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Tese de Doutorado

Papel da micróglia nas disfunções cognitivas relacionadas às alterações no metabolismo do colesterol: evidências experimentais

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"*A ignorância gera mais confiança do que o conhecimento: são os que sabem pouco, e não os que sabem muito, que afirmam positivamente que esse ou aquele problema nunca pode ser resolvido pela ciência.*"

Charles Darwin

À minha mãe e ao meu pai,

por todo amor, educação, carinho,

compreensão e pelos puxões de orelha

que me fazem ser uma pessoa melhor.

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APRESENTAÇÃO

Esta tese está organizada em três **Partes**, cada uma sendo constituída dos seguintes itens:

Parte I: Resumo, Resumo em inglês (Abstract), Lista de abreviações, Introdução e Objetivos;

Parte II: Resultados escritos na forma de artigo, precedidos de um breve prefácio introdutório.

Parte III: Discussão, Conclusão, Anexos e Referências bibliográficas citadas na Introdução da Parte I e Discussão da Parte III.

Os trabalhos que resultaram na elaboração desta tese foram desenvolvidos no Laboratório de Investigação em Doenças Metabólicas e Doenças Neurodegenerativas (LABIMN), no Departamento de Bioquímica da Universidade Federal do Rio Grande do Sul (UFRGS), sob orientação da Professora Drª. Jade de Oliveira, como também no Engblom's Laboratory da Universidade de Linköping (Linköping, Suécia), sob orientação da Professora Drª. Andreza Fabro de Bem e do Professor Dr. David Engblom.

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PARTE I

Resumo

O colesterol é um álcool não saturado pertencente à classe dos compostos esteroides que desempenham inúmeras funções biológicas. No entanto, em torno de 40% dos adultos têm níveis plasmáticos altos de colesterol, tornando a hipercolesterolemia um problema de saúde pública. A hipercolesterolemia pode ser adquirida ou de origem genética. Evidências clínicas e experimentais mostram que a hipercolesterolemia contribui para o desenvolvimento de neuropatologias, como a doença de Alzheimer (DA). Mecanismos como a disfunção da barreira hematoencefálica (BHE) e o aumento da reatividade microglial parecem conectar as alterações na homeostase do colesterol com as disfunções cerebrais características das doenças neurodegenerativas. As micróglias são as células imunes inatas cerebrais e o aumento de sua reatividade desempenha um papel crucial no desenvolvimento da neuroinflamação, e posteriormente da morte neuronal característica dos processos neurodegenerativos. Por este fato, a modulação das micróglias se mostrou benéfica nas neuropatologias, incluindo a DA. De particular importância, a hipercolesterolemia parece exercer efeito direto sobre as células microgliais. Sendo assim, a presente tese teve como principal objetivo estudar o papel das micróglias nos danos cognitivos relacionados à hipercolesterolemia adquirida e genética. Para isso, em um primeiro momento estudou-se o papel da micróglia nos danos de memória em camundongos nocautes para o receptor da lipoproteína de baixa densidade ($LDLr^{-/-}$) adultos jovens e de meia-idade. Posteriormente, administramos a minociclina, um potencial modulador farmacológico das micróglias, em camundongos $LDLr^{-/-}$ adultos. Por fim, camundongos CF-1 adultos jovens expostos à dieta rica em colesterol, foram tratados durante 4 semanas com a minociclina. Parâmetros metabólicos, bioquímicos, e comportamentais foram avaliados para melhor investigar o envolvimento das micróglias nos efeitos deletérios da hipercolesterolemia, seja ela de origem genética ou adquirida, no sistema nervoso central (SNC). Camundongos $LDLr^{-/-}$ adultos jovens e de meia-idade apresentaram aumento da microgliose e alterações morfológicas nas micróglias, compatíveis com fenótipos mais reativos em estruturas cerebrais importantes para o processo de memória. Além disso, foi observado um aumento da presença das micróglias na região perivascular hipocampal nos camundongos $LDLr^{-/-}$. Este processo esteve associado a alterações no imunoconteudo de proteínas sinápticas e de integridade da BHE. Ainda, a intervenção farmacológica com a minociclina melhorou a memória dependente do hipocampo dos animais $LDLr^{-/-}$ adultos, e reduziu a presença da micróglia na região perivascular. Efeitos benéficos do tratamento com a minociclina também puderam ser observados nos camundongos CF-1 com hipercolesterolemia induzida por dieta, onde observamos melhora na memória, em marcadores de integridade da BHE e na neuroinflamação, mas não na densidade, morfologia e atividade microglial. Portanto, nossos resultados sugerem que as micróglias contribuem para os déficits de memória relacionados aos distúrbios do metabolismo do colesterol, principalmente nos camundongos $LDLr^{-/-}$.

Palavras-chaves: Camundongos $LDLr^{-/-}$, Cognição, Hipercolesterolemia, Neuroinflamação, Micróglias, Receptores de LDL.

Abstract

Cholesterol is an unsaturated alcohol belonging to the class of steroid compounds that play numerous biological roles. However, around 40% of adults have high plasma cholesterol levels, making hypercholesterolemia a public health issue. Hypercholesterolemia can be acquired or of genetic origin. Clinical and experimental evidence indicates that hypercholesterolemia contributes to the development of neuropathologies, such as Alzheimer's disease (AD) and Parkinson's disease (PD). Mechanisms such as blood-brain barrier (BBB) dysfunction and increased microglial reactivity appear to link changes in cholesterol homeostasis with the cerebral dysfunctions' characteristic of neurodegenerative diseases. Specifically, microglia are innate immune cells in the brain, and their reactivity plays a crucial role in the development of neuroinflammation, followed by the characteristic neuronal death in neurodegenerative processes. Because of this, modulating microglial reactivity has proven beneficial in neuropathologies, including AD. Of particular importance, hypercholesterolemia seems to directly affect microglial cells. Therefore, the main objective of this thesis was to study the role of microglial reactivity in cognitive impairments related to acquired and genetic hypercholesterolemia. To achieve this, we initially investigated the role of microglia in memory impairments in young and middle-aged adult mice with a knockout of the low-density lipoprotein receptor ($LDLr^{-/-}$). Subsequently, we administered minocycline, a pharmacological modulator of microglial reactivity, to adult $LDLr^{-/-}$ mice. Finally, young adult CF-1 mice exposed to a high-cholesterol diet were treated with minocycline for 4 weeks. Metabolic, biochemical, and behavioral parameters were assessed to further investigate the involvement of microglia in the detrimental effects of hypercholesterolemia, whether of genetic or acquired origin, on the central nervous system (CNS). Young and middle-aged adult $LDLr^{-/-}$ mice exhibited increased microgliosis and morphological changes in microglia, consistent with more reactive phenotypes, in brain structures important for memory processes. Furthermore, adult $LDLr^{-/-}$ presented increased presence of microglia in the hippocampal perivascular area. This process was associated with alterations in the immunocontent of synaptic proteins and claudin-5, a tight-junction protein. Furthermore, pharmacological intervention with minocycline improved hippocampal-dependent memory performance in adult $LDLr^{-/-}$ mice and reduced microglial presence in the perivascular region. Beneficial effects of minocycline treatment were also observed in CF-1 mice with diet-induced hypercholesterolemia, where improvements in memory, BBB integrity markers, and neuroinflammation were observed. Thus, our findings suggest that microglial reactivity contributes to the progression and establishment of memory deficits related to cholesterol metabolism disorders.

Keywords: Cognition, Hypercholesterolemia, LDL receptors, $LDLr^{-/-}$ mice, Microglia, Neuroinflammation.

Lista de abreviaturas

Aβ	Peptídeo β-amiloide
ACAT	Acil-CoA colesteril aciltransferase
APP	Proteína precursora amiloide
AQP4	Aquaporina - 4
ATP	Adenosina trifosfato
BAX	Proteína X associada ao BCL-2
BCL-2	Linfoma de células B2
BHE	Barreira Hematoencefálica
CA1	*Cornu Ammonis* 1
CA3	*Cornu Ammonis* 3
CCL	Comprometimento cognitivo leve
CD68	Cluster de diferenciação 68
CPF	Córtex pré-frontal
DA	Doença de Alzheimer
DCV	Doença cardiovascular
DG	Giro denteado
EC	Éster de colesterol
EM	Esqualeno mono-oxigenase
GFAP	Proteína ácida fibrilar glial
HDL	Lipoproteína de alta densidade

HF	Hipercolesterolemia familiar
HMG COA	3-hidroxi-3-metilglutaril-CoA
IBA-1	Proteína adaptadora ionizada 1 de ligação ao cálcio
IDL	Lipoproteína de densidade intermediária
IL6	Interleucina 6
LDL	Lipoproteína de baixa densidade
LDLr	Receptor da Lipoproteína de baixa densidade
$LDLr^{-/-}$	Camundongos nocautes para receptor da lipoproteína de baixa densidade
LRP1	Proteína relacionada ao receptor de lipoproteína de baixa densidade
LTP	Potenciação de longa duração
mTOR	Proteina alvo da rapamicina
NADH	Nicotinamida Adenina Dinucleotídeo
NADPH	Fosfato de Dinucleotídeo de Nicotinamida e Adenina
PCSK9	Pró-proteína convertase subtilisina kexina tipo 9
PSD95	Proteína de densidade pós-sináptica 95
RAGE	Receptor para produtos finais de glicação avançada
SNC	Sistema nervoso central
SREBP2	Proteína de ligação ao elemento regulador de esterol 2
VLDL	Lipoproteína de muito baixa densidade

Introdução

Os lipídios compreendem um grupo de macromoléculas, na qual a característica em comum é a insolubilidade em água. Os lipídios de maior relevância clínica são os triglicerídeos e o colesterol (Nelson and Cox 2014). O colesterol ($C_{27}H_{46}O$) é um álcool não saturado pertencente à família dos compostos esteroides que, desde que fora isolado pela primeira vez a partir de cálculos biliares em 1784, vem fascinando e sendo um alvo de estudos por cientistas nas mais diversas áreas (Brown and Goldstein 1986; Vance and Van Den Bosch 2000; Mathew and Daniel 2008; Orth and Bellosta 2012; Goldstein and Brown 2015).

As funções do colesterol englobam desde participar da constituição e estabilização das membranas celulares, até atuar como precursor biossintetico de hormônios esteroides, ácidos biliares e vitamina D. Além disso, o colesterol desempenha papéis críticos em processos de sinalização e proliferação celular (Goedeke and Fernández-Hernando 2011; McLean *et al.* 2012). De particular relevância, o colesterol é o principal componente das bainhas de mielina, sendo essencial para uma eficiente transmissão de impulsos eletroquímicos entre os neurônios (Dietschy and Turley 2004). No entanto, o excesso de colesterol pode ser nocivo à saúde, favorecendo o desenvolvimento de doenças. Os defeitos na captação e metabolização do colesterol levam ao acúmulo deste lipídio na corrente sanguínea, i.e, hipercolesterolemia (Brown and Goldstein 1984). As células precisam de suprimento contínuo e controle preciso do metabolismo do colesterol. Tal regulação ocorre tanto no processo de biossíntese, quanto durante o seu transporte e metabolização. A biossíntese do colesterol ocorre em praticamente todas as células, mas cerca de 50% de sua síntese é concentrada apenas nas células hepáticas. O fígado tem papel central no metabolismo periférico do colesterol (Repa and Mangelsdorf 2003). Em

humanos, aproximadamente 80% de todo o colesterol é sintetizado endogenamente (síntese *de novo)* enquanto o restante é proveniente da dieta (Nelson and Cox 2014).

O processo de síntese endógena de colesterol é um processo de alto custo energético que requer um suprimento considerável de acetilcoenzima A (acetil-CoA), adenosina trifosfato (ATP), oxigênio e dos fatores redutores Fosfato de Dinucleotídeo de Nicotinamida e Adenina (NADPH) e Nicotinamida Adenina Dinucleotídeo (NADH). A sua síntese inicia-se com uma molécula de acetil-CoA, a qual através de ao menos trinta reações enzimáticas diferentes e com a participação de cerca de quinze enzimas presentes no citosol e retículo endoplasmático, serve como precursor para a formação da molécula de colesterol (Sharpe and Brown 2013; Nelson and Cox 2014) (Figura 1). Ao longo desse processo, a biossíntese do colesterol é regulada principalmente pela proteína de ligação ao elemento regulador de esterol 2 (SREBP-2), o qual funciona como um regulador transcricional da síntese de colesterol, e pelas enzimas 3-hidroxi-3-metilglutaril-CoA (HMGCoA) redutase e esqualeno mono-oxigenase (EM). Uma vez sintetizado, o colesterol pode ser esterificado e convertido em ésteres de colesterol por meio da ação da enzima acil-CoA colesteril aciltransferase (ACAT). A esterificação do colesterol é uma etapa importante para o armazenamento do excesso de colesterol, ou para a sua saída da célula associado às lipoproteínas (Luo *et al.* 2020).

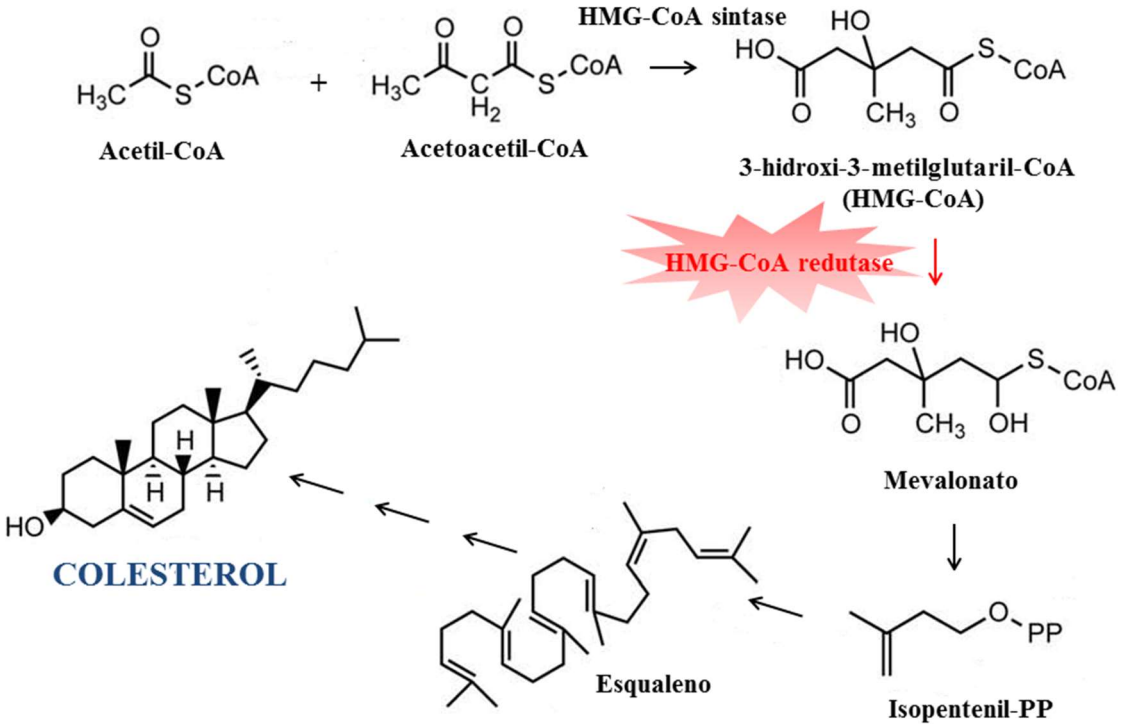
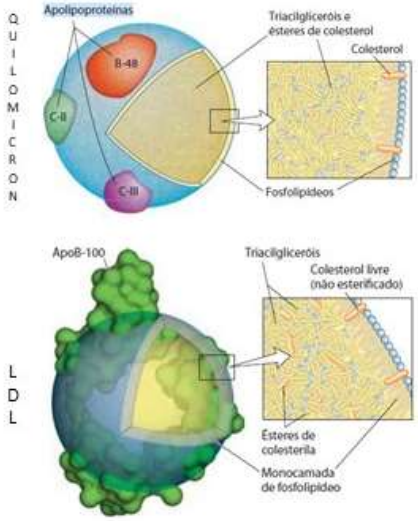


Figura 1. Principais etapas da síntese endógena de colesterol na periferia. A síntese endógena do colesterol na periferia, ocorre principalmente nas celulas hepáticas a partir de moléculas de Acetil-CoA. Duas moléculas de acetil-CoA se condensam para formar acetoacetil-CoA, que por sua vez se condensa novamente com uma nova molécula de acetil-CoA formando a molécula 3-hidroxi-3metilglutaril-CoA (HMG-CoA). A reação subsequente, na qual o HMG-CoA é reduzida à mevalonato por intermédio da ação da enzima HMG-CoA redutase, é a reação limitante da síntese do colesterol e o principal ponto de regulação de sua síntese. Uma vez sintetizado, o mevalonato é convertido em dois isoprenos ativados e posteriormente em isopentenil pirofosfato (Isopentanil-PP). Então seis unidades de isoprenos ativos são utilizadas para formar o esqualeno linear, com a respectiva eliminação dos grupos pirofosfatos. Por fim, o esqualeno passa pelo processo de ciclização dando origem ao lanosterol que por intermédio de aproximadamente 20 reações catalisadas por enzimas, é convertido em uma molécula de colesterol (Adaptado de Song et al. 2013).

