 21, and 28 days after receiving the ICV injection of AβOs. All the groups presented similar total distance traveled and mobility time in the open field task (OFT) (Fig. 1a and b) indicating preserved locomotor activity and exploratory behavior. All groups explored both objects equally in the OR training session (Fig. 1c). However, in the OR test session, the ability to recognize the novel object (NO) was impaired from 24 h to the 14th day after AβOs treatment, pointing to a long-term impairment of OR short-term memory (Fig. 1d). On the 21st day, animals spontaneously recovered short-term memory, indicating the transient effect of AβOs on the OR short-term memory. Based on this time-dependent effect, the subsequent behavioral experiments and biochemical analyses were performed at 24 h and 48 h after the injection of AβOs, respectively.

GUO Levels in Naive Mice

In the group receiving a single GUO dose, traced [8-^{3}H]GUO was detected in plasma in 5 min, maintaining similar levels at 15 and 60 min. In the group receiving 4 doses, the [8-^{3}H]GUO plasmatic levels, measured 1 h after the last gavage, were 5 times greater than the levels after one dose (Fig. 1e). [8-^{3}H]GUO was not detected in erythrocytes at any time (data not shown). In brain structures, [8-^{3}H]GUO levels were measured and detected 60 min after 1 dose and had the levels elevated around 4 times after 4 doses (Fig. 1f). These data indicate important features of GUO: the capacity to be quickly available and transported in plasma and the ability to cross the blood-brain barrier (BBB). Although traced GUO was elevated in plasma and brain structures after oral administration following 1 or 4 doses, this administered GUO was not enough to change the total GUO concentration present in the brain structures (data not shown).

GUO Recovered Short-Term Memory Impairment Caused by AβOS

In the OFT, all groups presented similar traveled distance and mobility time (Fig. 1g and h) and equally explored both objects in the OR training session (Fig. 1i). AβOs impaired the capacity to discriminate the novel object from the familiar object in the OR test session (short-term memory), as verified in the previous experiment. This ability was recovered by GUO treatment (Fig. 1j), thus indicating a neuroprotective role of GUO when confronted with AβOs toxicity.

Experiments with the Whole Hippocampus

a. GUO recovered glutamate uptake reduced by AβOs but did not affect glutamate oxidation

AβOs decreased both glutamate uptake (Fig. 2a) and glutamate oxidation to CO_2 (Fig. 2b) in hippocampal slices. GUO

treatment reduced glutamate uptake per se and reversed the AβOs effect on uptake (Fig. 2a), without effect on oxidation.

b. AβOs diminished antioxidant defenses but did not cause oxidative damage

AβOs decreased hippocampal levels of reduced glutathione (GSH) (Fig. 2c) and sulfhydryl content (Fig. 2d), which are considered antioxidant defenses, but AβOs did not affect either lipid peroxidation (TBARS) levels (Fig. 2e) or total ROS levels on the DCFH oxidation assay (Fig. 2f). GUO treatment recovered sulfhydryl content (Fig. 2d) and presented a tendency to recover GSH content (Fig. 2c).

c. AβOs did not cause changes in gene expression or protein content

Gene expression related to synaptic integrity (PSD-95 and SNAP-25), mitochondrial dynamics and bioenergetics (OPA1, DMN1L, MFF, MFN2, PGC-1α, VDAC1, SIRT3, SIRT1, NRF2, GSK-3β), and cellular apoptosis (Bax, GSK-3β) were evaluated to verify if the damage caused by AβOs was leading to changes in gene expression of these proteins, which are known to be involved in synaptic functionality. However, there was no difference among groups (Fig. 3a). AβOs also did not affect the content of synaptic proteins PSD-95, Syntaxin-1, Synaptotagmin, and SNAP-25; kinase GSK-3β, the proteins related to apoptosis Bcl-2 and p53; or the lipid peroxidation product 4-HNE (4-hydroxynonenal) (Fig. 3b).

Hippocampal Presynaptic Mitochondrial Parameters

a. AβOs reduced the mitochondrial spare respiratory capacity in synaptosomes

AβOs led to a decreased spare respiratory capacity (SRC) (Fig. 4c), which was not recovered by GUO. However, AβOs did not affect respiratory control ratio (RCR), OXPHOS, or proton leak flux (Fig. 4a, b, and d). Since the SRC is an essential reserve to support increased neuronal activity at times of high ATP requirement, thus allowing cells to survive stressful conditions, a decline in this respiratory parameter implies a raised vulnerability to oxidative stress, energy depletion, increased neuronal Ca^{2+} entrance, and cell death [23]. Taking these aspects into consideration, other mitochondrial and synaptosomal functions potentially affected by AβOs were evaluated.

b. AβOs decreased ATP levels

The synaptosomal ATP levels measured during respiration were lowered by AβOs (Fig. 4e) despite the maintenance of similar O_2 flux during the OXPHOS phase respirometry phase linked to ATP production. The ATP levels were not recovered by GUO. Yet, a large amount of ATP was produced by all groups after ADP addition to the chamber (Fig. 4f), thus suggesting that the ATP yield was derived through other non-OXPHOS pathways in the synaptosomal preparation which are not restricted to OXPHOS O_2 flux, and these non-OXPHOS sources might be affected by AβOs. ATP depletion is consistently found in patients and animal models of AD [24] which indicates this energetic failure as having an important role in synaptic impairment.

c. AβOs elevated peroxide production

AβOs increased H_2O_2 levels (Fig. 4g), a result that is in agreement with other findings in the literature [25]. Moreover, the H_2O_2 production was increased mainly after complex III inhibition by antimycin A (Fig. 4h). GUO did not change this imbalance.

d. GUO recovered mitochondria from AβOs impairment of Ca^{2+} homeostasis

AβOs led to higher Ca^{2+} concentration in the cytosol, while GUO maintained a Ca^{2+} concentration similar to the control group (Fig. 5a). Furthermore, Ca^{2+} was significantly elevated in synaptosomes in the basal condition in the AβOs group (Fig. 5b) and after the addition of $CaCl_2$ (Fig. 5c), but not after the addition of KCl (Fig. 5d). GUO treatment provided a remarkably sustained Ca^{2+} buffering (Fig. 5a–d). These results reinforce the involvement of Ca^{2+} homeostasis disruption caused by the in vivo injection of AβOs and the neuroprotective effects of GUO.

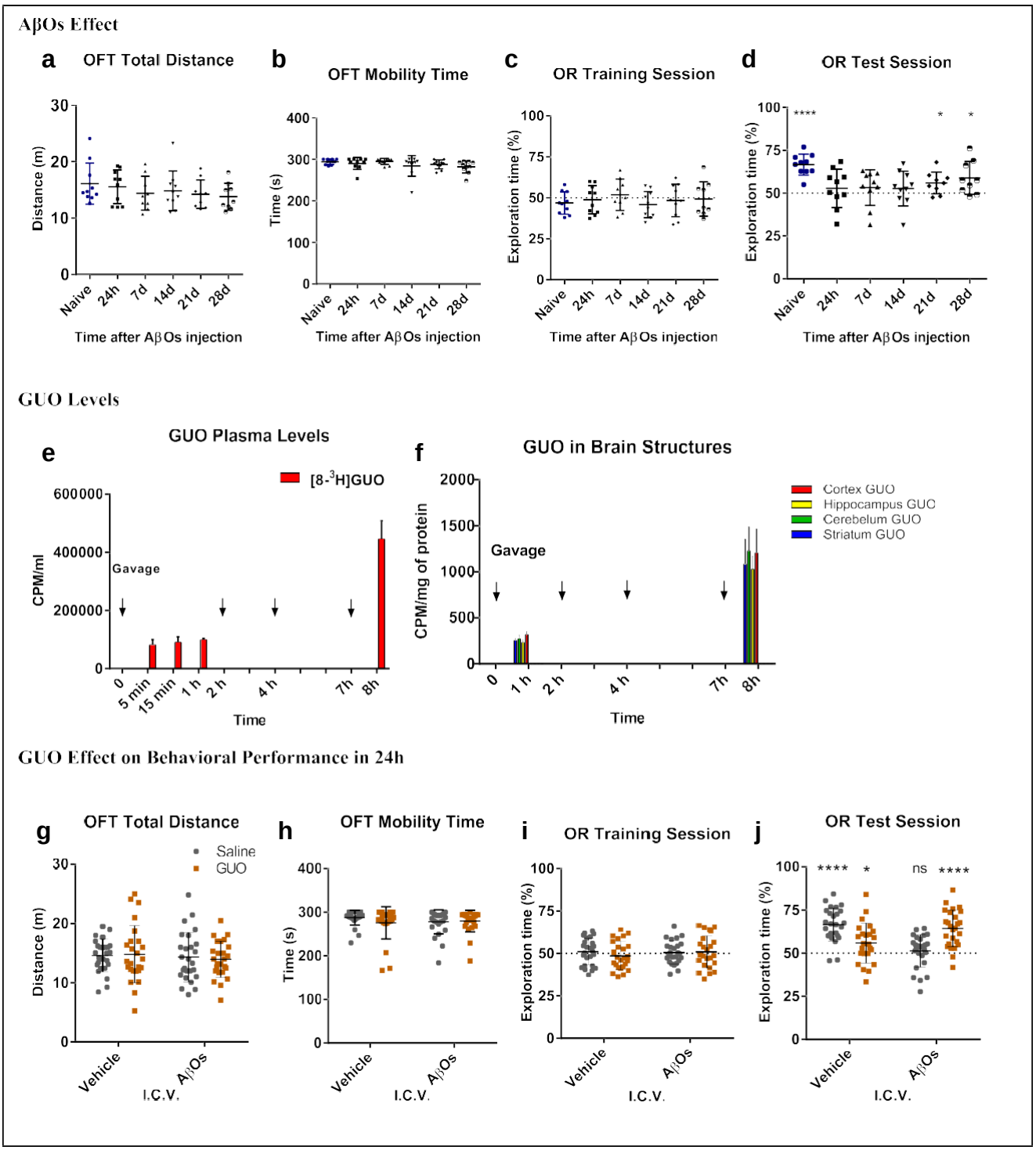
e. GUO reversed presynaptic mitochondrial Ca^{2+} retention capacity disrupted by AβOs

The measurement of cytosolic Ca^{2+} generated data on the condition of total Ca^{2+} levels inside the synaptosomes but did not provide clues regarding the source of Ca^{2+} distress. Indeed, the alteration of Ca^{2+} concentration could have originated in impaired mitochondrial Ca^{2+} buffering, increased permeability allowing Ca^{2+} leakage, or even Ca^{2+} release from the endoplasmic reticulum (ER). To address this matter, mitochondria were isolated from synaptosomes, and their capacity to retain Ca^{2+} was assayed using sequential Ca^{2+} pulses. This thorough experiment showed that AβOs decreased the capacity of isolated mitochondria to retain Ca^{2+}, with a marked reduction in Ca^{2+} influx after the 5th pulse and flux disruption after the 8th pulse (Fig. 5e), which might indicate the opening of the mitochondrial permeability transition pore (mPTP). At the same time, GUO treatment supported the capacity to retain Ca^{2+} as much as the control group did and

resisted Ca^{2+} uptake for more than 10 pulses. Likewise, the Ca^{2+} influx rate, which is associated with the capacity of the mitochondrial calcium uniporter (MCU) to take up Ca^{2+}, was reduced by AβOs after 80 μM and 90 μM Ca^{2+} concentrations, and GUO preserved the inward flow (Fig. 5f).

f. GUO partially recovered mitochondrial morphology altered by AβOs

All groups had an identical number of mitochondria in the electron microscopy (EM) images. This result was reinforced by citrate synthase assay, which demonstrated similar activity in all groups (Fig. 6c). Regarding morphology analysis, however, the AβOs group presented swelling in 63% of total mitochondria analyzed versus 23% in the control group and 45% in the AβOs: GUO group, indicating a partial reduction of 28% of the swollen mitochondria by GUO (Fig. 6a and b).



Discussion

In this study, the in vivo injection of AβOs caused short-term memory impairment in mice evaluated in OR task 24 h after the induction. In the hippocampus, AβOs reduced glutamate uptake and oxidation and led to a slight imbalance in redox homeostasis. In the presynaptic region, AβOs affected the mitochondrial SRC, increased H_2O_2 production, and reduced ATP levels but not the ATP synthesis. AβOs markedly harmed the ability of presynaptic mitochondria to handle Ca^{2+}, resulting in impaired cytosolic Ca^{2+} buffering. GUO recovered short-term memory, prevented glutamate uptake reduction, and recovered presynaptic Ca^{2+} homeostasis. AβOs did not change other respiratory parameters, presynaptic and mitochondrial protein content, and gene expression nor did induce oxidative injuries.

The behavioral tasks performed indicated that the transient impairment of AβOs on OR short-memory persisted for 14 days until spontaneous memory recovery, which is a find- ing similar to another study [3]. The factors underlying this memory loss induced by AβOs include impairment of synaptic plasticity, dendritic spine loss, inhibition of LTP, and mitochondrial dysfunction with Ca^{2+} dysregulation [15, 26–28]. Importantly, the hippocampus is considered an important structure involved in short-term memory modulation [29] and is one of the most affected structures by AβOs toxicity in AD [30].