O colesterol, particularmente o colesterol esterificado, devido a seu caráter hidrofóbico, precisa ser acoplado a moléculas chamadas lipoproteínas para ser transportado na corrente sanguínea. As lipoproteínas são macromoléculas esferoidais complexas formadas por uma porção proteica denominada de Apolipoproteína (Apo), e por uma porção lipídica composta por triglicerídeos, colesterol esterificado, colesterol livre e fosfolipídios (Hegele 2009). As principais classes de lipoproteínas diferem entre si pela sua composição, função e densidade (Kritchevsky 1976). Em conjunto, as lipoproteínas regulam o transporte de colesterol no organismo. Dentre as lipoproteínas, a lipoproteína de alta densidade (HDL) e a lipoproteína de baixa densidade (LDL) se destacam por suas funções antagônicas. A HDL participa do transporte reverso do colesterol, isto é, a captação do excesso de colesterol armazenado em tecidos extra-hepáticos, o que inclui as íntimas das artérias, e o seu transporte em direção ao fígado para que seja reutilizado ou excretado através da bile. Por outro lado, a LDL é a principal forma de carregar o colesterol no sangue para os tecidos periféricos ou de volta ao fígado (Nelson and Cox 2014) (Figura 2).

A



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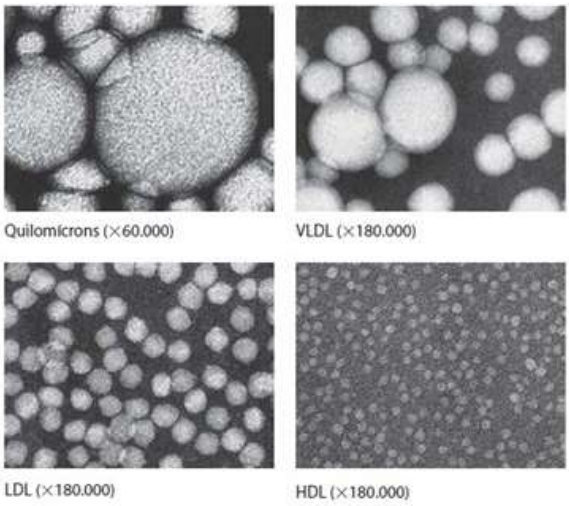

C

		Composição (% do peso)				
Lipoproteínas	Apolipoproteínas	Fosfolipídeos	Colesterol Livre	Ésteres de colesterila	Triacilgliceróis	Proteínas
Quilomícrons	ApoA, ApoB-48, ApoC, ApoE	9	1	3	85	2
VLDL	ApoB-100, ApoC, ApoE	18	7	12	50	10
LDL	ApoB-100	20	8	37	10	23
HDL	ApoA, ApoC, ApoE, ApoD	24	2	15	4	55

Figura 2. Composição estrutural das principais lipoproteínas plasmáticas humanas. (A) Representação gráfica da estrutura das lipoproteínas, ressaltando as suas diferenças. Os quilomícrons, moléculas maiores contendo altos teores de triacilgliceróis e as apolipoproteínas (Apo) ApoB48, ApoCII e ApoCIII, são diferentes em composição e em estrutura em relação as moléculas de lipoproteína de baixa densidade (LDL), moléculas menores com baixo teor de triacilgliceróis, altos teores de colesterol e ésteres de colesterila que contêm a ApoB100 em sua estrutura. (B) As diferenças nas composições das lipoproteínas determinam sua função, sua densidade e tambem seu tamanho. Os quilomícrons são os principais responsáveis pela via exógena do transporte de lipídeos no organismo, participando do empacotamento dos lipídeos da dieta e de seu transporte ao figado. Na via endógena, os lipídeos sintetizados ou empacotados no figado são distribuídos aos tecidos periféricos pela lipoproteína de muito baixa densidade (VLDL), a qual posteriormente é convertida em LDL. A LDL, lipoproteína com maior teor de colesterol, participa do transporte de colesterol para os tecidos extra-hepáticos ou de volta para o figado. O excesso de colesterol e de lipídeos presente nos tecidos extra-hepáticos retorna ao figado para ser metabolizado por intermédio da lipoproteína de alta densidade (HDL), a qual participa diretamente do chamado transporte reverso do colesterol. Imagens de microscopia eletrônica mostram que a lipoproteína de maior tamanho é os quilomícrons, seguido pela VLDL, LDL e por fim pela HDL – lipoproteína de menor tamanho, maior densidade e com maior conteúdo proteico. (C) Composição detalhada das principais classes de lipoproteínas plasmáticas humanas (Adaptado de Nelson e Cox, 2014).

A captação e posterior metabolização do colesterol associado às lipoproteínas, é regulada principalmente pelo receptor de LDL (LDLr), uma vez que ele medeia à remoção e o catabolismo de moléculas de lipoproteínas contendo colesterol da circulação sanguínea (Brown and Goldstein 1984; Hobbs *et al.* 1992). O LDLr, protótipo da família de receptores de LDL, é um receptor de superfície celular formado por ao menos cinco domínios estruturais, cada qual com a sua finalidade específica, crucial para a realização da endocitose mediada por receptor das lipoproteínas (Yamamoto *et al.* 1984). Em particular, o domínio amino-terminal do LDLr, possui resíduos de cisteína os quais reconhecem e interagem com os ligantes, incluindo a ApoB100 (principal Apo da LDL) e a Apo E (presente em lipoproteínas, tais como a lipoproteína de muito baixa densidade (VLDL), lipoproteína de densidade intermediária (IDL) e a HDL), possibilitando a posterior endocitose e metabolização do colesterol associado às lipoproteínas (Russell *et al.* 1984; Brown and Goldstein 1984; Hobbs *et al.* 1992). Desta forma, o correto funcionamento dos LDLr se torna importante para regular o conteúdo de colesterol celular e sanguíneo (Jeon and Blacklow 2005; Nelson and Cox 2014). As etapas que compõem a síntese de colesterol nos hepatócitos, assim como seus mecanismos regulatórios estão melhor apresentados na figura 3.

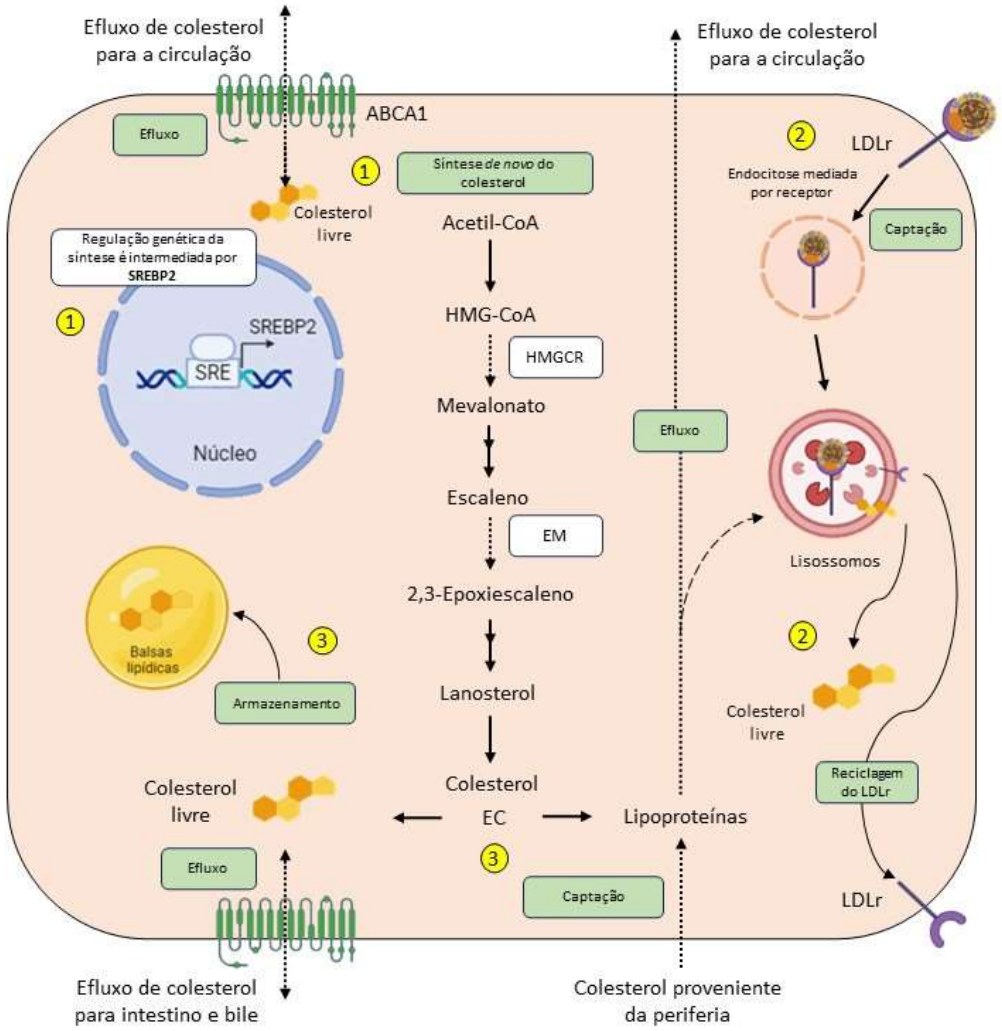

Figura 3. Metabolismo do colesterol nas células hepáticas. (1) O colesterol na periferia é sintetizado principalmente nas células hepáticas, por meio de uma síntese *de novo* a partir de uma molécula de acetil-CoA. A síntese *de novo* do colesterol compreende uma série de 30 reações intermediada por enzimas, regulada pelas enzimas 3-hidroxi-3-metilglutaril-CoA redutase (HMGCR), esqualeno mono-oxigenase (EM) e pela regulação da proteína de ligação ao elemento regulador de esterol 2 (SREBP-2), a qual funciona como uma reguladora transcricional da síntese de colesterol. (2) Além disso, o colesterol associado às lipoproteínas, principalmente na LDL, pode ser captado via LDLr pelas células hepáticas em um processo chamado de endocitose mediada por receptor. Uma vez dentro da célula, o complexo LDLr e LDL é direcionado aos lisossomos onde ocorre a metabolização da lipoproteína, com a subsequente liberação de colesterol livre, e a reciclagem do LDLr que retorna a membrana plasmática. O colesterol na sua forma livre também pode ser captado da circulação entero-hepática ou da corrente sanguínea, via transportadores do tipo ABC. (3) Por fim, o colesterol pode ser convertido a ésteres de colesterol (EC), via enzima colesterol aciltransferase (ACAT), para ser armazenado nas balsas lipídicas ou para ser excretado das células, associado às lipoproteínas. Adaptado de Luo, Yang and Song, 2020.

1.1 Os receptores de LDL e o metabolismo do colesterol no cérebro

Estima-se que aproximadamente 25% do colesterol corporal total em seres humanos adultos seja encontrado no cérebro, principalmente na sua forma não esterificada (Dietschy and Turley 2004). No cérebro, o colesterol é componente estrutural das membranas neuronais e gliais. Além disso, este lipídio é um constituinte significativo da bainha de mielina, sendo um componente essencial para uma eficiente transmissão de impulsos eletroquímicos. Por este fato, a manutenção do metabolismo do colesterol no SNC se faz de extrema importância. Os principais reguladores do metabolismo cerebral do colesterol são a ApoE, os transportadores do tipo ABC e os receptores da família do LDLr (Zhang and Liu 2015) (Figura 4).

Cabe ressaltar que o metabolismo cerebral do colesterol é independente de seu respectivo metabolismo periférico. Isso ocorre devido a seletividade da barreira hematoencefálica (BHE), a qual impossibilita a captação de moléculas de colesterol provenientes das lipoproteínas de circulação periférica (Björkhem *et al.* 2004). Portanto, praticamente todo o suprimento de colesterol no cérebro, precisa ser produzido no próprio parênquima cerebral via síntese *de novo* (Dietschy and Turley 2004; Nieweg *et al.* 2009).

A produção de colesterol no cérebro varia de acordo com a fase de desenvolvimento, atingindo um pico durante a fase de mielinização e diminuindo à medida que o tecido cerebral amadurece. Os astrócitos são as principais células responsáveis pela síntese de colesterol no cérebro desenvolvido (Dietschy and Turley 2004; Nieweg et al. 2009). Os astrócitos produzem colesterol por meio da via de Bloch, a partir de precursores esteroides específicos tais como o desmosterol (Nieweg *et al.* 2009). Uma vez produzido, o colesterol nascente é associado ainda nos astrócitos com moléculas de ApoE e fosfolipídeos, formando lipoproteínas similares às HDL plasmáticas (Boyles *et al.* 1985). O complexo colesterol-ApoE é posteriormente secretado das células

astrocitárias, via transportadores ABC (principalmente ABCA1). Uma vez no parênquima cerebral, as lipoproteínas são captadas pelos neurônios por intermédio dos LDLr, em um processo de endocitose mediada por receptor (Zhang and Liu 2015)

Nos neurônios, as lipoproteínas contendo colesterol são hidrolisadas nos lisossomos neuronais permitindo a liberação de colesterol livre. O colesterol pode ainda ser armazenado nas balsas lipídicas ou ser convertido a oxisteróis (principalmente em 24-hidroxicolesterol, ou 24-OH), os quais conseguem atravessar a BHE e serem metabolizados no fígado (Zhang and Liu 2015). A conversão de colesterol em oxisteróis é, portanto, um mecanismo importante para a eliminação do excesso de colesterol presente no cérebro.

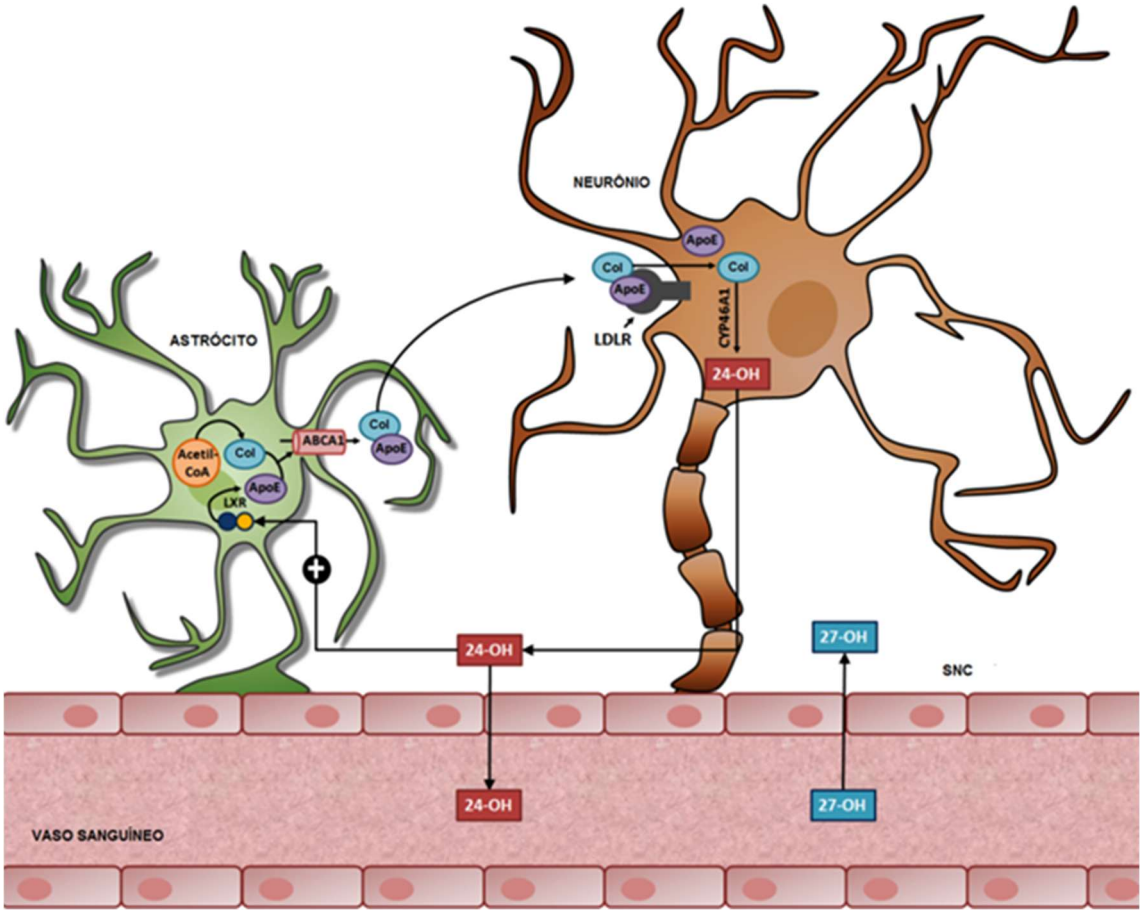

Figura 4. Metabolismo do colesterol no sistema nervoso central (SNC). Por conta da seletividade da barreira hematoencefálica (BHE), a metabolização do colesterol no SNC ocorre de maneira independente do metabolismo periférico. No cérebro, o colesterol, assim como a apolipoproteína E (ApoE), é sintetizado principalmente pelos astrócitos por meio da via Bloch, a partir de precursores esteroides como o desmosterol. O complexo colesterol-ApoE sai dos astrócitos por intermédio dos transportadores do tipo ABC 1 (ABCA1), e são captados pelos neurônios via endocitose mediada por receptor, processo este realizado por receptores da família dos receptores da lipoproteína de baixa densidade (LDLr). Uma vez nos neurônios, o colesterol pode ter diferentes destinos os quais incluem a sua conversão em 24 hidroxicolesterol (24-OH), o qual posteriormente é excretado através da BHE, e por fim metabolizado no fígado. A conversão de colesterol em 24-OH é a principal causa de eliminação do excesso de colesterol cerebral. Adaptado de Moreira e colaboradores (2015).

1.2 Hipercolesterolemia como fator de risco cardiovascular

Existem duas formas de hipercolesterolemia: a de origem genética, representada principalmente pela hipercolesterolemia familiar (HF), ou a forma adquirida, na qual os níveis elevados de colesterol ocorrem devido a maus hábitos de vida e de alimentação (Ibrahim *et al.* 2021). Aproximadamente 40% da população adulta mundial tem hipercolesterolemia, prevalência esta que pode ser ainda maior em determinadas populações (World Health Organization 2008; Danese et al. 2018; Ibrahim et al. 2021).

A HF é causada principalmente por mutações no gene que codifica o LDLr (Santos 2016). Estima-se que haja mais de 1.700 mutações no gene do LDLr já descritas na população (Jiang *et al.* 2015). A HF é possivelmente a doença de origem genética mais comum em humanos, classificada em duas formas de acordo com a presença de mutações em um ou dois alelos no gene do LDLr. Os portadores de HF heterozigótica herdam a mutação genética de um único progenitor, o que gera ausência parcial dos LDLr no organismo e níveis de colesterol elevados em torno de 310-580 mg/dL. Os portadores da forma homozigótica, por outro lado, herdam a mutação no receptor de ambos os progenitores e apresentam ausência total de LDLr nas células. Esta forma da doença é, portanto, mais rara e acarreta níveis de colesterol muito acima dos valores de referência (460-1160 mg/dL) (Santos 2016).

A prevalência da HF varia entre populações e deve ser interpretada com cuidado, uma vez que apenas 9% dos países em todo o mundo de fato avaliam a prevalência de HF em suas populações (Beheshti *et al.* 2020). A prevalência da HF na população mundial foi estimada por intermédio de estudos de metanálises, os quais relataram que a HF está presente em 1 a cada 313 pessoas, enquanto a prevalência da forma homozigótica ficou estimada em 1 a cada 300.000 pessoas (Defesche *et al.* 2017; Beheshti *et al.* 2020). Apesar de sua alta prevalência, a HF é frequentemente não diagnosticada e não tratada,

mesmo seu principal biomarcador sendo facilmente mensurado (colesterol presente na LDL, LDL-colesterol).

O colesterol plasmático, particularmente o LDL-colesterol, acima dos níveis desejáveis é um grande fator de risco para as doenças cardiovasculares (DCV) associadas à aterosclerose, principalmente a doença arterial coronariana (Libby 2000; World Health Organization 2008; Mozaffarian et al. 2015). Isto porque o excesso de colesterol no plasma acaba se acumulando nos tecidos, principalmente nas paredes das artérias. Devido a exposição crônica a altos níveis de LDL-colesterol, portadores de HF apresentam três a treze vezes mais chances de desenvolver DCV comparados a indivíduos com concentração normal de colesterol. Os pacientes portadores de HF homozigótica não tratados adequadamente apresentam taxas muito altas de mortalidade já aos trinta anos de idade, e pacientes heterozigotos começam a sofrer de DCV na quarta década de vida (Mytilinaiou et al. 2018; Iyen et al. 2020; Coutinho et al. 2021) (Figura 5).

Considerando os riscos associados aos níveis altos de LDL plasmáticos, a detecção precoce e o respectivo tratamento da HF são de suma importância. Atualmente existem estratégias farmacológicas e não farmacológicas para o tratamento da HF, sendo as estatinas (inibidores da enzima HMG-CoA redutase) os fármacos de primeira escolha para o tratamento desses pacientes (Pajak *et al.* 2016). O principal objetivo associado ao tratamento da HF é prevenir e reduzir a mortalidade prematura associada às DCV e a incidência de infarto do miocárdio (Watts *et al.* 2015). De fato, com o passar dos anos e com o advento das estatinas, a expectativa de vida de portadores da HF aumentou, principalmente os indivíduos portadores da forma heterozigótica (Mytilinaiou *et al.* 2018; Harada *et al.* 2018). Atualmente, terapias baseadas na inibição da ação da proteína convertase subtilisina kexina tipo 9 (PCSK9), uma proteína que se liga aos LDLr e catalisa a sua respectiva metabolização e reciclagem (Luo *et al.* 2020), auxiliam o

tratamento da HF principalmente nos pacientes com histórico de DCV (Santosa and Jensen 2015).

As intervenções terapêuticas no tratamento da HF têm se mostrado de fato eficientes. Neil e colaboradores (Holven *et al.* 2018) acompanharam 3.382 pacientes portadores da forma heterozigótica da HF entre os anos de 1980 e 2006, antes e após intervenção terapêutica com as estatinas. Os autores verificaram que a mortalidade por DCV foi significativamente menor nos pacientes tratados com as estatinas, com uma porcentagem de redução de 48% em pacientes sem histórico prévio de DCV e 25% naqueles com DCV estabelecida. Resultados similares foram observados em um grande estudo de coorte conduzido por pesquisadores na Holanda, os quais acompanharam os pacientes por dez anos. Outro estudo realizado com indivíduos portadores de HF homozigótica na África do Sul, demonstrou aumento na expectativa de vida e atenuação na ocorrência de mortes em pacientes HF homozigotos em terapia farmacológica (Cuchel *et al.* 2014).

Com isso, a expectativa de vida de pacientes com HF elevou-se significativamente, permitindo com que seja possível estudar os impactos da HF em complicações relacionadas ao envelhecimento, tais como alterações na cognição. Neste sentido, um estudo interessante mostrou que indivíduos portadores de HF com idade avançada tratados por mais de 15 anos com estatinas, apresentaram melhor memória episódica do que pacientes portadores de HF não tratados com as estatinas (Hyttinen *et al.* 2011). Esses dados representam uma coorte particular de 37 portadores de HF com a mesma mutação no gene que codifica o LDLr moradores da região de North Karelia (Finlândia). Nesta coorte, os pacientes reportaram que se exercitavam ao menos três vezes na semana. O estilo de vida saudável aliado ao controle dos níveis de colesterol plasmáticos provavelmente contribuíram para o prolongamento da expectativa de vida e

para a preservação das funções cognitivas nesta população estudada. Tais evidências atraíram a atenção de pesquisadores que passaram a investigar com mais afinco a relação da HF com prejuízos cognitivos.

Desde então, estudos vêm demonstrando que a ausência de LDLr, bem como o consequente aumento nos níveis de colesterol, parecem contribuir e atuar como um fator de risco para o desenvolvimento de comprometimentos cognitivos associados à neurodegeneração (Figura 5) (Kivipelto *et al.* 2001; Kivipelto *et al.* 2005; Zambón *et al.* 2010; Ariza *et al.* 2016). Logo, modificações na homeostase do colesterol mostram ser muito além de um simples fator de risco cardiovascular, contribuindo inclusive para o desenvolvimento de doenças do sistema nervoso central (SNC).

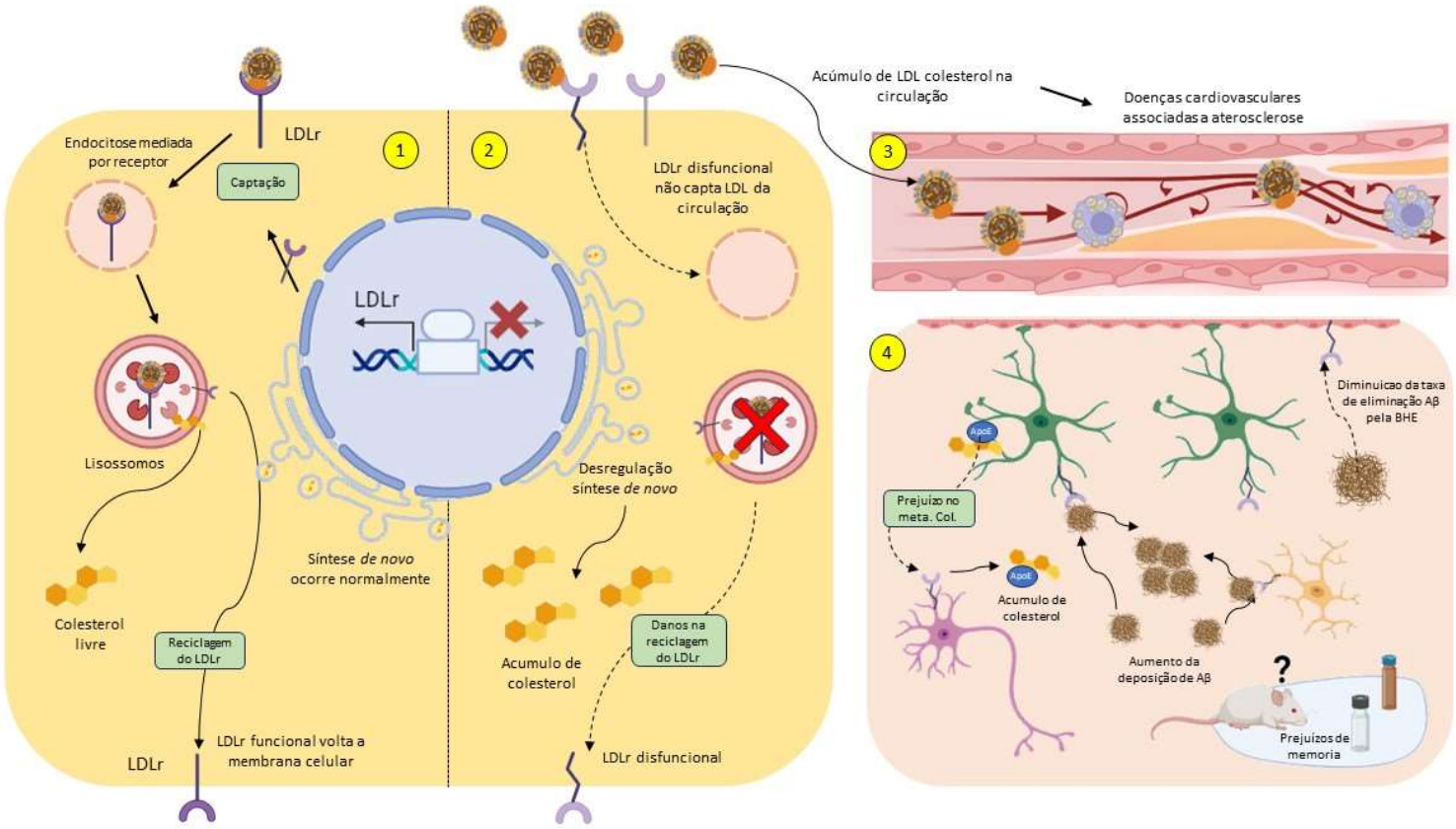

Figura 5. Consequências periféricas e centrais relacionadas à disfunção dos LDLr. (1) Em condições não patológicas, a transcrição dos receptores da lipoproteína de baixa densidade (LDLr) ocorre normalmente no núcleo gerando LDLr funcionais, os quais migram para a membrana plasmática e realizam a captação do colesterol associado às lipoproteínas. Uma vez dentro da célula, o complexo LDLr e lipoproteína de baixa densidade (LDL) é direcionado aos lisossomos onde ocorre a metabolização da lipoproteína, com a subsequente liberação de colesterol livre, e a reciclagem do LDLr que retorna a membrana plasmática. (2) No entanto, na hipercolesterolemia familiar, há uma desregulação nos genes que codificam os LDLr no núcleo, gerando alterações na produção e funcionalidade destes receptores. Por consequência, ocorre uma desregulação da síntese *de novo* do colesterol, na reciclagem dos LDLr nos lisossomos e na diminuição da captação de colesterol associado às lipoproteínas, gerando o acúmulo delas na corrente sanguínea. (3) O aumento nos níveis plasmáticos de LDL, resultante da disfunção dos LDLr na periferia, contribui para a ocorrência de doenças cardiovasculares associadas à aterosclerose. (4) Ainda, a disfunção / falta de LDLr no cérebro, gera prejuízos no metabolismo e captação de colesterol pelas células. Disfunção dos LDLr prejudicam a degradação do peptídeo β-amiloide (Aβ) pelas células da glia e através da barreira hematoencefálica (BHE), processo que resulta em aumento na deposição do Aβ no cérebro. Por fim, a ausência de LDLr está relacionada à ocorrência de danos de memória, atuando com um fator de risco em potencial para prejuízos de memória associados às neuropatologias.

1.3 Relação dos LDLr e da hipercolesterolemia com as doenças do sistema nervoso central

Além de causar doenças cardiovasculares, particularmente a doença arterial coronariana, e estar associada a um índice elevado de mortalidade, evidências clínicas e experimentais apontam que a hipercolesterolemia é um fator de risco em potencial para o desenvolvimento de déficits cognitivos, incluindo aqueles relacionados às doenças neurodegenerativas (Moreira et al. 2012a; Moreira et al. 2012b; de Oliveira et al. 2020a; de Oliveira et al. 2020b). Estudos pioneiros apontaram a hipercolesterolemia como um possível desencadeador do acúmulo do Aβ, na doença de Alzheimer (DA) de origem esporádica (Sparks *et al.* 1990; Sparks *et al.* 1994; Solomon *et al.* 2009). O interesse na relação entre os níveis de colesterol no plasma e a DA, iniciou-se a partir de uma observação de Sparks e colaboradores (1990). Os pesquisadores identificaram placas amiloides cerebrais em pacientes que morreram por doença arterial coronariana. Posteriormente, os mesmos pesquisadores (1994) demonstraram por meio de tratamento crônico com dieta rica em colesterol a coelhos, que a conexão entre hipercolesterolemia e DA parece envolver o aumento na formação de placas amiloides cerebrais. Do mesmo modo, Refolo e colaboradores (2000) demonstraram que a exposição a uma dieta hipercolesterolêmica resulta em um aumento significativo no acúmulo de Aβ no SNC de camundongos transgênicos modelos de DA, e que esse aumento se correlaciona fortemente com os níveis de colesterol plasmático (Refolo *et al.* 2000).

Na sequência, estudos epidemiológicos e clínicos apontaram que indivíduos hipercolesterolêmicos são mais suscetíveis a desenvolver a DA e prejuízos cognitivos associados (Kivipelto et al. 2001; Kivipelto et al. 2005; Kivipelto and Solomon 2006). Por exemplo, em um estudo prospectivo de coorte, Kivipelto e colaboradores (2001) verificaram que indivíduos que apresentam níveis elevados de colesterol plasmático

durante a meia-idade, apresentam um risco aumentado de desenvolver DA em idades mais avançadas. Além disso, Sparks e colaboradores em 2005 demonstraram que quando indivíduos com hipercolesterolemia são tratados com fármacos que reduzem os níveis de colesterol, i.e., estatinas, apresentam uma menor prevalência da DA e diminuição na deterioração cognitiva (Sparks et al., 2005).

Alterações cognitivas também foram observadas em roedores com hipercolesterolemia induzida por dieta. Moreira e colaboradores (2014) observaram que camundongos *Swiss* expostos a uma dieta rica em colesterol por oito semanas, apresentaram prejuízos de memória e alterações na atividade da enzima acetilcolinesterase (Moreira *et al.* 2014). Nesta mesma linha de evidência, ratos hipercolesterolêmicos apresentaram prejuízos de memória espacial, o que foi associado à diminuição em neurônios colinérgicos, redução nos níveis de acetilcolina na região cortical, aumento nos níveis de Aβ, neuroinflamação caracterizada por aumento da densidade de micróglias e alterações vasculares as quais indicam disfunção da BHE (Ullrich *et al.* 2010).

Nós recentemente, investigando os efeitos da exposição de camundongos a uma dieta com alto teor de colesterol, verificamos que os animais alimentados com a dieta por oito semanas além de desenvolverem hipercolesterolemia, apresentaram prejuízos na memória de reconhecimento e comportamento do tipo-depressivo. Os prejuízos cognitivos nos camundongos hipercolesterolêmicos foram associados a maior permeabilidade da BHE no hipocampo. Além disso, as alterações comportamentais e o dano da BHE foram atenuados quando os camundongos expostos à dieta hipercolesterolêmica receberam concomitantemente um tratamento anti-inflamatório (Rodrigues *et al.* 2021). De fato, estudos experimentais utilizando modelos animais de hipercolesterolemia apontam que a neuroinflamação, uma resposta imune inata complexa

do SNC intermediada por vários grupos de células gliais e por células imunes periféricas (Ransohoff 2016), e a quebra da BHE, parecem preceder as alterações cerebrais características da DA (Moreira et al. 2012a; Lane-Donovan et al. 2014; Watts et al. 2015; Santosa and Jensen 2015; Pajak et al. 2016; Harada et al. 2018).

Além disso, alterações na funcionalidade dos LDLr também parecem estar implicadas com o desenvolvimento de alterações no SNC. Conforme mencionado no tópico anterior, os LDLr são indispensáveis para a correta metabolização do colesterol e da ApoE no SNC (Zhang and Liu 2015; Luo *et al.* 2020). Em modelos animais de amiloidose, a deleção do LDLr resultou em um aumento significativo na deposição amiloide cerebral e dos prejuízos cognitivos associados (Cao *et al.* 2006; Katsouri and Georgopoulos 2011). Por outro lado, a superexpressao de LDLr cerebral, reduziu o conteúdo de Aβ no córtex e no hipocampo em um animal modelo para a DA (camundongos APP/PS1)(Kim *et al.* 2009b). A superexpressao de LDLr no cérebro eleva as taxas de eliminação de Aβ através da BHE, reduzindo assim a deposição deste peptídeo no cérebro em camundongos PDAPP, um modelo animal de amiloidose (Castellano *et al.* 2012) (Figura 5).