Here, GUO caused an amnesic effect on OR short-term memory per se. Studies on inhibitory avoidance tasks attribute this effect to the modulation of the glutamatergic system through activation of astrocytic glutamate transporters and reduction in the amount of glutamate available in the synaptic cleft, thereby decreasing the physiological activation of glutamate receptors, mainly NMDA receptors [20, 31–35], a process involved in memory modulation and with a prominent role in the hippocampus [36–38]. Moreover, in cultured astrocytes not submitted to injury, both GMP and GUO increase glutamate uptake, and the blockage of GMP hydrolysis to GUO abolishes the GMP effect, indicating that GUO is the guanine purine responsible for increasing glutamate uptake [39]. Furthermore, this same study found that GMP administration was amnesic on inhibitory avoidance tasks only by its conversion to GUO, reinforcing the GUO role on the amnesic effects [40]. At the same time, in experimental models of brain injury involving excitotoxicity, the neuroprotective effect of GUO comprehends a decrease in the glutamate levels by stimulating astrocytic glutamate uptake [19, 33], as shown in our study. Then, both the amnesic and the neuroprotective effects of GUO seem to involve a decrease of glutamate on the synaptic cleft. However, it is important to emphasize that the amnesic effect is transient, while the neuroprotective effect tends to stay longer.

In this study, both GUO administration and AβOs injection in vivo decreased glutamate uptake measured in vitro 48 h after the model induction. However, the phenomena are distinct. While GUO administration reduced glutamate uptake per se in control mice, it counteracted the decline caused by AβOs injection. This new finding of the GUO effect per se might be attributed to the protocol using in vivo administration of GUO 48 h before the evaluation of glutamate uptake in vitro, which was a different protocol from most studies using acute in vivo GUO administration or in vitro GUO incubation, in which GUO increases glutamate uptake [20, 39, 41]. Conversely to the previous results found in the short time exposure protocols, a previous study indicates that chronic oral administration of GMP in control animals decreases the glutamate uptake [42], similarly to the GUO effects observed in our study. All these data suggest that GUO modulation on glutamate uptake in control conditions depends on the used protocol, although the mechanisms of these distinct effects are still unknown and deserve further investigation.

In addition to the conceivable mechanisms of GUO neuroprotection, some studies suggest the involvement of adenosine receptors on the GUO modulation of glutamate release and uptake from the synaptic cleft, besides decreasing cell damage and enhancing synaptic plasticity, mainly by the interaction of GUO with the adenosine receptors A_1R, $A_{2A}R$, and the

Fig. 1 GUO administration recovered OR short-term memory impairment caused by AβOs injection. (a–d) AβOs effect: OFT total distance (a) and mobility time (b) were similar among groups evaluated 24 h and 7, 14, 21, and 28 days after the injection of AβOs compared with the naive group (one-way ANOVA, followed by Dunnett's multiple comparison test, $n = 9–10$ animals/group). All groups equally explored both objects on OR training session (c). Naive group discriminates the new object (NO) from the familiar object (FO), exploring preferentially the NO (****$P < .0001$) in the OR test session (d), while groups evaluated in 24 h and 7 and 14 days did not discriminate the NO from the FO but did in 21 ($P = .0237$) and 28 ($P = .0249$) days, again exploring preferentially the NO (column statistics, one sample *t* test with hypothetical value 50%, $n = 9–10$ animals/group). e, f [8-^{3}H]GUO levels in plasma (e) 5, 15 min, and 1 h after a single oral dose of [8-^{3}H]GUO and 1 h after receiving 4 doses (distributed according to the protocol; see the experimental design in Fig. 7). [8-^{3}H]GUO was also present in brain structures (f) 1 h after a single [8-^{3}H]GUO dose 1 h after 4 doses ($n = 4$ [8-^{3}H]GUO 1 dose, $n = 4$ [8-^{3}H]GUO 4 doses, $n = 2$ saline 1 dose, $n = 2$ saline 4 doses). Samples from saline groups (plasma and brain structures) had undetectable radioactivity. g–j GUO effect in 24 h after the injection of AβOs. Total distance (g) and mobility time (h) were not changed by AβOs or GUO on OFT (two-way ANOVA, followed by Tukey's multiple comparison test, $n = 25$ animals/group from 5 independent experiments). All groups had similar exploration activity on OR training session (i). In the OR test session (j), control group and control GUO discriminated the NO from the FO ($P < .0001$ and $P = .0224$, respectively), AβOs group did not discriminate the NO from the FO, exploring both objects equally ($P = .49$), while AβOs GUO discriminated the NO from the FO ($P < .0001$).(column statistics, one-sample *t* test with hypothetical value 50%, $n = 25$ animals/group from 5 independent experiments). Data are plotted as mean ± SD. OFT, open field test; OR, object recognition; GUO, guanosine; AβOs, amyloid-β oligomers; NO, new object; FO, familiar object

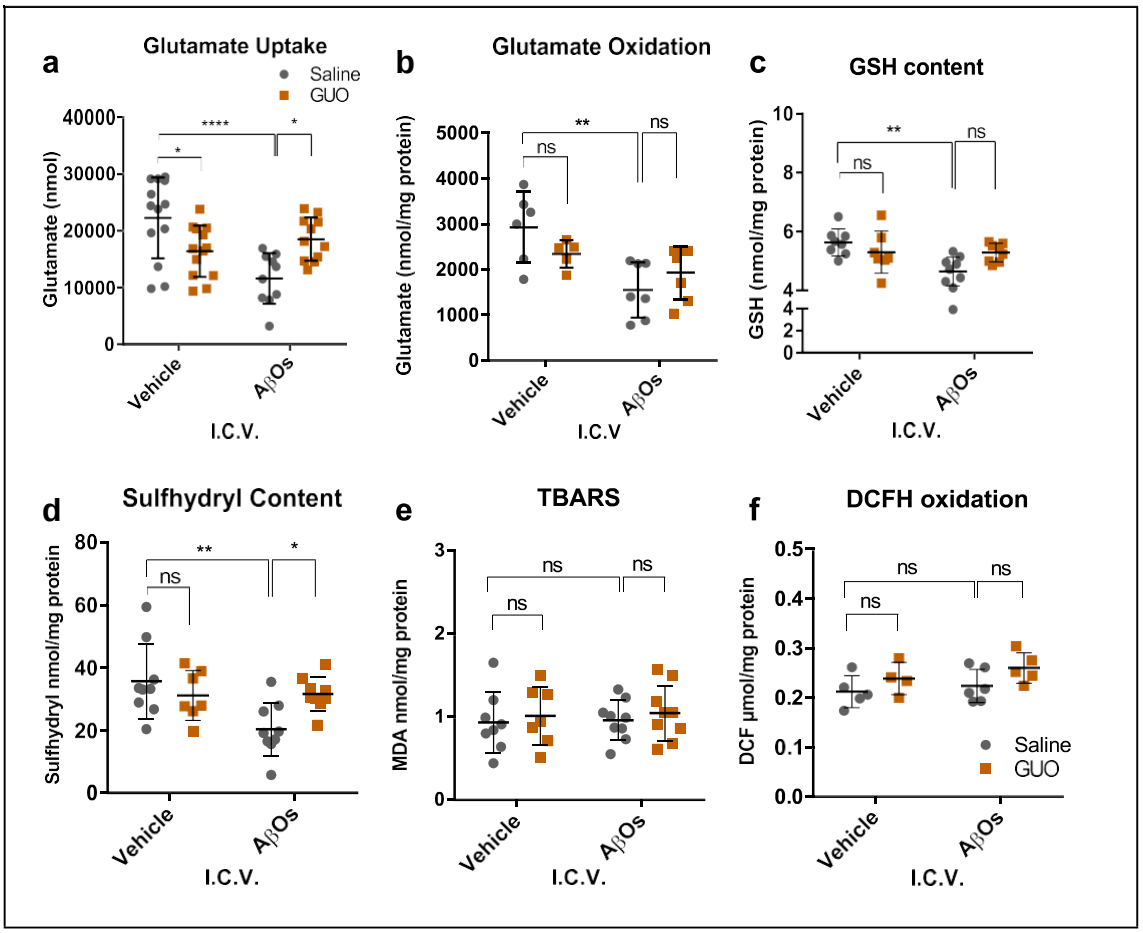


Fig. 2 AβOs injection changed hippocampal glutamate metabolism and oxidative stress profile. Glutamate uptake (a) was reduced by GUO per se (*P = .0315). The reduction caused by AβOs (****P < .0001) was recovered by GUO (*P = .0207) (two-way ANOVA, Tukey's multiple correction test, n = 10–13 animals/group from 3 independent experiments). Glutamate oxidation (b) was reduced by AβOs (**P = .0023); GUO had no effect (two-way ANOVA, followed by Tukey's multiple correction test, n = 5–7 animals/group from 2 independent experiments). GSH content (c) was reduced by AβOs (**P = .0020), and GUO presented a tendency to recover this effect (P = .0628) (two-way ANOVA, followed by Tukey's multiple correction test n = 7–9 animals/group from 2 independent experiments). Sulfhydryl content (d) was reduced by AβOs (**P = .0045), an effect recovered by GUO (*P = .0491); GUO had no effect (two-way ANOVA, followed by Tukey's multiple correction test, n = 7–9 animals/group from 2 independent experiments). Thiobarbituric acid reactive substances (TBARS) (e) levels were not affected by GUO or AβOs (two-way ANOVA, followed by Tukey's multiple correction test, n = 7–9 animals/group from 2 independent experiments). DCFH oxidation (f) was not affected by GUO or AβOs (two-way ANOVA, Tukey's multiple correction test, n = 4–6 mice from 2 independent experiments). Data are plotted as mean ± SD

heteromeric form $A_1R/A_{2A}R$ [43–47]. Therefore, it is relevant to highlight that some of the GUO neuroprotective effects found in our study might be mediated by or involved with adenosine receptors.

The behavioral data also pointed to significant protection promoted by GUO, repairing the short-term memory impairment caused by AβOs. This effect was also found in a previous study from our group, using intraperitoneal (IP) administration of GUO [48]. However, there was no explanation for the short-term memory effect nor information about the presence of exogenous GUO in the mice brain. Here, the quantification of traced GUO showed a rapid increase in plasma just 5 min after the administration, maintaining a stable concentration of [8-3H]GUO 1 h after the gavage. Also, traced GUO reached the brain structures and presented an elevation in plasma and brain [8-3H]GUO concentration proportionally to the administered doses. Therefore, the presence of [8-3H]GUO in the brain structures indicates that this drug crossed the BBB, which is an important pharmacokinetic feature for the treatment of neurodegenerative diseases [49], and might be the direct agent involved with neuroprotection in this model.

Among the factors leading to neural cell damage, glutamate excitotoxicity is known to be a driving mechanism in cellular dysfunction in AD [50]. In our study, the in vivo injection of AβOs caused a significant decrease in glutamate uptake in hippocampal slices measured 48 h after the injection. This reduced glutamate uptake can intensify the glutamate excitotoxicity through potential glutamate accumulation in the synaptic cleft, turning the cells more vulnerable to the early toxicity of AβOs. According to Alberdi and