A ausência de LDLr também eleva a expressão de apoE (Katsouri and Georgopoulos 2011). A ApoE se mostra uma importante molécula com atividade imunomodulatória no cérebro. Ademais, a presença de alelos da ApoE, principalmente a ApoE4, é um conhecido fator de risco para DA de início tardio (Shi *et al.* 2017; Krasemann *et al.* 2017). Shi e colaboradores em 2019 observaram que em modelos animais onde os LDLr foram superexpressos, houve uma redução significativa nos níveis de ApoE, na reatividade microglial e diminuição da neurodegeneração associada à taupatia (Shi *et al.* 2019). Na sequência, os mesmos autores investigando os possíveis mecanismos por trás dos efeitos neuroprotetores da superexpressão de LDLr, verificaram

que houve melhora em marcadores sinápticos e diminuição significativa da reatividade astrocitária e microglial no hipocampo em camundongos modelos de taupatia (camundongos P301S). Ainda, análises de expressão diferencial mostraram que a superexpressão de LDLr na micróglia elevou a expressão de genes associados às vias catabólicas, ao mesmo tempo em que reduziu a expressão de genes anabólicos e da proteína mTOR (Shi *et al.* 2021). Estes dados mais recentes destacam outro mecanismo crítico por meio do qual o LDLr participa no funcionamento do cérebro.

Neste sentido, a HF é uma condição que propicia uma observação única entre as alterações no metabolismo do colesterol e o desenvolvimento de disfunção cerebral. No caso da HF, não só os níveis aumentados de colesterol no plasma, mas também a perda de função dos LDLr nas células cerebrais parece contribuir com o desenvolvimento de prejuízos cognitivos (Defesche *et al.* 2017; Coutinho *et al.* 2021). Um importante estudo clínico realizado por Zambón e coautores (2010), mostrou que pacientes portadores de HF com idade média de 60 anos (meia-idade) são mais suscetíveis a desenvolver comprometimento cognitivo leve (CCL) (Zambón *et al.* 2010). Tal incidência se mostrou maior em pacientes portadores de HF do que a prevalência estimada de CCL em pacientes com hipercolesterolemia adquirida de mesma faixa etária (Kivipelto *et al.* 2001). Evidências mais recentes relatam danos neuropsicológicos, incluindo perda de memória, em portadores de HF já na idade entre 18 e 40 anos (Ariza *et al.* 2016).

Corroborando com estas observações clínicas, pesquisas experimentais utilizando camundongos nocautes para o LDLr ($LDLr^{-/-}$), um modelo experimental de HF e aterosclerose, mostraram que estes animais também apresentam danos cognitivos (De Oliveira *et al.* 2011; Moreira *et al.* 2012a; de Oliveira *et al.* 2020b). Os camundongos $LDLr^{-/-}$ já aos três meses de idade apresentam prejuízos de memória espacial e de trabalho (Moreira et al. 2012b; de Oliveira et al. 2014; de Oliveira et al. 2020b), o que se torna

mais severo na meia-idade (quatorze meses) (de Oliveira *et al.* 2020a; Moreira *et al.* 2012a). Ainda, animais LDLr$^{-/-}$ se mostraram mais suscetíveis a neurotoxicidade associada ao Aβ, apresentando maior atividade da enzima acetilcolinesterase, desbalanço oxidativo e astrogliose quando comparados aos camundongos selvagens também expostos ao peptídeo (de Oliveira *et al.* 2014). Apesar de serem mais suscetíveis a neurotoxicidade associada ao Aβ, camundongos LDLr$^{-/-}$ não apresentam alterações nos níveis de Aβ no cérebro nem mesmo na meia-idade, mas parecem ter um aumento na apoptose neuronal. Os camundongos LDLr$^{-/-}$ aos três meses apresentam tanto no hipocampo quanto no córtex pré-frontal uma exacerbação da apoptose, caracterizada por modificações nos níveis de RNA mensageiro das proteínas Linfoma de células B2 (Bcl-2) e Proteína X associado ao BCL-2 (Bax), e por um aumento dos níveis de caspase -3 clivada. Os níveis de caspase-3 ativada se colocalizam com células neuronais e não com astrócitos (de Oliveira *et al.* 2020a). Ademais, os camundongos LDLr$^{-/-}$ apresentam danos na neurogênese hipocampal adulta (Engel *et al.* 2019).

Os camundongos LDLr$^{-/-}$ hoje em dia considerados também modelos de prejuízo cognitivo apresentam mecanismos mais complexos que levam aos déficits de memória quando comparados com camundongos transgênicos para a DA. Nos modelos clássicos de DA familiar, as alterações de memória estão associadas a uma maior produção de Aβ (de Bem *et al.* 2021). *Então, quais seriam os mecanismos moleculares que explicam os prejuízos cognitivos e morte neuronal nos LDLr$^{-/-}$?*

Camundongos LDLr$^{-/-}$ de três meses de idade apresentam aumento da permeabilidade da BHE e neuroinflamação (Thirumangalakudi *et al.* 2008; de Oliveira *et al.* 2014; de Oliveira *et al.* 2020b). A neuroinflamação caracterizada por astrogliose foi visualizada no hipocampo de camundongos LDLr$^{-/-}$ com 3 meses de idade. O aumento do número de astrócitos no hipocampo de camundongos LDLr$^{-/-}$ foi associado ao aumento

da imunorreatividade da aquaporina-4 (AQP-4), o que indica disfunção da BHE (de Oliveira *et al.* 2014). Em um trabalho mais recente, de Oliveira e coautores (2020b) submeteram camundongos selvagens e LDLr$^{-/-}$ com 3 meses de idade a uma dieta rica em colesterol por trinta dias e observaram que a permeabilidade da BHE nos camundongos modelos de HF foi mais intensa. A disfunção da BHE no hipocampo e no córtex pré-frontal em camundongos LDLr$^{-/-}$ foi associada ao declínio cognitivo, enquanto os camundongos tipo selvagens alimentados com uma dieta rica em colesterol exibiram deficiências na função da BHE, mas não na cognição. Além disso, camundongos LDLr$^{-/-}$ apresentaram astrogliose intensa, aumento do conteúdo de microvasos e diminuição dos níveis de IL-6 no hipocampo (de Oliveira *et al.* 2020b). A neuroinflamação em camundongos LDLr$^{-/-}$ expostos à dieta rica em colesterol também é caracterizada por aumento na densidade de micróglias (Thirumangalakudi *et al.* 2008).

A neuroinflamação caracterizada pela participação das micróglias parece ter papel tanto nas alterações cerebrais induzidas pela dieta rica em colesterol em camundongos como nos camundongos LDLr$^{-/-}$, modelos de HF. Tais evidências demonstram que não somente a funcionalidade dos LDLr, mas também a hipercolesterolemia per se são capazes de modular a atividade microglial e atuar como um fator de risco para o desenvolvimento de neuropatologias e prejuízos cognitivos associados.

1.4 A micróglia como um agente nos déficits cognitivos relacionados a hipercolesterolemia

As micróglias são as células de maior mobilidade no SNC e constantemente monitoram o parênquima cerebral. Durante muito tempo, acreditou-se em uma visão dicotômica sobre os estados fisiológicos da micróglia no cérebro (Streit *et al.* 1988). Esta caracterização rígida descrevia a existência de uma micróglia “ativada", conhecida como

micróglia M1 ou micróglia “má”. Nesse estado, as micróglias adquiriam um hipotético estado ativado, com alta atividade fagocítica e perfil pro-inflamatório. Alterações morfológicas tais como encurtamento de processos, diminuição no número de ramificações e aumento do tamanho do núcleo, caracterizavam a micróglia em seu fenótipo M1. Por outro lado, haveria a micróglia em seu estado homeostático ou de “repouso”, também conhecida como micróglia M2 ou micróglia “boa”. Nesse estado, as micróglias estariam em um estado de repouso, com baixa atividade fagocítica e perfil anti-inflamatório. A morfologia desta célula caracteriza-se por um perfil hiper ramificado, alongamento das ramificações e pelo núcleo pequeno (Sierra *et al.* 2016; Sierra *et al.* 2019). No entanto, conforme as pesquisas na área foram avançando e com o uso de técnicas mais sensíveis, verificou-se que as micróglias na verdade são células dinâmicas, as quais estão constantemente se movimentando e desenvolvendo as mais diversas funções e, portanto, nunca estariam em um estado de repouso no parênquima cerebral (Paolicelli *et al.* 2022).

Em um elegante estudo, Paolicelli e colaboradores (2022) propõem que a micróglia de fato está constantemente “ativa”, respondendo de maneiras diferentes a alterações no parênquima cerebral, até mesmo em condições fisiológicas normais (Paolicelli *et al.* 2022). Portanto, a micróglia coexiste em múltiplos estados no cérebro, e suas funções são determinadas por variações no contexto fisiológico. Em condições normais, as micróglias auxiliam na manutenção da homeostase sináptica, oferecem suporte para o desenvolvimento do SNC, alertam o SNC da presença de agentes estranhos e ajudam na sua respectiva eliminação por meio da fagocitose (Sierra et al. 2010; Yang and Zhou 2019; Sierra et al. 2019). Já, em condições patológicas, as micróglias estariam reativas e elevam a produção de citocinas pró-inflamatórias e óxido nítrico contribuindo

assim com a propagação da neuroinflamação e com a morte neuronal característica das doenças neurodegenerativas (Wang *et al.* 2019; Zhao *et al.* 2019; Nordengen *et al.* 2019).

A atividade microglial no SNC pode ser modulada de diversas maneiras. De particular importância, a utilização de inibidores das micróglias se mostrou benéfica no contexto de patologias neurodegenerativas, incluindo a DA (Biscaro et al. 2012; Dhawan and Combs 2012; Cui et al. 2020). Apesar de seu mecanismo de ação não estar totalmente elucidado, a minociclina, um fármaco antibiótico de segunda geração pertencente à classe das tetraciclinas (Garrido-Mesa *et al.* 2013), se mostrou um inibidor das micróglias (Kobayashi *et al.* 2013). A exemplo de outros inibidores, suas propriedades neuroprotetoras vêm sendo confirmadas em vários modelos experimentais de neuropatologias (Du et al. 2001; Jackson-Lewis et al. 2002; Choi et al. 2007; Biscaro et al. 2012; Ferretti et al. 2012; Garcez et al. 2017). Em modelos animais de doenças metabólicas, a diminuição da reatividade microglial atenuou a neuroinflamação e preveniu a ocorrência de déficits cognitivos (Jackson *et al.* 2020). Ainda, camundongos obesos tratados com a minociclina apresentaram melhora na performance cognitiva em tarefas dependentes do hipocampo, e redução da atividade fagocítica microglial nessa região cerebral (Cope *et al.* 2018).

No que diz respeito a hipercolesterolemia, coelhos alimentados com uma dieta contendo 2% de colesterol, apresentaram alterações microgliais no hipocampo, em um processo independente da deposição de Aβ (Xue *et al.* 2007). Em um estudo anterior, Thirumangalakudi e colaboradores (2008) mostraram que camundongos $LDLr^{-/-}$ apresentaram aumento da microgliose no hipocampo em comparação aos camundongos $LDLr^{-/-}$ alimentados com uma dieta normal (Thirumangalakudi *et al.* 2008). No entanto, os autores não investigaram a influência do genótipo sob a densidade microglial.

Logo, pouco ainda se conhece sobre o papel de alterações morfológicas e funcionais das micróglias nos déficits de memória e alterações neuroquímicas relacionados à hipercolesterolemia. Nós hipotetizamos que a modulação das micróglias, por intermédio do fármaco minociclina, seja uma estrategia interessante para estudar o envolvimento da reatividade microglial nos danos de memória associados às alterações na homeostase do metabolismo do colesterol. Para comprovar esta hipótese, desenvolvemos protocolos experimentais para estudar os efeitos do envelhecimento e da modulação da atividade microglial nos déficits cognitivos relacionados à HF - Parte II, Capítulo 1. Além disso, o impacto da modulação farmacológica da reatividade microglial sob a cognição em um modelo animal de hipercolesterolemia induzida por dieta também foi investigado - Parte II, Capítulo 2.

Objetivos

Objetivo principal

Analisar a contribuição das micróglias nas alterações cerebrais relacionadas à hipercolesterolemia induzida por dieta ou de origem genética, em especial se a modulação farmacológica dessas células pela minociclina resulta em efeitos neuroprotetores.

Objetivos específicos

- Pesquisar os efeitos da HF e do envelhecimento na densidade e morfologia das células microgliais em córtex pré-frontal e hipocampo de camundongos C57BL/6 selvagens e camundongos LDLr$^{-/-}$.
- Estudar em camundongos C57BL/6 selvagens e LDLr$^{-/-}$ adultos jovens e de meia-idade o imunoconteúdo de proteínas sinápticas, proteínas estruturais de BHE e mTOR no córtex pré-frontal e no hipocampo.
- Analisar os efeitos da HF e da modulação farmacológica das micróglias, por intermédio da minociclina, sob parâmetros cognitivos e metabólicos em camundongos LDLr$^{-/-}$adultos.
- Pesquisar os efeitos da HF e do tratamento com minociclina sob a microgliose, morfologia microglial, sua respectiva atividade fagocítica e interação com microvasos no córtex pré-frontal e hipocampo de camundongos LDLr$^{-/-}$adultos.
- Investigar se a dieta rica em colesterol e a intervenção farmacológica com a minociclina provocam alterações em parâmetros comportamentais e metabólicos em camundongos CF-1 adultos jovens.
- Avaliar se a hipercolesterolemia induzida por dieta e a modulação farmacológica da atividade microglial induzem alterações nas proteínas associadas à integridade da BHE, no conteúdo de células positivas para lectina-tomato, na morfologia e em

parâmetros de reatividade microglial em estruturas cerebrais importantes para a cognição, em camundongos CF-1 adultos jovens.

PARTE II

Nesta seção os materiais e métodos, bem como os resultados serão apresentados em formato de artigos, subdivididos em capítulos precedidos por um breve prefácio. No capítulo I, estão demonstrados os resultados obtidos a partir de experimentos em animais $LDLr^{-/-}$, cujo principal objetivo foi estudar qual o papel das micróglias na disfunção cognitiva, neste modelo animal. No capítulo II, constam os resultados de experimentos realizados para o entendimento da influência da reatividade microglial sob os déficits cognitivos em camundongos submetidos a um modelo experimental de hipercolesterolemia induzida por dieta.

Capitulo I. *Microglia contribute to cognitive decline in hypercholesterolemic LDLr-/- mice.*

No **capítulo I**, apresentamos os nossos achados em forma de artigo aceito para publicação no *Journal of Neurochemistry*.

Neste estudo, procuramos entender primeiramente como a HF afeta a morfologia e atividade microglial com o envelhecimento. Para isso, avaliamos a densidade e morfologia microglial no córtex pré-frontal e hipocampo de animais C57BL/6 selvagens e $LDLr^{-/-}$ adultos jovens e de meia-idade. Verificamos que a microgliose foi mais severa no hipocampo de camundongos $LDLr^{-/-}$ de meia-idade, o que esteve associado a alterações morfológicas nas células microgliais e com alterações no imunoconteudo de proteínas sinápticas. Interessantemente, animais $LDLr^{-/-}$ adultos jovens já apresentavam aumento da microgliose e diminuição do imunoconteudo de claudina-5 no córtex pré-frontal. Tendo em mente que a morfologia microglial adquire um estado mais alterado e característico de micróglias em um estado mais reativo quando camundongos $LDLr^{-/-}$ atingem a meia-idade, na sequência tratamos camundongos C57BL/6 selvagens e $LDLr^{-/-}$ adultos (6 meses de idade) com a minociclina (um modulador farmacológico da atividade microglial). Verificamos que a modulação farmacológica da micróglia, melhorou a cognição, reduziu a presença da micróglia na região perivascular, causou alterações na morfologia microglial sem alterar a sua respectiva atividade fagocítica no hipocampo e no córtex pré-frontal dos animais $LDLr^{-/-}$.

Capitulo II. *Role of glial cells reactivity on the cognitive decline linked to diet- induced hypercholesterolemia.*

No **capítulo II** apresentamos os nossos achados em forma de artigo em processo de preparação para submissão em periódico.

Neste estudo, procuramos entender como a reatividade microglial e astrocitária contribuem para os déficits cognitivos associados a hipercolesterolemia induzida por dieta. Para tal, camundongos CF-1 de três meses de idade foram expostos a uma dieta rica em colesterol durante 8 semanas, sendo que nas últimas 4 semanas os animais foram concomitantemente tratados com a minociclina. Camundongos alimentados durante 8 semanas com uma dieta rica em colesterol apresentaram aumento significativo nos níveis de colesterol total plasmático, o que não foi afetado pelo tratamento com a minociclina. Por outro lado, a dieta rica em colesterol e a minociclina não alteraram a tolerância à glicose nos animais. Ainda, animais hipercolesterolêmicos apresentaram maior peso corporal e ganho de peso em relação aos animais que receberam dieta normal, o que não foi atenuado pelo tratamento com a minociclina. Analisando a performance dos animais em tarefas comportamentais, percebemos que os animais hipercolesterolêmicos apresentaram prejuízos na memória de habituação e em tarefas comportamentais dependentes do hipocampo. Interessantemente, o tratamento com a minociclina melhorou o desempenho cognitivo dos animais hipercolesterolêmicos. Além disso, verificamos que a minociclina elevou o imunoconteúdo da proteína claudina-5 no córtex pré-frontal e no hipocampo, antes reduzida no grupo hipercolesterolêmico não tratado. Ao realizar a avaliação da quantidade de microvasos usando a Lectina de Tomate nestas mesmas estruturas cerebrais, notamos uma redução significativa na coloração para Lectina de Tomate no córtex pré-frontal, mas não no hipocampo, dos animais hipercolesterolêmicos.

Animais hipercolesterolêmicos apresentaram aumento da imunoreatividade para IBA-1 na sub-região hipocampal CA3, o que não esteve relacionado a alterações na morfologia e atividade fagocítica microglial. Ademais, verificamos que o tratamento com a minociclina reduziu a imunorreatividade para GFAP nos animais hipercolesterolêmicos, mas não alterou parâmetros relacionados à reatividade microglial.

PARTE III

Discussão

Atualmente existem cerca de 55 milhões de pessoas que vivem com demência no mundo, e devido ao aumento da expectativa de vida é estimado que este número aumente para aproximadamente 139 milhões de pessoas em 2050 (World Health Organization 2023). De particular importância, alterações no metabolismo do colesterol vêm sendo consideradas como fatores de risco para o desenvolvimento de prejuízos cognitivos (Kivipelto *et al.* 2001; Sparks *et al.* 2005; Zambón *et al.* 2010; Ariza *et al.* 2016; Anstey *et al.* 2017; Wee *et al.* 2023). Por exemplo, a APOE ε4 é fortemente implicada na DA (Tokuda *et al.* 2000; Kim *et al.* 2009a; Safieh *et al.* 2019). Além disso, o aumento dos níveis de colesterol no sangue, i.e., a hipercolesterolemia, bem como a falta do LDLr podem causar alterações neuroquímicas e comportamentais características de demência (De Oliveira et al. 2011; Moreira et al. 2012b; de Oliveira et al. 2014; de Oliveira et al. 2020a; de Oliveira et al. 2020b). Tendo em vista, a importância epidemiológica dos distúrbios do metabolismo do colesterol, nas últimas décadas inúmeros pesquisadores incluindo nosso grupo de pesquisa vêm se dedicando ao estudo dos eventos celulares e moleculares envolvidos nesta conexão. Nesta tese investigamos experimentalmente o envolvimento da micróglia nos prejuízos cognitivos e disfunção cerebral associados à hipercolesterolemia, seja ela de origem genética ou de origem adquirida. Para tal, utilizamos na **parte II - capítulo 1** um modelo animal de HF (camundongos LDLr$^{-/-}$). Ademais, na **parte II - capítulo 2,** investigamos a participação da reatividade microglial nos déficits cognitivos e mecanismos associados, em um modelo animal de hipercolesterolemia induzida por dieta. Com o intuito de modular a micróglia *in vivo*, utilizamos o tratamento com a minociclina, um fármaco conhecidamente capaz de alterar a reatividade microglial em modelos experimentais.

Os animais LDLr$^{-/-}$ são um modelo animal de extrema importância para o estudo experimental da HF. Esta linhagem de camundongos foi desenvolvida primeiramente por Ishibashi e colaboradores no início dos anos 90, e mimetiza os defeitos genéticos observados em humanos portadores de HF. Animais LDLr$^{-/-}$ apresentam a deleção do gene que codifica os receptores de LDL e, como consequência, apresentam níveis de colesterol plasmático elevados similar a aqueles encontrados em pacientes portadores de HF (Ishibashi *et al.* 1993). Os níveis de colesterol em camundongos LDLr$^{-/-}$ giram em torno de 250 mg/dL quando eles são mantidos sob dieta normal, níveis estes duas vezes maiores comparados aos animais selvagens sem alterações genéticas. Quando alimentados com dieta rica em gordura e colesterol, os níveis de colesterol se elevam substancialmente nesses animais, podendo atingir os valores de 1500 mg/dL (Ishibashi *et al.* 1993). Por outro lado, ao contrário de pacientes portadores de HF, animais com deficiência heterozigótica no LDLr apresentam níveis normais de colesterol plasmático, sugerindo que uma única cópia do LDLr se mostra capaz de manter os níveis de colesterol normais em camundongos (He *et al.* 2019). Camundongos da linhagem LDLr$^{-/-}$ são comumente utilizados experimentalmente no estudo de DCV e da aterosclerose (Oppi *et al.* 2019). No entanto, recentemente este modelo animal vem sendo usado também no estudo dos mecanismos de disfunção cerebral e prejuízos cognitivos associados à HF (de Bem *et al.* 2021).

O modelo animal de hipercolesterolemia utilizado no segundo estudo que compõem essa tese, foi induzido por meio da oferta de uma dieta rica em colesterol aos animais, pelo período de oito semanas. A exposição de roedores (e.g., camundongos e ratos) a dietas com alto teor de colesterol, é uma estratégia experimental que se mostrou eficaz na indução de um modelo animal de hipercolesterolemia (Ullrich et al. 2010; de Oliveira et al. 2014; Paul et al. 2017; de Oliveira et al. 2020b). A escolha do período de

oferta da dieta foi baseada em estudos anteriores do nosso grupo de pesquisa, onde a oferta de uma dieta rica em colesterol por oito semanas se mostrou eficiente em elevar os níveis de colesterol plasmático em camundongos (Moreira *et al.* 2014; Rodrigues *et al.* 2021; Rodrigues *et al.* 2022). Nestes estudos, o consumo da dieta rica em colesterol elevou os níveis de colesterol total, sem alterar a homeostase da glicose. Efeito similar foi observado em nosso estudo, onde os animais se tornaram hipercolesterolêmicos sem sofrer alterações nos níveis de glicose em jejum ou na tolerância à glicose. Ademais, percebemos que os animais alimentados com a dieta rica em colesterol, apresentaram aumento de peso comparados aos animais alimentados com dieta normal para roedores. Tais alterações foram relatadas em estudos anteriores (de Souza *et al.* 2019; Rodrigues *et al.* 2021) e se dá em decorrência do teor de gordura saturada presente na dieta rica em colesterol. É importante ressaltar que o teor de gordura presente na dieta utilizada (45%), é menor do que o teor de gordura geralmente utilizado em modelos animais de obesidade e diabetes, nos quais geralmente também é adicionado teores elevados de açúcar à dieta (Marques et al. 2016; de Moura e Dias et al. 2021). Ademais, dietas chamadas de ricas em gordura e ocidentais contém pouco colesterol comparado a dieta utilizada em nosso estudo.

As micróglias são células com um perfil muito dinâmico, alterando o seu estado de acordo com o contexto e sob a influência do meio em que está inserida. Sendo assim, é possível que as micróglias apresentem um perfil inflamatório associado a doenças, ou um perfil resolutivo em que há o favorecimento de mecanismos relacionados à manutenção da homeostase (Wolf et al. 2017; Paolicelli et al. 2022). De fato, células gliais reativas desempenham um importante papel na resposta cerebral a insultos externos e na subsequente resposta neuroinflamatória (Yang and Zhou 2019). Uma vez desafiadas, células gliais como a micróglia e os astrócitos elevam a secreção de substâncias chamadas

citocinas, as quais funcionam como mensageiros químicos e intermedeiam a comunicação entre micróglia e astrócitos, e das células gliais com os neurônios (Kölliker-Frers *et al.* 2021). Portanto, alterações no estado das células da glia potencialmente afetam o funcionamento e a integridade neuronal (Ransohoff 2016; Kölliker-Frers et al. 2021). Em contexto patológicos, micróglia e astrócitos alteram sua expressão gênica para perfis associados a doenças, adquirindo características que prejudicam a integridade neuronal e que favorecem a ocorrência de neuropatologias (Liddelow *et al.* 2020). Ademais, alterações no metabolismo do colesterol astrocitário e na captação de colesterol pelas micróglias, afetam o estado fisiológico microglial (Bohlen *et al.* 2017). Mais precisamente, o acúmulo de colesterol na micróglia, gera aumento da deposição de colesterol nas balsas lipídicas e alterações na atividade fagocítica microglial (Marschallinger *et al.* 2020). Micróglias com altos teores de colesterol captam mais lipídios do meio extracelular, gerando um efeito de ativação crônica celular associado à exacerbação da neuroinflamação (Colombo *et al.* 2022).

Estudos demonstram que fatores como o envelhecimento e as doenças metabólicas, incluindo a hipercolesterolemia, podem contribuir para a exacerbação da reatividade microglial (Streit and Sparks 1997; Koellhoffer *et al.* 2017; Chunchai *et al.* 2018). Em um contexto de envelhecimento, as micróglias parecem adquirir um estado disfuncional caracterizado por alta deposição de gotículas lipídicas e baixa atividade fagocítica (Marschallinger et al., 2020). Já um estudo de revisão chegou à conclusão de que alterações metabólicas periféricas resultantes da obesidade, modulam a atividade microglial no cérebro. Ainda segundo o estudo, a micróglia quando tem sua função alterada favorece a ocorrência de déficits cognitivos em modelos animais de obesidade (Chunchai *et al.* 2018). Nós acreditamos que efeito similar ocorre com os altos níveis de colesterol plasmático. Um estudo pioneiro mostrou que pacientes que morreram em

decorrência de DCV, apresentaram maior número de micróglias em seu fenótipo reativo no cérebro. Tal processo, parece favorecer o envelhecimento cerebral e suas desordens neuropatológicas associadas (Streit and Sparks 1997).

Por outro lado, um estudo recente mostrou que o LDLr está envolvido na neurodegeneração associada à proteína TAU e parece compartilhar mecanismos com a ApoE. Shi e colaboradores (2021) realizaram o cruzamento de animais transgênicos que superexpressam o LDLr com camundongos P301S, um modelo experimental de tauopatia (Shi *et al.* 2021). É importante mencionar que aos 9 meses de idade, camundongos P301S exibem considerável perda de volume cerebral. Neste sentido, os autores observaram que aos 9 meses de idade, camundongos P301S superexpressando o LDLr apresentaram redução da neurodegeneração hipocampal, diminuição na perda sináptica e nos níveis de proteína TAU fosforilada. A redução nos danos cerebrais associados à proteína TAU esteve relacionada à redução nos níveis de ApoE no líquido cefalorraquidiano e na área cortical. Em adição, os autores por meio de achados provenientes de experimentos *in vitro* e *in vivo* mostraram que a redução na expressão de ApoE e de seu respectivo conteúdo intracelular, parecem ser importantes eventos que levam a redução da reatividade microglial, o que parecer uma causa primária da neurodegeneração nos camundongos P301S super expressando LDLr. Ademais, a redução da reatividade microglial esteve associada com modulações no imuno metabolismo celular. Portanto, tais evidências demonstram um outro importante mecanismo pelo qual os LDLr participam do funcionamento cerebral via modulação da atividade microglial (Shi et al, 2021).

No entanto, pouco ainda se conhece sobre a influência da HF, doença genética na qual ocorre a associação entre altos níveis de colesterol no sangue e disfunção dos LDLr, na reatividade microglial. Em um estudo anterior, Thirumangalakudi e colaboradores (2008) mostraram que camundongos $LDLr^{-/-}$ aos 6 meses de idade, quando desafiados

com uma dieta rica em colesterol, apresentaram aumento da microgliose no hipocampo. No entanto, os autores não investigaram a influência do genótipo dos animais sob a densidade microglial (Thirumangalakudi *et al.* 2008). Portanto, em um primeiro momento, procuramos entender como o envelhecimento e a ausência dos LDLr afetam a densidade de micróglias em camundongos LDLr$^{-/-}$. Nossos achados demonstram que animais camundongos modelos de HF apresentam maior microgliose em regiões cerebrais responsáveis pela formação e consolidação de memória já aos três meses de idade, mesmo sem terem sido desafiados com uma dieta com alto teor de colesterol. Ainda, verificamos que a microgliose estava exacerbada no hipocampo dos animais LDLr$^{-/-}$ de meia-idade (14 meses de idade), mas não nos camundongos C57BL/6 selvagens de mesma idade. Tomados em conjunto, nossos resultados demonstram que a reatividade microglial associada à HF nos animais LDLr$^{-/-}$ é um processo precoce, que se inicia aos 3 meses de idade, e se agrava quando os animais envelhecem.

Animais envelhecidos e alimentados com dieta rica em colesterol, apresentaram aumento da microgliose em regiões cerebrais cruciais para a memória (Chen *et al.* 2018). Neste trabalho investigamos também a influência da hipercolesterolemia induzida por dieta na densidade das micróglias no hipocampo de camundongos adultos. Camundongos com hipercolesterolemia induzida por dieta apresentaram aumento na microgliose na região CA3 do hipocampo. Nossos dados estão de acordo com estudos anteriores, onde demonstrou-se que animais alimentados com dietas ricas em colesterol apresentam aumento na densidade de micróglias no cérebro, comparados ao grupo controle alimentado com uma dieta normal (Xue *et al.* 2007; Ullrich *et al.* 2010). O aumento na quantidade destas células gliais parece ocorrer de maneira independente de patologias associadas, tais como a deposição amiloide cerebral (Xue *et al.* 2007), demonstrando que

a hipercolesterolemia de origem adquirida *per se*, exerce efeitos deletérios no cérebro favorecendo a microgliose e mecanismos associados.

A fim de melhor investigar a reatividade glial em camundongos LDLr$^{-/-}$ em diferentes idades, assim como também nos animais CF-1 com hipercolesterolemia induzida por dieta, realizamos a avaliação da morfologia microglial em diferentes áreas hipocampais e córtex pré-frontal usando os plugins Skeletonize e Simple Neurite Tracer para ImageJ. Ainda, avaliamos a atividade fagocítica microglial por meio da quantificação do cluster de diferenciação 68 (cd68), um marcador lisossomal, dentro da micróglia. Verificamos que os animais LDLr$^{-/-}$ aos 3 meses de idade, não apresentam alterações morfológicas relevantes nas células microgliais. Já aos 6 meses de idade verificamos nos animais LDLr$^{-/-}$ uma tendência à hiper ramificação microglial na região hipocampal CA3 e um encurtamento significativo das ramificações microgliais no giro denteado (DG). O encurtamento das ramificações microgliais é um indicativo de micróglias mais reativas (Sierra *et al.* 2016; Sierra *et al.* 2019). O possível aumento da reatividade microglial na região do DG é particularmente relevante, tendo em vista o papel desta região na neurogênese hipocampal adulta (Kempermann *et al.* 2015; Abbott and Nigussie 2020). De particular importância, camundongos LDLr$^{-/-}$ apresentam prejuízos na neurogênese hipocampal adulta, já aos três meses de idade (Engel *et al.* 2019). Apesar de não termos visualizada diferença na complexidade das células microgliais e sua respectiva atividade fagocítica nas regiões hipocampal do DG aos três meses de idade, hipotetizamos que a micróglia pode estar contribuindo para a desregulação da neurogênese nesse modelo animal. No entanto, mais estudos são necessários para melhor investigar o papel da reatividade microglial na neurogênese hipocampal adulta em camundongos LDLr$^{-/-}$. Assim como no DG, a análise morfológica da micróglia não detectou diferenças significativas na complexidade e atividade

fagocítica microglial nas demais regiões hipocampais analisadas. Isso demonstra que as alterações na morfologia e reatividade microglial em camundongos LDLr$^{-/-}$ adultos variam de acordo com a região cerebral analisada. As micróglias, portanto, apresentam um perfil heterogêneo no cérebro de camundongos LDLr$^{-/-}$. Colombo e colaboradores (2022) encontraram um perfil semelhante ao analisar a presença de diferentes fenótipos microgliais no cérebro de camundongos. Os autores notaram que o fenótipo microglial varia de acordo com a região cerebral analisada, sexo e idade do animal e também de acordo com o contexto patológico, a qual contribui significativamente na mudança do fenótipo microglial em camundongos (Colombo *et al.* 2022).

Os efeitos do envelhecimento na HF na morfologia e atividade fagocítica das células microgliais também foram avaliados. Verificamos que camundongos LDLr$^{-/-}$ aos 14 meses de idade apresentam alterações morfológicas no hipocampo e córtex pré-frontal, i.e., diminuição do tamanho das ramificações microgliais no hipocampo e diminuição no número de processos no córtex pré-frontal. Essas evidências nos levaram a hipotetizar que as alterações morfológicas da micróglia nesse modelo animal de HF tem início na fase adulta, é região-específica e se agrava em função do envelhecimento. Animais LDLr$^{-/-}$ se mostram mais suscetíveis ao processo de envelhecimento. Em um estudo prévio, aos 14 meses de idade, camundongos LDLr$^{-/-}$ apresentaram comprometimento do sistema colinérgico, desbalanço antioxidante, desbalanço da sinalização apoptótica e possível aumento do apoptose neuronal, levando a um prejuízo de memória mais severo (Moreira *et al.* 2012a; de Oliveira *et al.* 2020a). Entendemos, portanto, que animais LDLr$^{-/-}$ também se mostram mais suscetíveis a alterações na reatividade microglial comparados a animais selvagens de mesma idade. Entretanto, não podemos afirmar que animais LDLr$^{-/-}$ apresentam aos 14 meses de idade, alterações na funcionalidade/atividade microglial. Isto porque, conforme descrito por Paolicelli et al. (2022), alterações na morfologia microglial

servem apenas para gerar hipóteses sobre o estado funcional das micróglias, o qual deveria ser confirmado com técnicas sensíveis, visando detectar alterações de transcriptoma e proteoma nas micróglias em resposta ao contexto em que elas estão inseridas (Paolicelli *et al.* 2022).

Por sua vez, no modelo animal de hipercolesterolemia esporádica induzida por dieta, verificamos que os níveis aumentados de colesterol elevaram a microgliose na região hipocampal CA3, mas não alteraram a morfologia, a complexidade e a atividade fagocítica das micróglias no hipocampo. Dessa forma, a morfologia e densidade microglial, mas não sua funcionalidade, são mais afetadas no contexto da HF, onde os animais são expostos a altos níveis de colesterol e a disfuncionalidade do LDLr desde o nascimento em uma condição que se agrava com o envelhecimento, do que no contexto da hipercolesterolemia induzida por dieta, uma condição metabólica adquirida ao decorrer da vida do animal.