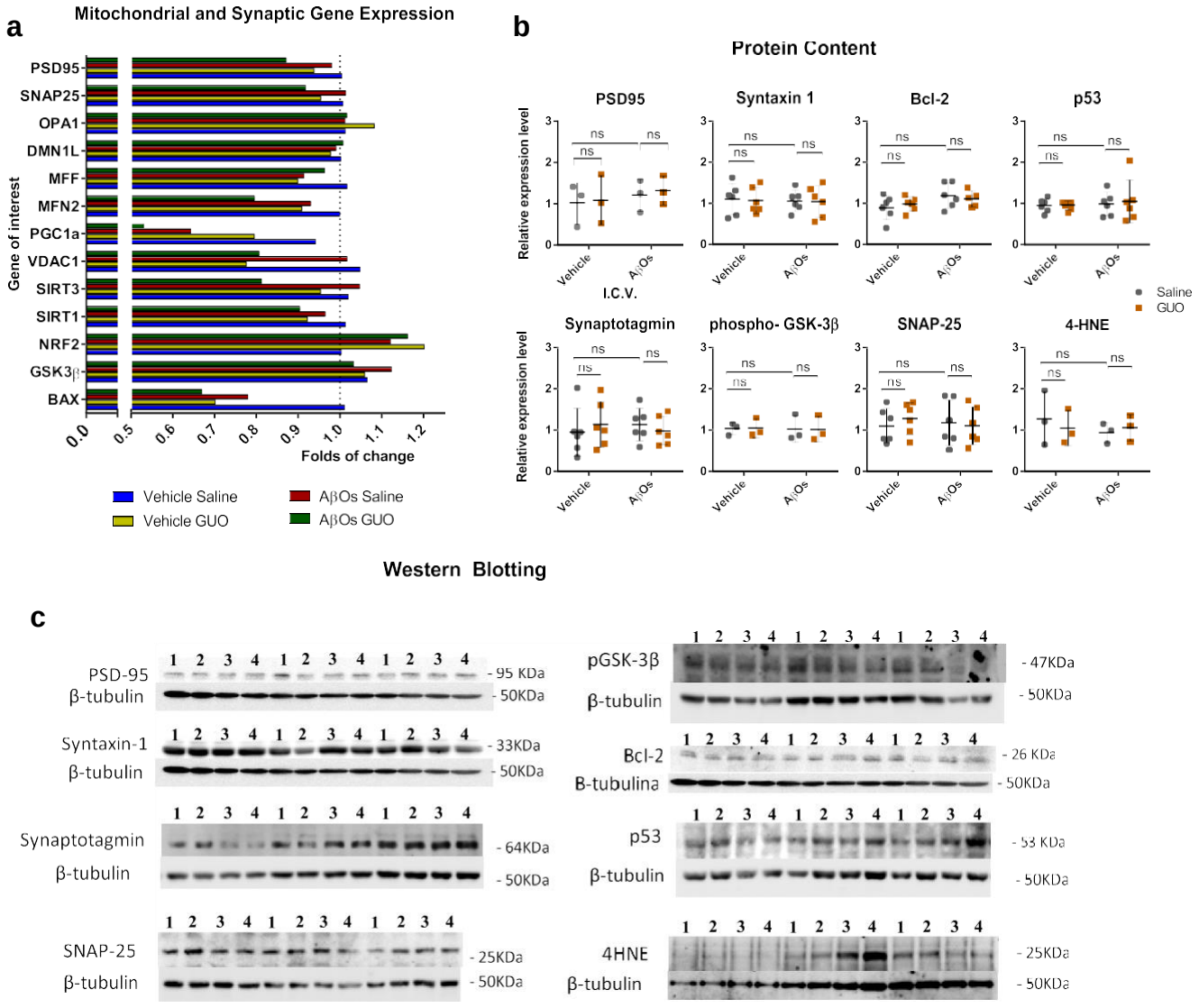


Fig. 3 Gene expression and protein content. Gene expression (a) related to synaptic function, mitochondrial biogenesis, and cellular apoptosis obtained by qPCR were not significantly affected by AβOs or GUO; results are expressed as fold of change, ΔΔCT, normalized for the beta-actin gene (two-way ANOVA followed by Tukey's multiple correc- tion test, $n = 5$ animals/group). Contents of proteins (b and c) related to synaptic function, apoptosis, and oxidative stress obtained by Western blotting were not significantly affected by AβOs or GUO (two-way ANOVA followed by Tukey's multiple correction test, $n = 3$–6 animals/group). Data are plotted as mean ± SD. Representative images are shown below the data

collaborators [50], the excitotoxicity triggered by AβOs overactivation of glutamate receptors arouses an intracellular Ca^{2+} overload in the synaptic region. Ca^{2+} overload, in turn, is responsible for mitochondrial dysfunction and thus promotes ROS generation and the release of proapoptotic factors, culminating in cell death. In vitro experiments have pointed to the displacement of astrocyte glutamate transporter (GLT-1) caused by exposure to AβOs, thus impairing glutamate uptake from the synaptic cleft, which is postulated as a mechanism involved in glutamate accumulation and excitotoxicity in AD [51].

GUO reversed the decrease in glutamate uptake caused by AβOs injection, potentially preventing AβOs excitotoxicity. This GUO effect is attributed to the modulation of the glutamatergic system through enhanced activity of astrocytic glutamate transporters without blockage of the physiological glutamate effects [19, 33], thus favoring the improvements found on behavioral tasks. However, AβOs decreased glutamate oxidation, and GUO did not change this parameter. Here, the role of GUO might be related to the shift of glutamate carbons from the TCA cycle to the synthesis of GSH and in this way seeing as a neuroprotective mechanism [52].

Another consequence of glutamate excitotoxicity is mitochondrial dysfunction, which leads to Ca^{2+} overload, oxidative stress, and reduced SRC [13]. In this study, AβOs impaired mitochondrial function through the reduction of SRC in the synaptosomal preparation. Since the OXPHOS capacity maintained constant O_2 flux but reduced the ATP levels, this disparity might be attributed to other non-OXPHOS ATP sources affected by AβOs in the synaptosomal preparation, which are not restricted to OXPHOS O_2 flux. Besides, in the context of AβOs synaptotoxicity, the high ATP needs in the synaptic region, including the usage of ATP to extrude Ca^{2+} and return it to the cytosol, can lead to the reduction of ATP levels [13]. Nevertheless, the capacity to deal with conditions of high energy demand is hindered once SRC is reduced, triggering ROS generation and Ca^{2+} overload as the first manifestations of this energetic failure [10, 13]. Therefore, with restricted SRC and lower ATP levels, the bioenergetic metabolic dysfunction caused by AβOs puts the synaptic region in a condition of high vulnerability.

In AD, the biomolecular damage caused by lipid peroxidation in the synaptic regions is related to neuronal loss and mitochondrial and synaptic dysfunction [53, 54]. In this study, AβOs raised H_2O_2 levels mainly after the addition of antimycin A. Following complex III inhibition, H_2O_2 generation increases, thus redirecting H_2O_2 to the intermembrane space where GSH content and activity are low [55]. Consequently, the scavenging of H_2O_2 reduces while the outer membrane mitochondrial permeability increases, allowing easy diffusion of H_2O_2 to the cytosol [55]. Despite the elevated H_2O_2 generation, the AβOs did not cause oxidative damage in the biomolecules evaluated, as the levels of malondialdehyde and DCFH were similar in all groups. On the other hand, AβOs reduced GSH and sulfhydryl levels, whereas GUO recovered the sulfhydryl levels and partially recovered GSH levels. Then, these results indicate that GUO protected the synaptic region through the maintenance of antioxidant levels, which has been recognized as a neuroprotective mechanism in the aging process [56].

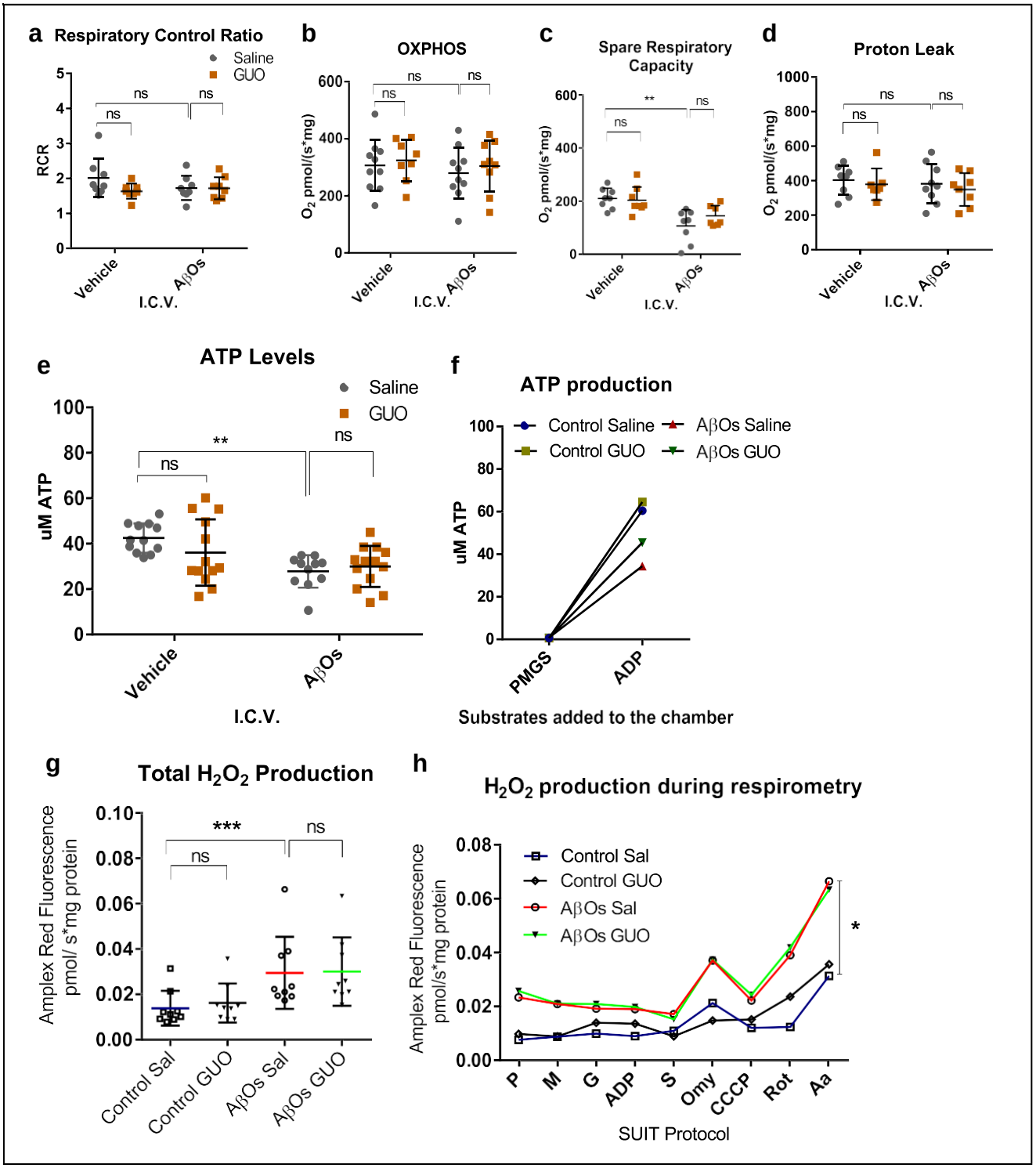
AβOs increased the cytosolic Ca^{2+} concentration in synaptosomes, an alteration associated with higher vulnerability to glutamatergic excitotoxicity, further ROS damage, energetic collapse, and the opening of the mPTP [57, 58]. Moreover, the restricted ability of the isolated presynaptic mitochondria to take up Ca^{2+} pulses sustains the hypothesis of AβOs causing mitochondrial injury, even evaluated 48 h after the in vivo approach. Besides, the reduction in the rate of Ca^{2+} flow, which we reasoned to be caused by a restriction on the MCU transport, is possibly leading to the premature opening of the mPTP, then contributing to the Ca^{2+} overload and mitochondrial failure found in AD [50, 59]. In this experiment, GUO was able to recover Ca^{2+} uptake, possibly by maintaining an MCU rate similar to that of the control group, and also able to avoid early mPTP opening. This effect of GUO on Ca^{2+} uptake was not recorded in any previous study, and this might be the determining factor in short-term memory recovery.

Synaptic plasticity modulation relies on mitochondrial Ca^{2+} release from its stores and depends on precisely regulated homeostasis and the Ca^{2+} buffering mechanism, including uptake by MCU [60]. Also, the close structural association of the mPTP with the c-subunit portion of ATP synthase determines efficient physiological coupling and adequate ATP production. However, this structural association can potentiate the mitochondrial vulnerability to the effects of AβOs. In a condition of glutamatergic excitotoxicity triggered by AβOs, the mitochondria have to deal with Ca^{2+} overload and simultaneously higher ATP production. Consequently, under elevated mitochondrial Ca^{2+} levels and ROS, mPTP is opened early, resulting in reduced ATP formation, membrane peroxidation, and the activation of signaling cascades that lead to apoptosis [61]. Hence, the damage caused by AβOs might be one of the primary actors in the synaptic failure and cognitive dysfunction seen in AD. The data about Ca^{2+} uptake reinforce the neuroprotective effect of GUO in confronting mitochondrial failure caused by AβOs in the synaptic region.