Estudos mostram que a reatividade microglial influência e modula os astrócitos, tornando-os igualmente reativos (Liddelow *et al.* 2020). Micróglia e astrócitos interagem por meio de contato célula-célula ou por intermédio da liberação de citocinas específicas. Tendo em vista que os astrócitos são as células gliais mais abundantes no parênquima cerebral, a sua atividade é fundamental para o funcionamento normal do SNC (Sofroniew and Vinters 2010). Entretanto, a elevação da reatividade astrocitária gera diversas respostas imunológicas, as quais podem favorecer a progressão de doenças, incluindo a DA (Sofroniew 2020; Bellaver *et al.* 2023). Camundongos $LDLr^{-/-}$ apresentaram um aumento basal da astrogliose no hipocampo, o que foi exacerbado quando os animais foram expostos ao Aβ (de Oliveira *et al.* 2014). Sabendo disso, realizamos a análise da astrogliose no hipocampo de animais com hipercolesterolemia induzida por dieta. Nossos dados mostram que os animais hipercolesterolêmicos não apresentaram aumento na

astrogliose hipocampal, ao menos na região CA3 do hipocampo. Tais resultados contrastam com estudos anteriores, onde a exposição de camundongos a uma dieta rica em colesterol por oito semanas aumentou a imunorreatividade para GFAP no hipocampo (Chen *et al.* 2016b). Ademais, camundongos fêmeas alimentados com dieta hipercolesterolêmica mostraram aumento na imunorreatividade para GFAP no hipocampo, em um processo que foi agravado pelo envelhecimento (Chen *et al.* 2018). Alguns fatores ajudam a explicar a discrepância entre os nossos achados e os artigos citados. Os estudos de Chen e colaboradores (2016, 2018) investigaram os efeitos de uma dieta contendo 3% de colesterol sobre a astrogliose hipocampal, em camundongos fêmeas com 6 meses de idade (Chen et al. 2016b; Chen et al. 2018). Desta forma, os fatores teor de colesterol na dieta, idade e sexo do animal podem ter influenciado no aumento da astrogliose hipocampal, processo este não visualizado pelo nosso estudo ao investigar a astrogliose hipocampal em camundongos machos e femeas de três meses de idade.

Astrócitos e micróglia frequentemente interagem com os neurônios. Neste sentido, as células microgliais por intermédio de moléculas de sinalização, tais como fatores neurotróficos, se comunicam e regulam a plasticidade sináptica além de realizarem a poda sináptica (Cornell *et al.* 2022). Sendo assim, nesta tese investigamos a relação da micróglia com a integridade sináptica por meio da avaliação do imunoconteúdo das proteínas sinápticas PSD-95 e sinaptofisina-1. Nossos dados mostram que animais LDLr$^{-/-}$ apresentaram, aos três meses de idade, redução no imunoconteúdo de PSD-95 no hipocampo. Já aos 14 meses de idade, animais LDLr$^{-/-}$ apresentaram uma redução significativa no imunoconteúdo de sinaptofisina hipocampal. Nesta mesma linha de evidência, Mulder et al., (2004) mostraram que animais LDLr$^{-/-}$ aos 13 meses de idade apresentam redução na quantidade de botões sinápticos contendo sinaptofisina no hipocampo (Mulder *et al.* 2004). Cabe ressaltar que fomos os primeiros a relatar

alterações sinápticas em camundongos LDLr$^{-/-}$ já aos três meses de idade. Por outro lado, não encontramos alterações no imunoconteúdo dessas proteínas sinápticas no córtex pré-frontal, mostrando que animais LDLr$^{-/-}$ apresentam respostas diferentes no que diz respeito ao imunoconteúdo de proteínas sinápticas, de acordo com a idade e região cerebral analisada. Nossos achados estão de acordo com os estudos de Mulder et., (2004; 2007), onde demonstrou-se que a funcionalidade sináptica e neuronal está alterada neste modelo animal de HF e parece estar mais concentrada no hipocampo (Mulder *et al.* 2004; Mulder *et al.* 2007).

A disfunção sináptica pode ser desencadeada por inúmeros mecanismos, dentre os quais está a alteração da permeabilidade da BHE e consequente neuroinflamação (Takechi *et al.* 2017; Nation *et al.* 2019). Em camundongos LDLr$^{-/-}$ aos três meses de idade, ocorre uma perda da integridade da BHE no córtex pré-frontal e hipocampo, caracterizada pela diminuição significativa na expressão gênica de proteínas associadas às junções oclusivas no hipocampo, bem como pelo aumento do extravasamento cortical e hipocampal do corante fluoresceína de sódio injetado perifericamente nos animais (de Oliveira *et al.* 2020b). No primeiro artigo que compõe este documento, verificamos que animais LDLr$^{-/-}$ também aos três meses de idade, apresentaram redução no imunoconteúdo de claudina-5 no córtex pré-frontal, mas não no hipocampo. Embora os dados referentes aos efeitos da HF na expressão de claudina-5 sejam conflitantes, nossos achados reforçam que a HF ou a ausência de LDLr alteram o conteúdo de proteínas das junções oclusivas no cérebro de animais hipercolesterolêmicos. Neste sentido, resultados prévios do nosso grupo de pesquisa apontaram que a disfunção da BHE é exacerbada quando camundongos LDLr$^{-/-}$ são alimentados com dieta rica em colesterol (de Oliveira *et al.* 2020b). Animais com hipercolesterolemia induzida por dieta apresentam disfunção da BHE no hipocampo, o que contribuiu para o comprometimento de memória nesses

animais (Rodrigues *et al.* 2021). Deste modo, verificamos que a hipercolesterolemia adquirida exerceu uma diminuição do imunoconteúdo da proteína claudina-5 no córtex pré-frontal. Ademais, foi observado uma redução no número de células positivas para Lectina de tomate nessa mesma estrutura cerebral nos animais com hipercolesterolemia induzida por dieta. Esses dados referentes à lectina de tomate, contrastam com resultados observados em animais LDLr$^{-/-}$, onde a HF elevou a quantidade de microvasos no cérebro (de Oliveira *et al.* 2020b). Aumento da quantidade de microvasos no cérebro, serve como um indicativo de um processo chamado angiogênese patológica onde as células que compõem a unidade neurovascular secretam fatores angiogênicos em demasia, alterando a permeabilidade da BHE e exacerbando o processo neuroinflamatório associado (Jeong *et al.* 2021).

Resultados prévios do nosso grupo de pesquisa apontaram que camundongos LDLr$^{-/-}$ apresentam uma neuroinflamação e quebra de BHE ainda mais intensa quando expostos à dieta hipercolesterolêmica, se comparados aos animais que foram expostos à dieta padrão e camundongos controles expostos à dieta rica em colesterol (de Oliveira *et al.* 2020b). Levando em consideração estas evidências prévias, nossa próxima pergunta foi: *A reatividade microglial está influenciando na manutenção da integridade da BHE em animais hipercolesterolêmicos?* Para responder esta questão, investigamos a presença da micróglia na região perivascular no cérebro de camundongos adultos C57BL/6 selvagens e LDLr$^{-/-}$. Nós observamos que os animais LDLr$^{-/-}$ apresentaram maior presença da micróglia na região perivascular no hipocampo, mas não no córtex pré-frontal, em comparação aos animais C57BL/6 selvagens de mesma idade. A presença das micróglias na região perivascular se faz importante para a atuação da micróglia como primeira linha de defesa contra estressores externos (Dudvarski Stankovic *et al.* 2015). No entanto, em condições patológicas, o aumento de micróglias nesta região pode ser

prejudicial e elevar a disfunção da BHE (Zhao et al. 2018; Haruwaka et al. 2019). Haruwaka e colaboradores (2019), investigando os efeitos de uma inflamação sistêmica persistente e da micróglia perivascular na integridade da BHE em um modelo animal, demonstraram que a micróglia em um momento inicial se mostrou importante para a manutenção da integridade da BHE, elevando de maneira interessante a expressão microglial de claudina-5 e mantendo contato físico com o endotélio. Todavia, os autores também reportaram que à medida que a inflamação sistêmica se torna persistente e não resolvida, a micróglia associada aos microvasos se torna mais reativa, elevando sua atividade fagocítica e reduzindo a expressão de claudina-5 na região cortical (Haruwaka *et al.* 2019).

Deste modo, a presença da micróglia na região perivascular exerce efeitos dúbios sobre a manutenção da BHE, em um processo que envolve a expressão da proteína claudina-5. Em nosso estudo, percebemos que ocorreu a diminuição do imunoconteúdo da proteína claudina-5 no córtex pré-frontal de animais $LDLr^{-/-}$ e de animais CF-1 com hipercolesterolemia induzida por dieta. Interessantemente, tais processos estiveram acompanhados por aumento da microgliose, mas não de alterações em sua reatividade e respectiva atividade fagocítica. Apesar de mais investigações serem necessárias, nós acreditamos que na hipercolesterolemia (principalmente na HF), o aumento da presença da micróglia na região perivascular contribui para a disfunção da BHE, também por intermédio da redução na expressão de proteínas associadas as junções oclusivas. Deste modo, mecanismos que visem reduzir a presença da micróglia reativa na região perivascular, se tornam uma estratégia em potencial para mitigar a disfunção da BHE associada a hipercolesterolemia.

Ademais, a proteína alvo da rapamicina (mTOR) se mostra importante na regulação da reatividade microglial decorrente de estímulos inflamatórios (Dello Russo

et al. 2009; Cappoli et al. 2019; Keane et al. 2021). Em relação ao papel da mTOR na resposta microglial à inflamação, Dello Russo e colaboradores (2009) demonstraram que a mTOR se torna ativa quando estimulada com indutores inflamatórios. Por outro lado, a inibição da mTOR reduziu a proliferação microglial em um processo que favoreceu a resolução da resposta neuroinflamatória (Dello Russo *et al.* 2009). No que diz respeito ao envolvimento das doenças metabólicas na ativação da mTOR, evidências mostram que camundongos obesos apresentam aumento na expressão de pmTOR no cérebro (Dasuri *et al.* 2016). Além disso, inibição da mTOR foi associada à uma melhora na permeabilidade da BHE em camundongos $LDLr^{-/-}$ (Van Skike *et al.* 2018). Sabendo que a desregulação da sinalização da mTOR está associado com a ocorrência de desordens metabólicas, disfunção da BHE e neuropatologias (Van Skike *et al.* 2018; Mao and Zhang 2018), resolvemos avaliar o imunoconteúdo da proteína mTOR no cérebro de camundongos $LDLr^{-/-}$. Nossos dados demonstram que os animais $LDLr^{-/-}$ aos três meses de idade apresentaram aumento no imunoconteúdo da mTOR no córtex pré-frontal, o que foi reduzido pelo envelhecimento. Por outro lado, o envelhecimento elevou o imunoconteúdo de mTOR no hipocampo, demonstrando que os conteúdos da proteína mTOR variam de acordo com a região cerebral analisada e com a idade no modelo animal de HF.

A quebra da integridade da BHE e a subsequente neuroinflamação caracterizada pela reatividade das células gliais, parecem ser eventos conectivos entre as desordens da homeostase do colesterol e a ocorrência de déficits cognitivos (Streit and Sparks 1997; Sparks et al. 2000; Ullrich et al. 2010; de Oliveira et al. 2014). Indivíduos com altos níveis de colesterol plasmático durante a meia-idade, apresentam um risco aumentado de apresentarem DA e prejuízos cognitivos em idades mais avançadas (Kivipelto et al. 2001; Kivipelto and Solomon 2006; Solomon et al. 2009). Além disso, um importante estudo

clínico realizado por Zambón e coautores (2010), mostrou que pacientes portadores da HF de meia-idade são particularmente mais suscetíveis a desenvolver CCL (Zambón *et al.* 2010). Evidências mais recentes relatam danos neuropsicológicos, incluindo perda de memória, em portadores de HF com idade entre 18 e 40 anos (Ariza *et al.* 2016). Nesta tese, utilizamos testes comportamentais para estudar a relação da hipercolesterolemia adquirida e de origem genética com déficits cognitivos. De acordo com dados prévios do nosso e de outros grupos de pesquisa (Ramírez et al. 2011; Moreira et al. 2012a; Lopes et al. 2015; de Oliveira et al. 2020a), observamos que os camundongos LDLr$^{-/-}$ já aos três meses de idade apresentaram pior performance nos testes de realocação e reconhecimento do objeto, evidenciando um prejuízo na função hipocampal. Além disso, notamos que os animais LDLr$^{-/-}$ apresentaram um padrão de hiper locomoção, evidenciado pelo aumento na distância percorrida no campo aberto. Nossos dados estão de acordo com estudos anteriores, nos quais a atividade hiper locomotora dos camundongos LDLr$^{-/-}$ pode ser visualizada já aos três meses de idade (Elder *et al.* 2008; Moreira *et al.* 2012a; de Oliveira *et al.* 2020b). Tal efeito persiste quando os animais envelhecem (Moreira *et al.* 2012a).

Efeitos similares na memória puderam ser observados em nosso modelo de hipercolesterolemia induzida por dieta, onde animais hipercolesterolêmicos tiveram baixa performance no teste de realocação do objeto, além de prejuízos na memória de habituação no campo aberto. Nossos dados estão de acordo com estudos anteriores do nosso grupo de pesquisa, os quais mostraram que camundongos alimentados por oito semanas com uma dieta rica em colesterol, tiveram déficit em memórias dependentes do hipocampo e comportamento do tipo-depressivo (Moreira *et al.* 2014; Rodrigues *et al.* 2021). No entanto, nenhuma alteração em padrões de locomoção foi observada em

animais com hipercolesterolemia induzida por dieta, demonstrando que o padrão de hiper locomoção é restrito aos camundongos LDLr$^{-/-}$.

Na sequência, com o intuito de melhor investigar o envolvimento da neuroinflamação e da reatividade microglial nos déficits cognitivos relacionados às alterações na homeostase do colesterol, tratamos tanto animais LDLr$^{-/-}$ quanto CF-1 hipercolesterolêmicos durante quatro semanas com a minociclina. A minociclina é um fármaco pertencente à classe das tetraciclinas, com importantes propriedades anti-inflamatórias e neuroprotetoras já descritas na literatura (Du et al. 2001; Jackson-Lewis et al. 2002; Choi et al. 2007; Ferretti et al. 2012; Garcez et al. 2017). O principal efeito biológico associado a este composto no SNC parece ser a redução da reatividade microglial (Du *et al.* 2001; Scholz *et al.* 2015). A escolha da dose e período de tratamento, 50 mg/kg e quatro semanas, foi baseado em estudos prévios onde foi demonstrado que esta estratégia de tratamento exerce importantes efeitos terapêuticos e anti-inflamatórios em modelos animais de doenças neurodegenerativas (Ferretti *et al.* 2012; Garcez *et al.* 2017). Nossos resultados demonstraram que a modulação farmacológica das micróglias exerceu efeito benéfico sobre a cognição em tarefas comportamentais dependentes do hipocampo, tanto em camundongos LDLr$^{-/-}$ quanto em camundongos CF-1 hipercolesterolêmicos. Nossos dados corroboram com evidências experimentais provenientes de estudos com animais modelo de doenças metabólicas e de amiloidose. A modulação farmacológica da atividade microglial aliviou a neuroinflamação e preveniu o declínio cognitivo em animais diabéticos (Jackson *et al.* 2020). Efeito semelhante pode ser observado em modelos animais de amiloidose, onde o tratamento com a minociclina melhorou a memória dependente do hipocampo, reduziu marcadores inflamatórios e o processamento da APP (Ferretti *et al.* 2012; Garcez *et al.* 2017). De particular interesse, camundongos com obesidade induzida por dieta ao receberem a minociclina,

apresentaram redução da reatividade microglial, evidenciado pela diminuição da imunorreatividade para CD68 nas células microgliais, e melhora na memória dependente do hipocampo (Cope *et al.* 2018).

No que diz respeito aos camundongos $LDLr^{-/-}$, a escolha de realizar a intervenção farmacológica com a minociclina aos 6 meses de idade, foi com o intuito de tentar verificar melhora nos parâmetros de reatividade microglial visualizados no cérebro desses animais ao longo do envelhecimento. No córtex pré-frontal, o tratamento de camundongos modelo de HF, reduziu a imunorreatividade para IBA-1 sem alterar a morfologia, a complexidade ou a atividade fagocítica dessas células. Já no hipocampo, o tratamento com a minociclina não reduziu a densidade microglial, mas causou alterações morfológicas pontuais, as quais variaram de acordo com o genótipo do animal e a região hipocampal analisada. A exemplo da densidade microglial, a intervenção farmacológica com a minociclina também não alterou a sua atividade fagocítica no hipocampo. Efeito similar foi encontrado no hipocampo de animais com hipercolesterolemia induzida, onde a minociclina não alterou os parâmetros de reatividade microglial avaliados. Tomados em conjunto, nossos dados mostram que a minociclina não alterou de forma significativa a reatividade microglial em animais hipercolesterolêmicos. Nesta mesma linha de evidência, a minociclina exerceu efeitos heterogêneos sobre a reatividade microglial em um modelo animal de lesão cerebral (Hanlon *et al.* 2017), e não se mostrou eficiente em alterar a morfologia microglial e sua reatividade no contexto de isquemia e reperfusão (Ahmed *et al.* 2017). Efeito similar foi relatado em um modelo animal de hemorragia cerebral, onde a minociclina não alterou a morfologia microglial na região do tálamo e hipotálamo (Blecharz-Lang *et al.* 2022).

De particular interesse, nossos dados apontaram importantes ações da minociclina sobre o imunoconteúdo de proteína associada a integridade da BHE e na presença da

micróglia na região perivascular. Especificamente, o tratamento com a minociclina elevou o imunoconteúdo da proteína claudina-5 tanto no córtex pré-frontal quanto no hipocampo de camundongos com hipercolesterolemia induzida por dieta. Tendo em vista o papel da micróglia na regulação e elevação dos níveis das proteínas de junção oclusiva na BHE (Yang et al. 2015b; Yan et al. 2015; Godinho-Pereira et al. 2022; Lu et al. 2022), nós investigamos os efeitos da minociclina sobre a presença da micróglia na região perivascular em animais $LDLr^{-/-}$. Nossos resultados mostram que a minociclina reduziu significativamente a quantidade de micróglias na região perivascular, tanto no córtex pré-frontal quanto no hipocampo dos camundongos $LDLr^{-/-}$. Em um modelo animal de isquemia e reperfusão, o tratamento com a minociclina reduziu a presença de micróglias reativas na região perivascular, auxiliando no remodelamento neurovascular após o evento isquêmico (Yang *et al.* 2015a). Sabendo que animais $LDLr^{-/-}$ apresentam disfunção da BHE já aos três meses de idade, e que isso pode provocar uma maior migração da micróglia para a região perivascular (Haruwaka et al. 2019; Kang et al. 2020), podemos propor que a redução da micróglia perivascular pela minociclina ajude na manutenção da integridade da BHE nesse modelo animal e consequentemente melhore a cognição dos animais. No entanto, novos estudos se fazem necessários para melhor investigar essa questão.

Apesar do efeito da minociclina ser mais estudado em micróglias, estudos mostram que este fármaco também exerce efeitos sobre a reatividade astrocitária, evidenciando que a ação da minociclina no SNC não fica restrita à micróglia (Cai *et al.* 2010; Stokes *et al.* 2017). Tal efeito foi percebido em nosso segundo estudo, onde a minociclina modulou a imunorreatividade astrocitária em camundongos hipercolesterolêmicos.

Células gliais reativas podem liberar uma série de fatores pró-inflamatórios e neurotóxicos, o que intensifica a cascata de neuroinflamação (Chen *et al.* 2016a). Dentre esses eventos, está a cascata de sinalização inflamatória desencadeada pelo RAGE. Um importante estudo demonstrou previamente que a exacerbação da sinalização inflamatória mediada por RAGE nas micróglias afeta as funções neuronais prejudicando a cognição em animais modelo para a DA (Fang *et al.* 2010). A hipercolesterolemia elevou a expressão de RAGE no cérebro em camundongos modelo para DA alimentados com dieta rica em colesterol (Zhou *et al.* 2021). Nossos dados demonstraram que na ausência de patologias a hipercolesterolemia não alterou o imunoconteúdo de RAGE em regiões cerebrais importantes para a cognição dos camundongos. Por outro lado, o tratamento com a minociclina reduziu o imunoconteúdo da proteína RAGE no córtex pré-frontal, o que pode ter levado à diminuição da resposta inflamatória por ele mediada. Apesar de mais estudos serem necessários, nossos resultados mostram que a cascata inflamatória desencadeada pelo RAGE pode ser modulada pela minociclina.

Conclusão e Perspectivas

Nesta tese observamos experimentalmente que alterações na densidade e morfologia, mas não a sua respectiva atividade fagocítica, estão envolvidas nos danos cognitivos associados à hipercolesterolemia, principalmente na HF. Ademais, percebemos que alterações no imunoconteúdo de proteínas sinápticas, de claudina-5 e na presença da micróglia na região perivascular, acompanharam os efeitos negativos na cognição relacionados aos altos níveis de colesterol e disfunção do receptor de LDL (Figura 4). Por fim, notamos que o tratamento com a minociclina melhorou o desempenho cognitivo dos animais hipercolesterolêmicos, independentemente de sua etiologia. Mecanismos como a redução na presença da micróglia na região perivascular, redução da

microgliose e da astrogliose e aumento do imunoconteúdo de claudina-5, acompanharam a melhora na cognição observado nos animais hipercolesterolêmicos tratados com a minociclina. No entanto, mais dados são necessários para melhor entender os mecanismos relacionados ao impacto das alterações na homeostase do colesterol no cérebro. Portanto, temos como perspectivas futuras:

- Analisar se a morfologia e a atividade fagocítica da micróglia localizada na região perivascular, muda em resposta a ausência dos LDLr e aos altos níveis de colesterol;
- Investigar a influência da reatividade microglial sobre a reatividade astrocitária, sua presença na região perivascular e suas respectivas relações com a disfunção da BHE observada em animais $LDLr^{-/-}$;
- Estudar os efeitos do dimorfismo sexual nos prejuízos cognitivos e mecanismos associados, em um modelo animal de hipercolesterolemia induzida por dieta;
- Verificar se o soro de animais hipercolesterolêmicos gera alterações na complexidade, na atividade fagocítica e no metabolismo de células da linhagem BV2 e se isso sofre variação de acordo com a etiologia da hipercolesterolemia;
- Pesquisar se as alterações descritas no item anterior, afetam a reatividade e o metabolismo astrocitário através do tratamento de células da linhagem C6 com o meio condicionado da BV2, expostas ao soro hipercolesterolêmico.

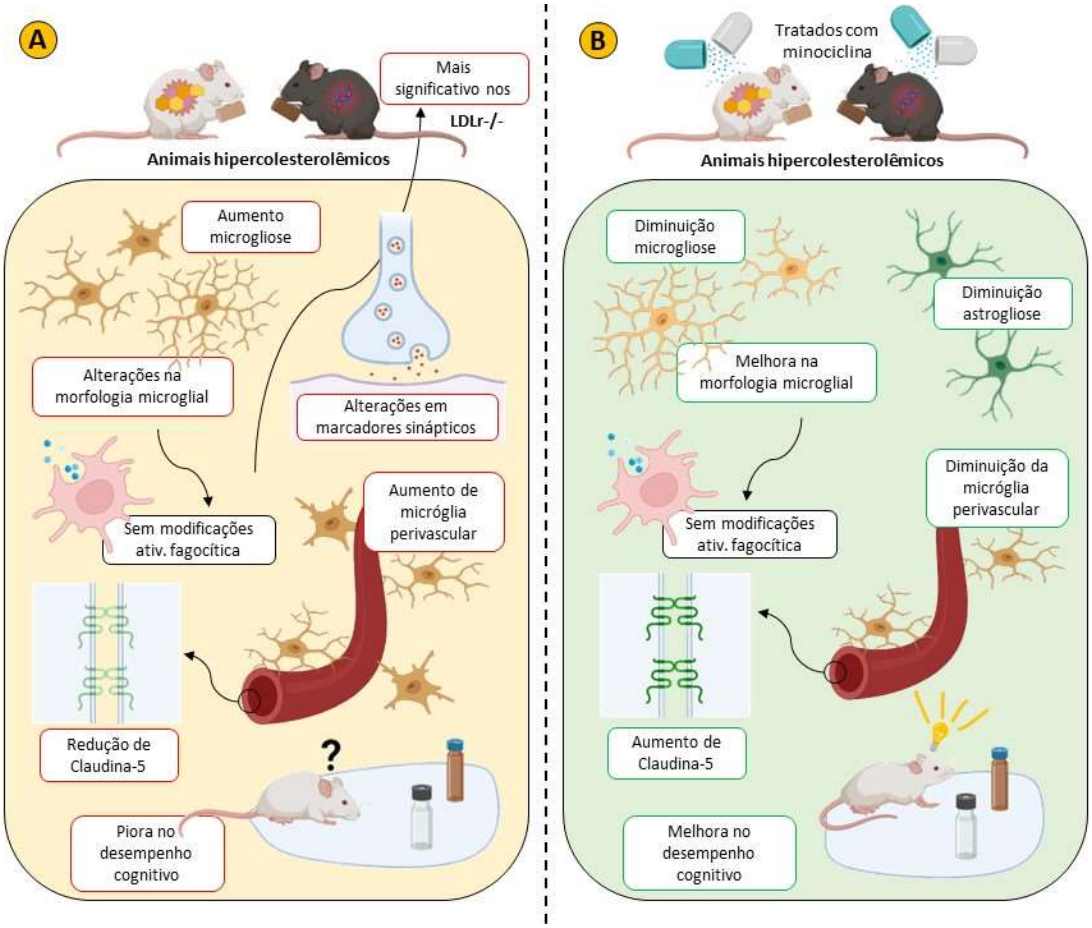

Figura 4. Representação esquemática contendo os principais resultados dos estudos que compõem esta tese. (A) Animais hipercolesterolêmicos apresentaram aumento da microgliose, da presença da micróglia na região perivascular, alterações na morfologia microglial e em marcadores sinápticos e de junções oclusivas (claudina-5). Tais alterações parecem ser mais significantes em animais modelo para a hipercolesterolemia familiar (camundongos nocautes para o receptor da lipoproteína de baixa densidade - $LDLr^{-/-}$), e acarretaram piora no desempenho cognitivo dos animais hipercolesterolêmicos (independente da etiologia). (B) A modulação da reatividade microglial com a minociclina, por outro lado, esteve associada à redução na microgliose, na astrogliose e na diminuição da presença da micróglia na região perivascular. O tratamento com a minociclina ainda elevou o imunoconteúdo de claudina-5 e melhorou o desempenho cognitivo nos animais hipercolesterolêmicos, independentemente de sua etiologia. Por fim, nenhum efeito da hipercolesterolemia ou do tratamento com a minociclina, fora observado em relação à atividade fagocítica microglial.

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Anexos

Anexo I: Cartas de aprovação dos estudos no comitê de ética no uso de animais (CEUA).

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

PRO-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 37626
Título: HIPERCOLESTEROLEMIA COMO FATOR DE RISCO PARA DOENCAS NEURODEGENERATIVAS: ESTUDOS DE POSSIVEIS ESTRATEGIAS NEUROPROTETORAS

Vigência: 01/11/2019 à 01/09/2022

Pesquisadores:

Equipe UFRGS:

Jade De Oliveira - coordenador desde 01/11/2019
Rachel Krolow Santos Silva Bast - pesquisador desde 01/11/2019
Alessandra Gonçalves Machado - Aluno de Doutorado desde 01/11/2019
Ariadni Mesquita Peres - Aluno de Mestrado desde 01/11/2019

Comissão De Ética No Uso De Animais aprovou o mesmo em seus aspectos éticos e metodológicos, para a utilização de 280 animais (140 machos e 140 fêmeas), três meses de idade da linhagem Swiss CF-1 pesando cerca de 25-30g provenientes do Biotério de Criação do Departamento de Bioquímica da Universidade Federal do Rio Grande do Sul (UFRGS); de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Quinta-Feira, 21 de Novembro de 2019

ALEXANDRE TAVARES DUARTE DE OLIVEIRA
Coordenador da comissão de ética

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

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 39781
Titulo: MINOCICLINA COMO ESTRATEGIA TERAPEUTICA CONTRA AS DISFUNÇÕES CEREBRAIS INDUZIDAS PELA HIPERCOLESTEROLEMIA

Vigência: 01/11/2020 à 01/11/2024

Pesquisadores:

Equipe UFRGS:

Jade De Oliveira - coordenador desde 01/11/2020
Matheus Scarpatto Rodrigues - desde 01/11/2020
Hémelin Resende Farias - desde 01/11/2020

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 23/11/2020 - Reunião via Webconferência - Mconf UFRGS, em seus aspectos éticos e metodológicos, para a utilização de 504 camundongos da linhagem CF-1 (machos e fêmeas), com 3 e 14 meses advindos do CREAL. Além disso, irão utilizar 504 animais (252 C57BL/6 e 252 LDLr -/-), machos e fêmeas, com 3 e 14 meses, provenientes do biotério setorial do Departamento de Bioquímica/ICBS/UFRGS, de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Sexta-Feira, 4 de Dezembro de 2020

ALEXANDRE TAVARES DUARTE DE OLIVEIRA
Coordenador da comissão de ética

Anexo II: Lista de artigos publicados durante o período de doutoramento.

Rodrigues, M. S., Pieri, B. L. D. S., Silveira, G. B., Zaccaron, R. P., Venturini, L. M., Comin, V. H., Luiz, K. D., & Silveira, P. C. L. (2020). Reduction of oxidative stress improves insulin signaling in cardiac tissue of obese mice. *Einstein (São Paulo, Brazil)*, *18*, eAO5022. https://doi.org/10.31744/einstein_journal/2020AO5022

Streck, E. L., Bussular, F. P., Wessler, L. B., Duarte, M. B., Rezende, V. L., **Rodrigues, M. S.**, Torres, C. A., Lemos, I. S., Candiotto, G., Gava, F. F., de Oliveira, J., & Valvassori, S. S. (2021). Administration of branched-chain amino acids alters epigenetic regulatory enzymes in an animal model of Maple Syrup Urine Disease. *Metabolic brain disease*, *36*(2), 247–254. https://doi.org/10.1007/s11011-020-00631-1

Rodrigues, M. S., de Paula, G. C., Duarte, M. B., de Rezende, V. L., Possato, J. C., Farias, H. R., Medeiros, E. B., Feuser, P. E., Streck, E. L., de Ávila, R. A. M., Bast, R. K. S. S., Budni, J., de Bem, A. F., Silveira, P. C. L., & de Oliveira, J. (2021). Nanotechnology as a therapeutic strategy to prevent neuropsychomotor alterations associated with hypercholesterolemia. *Colloids and surfaces. B, Biointerfaces*, *201*, 111608. https://doi.org/10.1016/j.colsurfb.2021.111608

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Silveira, P. C. L., **Rodrigues, M. S.**, Gelain, D. P., & de Oliveira, J. (2023). Gold nanoparticles application to the treatment of brain dysfunctions related to metabolic diseases: evidence from experimental studies. *Metabolic brain disease*, *38*(1), 123–135. https://doi.org/10.1007/s11011-022-00929-2

Vieira, A. D. C., Medeiros, E. B., Zabot, G. C., Pereira, N. S., do Nascimento, N. B., Lidio, A. V., Scheffer, Â. K., Rempel, L. C. T., Macarini, B. M. N., Costa, M. A., Gonçalves, C. L., Kucharska, E., **Rodrigues, M. S.**, Moreira, J. C. F., de Oliveira, J., & Budni, J. (2023). Neuroprotective effects of combined therapy with memantine, donepezil, and vitamin D in ovariectomized female mice subjected to dementia model. *Progress in neuro-psychopharmacology & biological psychiatry*, *122*, 110653. https://doi.org/10.1016/j.pnpbp.2022.110653

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Anexo III: Artigos publicados durante o período de doutoramento cujos temas se relacionam a esta tese, mas não foram incluídos no corpo principal da tese.

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Nanotechnology as a therapeutic strategy to prevent neuropsychomotor alterations associated with hypercholesterolemia

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ABSTRACT

Hypercholesterolemia has been linked to neurodegenerative disease development. Previously others and we demonstrated that high levels of plasma cholesterol-induced memory impairments and depressive-like behavior in mice. More recently, some evidence reported that a hypercholesterolemic diet led to motor alterations in rodents. Peripheral inflammation, blood-brain barrier (BBB) dysfunction, and neuroinflammation seem to be the connective factors between hypercholesterolemia and brain disorders. Herein, we aimed to investigate whether treatment with gold nanoparticles (GNPs) can prevent the inflammation, BBB disruption, and behavioral changes related to neurodegenerative diseases and depression, induced by hypercholesterolemic diet intake in mice. Adult Swiss mice were fed a standard or a high cholesterol diet for eight weeks and concomitantly treated with either vehicle or GNPs by the oral route. At the end of treatments, mice were subjected to behavioral tests. After that, the blood, liver, and brain structures were collected for biochemical analysis. The high cholesterol diet-induced an increase in the plasma cholesterol levels and body weight of mice, which were not modified by GNPs treatment. Hypercholesterolemia was associated with enhanced liver tumor necrosis factor- α (TNF-α), BBB dysfunction in the hippocampus and olfactory bulb, memory impairment, cataleptic posture, and depressive-like behavior. Notably, GNPs administration attenuated liver inflammation, BBB dysfunction, and improved behavioral and memory deficits in hypercholesterolemic mice. Also, GNPs increased mitochondrial complex I activity in the prefrontal cortex of mice. It is worth highlight that GNPs' administration did not cause toxic effects in the liver and kidney of mice. Overall, our results indicated that GNPs treatment potentially mitigated peripheral, brain, and memory impairments related to hypercholesterolemia.

1. Introduction

Cholesterol, which is essential to all animal life, is an important cell membrane component and a precursor of bile acids and steroid hormones [1]. However, imbalances on cholesterol metabolism, i.e., hypercholesterolemia, have deleterious effects on biological tissues and are related to diseases development [2]. Around 40 % of the global adult population has hypercholesterolemia. Plasma cholesterol levels above the limits considered normal is a major cause of disease burden worldwide as a risk factor for atherosclerotic cardiovascular disease and stroke [3,4].

Also, high serum cholesterol levels have been considered a risk factor for the occurrence of cognitive decline and Alzheimer's disease (AD) [5,6]. Sparks and collaborators [7] reported the first association between hypercholesterolemia and AD. Using a post-mortem analysis, these authors found an increase in the senile plaques' content in brain specimens

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of individuals with cardiovascular disease. The enhanced amyloid plaque presence was also observed in rabbits fed a high cholesterol diet [8]. Of particular interest, Moreira and coauthors [9] observed that Swiss mice exposed to a high cholesterol diet for eight weeks displayed learning and memory impairments, which were associated with biochemical alterations, such as the increased activity of acetylcholinesterase, in the prefrontal cortex and hippocampus.

More recently, hypercholesterolemia has also been linked to Parkinson's disease (PD). For instance, Paul et al. [10] reported that mice exposed to a high cholesterol diet displayed psychomotor alterations, including depressive-like behavior, which were combined with a deficit of biogenic amines (serotonin and dopamine) in the cortex and striatum, and loss of neurons in the substantia nigra [11]. Moreover, the dopaminergic neurodegeneration and neurotoxicity were exacerbated by hypercholesterolemia in a rodent model of PD induced by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride) exposure [11].