In line with our findings, GUO has demonstrated neuroprotective effects in various experimental models of brain diseases, but the precise mechanisms that cause neuroprotection are still unknown. In permanent focal brain ischemia, GUO reduced the infarcted area allowing faster recovery from

Fig. 4 AβOs injection caused energetic failure in the hippocampal synaptosomes. (a–d) In high resolution respirometry, respiratory control ratio (a) was not changed by AβOs or GUO (two-way ANOVA with Tukey's multiple correction test, n = 14 biologically independent samples from 6 independent experiments); data are plotted as mean ± SD. Oxidative phosphorylation (OXPHOS) (b) was not modified by AβOs or GUO (two-way ANOVA with Tukey's multiple correction test, n = 14 biologically independent samples from 6 independent experiments); data are plotted as mean ± SD. Spare respiratory capacity (c) was decreased by AβOs (**P = .0011); GUO had no effect (two-way ANOVA with Tukey's multiple correction test, n = 8–9 biologically independent samples from 4 independent experiments); proton leak (d) was not modified by AβOs or GUO (two-way ANOVA with Tukey's multiple correction test, n = 8–9 biologically independent samples from 4 independent experiments); data are plotted as mean ± SD. ATP levels (e) were decreased by AβOs (**P = .0050), without recovery by GUO (two-way ANOVA, followed by Tukey's multiple correction test, n = 11 biologically independent samples from 5 independent experiments); data are plotted as mean ± SD. ATP production (f) with substrates PMGS before and after ADP addition to the chamber demonstrate that ATP was produced by synaptosomal preparation of all groups during the essay (preliminary data, n = 2 biologically independent samples). Total H_2O_2 production (g) was increased by AβOs (***P = .0004), without recovery by GUO (data are plotted as mean ± SD), and the H_2O_2 production during respirometry (h) was increased by AβOs mainly after antimycin addition (*P = 0.0139), without GUO effect (two-way ANOVA followed by Tukey's correction, n =3–5 biologically independent samples from 3 independent experiments); data are plotted as mean

ischemic damage and resulting in the improvement of front paw movement and gait symmetry [19]. Moreover, in a mouse model of Parkinson's disease, GUO treatment reduced mitochondrial damage, preserved the ATP production level in a model induced by alpha-synuclein [62], and reduced motor impairments induced by reserpine and 6-hydroxydopamine [63]. A dose-dependent administration of GUO acted as an anticonvulsant in a study of quinolinic acid-induced seizures in rats [20]. Likewise, in a previous study from our group in a mouse model of AD that received ICV injections of AβOs, GUO prevented behavioral deficits and anhedonia-like behavior and also partially recovered glutamatergic transmission by reestablishing glutamate uptake in the hippocampus [48]. Therefore, GUO plays



a meaningful neuroprotective role and promotes behavioral improvement in several experimental models of neurologic disorders.

In conclusion, GUO treatment recovered OR short-term memory impairment evaluated 24 h after the injection of AβOs and normalized glutamate uptake in the hippocampus

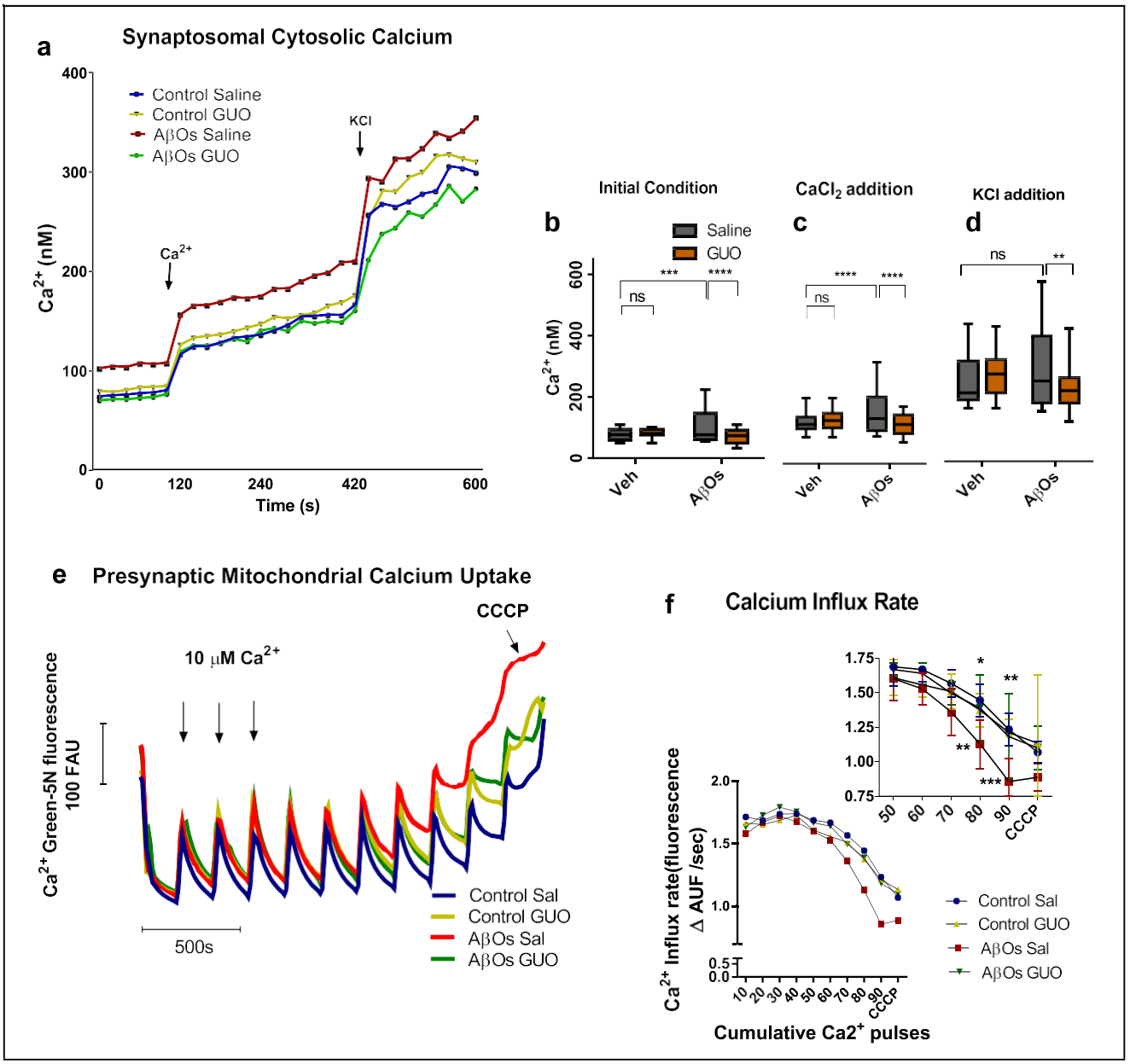


Fig. 5 GUO administration protected presynaptic mitochondria from calcium homeostasis disruption caused by AβOs injection. Synaptosomal cytosolic calcium (a) from the 4 groups increased during the experiment phases; the observed cytosolic Ca^{2+} increase caused by AβOs was abolished by GUO; data are plotted as mean. Each phase of this graphic is independently depicted and analyzed in b–d. In initial condition (b) (the first step of graphic A), AβOs increased synaptosomal cytosolic Ca^{2+} levels (***P= .0003), an effect abolished by GUO (****P < .0001); GUO had no effect; data are plotted as mean. After $CaCl_2$ addition (c), the cytosolic Ca^{2+} levels were additionally increased by AβOs (****P= < .0001), an effect abolished by GUO (****P= < .0001); data are plotted as mean ± SD. After depolarization with KCl addition (d), AβOs did not significantly affect the cytosolic Ca^{2+} levels (P= .0690); however, GUO reduced Ca^{2+} levels in AβOs group (P= .0010), without affecting cytosolic Ca^{2+} in control group (two-way ANOVA followed by Tukey's multiple correction test, n =5 biologically independent samples from 5 independent experiments); data are plotted as mean ± SD. Calcium uptake by isolated presynaptic mitochondria (e), using pulses of 10 μM Ca^{2+} every 3 min, represented by the peaks, while the influx of Ca^{2+} is represented by the slope (n =6 biologically independent samples from 5 independent experiments); data are plotted as mean. Calcium influx rate (f) was decreased by AβOs in 80 uM and 90 uM Ca^{2+} (**P= .0054 and ***P= .0006, respectively), an effect abolished by GUO (*P= .0404 and **P= .0057, respectively; two-way ANOVA followed by Tukey's multiple correction test, n =6 biologically independent samples from 6 independent experiments); data are plotted as mean ± SD

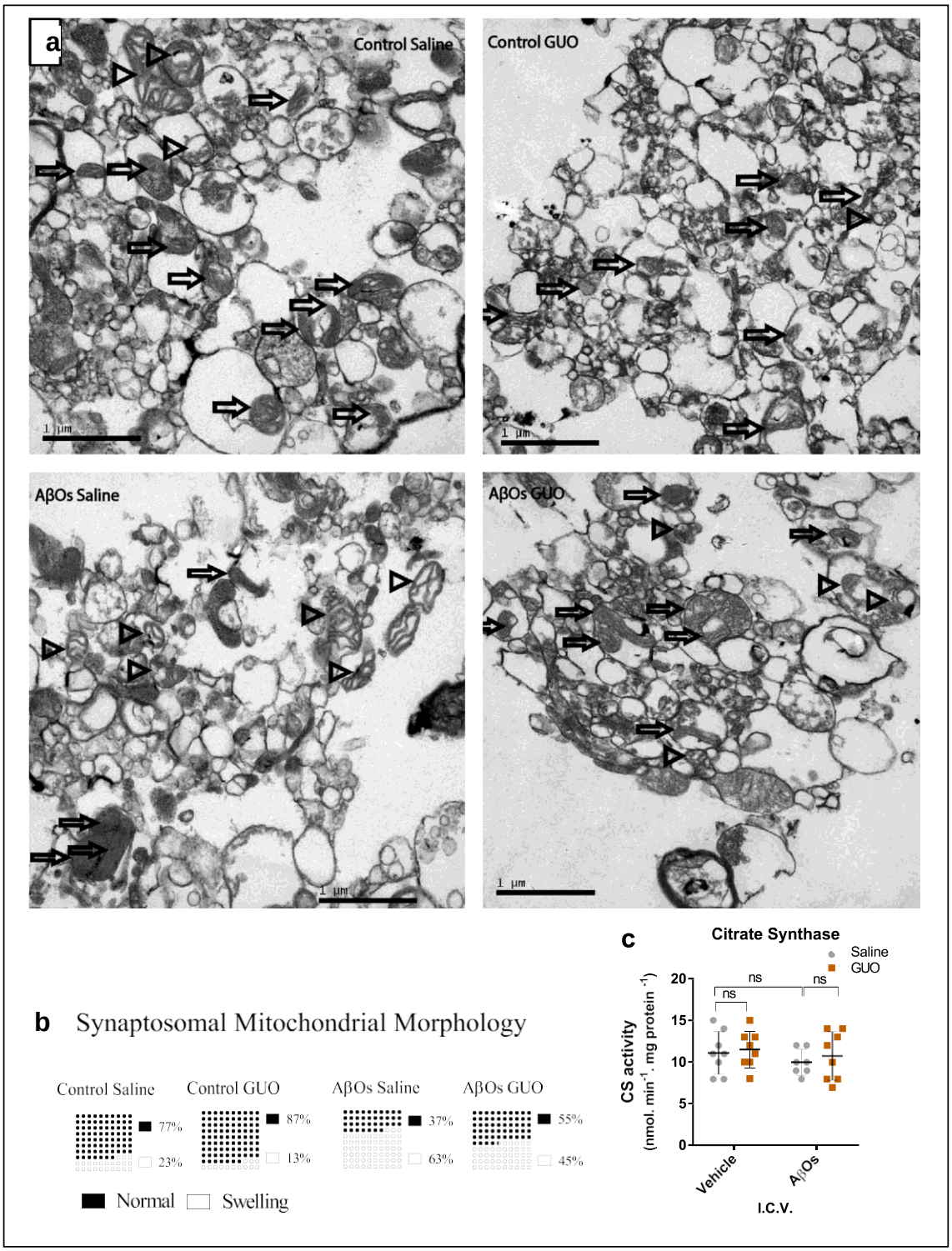


Fig. 6 GUO administration partially recovered mitochondrial swelling caused by AβOs injection. Mitochondrial morphology (a) from each group obtained by EM, showing mitochondrial with normal morphology (arrows) or with swelling (arrowhead). The analysis of EM images (b) points to an increased percentage of swelled mitochondria in the AβOs Sal group (63%) compared with control Sal (23%) and Control GUO (13%), an effect reduced by GUO (AβOs GUO group) (45%); 23 images from control saline ($n = 2$), 25 from AβOs saline ($n = 2$), 7 from GUO saline ($n = 1$), and 20 from AβOs GUO ($n = 2$) were analyzed. As indicated by citrate synthase activity (c), the total number of mitochondria in the images was similar among all groups (two-way ANOVA, followed by Tukey's multiple correction test, $n = 8$ biologically independent samples from 4 independent experiments); data are plotted as mean ± SD

while preserving antioxidant defense levels. Furthermore, GUO recovered mitochondrial Ca^{2+} buffering capacity by maintaining Ca^{2+} inflow, thus resulting in decreased Ca^{2+} cytosolic levels. For the first time, GUO, a purine with neuroprotective effects, was demonstrated to modulate the mitochondrial regulation of Ca^{2+} and reestablished presynaptic function related to OR learning and memory modulation, therefore counteracting the synaptotoxicity caused by AβOs. It is essential to highlight that this GUO neuroprotective effect was observed after oral administration, thus providing a feasible strategy for a translational study. Moreover, GUO presents possibilities for medications that can cross the BBB and especially those that could support the function of mitochondria in the synaptic region.

Materials and Methods

Animals

All animal experiments were performed under the principles of the laboratory of animal care from the National Research Institute and approved by the Federal University of Rio Grande do Sul (UFRGS) Animal Care and Use Committee (CEUA/UFRGS no. 30169). Swiss male mice (35–45 g), 3–4 months old, were housed in the animal room of the Federal University of Rio Grande do Sul (UFRGS), maintained in groups of 4 per individually ventilated cage (W × H × D: 391 × 199 × 160 mm), with free access to food and water, under a 12 h light/dark cycle, with controlled room temperature and humidity.

Preparation of Amyloid-β Oligomers

Oligomers were prepared using synthetic $A\beta_{1-42}$ peptide (genome) following the Lambert protocol [64]. Characterization of the oligomers was performed by HPLC size exclusion [3], and the preparations containing a mixture of high (80 to 150 kDa) and low (10 kDa) molecular weight were used for the subsequent experiments. The mobile phase consisted of PBS, pH 7.0, and the flow rate was 0.5 ml/min. Detection was performed using a fluorescence detector with excitation and emission wavelengths of 275 nm and 305 nm, respectively.

In Vivo Methodologies

a. Intracerebroventricular injection of AβOs, GUO treatment, and experimental design

Animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) before the intracerebroventricular (ICV) injection of AβOs, which was performed as previously described [3]. A final volume of 3.3 μl containing 10 pmol of AβOs or vehicle solution was injected into the brain's right second ventricle. Guanosine (Sigma, Cat# G6752) was diluted in a saline solution of 0.75 mg/ml, and 7.5 mg/kg was administered.

The determination of GUO dose was based on previous studies demonstrating 7.5 mg/kg as the lowest dose with efficient neuroprotective effect, with administration via gavage or i.p. in animal models of anxiety-related behavior [46, 65] and seizures [20, 66]. Animals received GUO or saline per gavage with a curved needle 1 h before the ICV injection of AβOs and then 1 h, 3 h, and 6 h afterward. Behavioral tasks and biochemical assays were performed 24 h and 48h after the injection of AβOs, respectively. The animals were euthanized by decapitation. The experimental design is illustrated in Fig. 7.

b. GUO levels

In naive animals, a 500 μl GUO solution containing 0.75 mg/ml GUO and 2 μl traced [8-^{3}H]GUO (American Radiolabeled Chemicals, Cat# ART0545, Conc. 1 mCi/ml) dissolved in saline was administered per gavage, while the control group received just saline. In a mouse group, blood samples were acquired under isoflurane anesthesia from the facial vein 5, 15, and 60 min after a single GUO dose, followed by euthanasia and brain dissection to obtain the hippocampus, cortex, cerebellum, and striatum. Other mouse group received 4 doses of 500 μl GUO solution in the same intervals used for treatment (Fig. 7) and had a blood sample and brain structures obtained only at 1 h after the last gavage. Blood samples were immediately centrifuged at 4 °C, 5000 rpm for 10 min. Plasma was separated from erythrocytes, and the last one was lysate using 200 μl of distilled water for 10 min and then frozen. Brain structures were homogenized in PBS, and protein was determined with a BCA assay kit (Thermo Fisher, Cat# 23225).

GUO was measured using 2 methodologies: (a) a scintillation counter into which samples of plasma, erythrocytes, and brain structures were transferred in vials containing liquid-scintillation for determination of [8-^{3}H]GUO levels (data were expressed as CPM/ml or CPM/mg) and (b) HPLC protocol in which samples were deproteinized with 1:5 volume methanol proportion, followed by lyophilization in a speed-vacuum centrifuge (10,000×*g*, 80 min, 4 °C), and then the pellet was diluted with saline for GUO determination in the HPLC as described previously [33].

Behavioral Tasks

Twenty-four hours after ICV injection of AβOs, the OR task was performed in an arena measuring 30 × 30 × 45 cm with reduced light intensity and recorded by a camera situated near the ceiling, using the ANY maze software (v6.17, Stoelting

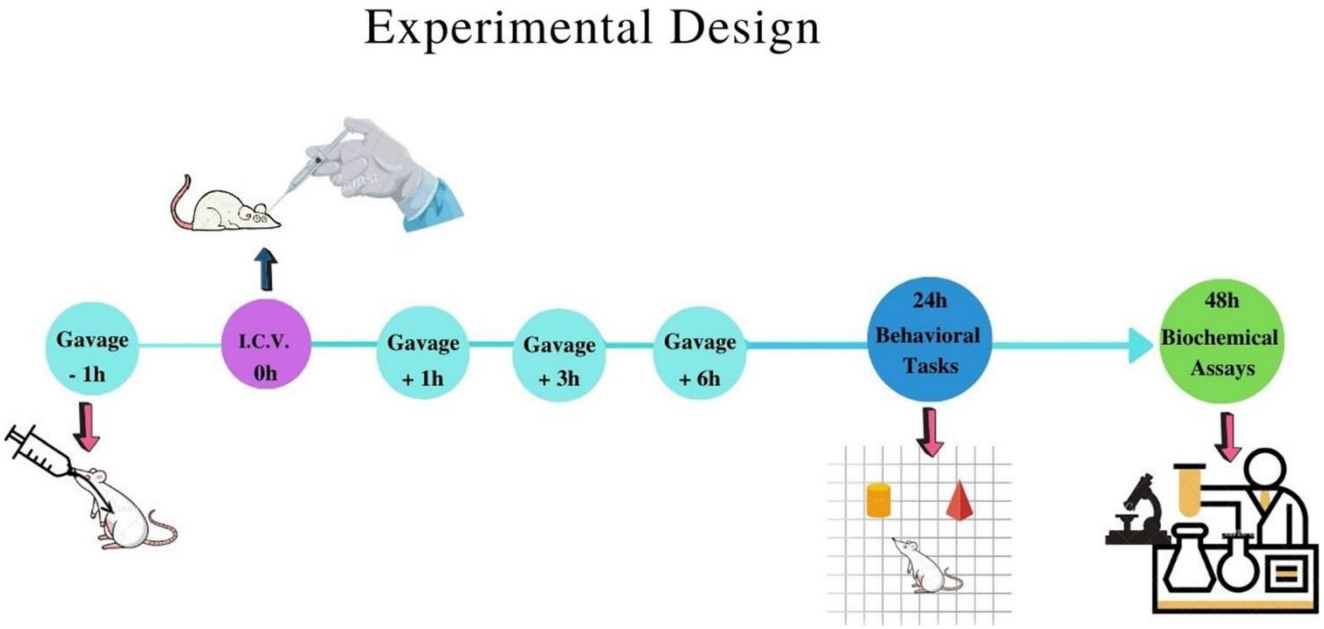


Fig. 7 Experimental design. Mice received an ICV injection in the left brain ventricle containing vehicle solution or AβOs. Treatment with saline or guanosine per gavage was administered: 1 h before (−) the ICV and then 1, 3, and 6 h after (+) the ICV. The behavioral tasks were performed 24 h after the ICV and the biochemical assays 48 h after the ICV

Co.). Animals were previously habituated to the behavioral room for at least 1 h. Afterward, they were placed into the arena for 5 min for habituation and space exploration (OFT). Then, the OR task was divided into two phases, each one lasting 5 min: (a) a training session in the presence of 2 similar objects that would not evoke natural preference for one of them and (b) a test session, 90 min after the training session, with 1 object from the previous phase (familiar object) and a new and different one (new object). Only animals that explored the objects for at least 20 s and did not present any preference for one of the objects in the training session were selected for the test session [67]. After the OR task, animals were returned to the home cage until the neurochemical experiments 24 h later.

Hippocampal Methodologies

a. Hippocampal slice preparation

Forty-eight hours after the injection of AβOs, the mice were euthanized, and hippocampi were rapidly dissected and immediately cut into 300 μm thick slices using a Mcllwain Tissue Chopper for subsequent measurement of glutamate uptake or glutamate oxidation to CO_2.

b. Glutamate uptake by hippocampal slices

Hippocampal slices were immediately immersed in HBSS solution with a pH of 7.2, at 4 °C, and glutamate uptake was performed following an adapted protocol [46]. Slices were pre-incubated with HBSS, at 37 °C for 15 min, followed by medium change and incubation in the presence of 0.2 μCi/ml L-[3,4-^{3}H] glutamic acid (American Radiolabeled Chemicals, Cat# 0132, Conc. 1 mCi/ml) for 5 min. The incubation was interrupted with 2 ice-cold washes using 1 ml of HBSS, followed by the immediate addition of 200 μl of 0.5 N NaOH and stored overnight. Na^+-independent uptake of glutamate was measured using an HBSS Na^+ free solution, and then Na^+-dependent glutamate uptake was considered the difference between the total uptake and the Na^+-independent uptake. Results are expressed as nMol of glutamate.

c. Glutamate oxidation by hippocampal slices

Glutamate oxidation was performed as previously described [68]. The whole sliced hippocampus was pre-incubated for 30 min at 37 °C in Dulbecco's buffer ($MgCl_2$ 0.5 mM; NaCl 108 mM; KCl 2.7 mM; NaH_2PO_4 17 mM; $CaCl_2$ 0.9 mM, Glucose 5 mM, pH 7.4), which was replaced by the same medium containing 0.2 μCi L-[^{14}C(U)] glutamic acid (PerkinElmer Radiochemicals, Cat# NEC290E250UC, Conc. 0.1 mCi/ml) and exposed to 95% O_2 and 5% CO_2 for 1 min. Before sealing the glass with a rubber cap, each flask received a glass well containing a filter paper strip 0.5 cm × 1 cm. This glass well was held by the cap over the solution with the tissue. The hippocampal slices were incubated for 1 h at 37 °C in a metabolic shaker (60 cycles/min). Without opening the flask, the reaction was interrupted with 0.2 ml of 50% tricarboxylic acid (TCA) added to the flask and 0.1 ml of 2 N NaOH in the central well to trap traced $^{14}CO_2$, and then the

entire structure was incubated for an additional 30 min. Thereafter, the central well content was transferred to a vial containing liquid-scintillation counter for $^{14}CO_2$ radioactivity reading. Data were expressed as nmol/mg of tissue.

d. Antioxidant defense levels and oxidative damage

The whole hippocampus was homogenized in PBS, pH 7.4, for the measurement of GSH, sulfhydryl content, DCFH, and TBARS, as described below.

Reduced glutathione (GSH) levels were measured according to the protocol [69]. Hippocampus homogenate (0.3 mg protein) was diluted in 20 volumes (1:20, v/v) of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA. One hundred microliters of this preparation was incubated with an equal volume of o-phthaldialdehyde (1 mg/ml methanol) at RT for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. The calibration curve was performed with standard GSH (0.001–0.1 mM), and GSH concentrations were reported as nmol/mg of protein.

The sulfhydryl content, a measure inversely correlated with protein oxidative damage, was measured by the generation of TNB from the reduction of 5-5-dithiobis (2-nitrobenzoic acid) (DTNB) by thiols, through the absorption at 412 nm as read in a spectrophotometer [70]. Shortly after, 30 μl of 10 mM DTNB and 980 μl of PBS were added to 50 μl of hippocampus homogenate (0.3 mg protein), followed by 30 min of incubation at RT while protected from light, and later the absorption was read at 412 nm. Results were reported as sulfhydryl content in nmol/mg of protein.

Total ROS production was evaluated by a 2′,7′-dihydrodichlorofluorescein (DCFH) oxidation assay following the previous protocol [71]. DCF-DA (5 μM) was prepared in the buffer (20 mM NaH_2PO_4, 140 mM KCl, pH 7.4), incubat- ed with the hippocampus homogenate for 60 min at 37 °C, and a calibration curve standard (from 0.25 to 10 μM DCF) was prepared. DCF-DA was enzymatically hydrolyzed by intracellular esterases generating non-fluorescent DCFH, which was rapidly oxidized into the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. Fluorescence was measured at 480 nm and 535 nm excitation and emission wavelengths, respectively. ROS levels were expressed as pmol DCF/mg of protein.

Lipid peroxidation was estimated as thiobarbituric acid reactive substances (TBA-RS) by measuring MDA levels following Yagi's method [72]. One hundred microliters of hippocampus homogenate was treated with 200 μl of 10% trichloroacetic acid and 300 μl of 0.67% thiobarbituric acid in 7.1% sodium sulfate and incubated for 2 h in a boiling water bath. After cooling down for 5 min, the resulting pink-stained mixture was extracted, and the fluorescence of the organic phase was read at 515 and 553 nm as excitation and emission wavelengths, respectively. A calibration curve was performed using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as the supernatants. TBA-RS levels were expressed as nmol MDA/mg protein.

e. Western blotting

Forty-eight hours after the injection of AβOs, the mice were euthanized and the hippocampi were rapidly dissected and immediately homogenized in RIPA buffer (Thermo Fischer Scientific, Waltham, USA) containing a protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, USA). The protein content was determined using the BCA assay kit, and samples were standardized in sample buffer (Bio-Rad, Hercules, USA) with the addition of 5% β-mercaptoethanol and heated at 90 °C for 10 min. Samples were separated by SDS-PAGE (10 μg protein/well) and transferred to a nitrocellulose membrane (GE Healthcare, Amersham, UK). Then, the membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 (TTBS) and 3% bovine serum albumin for 1 h at room temperature (RT). Subsequently, membranes were incubated with primary rabbit or mouse antibody overnight at 4 °C.

The antibodies used were Synaptotagmin (Abcam Cat# ab13259, 1:1000 dilution), SNAP-25 (Sigma Cat# ab9684, 1:1000 dilution), Syntaxin-1 (Abcam Cat# ab41453, 1:2000 dilution), p-GSK-3β (Cell Signaling Cat# 9336, 1:500 dilution), Bcl-2 (Cell Signaling Cat# 2870, 1: 500 dilution), PSD-95 (Abcam Cat# ab18258, 1:1000 dilution), 4HNE (Abcam Cat# ab48506, 1:1000 dilution), 3NT (Abcam Cat# ab61392, 1:1000 dilution), p-53 (Cell Signaling Cat# 9282, 1:1000 dilution), and β-tubulin (Abcam Cat# ab6046, 1:1000 dilution) and then washed with TTBS and incubated with secondary antibody horseradish peroxidase and conjugated donkey anti-rabbit (GE Healthcare Cat# NA934, 1:5000 dilution) or sheep anti-mouse (GE Healthcare Cat# NA9310, 1:5000 dilution) IgG 1 for 2 h at RT. Chemiluminescent bands were detected in the ImageQuant LAS4000 system (GE Healthcare, Amersham, UK) using an Immobilon™ Western chemiluminescence kit (Merck Millipore, Cat# WBKLS0500). Densitometric quantification was performed with ImageJ software (NIH). The results were expressed in percent of control levels after normalization using β-tubulin as an internal standard for protein load.

f. qPCR

For RNA purification, the hippocampus was homogenized in PBS and maintained in ice, and all RNA was extracted using Trizol (Thermo Fisher, Cat# 15596026) following the manufacture's protocol. First-strand cDNA was synthesized using a high-capacity cDNA transcription kit (Thermo Fisher, Cat# 4368814). After reverse transcription, the

cDNA was subjected to qPCR with a 7500 real-time PCR system (Applied Biosystems) using PowerUp SYBR green PCR master mix (Thermo Fisher, Cat# 4309155). Amplification was carried out in a total volume of 10 μl containing 0.2 μM of each primer, 5 μl of Power SYBR green master mix (2×), and 2 μl of cDNA (1:10). The PCRs were cycled 40 times after initial holding (50 °C, 2 min) and denaturation (95 °C, 2 min) with the following parameters: 95 °C for 3 s and 60 °C for 30 s. The absence of DNA contaminants was assessed in reverse transcriptase-negative samples. Product amplification and absence of primer-dimer were verified using the melting curve at the end of each run, by cooling the samples to 60 °C and then increasing the temperature to 95 °C at 0.1 °C/s. Relative quantification of gene expression was calculated with the $\Delta\Delta CT$ method, using the β-actin to normalize the data. Sequences of primers used are available upon request. Gene information was acquired from RefSeq (NIH, https://www.ncbi.nlm.nih.gov/refseq/). The primers PSD95, SNAP25, PGC1-α, SIRT3, SIRT1, OPA1, DMN1L, MFF, MFN2, GSK3β, VDAC1, NRF2, and BAX were designed using the design primer tool Primer-BLAST (NIH, https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and acquired from Microsynth (Switzerland). The characteristics of the primers are described in Table 1.

Mitochondrial Methodologies

a. Synaptosomal preparation

Forty-eight hours after injection of AβOs, the mice were euthanized, and the hippocampi were rapidly dissected. Hippocampal synaptosomal preparation was adapted from the protocol [73]. Each hippocampus was homogenized in a glass potter filled with 2 ml isolation sucrose buffer (0.32 M sucrose; 1 mM EDTA; 1 mM EGTA; 10 mM Tris-HCl; pH 7.4) and then centrifuged (1350×*g* for 3 min at 4 °C). The supernatant was diluted in isolation sucrose buffer and centrifuged (21,200×*g* for 10 min at 4 °C). The pellet was resuspended in 3 ml Percoll (GE Healthcare, Cat# 17089101) 15% and applied over a discontinuous Percoll gradient comprising 23% and 40% layers and centrifuged (30,700×*g* for 5 min at 4 °C, acceleration 6, deceleration 4). The enriched synaptosomal preparation band (between 15 and 23% layers—the presence of synaptosomes in this band can be seen in the electron microscopy image representation Fig. 6a) was transferred into another tube and rinsed with mannitol buffer (0.32 M mannitol, 1 mM EDTA; 1 mM EGTA; 10 mM Tris-HCl; BSA 0.2%; pH 7.4) in 2 more centrifugation at 4 °C (16,700×*g* for 10 min and 6900×*g* for 10 min), after which the pellet was collected. Samples (fresh synaptosomal preparations) were maintained in ice until the next experiments.

b. High resolution respirometry

The fresh synaptosomal preparation was transferred into a 2 ml chamber of the oxygraph-2k (O2k, Oroboros Instruments, Innsbruck, Austria) containing potassium buffer (100 mM KCl; 75 mM Mannitol; 25 mM sucrose; 5 mM KH_2PO_4; 0.05 mM EDTA; 10 mM Tris-HCl; BSA 0.1%; pH 7.4) in a final concentration of 50 μg protein/ml at 37 °C and initial O_2 concentration of around 200 μM. Respirometry was analyzed with the DatLab software (v6.1.0.7. Oroboros Instruments) Respiratory states were induced with the substrate-uncoupler inhibitor titration (SUIT) protocol, adapted from Pesta [74]: pyruvate 5 mM (P), malate 4 mM (M), and glutamate 10 mM (G). Oxidative phosphorylation (OXPHOS) activity was measured with ADP 500 μM and succinate 10 mM (S). Oligomycin 0.2 μg/ml (Omy, Sigma, Cat# O4876), an ATP synthase inhibitor, was used to determine O_2 consumption flux associated with ATP formation. Titration of the uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP, Sigma, Cat# C2759) 0.5 μM allowed verification of the electron transfer system (ETS), reaching maximum O_2 consumption. CI inhibition was obtained with rotenone 0.5 μM (R, Sigma, Cat# *R8875)*. Residual consumption (ROX) was induced with antimycin A 5 mM (Ama, Sigma, Cat# A8674).

Respiratory control ratio (RCR) was defined by the coupling state of mitochondria obtained by the division of the O_2 flux in the presence of substrates and ADP (OXPHOS) by the O_2 flux after Omy addition. OXPHOS capacity is referred to as oxidation coupled to phosphorylation in the presence of saturating O_2, ADP, and substrate concentration. Spare respiratory capacity (SRC) is the difference between the maximum respiratory capacity achieved under uncoupler titration (CCCP) and the OXPHOS state in the presence of substrates (PMGS) and ADP and is considered to have an ability to respond to increased energy demand in stress- ful conditions [75]. Proton leak is understood as respiration in the presence of substrates or incomplete coupled oxidative phosphorylation, without driving ATP synthesis.

c. H_2O_2 production

H_2O_2 production by fresh synaptosomal preparation was measured during the respirometry using the DatLab software. Amplex Red (Sigma, Cat# 90101) 10 μM was added to the chamber as an H_2O_2 sensitive probe together with horseradish peroxidase (HRP 1 U/ml) and superoxide dismutase (SOD 5 U/ml). H_2O_2 step addition of 0.1 μM was used for calibration at the beginning of the experiment [76]. Data were expressed as pmol fluorescence/mg protein.

Table 1 Primers description

Target	Gene reference	DNA sequence (5′-> 3′)	Product length (base pairs)	Tm (°C)
PPARGC1	XM_017320717.1	TGCTGAAACATGAAAGCAGTCAA	116	59.62
		ATTTTCAGGGAGTGTTGCCCA		60.13
VDAC1	NM_001362693.1	CCTACACGCCCTAGCCG	162	59.2
		GTATGGCTCCCTATGGGGTCT		60.49
DNM1L	XM_006522628.3	CCGGAGACCTCTCATTCTGC	95	59.90
		GGTTCCAGCTCTGAAATTTACCAT		59.29
MFN2	NM_001285920.1	AAACTTCTCCTCTGTTCCAGTTGT	111	60.08
		TCGAGAAAAGAGCAGGGACATC		60.09
MFF	XM_006496557.3	AGTAAACACCAGCAGGGTAGC	92	60.00
	XM_006496556.2	TGATGTCTCACTGCCCGTTT		59.60
SIRT1	NM_019812.3	TGTTGACCGATGGACTCCTCA	83	60.83
		ACTAGAGCTGGCGTGTGAC		59.41
BCL2	NM_009741.5	CTGAGTACCTGAACCGGCAT	70	59.46
		AGTTCCACAAAGGCATCCCA		59.52
OPA1	NM_001199177.1	GCTCAGAAGACCTTGCCAGT	140	59.96
		ACCTAACAAGAGAAGGGCCTC		59.09
SIRT3	NM_022433.2	ACTTCCGCTAAACTTCTCCCG	104	60.07
		GCTTGAGGCTACGTGACCAG		60.74
		GACTGGTCACGTAGCCTCAAG	104	60.4
		ACAGAGGGATATGGGCCTTCT		60.06
Bax	XM_011250780.2	AACTTCACAGGTTGGCATTAGGA	84	60.18
		TCCAGCCACAAAGATGGTCA		59.23
		TCAATAAACTTCACAGGTTGGCAT	91	59.17
		CTCCAGCCACAAAGATGGTCA		60.27
NRF2	NM_010902.4	AAAGCACAGCCAGCACATTC	85	59.68
		GGGATTCACGCATAGGAGCA		59.89
GSK3β	NM_001347232.1	CGGAGTTGTCCAGCCAATGA	84	60.32
	NM_019827.7	TTAAGGACGGCGAAACTCCC		60.04
		TTGGACAAAGGTCTTCCGGC	103	60.54
		AGCTTCCAGTGGTGTTAGCC		59.96
SNAP-25	NM_011428.3	ACGCATTGAGGAAGGGATGG	95	60.11
		ACACACAAAGCCCGCAGAAT		60.82
PSD-95 (Dlg4)	NM_007864.3	CTCCCCAACCAGGCCAATTC	86	60.97
		GTTCCGTTCACCTGCAACTC		59.41

PPARGC1 peroxisome proliferative activated receptor, gamma, coactivator 1 alpha, *VDAC1* voltage-dependent anion channel 1, *DNML1* Dynamin 1-like, *MFN2* Mitofusin 2, *MFF* mitochondrial fission factor, *SIRT1* Sirtuin 1, *BCL2* B cell leukemia/lymphoma 2; *OPA1* mitochondrial dynamin-like GTPase, *SIRT3* Sirtuin 3, *Bax* BCL2-associated X protein, *NRF2* nuclear factor, erythroid derived 2, *GSK3*β glycogen synthase kinase 3 beta, *SNAP 25* synaptosomal-associated protein 25, *PSD-95* postsynaptic density protein 95 (discs large MAGUK scaffold protein 4 (Dlg4)), *Tm* temperature melting in °C

d. ATP quantification

Net ATP produced by fresh synaptosomal preparation was quantified using HPLC. Samples were previously submitted to the respirometry assay in the O2k in an OXPHOS linked O_2 flux (P 5 mM; M 4 mM; G 10 mM; ADP 500 μM; S 10 mM). Then, the sample was promptly removed from the chamber 10 min after adding succinate and immersed in liquid nitrogen to stop ATP/ADP turnover reactions occurring in the synaptosome. Following sample deproteinization with $HClO_4$ (0.6 N), centrifugation (25,000×*g* for 10 min at 4 °C), pH adjustment to 7.0, and centrifugation (25,000×*g* for 30 min at 4 °C), samples were placed in an HPLC to determine the ATP levels. To determine the ATP source, a separate experiment was performed measuring ATP before and after ADP addition to the chamber (Fig. 4f).

e. Synaptosomal cytosolic calcium assay

Fresh synaptosomal preparations (0.35 mg protein/ml) were incubated for 30 min in buffer (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, 10 mM HEPES, 0.1% BSA, pH 7.4, and 0.1 mM $CaCl_2$) with Fura-2AM (Invitrogen, Cat# F1201)

5 μM at 37 °C and protected from light. After 30 min of incubation, samples were centrifuged (6900×*g*, for 2 min, at RT), rinsed with buffer, then pipetted in triplicate into a black bottom microplate, and read in a microplate fluoresce reader at excitation 340 and 380 nm/emission 505 nm wavelength. Five kinetic reading steps were followed: (1) basal, 2 min; (2) $CaCl_2$ 1.2 mM addition, 5 min; (3) KCl 30 mM addition, 3 min; (4) SDS 10%, 2 min; and (5) EGTA 100 mM, 2 min. Fura ratio was converted with the equation $(Ca^{2+}) = K_d \times [(R - R_{min})/(R_{max} - R)] \times Sf2/Sb2$, adapted from [77]. Data are expressed as nM Ca^{2+} concentration.

f. Calcium uptake by isolated presynaptic mitochondria

Mitochondria were isolated from synaptosomal preparations through permeabilization with digitonin (Sigma, Cat# D141), then centrifuged (12,000×*g* for 10 min at 4 °C), and rinsed with potassium buffer (KCl 150 mM, $MgCl_2$ 5 mM, HEPES 5 mM, KH_2PO_4 2 mM, BSA 0.1%). Then, these isolated mitochondria were added to a chamber containing buffer P 2.5 mM, M 2.5 mM, ADP 30 μM, calcium green (Invitrogen, Cat# C3737) 5 μM, and 10 μM EGTA [78]. Stepwise Ca^{2+} titration (10 μM) was used to analyze the presynaptic mitochondrial Ca^{2+} uptake capacity until its disruption or addition of uncoupler (CCCP 3 μM) to induce maximal Ca^{2+} release.

g. Citrate synthase (CS) activity

Quantification of CS activity was measured using synaptosomal preparation dilution 1:40, in Tris-HCl buffer 100 mM, DTNB 0.1 mM, acetyl-CoA 0.1 mM, Triton X-100 0.1%, and oxaloacetate 0.2 mM, in a reaction of DTNB, acetyl-CoA, and a thiol group, producing DTN as a fluorescent dye, read every 15 s in a 412 nm kinetic running experiment in a microplate reader (M5), at 25 °C. The result was adjusted for protein concentration. Data are expressed as nmol/min/mg protein.

h. Electron microscopy (EM)

Fresh synaptosomal preparation was fixed with a solution containing paraformaldehyde 4% and glutaraldehyde 2.5% in 0.1 M phosphate buffer (PB) at RT. Afterward, they were washed in PB and fixed in 1% osmium tetroxide, OsO_4 (Sigma) in PB, pH 7.4, for 1 h at RT, and then washed again with PB and gradually dehydrated with acetone (Merck) and soaked in EPON resin. The polymerization was carried out for 48 h at 60 °C. Ultrafine (70 nm) cuts were obtained in ultramicrotome for assembly on copper grids (200 mesh). The samples were counter- stained with 1% uranyl acetate (Merck) and then with 1% lead citrate (Merck) and examined in a transmission electron micrometer (JEM 1200 EXII, Japan).

Statistical Analysis

Comparisons were carried out using GraphPad Prism Software v6.0 (GraphPad), and one-way ANOVA followed by Dunnett's multiple comparison test was used for OFT analysis and column statistics, one sample *t* test with a hypothetical value 50% for OR task, and two-way ANOVA followed by Tukey's correction test for multiple comparisons for most of the experiments, as specified in the figure's legends. Data are represented as the mean ± SD. Differences were considered statistically significant at $P < .05$ (*), $P < .01$ (**), $P < .001$ (***), and $P < .0001$ (****).

Behavioral tasks were performed and analyzed by a blind investigator and preceded most of the biochemical experiments to certify the reproducibility of the model on short-term memory impairment. The other experiments were performed by not blinded investigators. Experiments were repeated at least 3 times, excepted for imaging acquisition in EM, which occurred just once using samples from a maximum of 2 independent experiments. Mitochondrial morphology was first evaluated by one blinded and one non-blind investigator.

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Compliance with Ethical Standards

All animal experiments were performed under the principles of the laboratory of animal care from the National Research Institute and approved by the Federal University of Rio Grande do Sul (UFRGS) Animal Care and Use Committee (CEUA/UFRGS no. 30169).

Conflict of Interests The authors declare that they have no conflict of interests.

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PARTE III

3. DISCUSSÃO

Nesse trabalho, buscamos verificar os efeitos da administração in vivo de oligômeros β-amiloide em tarefas comportamentais focadas na memória de objeto de curta duração e em parâmetros bioquímicos e energéticos do hipocampo e, de forma aprofundada, na mitocôndria pré-sináptica do hipocampo. Além disso, analisamos os efeitos neuroprotetores da GUO, a qual foi administrada por via oral (gavagem), medindo os níveis séricos da GUO e sua distribuição, de acordo com o tempo de administração e doses. Dessa forma, utilizamos um modelo experimental de DA que parte de uma abordagem in vivo, com injeção intracerebroventricular de AβOs e administração do tratamento in vivo com GUO por gavagem, passando pela avaliação comportamental em 24 h (em algumas abordagens até 28 dias) e análises bioquímicas em 48 h, explorando de forma gradativa e específica aspectos bioenergéticos do hipocampo, sinaptossoma e mitocôndria isolada da pré-sinapse envolvidos na disfunção causada pela toxicidade dos AβOs e nas vias de proteção da GUO.

No primeiro momento, avaliamos o tempo de ação dos AβOs na memória de RO de curto prazo e verificamos que o prejuízo dessa memória já está presente em 24h após a injeção ICV de AβOs e se estende por até 14 dias. Após esse período, observamos a recuperação espontânea da memória em 21 e 28 dias (quando ocorreu a última avaliação), permanecendo semelhante a dos animais controle. Trabalhos anteriores já haviam observado tempo de ação semelhante ao encontrado nesse estudo, utilizando a mesma dose de AβOs (10 ρMol) (FIGUEIREDO; CLARKE; LEDO; RIBEIRO *et al.*, 2013). A partir dessa curva de tempo de ação dos AβOs, passamos a utilizar o tempo de 24h após a indução para a avaliação

comportamental e 48h para as avaliações bioquímicas.

Para avaliar a absorção da GUO por via oral, utilizamos GUO marcada radioativamente ([8-^{3}H]GUO) e medimos os níveis no plasma e nas estruturas cerebrais. Nesse experimento, observamos que a GUO (e algum possível metabólito da GUO) está detectável no plasma já em 5 minutos após a administração oral, mantendo a mesma concentração 15 e 60 minutos após uma dose. Além disso, verificamos que ocorreu passagem de GUO radioativa pela BHE, chegando às diferentes estruturas: córtex cerebral, hipocampo, cerebelo e estriado, em concentrações semelhantes. Quando avaliamos a concentração de GUO radioativa após 4 doses (seguindo os mesmos intervalos utilizados com GUO não radioativa no protocolo de indução do modelo), encontramos níveis muito aumentados no plasma e nas estruturas cerebrais, em comparação aos níveis subsequentes a uma única dose. Com esses achados, conseguimos esclarecer que a GUO administrada por via oral tem rápida disponibilidade no plasma, mantém a concentração estável 1h após a administração e têm seus níveis aumentados com o aumento do número de administrações. Além disso, um aspecto muito importante para o nosso modelo e estratégia de tratamento, a GUO atravessa a barreira hematoencefálica e se distribui nas estruturas cerebrais.

Em tarefas comportamentais, a GUO recuperou o dano causado pelos AβOs na memória de curto prazo. Contudo, nesse mesmo experimento, observamos que a GUO per se causa amnésia. Esse efeito já havia sido apontado em trabalhos anteriores e atribuído à modulação do sistema glutamatérgico pela GUO, reduzindo o estímulo fisiológico dos receptores glutamatérgicos, principalmente NMDA (FRIZZO; ANTUNES SOARES; DALL'ONDER; LARA *et al.*, 2003; FRIZZO; SCHWALM; FRIZZO; SOARES *et al.*, 2005; ROESLER; VIANNA; LARA; IZQUIERDO *et al.*, 2000; SCHMIDT; LARA; DE FARIA MARASCHIN; DA SILVEIRA PERLA *et al.*, 2000; VINADÉ; SCHMIDT; FRIZZO; IZQUIERDO *et al.*,

2003; VINADÉ; SCHMIDT; FRIZZO; PORTELA *et al.*, 2005). Contudo, esses mesmos trabalhos e outros do nosso grupo mostraram que a GUO é protetora na presença de injúria cerebral, reduzindo ou evitando as consequências da excitotoxicidade, dano oxidativo e morte neuronal nos modelos de doenças cerebrais, além de recuperar prejuízos observados em tarefas comportamentais (ALMEIDA; COMASSETO; RAMOS; HANSEL *et al.*, 2017; CITTOLIN-SANTOS; DE ASSIS; GUAZZELLI; PANIZ *et al.*, 2017; HANSEL; RAMOS; DELGADO; SOUZA *et al.*, 2014; HANSEL; TONON; GUELLA; PETTENUZZO *et al.*, 2015; LANZNASTER; MACK; COELHO; GANZELLA *et al.*, 2017; PANIZ; CALCAGNOTTO; PANDOLFO; MACHADO *et al.*, 2014; TEIXEIRA; ALMEIDA; ROHDEN; MARTINS *et al.*, 2018). Nesse sentido, o efeito amnésico per se da GUO se diferencia do dano causado pelos AβOs na memória de curto prazo, pois não houve piora da memória causada pela GUO na presença de AβOs. Ao contrário, ocorreu recuperação da memória de curto prazo relacionada à tarefa de RO, indicando o efeito neuroprotetor.

Também observamos que em 48 h os AβOs reduziram a captação e a oxidação do glutamato. Estudos prévios indicam a excitotoxicidade glutamatérgica causada pelos AβOs como uma das vias de disfunção neuronal, dano mitocondrial, estresse oxidativo e morte celular na DA (ALBERDI; SANCHEZ-GOMEZ; CAVALIERE; PEREZ-SAMARTIN *et al.*, 2010; HUANG; TONG; LEI; ZHOU *et al.*, 2017; KABOGO; RAUW; AMRITRAJ; BAKER *et al.*, 2010; LI; HONG; SHEPARDSON; WALSH *et al.*, 2009; LI; JIN; KOEGLSPERGER; SHEPARDSON *et al.*, 2011b; PARAMESHWARAN; DHANASEKARAN; SUPPIRAMANIAM, 2008; SCIMEMI; MEABON; WOLTJER; SULLIVAN *et al.*, 2013; SIVANESAN; TAN; RAJADAS, 2013; TANAKA; SAKAGUCHI; HIRANO, 2019; TONG; ZHANG; MENG; XU *et al.*, 2017). Nesse contexto, a GUO recuperou a captação de glutamato que havia sido reduzida pelos AβOs, sem modificar a oxidação desse substrato. Porém, a GUO

também causou redução da captação de glutamato per se, efeito que também pode ser atribuído à modulação do sistema glutamatérgico com menor disponibilidade de glutamato na fenda sináptica (ALMEIDA; COMASSETO; RAMOS; HANSEL *et al.*, 2017; FRIZZO; LARA; PROKOPIUK; VARGAS *et al.*, 2002; VINADÉ; SCHMIDT; FRIZZO; IZQUIERDO *et al.*, 2003). Contudo, devemos ressaltar que o protocolo do nosso estudo utiliza um modelo in vivo, com análise ex vivo 48 h após a exposição aos AβOs e à GUO, ou seja, o tempo de exposição ao agente tóxico e ao tratamento é in vivo e mais prolongado do que a maioria dos protocolos e, dessa maneira, pode apresentar modulação diferente do sistema glutamatérgico.

Apesar das alterações no metabolismo do glutamato, não observamos dano oxidativo pelos AβOs nesse modelo de DA. Observamos que a oxidação de DCFH (2,7-Diclorofluoresceína) e subprodutos da peroxidação lipídica (TBARS - substâncias reativas ao ácido tiobarbitúrico) permaneceram semelhantes entre os grupos. Contudo, houve redução das defesas antioxidantes, na forma de GSH e do conteúdo de sulfidrilas, sendo que a GUO apresentou tendência de recuperação do conteúdo de GSH e recuperou os níveis de sulfidrilas. Conforme estudos anteriores, um dos mecanismos de neuroproteção da GUO ocorre por meio da ativação ou aumento dos níveis da enzima heme-oxigenase-1, a qual está envolvida na formação da glutationa, reforçando os efeitos antioxidantes da GUO (DAL-CIM; MOLZ; EGEA; PARADA *et al.*, 2012; SOUZA; BELLAVER; BOBERMIN; SOUZA *et al.*, 2016).

Uma vez que na DA ocorre perda substancial de proteínas sinápticas envolvidas na modulação de memória e plasticidade sináptica, como PSD-95, sinaptofisina, complexo SNARE, SNAP-25, bcl-2, bax, NRF-2, GSK-3β (ADAMS; SHEN; LEVENGA; BASTA *et al.*, 2017; BRADLEY; PEINEAU; TAGHIBIGLOU; NICOLAS *et al.*, 2012; CATTERALL; FEW, 2008; DAROCHA-SOUTO; COMA; PÉREZ-NIEVAS; SCOTTON *et al.*, 2012; DINKOVA-KOSTOVA; ABRAMOV, 2015; GYLYS; FEIN; YANG; WILEY *et al.*, 2004; KUDO; LEE;

SMITH; ZHU *et al.*, 2012; LLORENS-MARTIN; JURADO; HERNANDEZ; AVILA, 2014; POIREL; MELLA; VIDEAU; RAMET *et al.*, 2018; SIVANESAN; TAN; RAJADAS, 2013; TAMPELLINI; CAPETILLO-ZARATE; DUMONT; HUANG *et al.*, 2010; YUKI; SUGIURA; ZAIMA; AKATSU *et al.*, 2014) e nas funções de bioenergética e dinâmica mitocondrial, incluindo DRP1, OPA1, SIRT1, SIRT3, VDAC1, DNM1L, MFN2, MFF e BCL-2 (ALAVI; FUHRMANN, 2013; BAEK; PARK; JEONG; KIM *et al.*, 2017; BIRNBAUM; BALI; RAJENDRAN; NITSCH *et al.*, 2015; DOROSTKAR; ZOU; BLAZQUEZ-LLORCA; HERMS, 2015; EBENEZER; WEIDNER; LEVINE; MARKESBERY *et al.*, 2010; GYLYS; FEIN; YANG; WILEY *et al.*, 2004; LAUREN; GIMBEL; NYGAARD; GILBERT *et al.*, 2009; LEE; KIM; LIU; HWANG *et al.*, 2018; LIN; CHENG; TAMMINENI; XIE *et al.*, 2017; PARADIS; DOUILLARD; KOUTROUMANIS; GOODYER *et al.*, 1996; SHANKAR; BLOODGOOD; TOWNSEND; WALSH *et al.*, 2007; SIVANESAN; TAN; RAJADAS, 2013; SMILANSKY; DANGOOR; NAKDIMON; BEN-HAIL *et al.*, 2015; SU; WANG; BONDA; PERRY *et al.*, 2010; WANG; GUO; LU; SUN *et al.*, 2016), verificamos o conteúdo e/ou expressão gênica dessas proteínas em nosso modelo e observamos que não ocorreram alterações. Atribuímos esse resultado ao modelo de exposição aos AβOs com duração menor e características diferentes dos modelos animais transgênicos ou mesmo pacientes com DA. Além disso, podemos indicar que a dose utilizada (3,3 μL contendo 10 pmol) gerou alterações bioquímicas e sinápticas, inclusive disfunção mitocondrial (CAI; TAMMINENI, 2017), mas não foi suficiente para causar mudanças nos genes e proteínas que compõem a estrutura mitocondrial e sináptica.

Diversos estudos vêm evidenciando a disfunção mitocondrial como aspecto central no desenvolvimento da DA, apresentando perda do potencial de membrana, redução da eficiência respiratória, insuficiência energética e aumento da produção de espécies reativas de oxigênio,

além de desregulação da homeostase do Ca^{2+} (CADONIC; SABBIR; ALBENSI, 2016; CAI; TAMMINENI, 2017; HU; TAN; TAN; YU, 2016). Nesse sentido, modelos animais com DA indicam a toxicidade dos AβOs como evento desencadeante da disfunção mitocondrial, iniciando uma cascata de eventos que culmina em dano neuronal (DU, H.; GUO, L.; YAN, S.; SOSUNOV, A. A. *et al.*, 2010; REDDY; BEAL, 2008; WANG; GUO; LU; SUN *et al.*, 2016; YAO; IRWIN; ZHAO; NILSEN *et al.*, 2009).

Em nosso modelo de DA, observamos que os AβOs causaram a perda da capacidade respiratória reserva, que é um mecanismo mitocondrial de compensação energética na presença de fatores que levam à menor produção de ATP ou ineficiência energética, ocorrendo principalmente em condições de grande estresse celular, permitindo a sobrevivência da célula (NICHOLLS, 2008). O uso da capacidade reserva ocorre, por exemplo, em condições de excitotoxicidade glutamatérgica e sobrecarga de Ca^{2+} (NICHOLLS, 2009b). Contudo, a demanda energética de ATP pode suplantar o rendimento dessa capacidade, levando à exaustão mitocondrial e dano oxidativo e, dessa forma, aumentar a vulnerabilidade celular aos processos neurodegenerativos (DESLER; HANSEN; FREDERIKSEN; MARCKER *et al.*, 2012; NICHOLLS, 2008).

Além disso, os resultados mostram que os AβOs reduziram os níveis de ATP, o que sugerimos ser resultado de prejuízo na formação não-OXPHOS de ATP e maior consumo energético pelo sinaptossoma em outras funções mitocondriais e sinápticas (BILLUPS; FORSYTHE, 2002; FLYNN; CHOI; DAY; GERENCSER *et al.*, 2011; MNATSAKANYAN; BEUTNER; PORTER; ALAVIAN *et al.*, 2017), uma vez que a fosforilação oxidativa e outros estados respiratórios estão mantidos, exceto a capacidade reserva. Simultaneamente, houve aumento da produção de peróxido de hidrogênio pelo sinaptossoma durante a respirometria. Essa produção de ROS combinada com a redução dos níveis de ATP e uso da capacidade

reserva apontam para a disfunção mitocondrial em nosso modelo. Contudo a GUO não recuperou a capacidade reserva, a formação de ATP, nem evitou a produção de H_2O_2.

AβOs também causaram disfunção no tamponamento do Ca^{2+} pelas mitocôndrias pré-sinápticas, causando elevação dos níveis de Ca^{2+} no citosol do sinaptossoma. A atribuição específica às mitocôndrias pré-sinápticas pela desregulação da homeostase do Ca^{2+} pelos AβOs, administrados in vivo 48h antes, foi corroborada por experimento utilizando apenas mitocôndrias isoladas do sinaptossoma hipocampal. Essas mitocôndrias foram desafiadas com pulsos repetidos de Ca^{2+}. Nesse experimento observamos que os AβOs causaram redução do influxo de Ca^{2+} pelas mitocôndrias e essas organelas suportaram por menos tempo os pulsos de Ca^{2+} em comparação com o grupo controle, chegando ao extravasamento precoce do conteúdo de Ca^{2+}. De forma surpreendente, a GUO manteve o influxo de Ca^{2+} e o tempo de retenção de forma semelhante ao grupo controle, protegendo a função mitocondrial de homeostase do Ca^{2+}.

Vários estudos associam o influxo de Ca^{2+} principalmente ao MCU (LLORENTE-FOLCH; RUEDA; PARDO; SZABADKAI *et al.*, 2015; MARLAND; HASEL; BONNYCASTLE; COUSIN, 2016; RUEDA; LLORENTE-FOLCH; AMIGO; CONTRERAS *et al.*, 2014) e a liberação maciça de Ca^{2+} ao mPTP (JADIYA; KOLMETZKY; TOMAR; DI MECO *et al.*, 2019; MÜLLER; AHUMADA-CASTRO; SANHUEZA; GONZALEZ-BILLAULT *et al.*, 2018). Em nosso estudo não avaliamos diretamente o transportador e o poro, porém medimos a taxa de influxo de Ca^{2+}, função que sugerimos se relacionar à atividade do MCU, que mostrou redução gradativa pelos AβOs, enquanto a liberação antecipada de Ca^{2+} no grupo AβOs, pode ser relacionada à abertura do mPTP ou mesmo ruptura das membranas mitocondriais.

Uma vez que o Ca^{2+} está diretamente envolvido nos ciclos das vesículas pré-sinápticas e nas vias de sinalização de modulação da memória, inclusive as LTP e LTD (BILLUPS;

FORSYTHE, 2002; MARLAND; HASEL; BONNYCASTLE; COUSIN, 2016; SUDHOF, 2004; ZÜNDORF; REISER, 2011), o desbalanço causado pelos AβOs pode interferir em todas essas funções, contribuindo para o déficit cognitivo presente no nosso modelo animal (LI; HONG; SHEPARDSON; WALSH *et al.*, 2009; SIVANESAN; TAN; RAJADAS, 2013; ZÜNDORF; REISER, 2011). Por outro lado, a neuroproteção promovida pela GUO em nosso modelo teve o potencial de prevenir ou recuperar os danos causados pelos AβOs, protegendo a memória de curto prazo relacionada ao RO. Enfatizamos que esse é o primeiro estudo apontando o efeito neuroprotetor da GUO envolvendo a homeostase do Ca^{2+} pela mitocôndria pré-sináptica.

Outro aspecto relevante em nosso trabalho foi o estudo morfológico das mitocôndrias pré-sinápticas. Observamos importante edema de membrana e distorção da morfologia mitocondrial causados pelos AβOs, sendo que a GUO foi capaz de reduzir o percentual de mitocôndrias morfologicamente alteradas. Outros trabalhos com modelos de DA já haviam avaliado as mudanças morfológicas mitocondriais em modelos de DA, relacionando o edema mitocondrial com sobrecarga de Ca^{2+}, ruptura da membrana externa e liberação de citocromo c e fatores pró-apoptóticos (CALVO-RODRIGUEZ; HOU; SNYDER; KHARITONOVA *et al.*, 2020; DU, HENG; GUO, LAN; YAN, SHIQIANG; SOSUNOV, ALEXANDER A. *et al.*, 2010; DU, H.; GUO, L.; YAN, S.; SOSUNOV, A. A. *et al.*, 2010; DU; GUO; ZHANG; RYDZEWSKA *et al.*, 2011; DU; YAN, 2010; FAIZI; SEYDI; ABARGHUYI; SALIMI *et al.*, 2016; MOREIRA; CARVALHO; ZHU; SMITH *et al.*, 2010; WINBLAD; AMOUYEL; ANDRIEU; BALLARD *et al.*, 2016). Apesar dessa importante alteração morfológica, não identificamos mudanças significativas na expressão de genes e no conteúdo de proteínas relacionadas à dinâmica mitocondrial, adaptações bioenergéticas ou de indução de apoptose.

Em resumo, a dificuldade em manter o tamponamento de Ca^{2+} e os níveis de ATP, além

da redução da capacidade respiratória reserva com maior produção de H_2O_2 indicam a disfunção mitocondrial desencadeada pelos AβOs, culminando em importante alteração morfológica. Enquanto isso, a GUO foi capaz de proteger algumas funções mitocondriais essenciais para a memória e função sináptica.

Esquema final do trabalho

Os AβOs:	**A GUO:**
• causam déficit de memória de curto prazo em tarefas de RO; • promovem as condições que levam a excitotoxicidade glutamatérgica • reduzem as defesas antioxidantes no hipocampo; <u>Na região pré-sináptica:</u> • comprometem as funções mitocondriais com diminuição da capacidade respiratória reserva • reduzem os níveis de ATP • estimulam a formação de H_2O_2 durante a respiração • alteram a regulação do Ca^{2+}	• atravessa a BHE após a administração via oral • recupera o déficit de memória causado pelos AβOs; • restaura a captação de glutamato e os níveis de defesas antioxidantes no hipocampo; • na região sináptica: recupera a homeostase do Ca^{2+} e evita as alterações morfológicas (edema mitocondrial) induzidas pelos AβOs.

4. CONCLUSÃO

Nesse trabalho observamos que os AβOs administrados por injeção ICV causaram déficit na memória de curto prazo de reconhecimento de objeto (em 24 h) e a GUO recuperou esse dano. Em 48 h, observamos alteração no metabolismo do glutamato, com redução da captação de forma acentuada e redução na oxidação de glutamato pelos AβOs – ambos fatores que contribuem para a excitotoxicidade glutamatérgica -, além de causar redução nas defesas antioxidantes, sem causar dano oxidativo. Nas pré-sinapses de hipocampo, funções mitocondriais essenciais para o funcionamento adequado da sinapse foram afetadas pelos AβOs, apresentando redução da capacidade respiratória reserva, redução da formação de ATP, aumento da produção de peróxido de hidrogênio, desregulação da homeostase do Ca^{2+} e edema mitocondrial. A GUO, por sua vez, recuperou a captação de glutamato na presença dos AβOs, porém reduziu a captação per se e não alterou a oxidação de glutamato. Na avaliação mitocondrial, observamos que a GUO apresentou importante efeito protetor ao manter o influxo mitocondrial de Ca^{2+} e reduzir os níveis citosólicos desse íon, além de reduzir o edema de mitocôndrias sinaptossomais.

Dessa forma, entendemos que a GUO apresentou importante mecanismo de neuroproteção na mitocôndria pré-sináptica de hipocampo. Efeito que até então não havia sido verificado em outros modelos utilizando a GUO e que, combinado à recuperação de outros parâmetros bioquímicos observados, pode ter contribuído para a recuperação da memória de curto prazo observada em nosso modelo.

5. PERSPECTIVAS

- Verificar o tempo de ação dos AβOs na disfunção mitocondrial – 14 e 28 dias;
- Compreender a interação dos receptores de adenosina no efeito neuroprotetor da GUO nesse modelo;
- Avaliar o possível sítio de ação da GUO na mitocôndria e/ ou 2ºs mensageiros que integram as vias de neuroproteção da GUO;
- Medir os metabólitos da GUO no plasma, eritrócitos, e estruturas cerebrais;
- Investigar a disfunção mitocondrial, inclusive regulação do Ca^{2+}, potencial de membrana e uso de substratos energéticos em outros modelos de injúrias cerebrais envolvendo a excitotoxicidade glutamatérgica;
- Estudar o efeito da GUO na modulação do transportador uniporter de Ca^{2+} (MCU) e do poro de permeabilidade transitório mitocondrial (mPTP)
- Verificar a interação da GUO com ciclofilina D em relação ao funcionamento do mPTP (DU; GUO; ZHANG; RYDZEWSKA *et al.*, 2011);

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ANEXO I

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LISTA DE FIGURAS