Furthermore, a higher depression incidence was observed in hypercholesterolemic individuals [12–15]. Similar results were found in experimental models of familial hypercholesterolemia and diet-induced hypercholesterolemia. Hypercholesterolemic animals showed increased depressive-like behavior when compared to their respective healthy controls [16,17]. Importantly, depression is also comorbidity of neurodegenerative diseases, such as AD and PD [18]. Therefore, the management of hypercholesterolemia brain outcomes seems to be an essential strategy to prevent brain disorders.

Evidence has reported that hypercholesterolemia triggers neuroinflammation and oxidative stress associated with loss of blood-brain barrier (BBB) integrity [19–21]. Of note, inflammation and oxidative stress are biological processes closely connected to the development of neuropathologies such as PD, AD, and depression [22–24]. In this context, gold nanoparticles (GNPs) have been explored due to their antioxidant and anti-inflammatory properties, showing remarkable efficacy in non-toxic and non-immunogenic doses [25–28].

The GNPs beneficial effects were observed in both neurodegenerative diseases and metabolic disorders experimental models [26,28,29]. Previously, GNPs treatment prevented the cognitive decline and the brain oxidative damage and inflammatory response in a rodent model of dementia [26]. In addition, the GNPs administration reduced the neuroinflammation, TAU phosphorylation, and prevented the cognitive deficits induced by okadaic acid in an experimental model of AD [28]. In obese mice, GNPs treatment ameliorated metabolic profile and inflammation in the fat and hepatic tissue [29,30].

Taking into account the importance of controlling brain damage caused by hypercholesterolemia, as well as the potential neuroprotective effects of GNPs, herein, we hypothesized that GNPs could be an interesting therapeutic strategy to ameliorate hypercholesterolemia-induced neurodegenerative diseases and depression phenotypes in mice. Importantly, we have investigated the anti-inflammatory and neuroprotective actions of GNPs in a very low dosage, unlike previous works that have used a higher dosage.

2. Materials and methods

2.1. Animals

At three months of age, male Swiss mice from the Central Animal House of Universidade do Extremo Sul Catarinense (UNESC, Brazil) were used. The animals were maintained in groups of 9–10 animals per cage, under a 12 h light / 12 h dark cycle (light from 7 a.m. to 7 p.m.) and controlled temperature (23 ± 1 °C). All animals' procedures were performed following the local ethics committee and following the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). Process Number: 007/2018-1.

2.2. Gold nanoparticles synthesis and characterization

Gold nanoparticles (GNPs) of 20 nm of diameter and a 70 mg/L concentration were synthesized as described previously [31]. Briefly, an aqueous solution of sodium citrate ($Na_3C_6H_5O_7$. $2H_2O$, 38 mM, Nuclear, SP, Brazil), a reducing agent and stabilizer, was added to a hydrogen tetrachloroaurate ($HAuCl_4$, 4 mM Sigma-Aldrich, MO, USA) solution previously heated to 90 °C. The system was maintained under reflux with magnetic stirring for 20 min. A new 70 mg/L solution was synthesized weekly, and the viability of GNPs was accompanied by a UV–vis spectrum throughout the experimental period. Before each administration, the GNPs solution at 70 mg/L was diluted in water to reach the working concentration of 0.25 μg/mL, resulting in a dose equivalence of 2.5 μg/kg body weight in mice. GNPs were characterized by UV–vis performed in a Shimadzu UV-1800 spectrophotometer (Shimadzu, Tokyo, Japan) and by transmission electron microscopy (TEM) using a JEM-1011 (100 kV). A diagram of the preparation and characterization of GNPs is demonstrated in Fig. 1A.

2.3. Experimental protocol

Three-month-old male Swiss mice were fed a normal diet (Puro Trato®- Puro Lab 22bp) or a high cholesterol diet (Rhoster® - RH19539C) continuously for eight weeks [9]. The details regarding the caloric profile and compositions of experimental diets are shown in Table 1. Also, the animals received oral gavage of GNPs (2.5 μg/kg prepared in water) or vehicle (water) every two days (48 -h interval between each administration) throughout the experimental period. Considering diet manipulation and treatment, the animals were randomized into four experimental groups (Fig. 1D). During the experimental period, food consumption and body mass were measured. After that, animals were submitted to behavioral tests (n = 8 animals per experimental group in each experimental cohort) - open field, object recognition, catalepsy and tail suspension. In the first experimental cohort, animals were submitted to open field and object recognition test, while in the second experimental cohort, we performed the catalepsy, and then tail suspension test on mice. After the object recognition test, sodium fluorescein assay (n = 8) was performed to evaluate the BBB permeability. Following the catalepsy and tail suspension tests, mice were food-deprived for 6 h, and blood was collected from the heart to determine plasma cholesterol levels, tumor necrosis factor- α (TNF-α) levels, and toxicological parameters (n = 5 per experimental group). The liver was dissected for TNF-α content evaluation (n = 5). Finally, the hippocampus, prefrontal cortex, and striatum were also dissected to assess mitochondrial complex I activity (n = 5 animals per group). Fig. 1D presents a scheme of the study design.

2.4. Body weight, weight gain, and food consumption

Body mass was measured in alternate days for eight weeks and at the end of the experimental period, while weight gain was evaluated by subtraction of the final weight (last week) of each animal by their respective initial weight (first week). Food consumption (in g) was calculated by subtracting the initial feed weight by its respective final weight. To calculate calorie intake (in kcal), we multiplied the amount of feed consumed by their respective caloric values (regular diet 3.3 kcal/g and high cholesterol diet 4.7 kcal/g). It should be noted that food consumption was assessed on alternate days for eight weeks.

2.5. Plasma total cholesterol levels and toxicological markers

The animals were food-deprived for 6 h, deeply anesthetized under ketamine and xylazine mixture (75 and 10 mg/kg, respectively, i.p.), and blood was collected from the heart, immediately centrifuged at 1000 x g for 5 min, and the plasma was frozen at -80 °C. Total cholesterol levels were measured in plasma using the enzymatic kit according to the

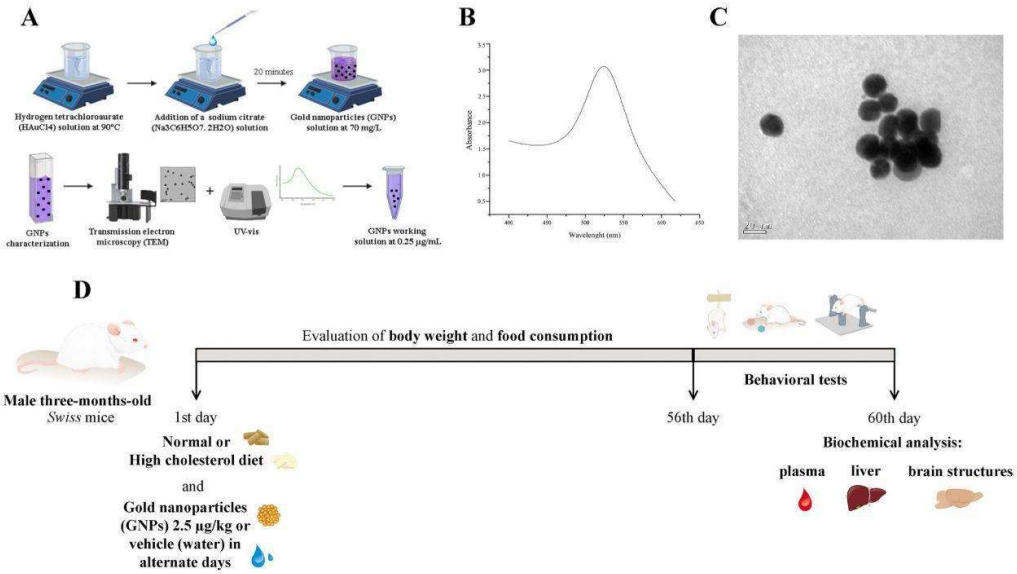

Fig. 1. Synthesis and characterization of gold nanoparticles (GNPs) and experimental protocol. (A) Diagram of the preparation and characterization of GNPs. GNPs of 20 nm of diameter (70 mg/L) were synthesized from hydrogen tetrachloroaurate ($HAuCl_4$) using sodium citrate ($Na_3C_6H_5O_7$. $2H_2O$) as reducing agent and stabilizer. Before each administration, the GNP solution at 70 mg/L was diluted in water to reach the working concentration of 0.25 µg/mL. GNPs were characterized by UV–vis and by transmission electron microscopy (TEM). (B) UV–vis absorption spectra in the visible region of GNPs and (C) TEM image, Scale bar = 20 nm (D) Experimental protocol. Three-month-old male *Swiss* mice were fed a normal diet or a high cholesterol diet for eight weeks. During this time, the animals received GNPs (2.5 µg/kg) or vehicle (water) by oral gavage in alternate days. Food consumption and mice's body mass were evaluated. On day 56th, the animals were submitted to behavioral tests. After that, on day 60th, the mice were anesthetized and euthanized for the posterior performance of biochemical analysis in the plasma, liver, and brain structures. We conducted two experimental cohorts.

Table 1
Composition and caloric profile of experimental diets.

Component	Normal diet	High cholesterol diet
Protein, %	22.53	14.79
Carbohydrate, %	41.02	50.19
Fat*, %	8.75	23.64
Cholesterol, %	–	1.25
Total, kcal/g	3.3	4.7

*Normal diet: fat content derived from vegetable source (rice bran and ground brown corn).
*High cholesterol diet: fat content derived from vegetable oils (soy oil and refined coconut oil).
Additional information: the composition of both diets followed the American Institute of Nutrition [32], and the levels of essential ingredients, such as vitamins and minerals, were adequate to the nutritional needs of adult animals (AIN-93 M diets).

manufacturer's instructions (Gold Analisa Diagnostica Ltda, Minas Gerais, Brazil). The data are expressed as mg/dL.

To analyze the safety of the GNPs administration, we evaluated the levels of alanine aminotransferase (ALT), gamma-glutamyl transferase (γ - GT) activities and creatinine, markers of hepatic and kidney damage. The ALT, γ – GT, and creatine levels were measured according to the manufacturer's instructions (Gold Analisa Diagnostica Ltda, Minas Gerais, Brazil). The ALT and γ - GT determination data are expressed as U/L, while the results from creatine measure are expressed as mg/dL [27].

2.6. Behavioral tests

For behavioral tests' performance, the animals have moved to the testing room at least 1 h before the start of behavioral tests to acclimate. The testing room used presented controlled temperature and humidity, similar to regular housing conditions. To minimize stress, we used diffuse and low lighting (red light), and the animals were maintained under normal environment noise. The room was also free from extraneous odors, including the emanating from the experimenter (i.e., the use of perfume, deodorant, or lotion was avoided). All behavioral tests were performed between 7 a.m. and 5 p.m. during the light phase of the animals' light / dark cycle.

2.7. Open field

The open-field test was used to investigate the spontaneous locomotor activity of mice. The open field apparatus consisted of a 45cm × 60cm brown plywood arena, which is surrounded by 50 cm-high wooden walls containing a frontal glass wall. The arena's floor was divided into nine rectangles (15cm × 20cm each) by black lines. For evaluation of locomotor activity, each animal was gently placed on the central quadrant, and the total crossings number was measured for 5 min. The animals were returned to their home cages immediately after the performance of the test session. It is essential to mention that the open field apparatus was cleaned with ethyl alcohol 10 % between each test session to avoid any influence of the anterior animal on the posterior animal's behavior. A crossing was recorded when the animal crossed the square with four legs [33].

2.8. Object recognition task

The recognition memory of mice was assessed using the object recognition task, according to Ainge et al. [34] protocol. The task is based on rodents' spontaneous tendency, previously exposed to two identical objects, to later explore a new object on open field apparatus. Exploration of the objects was recorded using a stopwatch when mice sniffed, whisked, or looked at the objects from no more than 1 cm away. Twenty-four hours before the training session's performance, the animals explored the open field apparatus for 5 min to adapt to that apparatus. During the training session, the animals were exposed to two identical objects for 5 min. After the training phase, the mice were removed from the apparatus and immediately returned to their home cages. One hour after the training, the animals returned to the open field. A new object was introduced into the apparatus in the same position as one of two old objects previously contained in the apparatus. After that, the animals' time exploring the new and old objects was recorded for over 5 min. The apparatus was cleaned with ethyl alcohol 10 % between each test session to avoid any influence of the anterior animal on the behavior of the posterior animal. A discriminatory index (total time spent with the new object/total time of objects exploration) was used to measure recognition memory. The object recognition task was used to assess the hippocampal-dependent memory.

2.9. Tail suspension test

Mice were individually suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Mice were visually isolated. This test is based on the fact that animals subjected to the short-term, inescapable stress of being suspended by their tail, will develop an immobile posture. Mice were considered immobile only when they hung passively and completely motionless. The immobility time was recorded for 6 min [35].

2.10. Catalepsy test

Catalepsy is a feature of PD. Catalepsy behavior was assessed by placing the animals' forelimbs individually over a horizontal bar (3 mm of diameter, 4.5 cm of height, and 10 cm of width), and the immobility time was measured up to a maximum of 180 s. The duration of catalepsy, which was defined as an immobile and cataleptic posture, was performed and calculated as described by Shiozaki et al. [36].

2.11. Blood-brain barrier (BBB) functional analysis

The BBB permeability to low molecular weight dye, sodium fluorescein, was evaluated in the hippocampus, prefrontal cortex, striatum, and olfactory bulb as an indicator of BBB function and integrity. For that, mice were anesthetized with isoflurane, and 4% sodium fluorescein (4 mL/kg) was injected into the penile vein. Thirty minutes after application, the animals were anesthetized (xylazine 10 mg/kg and ketamine 75 mg/kg, i.p.) and then perfused with 0.9 % saline. Followed by the perfusion, the brain structures were dissected and processed for the sodium fluorescein concentration analyses [37]. The brain structures, the hippocampus, prefrontal cortex, striatum, and olfactory bulb were diluted in trichloroacetic acid (TCA) 7.5 % (1:8, 1:7, 1:8, and 1:11, respectively), homogenized and centrifuged at 10,000 *g* for 10 min. Supernatants were diluted with 1:2.5 vol of 1 M phosphate buffer (TFK; pH 7.0) before spectrophotometric determination of sodium fluorescein (485 nm excitation/538 nm emission) fluorescence. Results are expressed as ng/mg tissue.

2.12. Cytokine content

The determination of the TNF-α cytokine content was performed evaluated in the plasma and liver samples by enzyme-linked immunosorbent assay (DuoSet ELISA) capture method (R&D System Inc., Minneapolis, USA). The samples of liver were homogenized with a specific buffer containing protease and phosphatase inhibitors diluted in Phosphate Buffer Sodium (8 g Sodium Chloride; 0.2 g Potassium Chloride; 1.44 g Disodium Phosphate; 0.24 g Potassium hypophosphite in 800 mL distilled water; pH = 7.4) and centrifuged at 1000 × *g* at 4 °C and the supernatant was maintained at -80 °C for further analysis. The plasma was obtained after centrifugation of blood at 3000 rpm at 4 °C for 10 min, and maintained at -80 °C for further analysis. After that, the samples were processed, and plates sensitized for incubation with the antibody. All experimental protocols were performed following the manufacturers' instructions (R&D System Inc., Minneapolis, USA). The results are expressed as pg/mg of protein.

2.13. Mitochondrial complex I activity

For the determination of mitochondrial complex I activity, the hippocampus, prefrontal cortex, and striatum were homogenized (1:10, w/v) in SETH buffer (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU/mL heparin), pH 7.4. The homogenates were centrifuged at 3000 rpm for 10 min, and the supernatants kept at −80 °C until the determination of complex I activity. The activity of complex I (NADPH dehydrogenase – EC 1.6.5.3) was evaluated by determining the rate of NADH-dependent ferricyanide reduction at 420 nm for 4 min at 25 °C, as described by Cassina and Radi [38]. Results are expressed as nmol/min/mg protein.

2.14. MTT test

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) test was used as cell integrity and GNPs toxicity parameter by evaluating mitochondrial tissue integrity. MTT reduction by enzyme succinate dehydrogenase occurs in the mitochondria. The MTT reduction was carried out according to Bhattacharya et al. [39], with minor modifications. Briefly, 100 mg of the liver slice was dissected, weighed, and homogenized in 1 mL of Phosphate-Buffered Saline (PBS) (pH 7.4). Next, the homogenate was centrifuged at 3000 × *g* for 5 min. After that, 250 μL of the supernatant was mixed with 750 μL of PBS and 200 μL of MTT solution (2.5 mg/mL in PBS), then the mixture was incubated in the dark at 37 °C for 90 min. During this time, the yellow-colored MTT was reduced to a purple formazan salt. The absorbance of the formazan salt was evaluated spectrophotometrically at 570 nm, and the values are expressed as A570/g wet tissue.

2.15. Protein content

The protein content of samples was determined using bovine serum albumin as a standard, as described by Lowry et al. [40]. Absorbance was measured in a Spectra Max M5 microplate reader (Molecular Devices, USA) at a wavelength of 700 nm.

2.16. Statistical analysis

All data were expressed as mean ± SEM. The statistical analysis was carried out using unpaired *t*-test or two-way ANOVA with multiple post-hoc comparisons performed using Duncan's test. The level of significance for all tests was $P < 0.05$. All tests were performed using the STATISTICA® software package.

3. Results

3.1. GNPs characterization and toxicological study

As shown in Fig. 1B, the electron spectrum showed an absorption peak at 528 nm, the characteristic peak of GNPs with a diameter of approximately 20 nm. TEM image (Fig. 1C) validated the diameter of

GNPs (20 nm). Also, TEM images presented GNPs with nearly spherical morphology. Toxicity parameters are shown in Table 2. GNPs treatment did not change the plasma activities of ALT and γ – GT, and the levels of creatinine. Moreover, the MTT and protein levels in the liver were not altered by GNP treatment, regardless of the diet (Table 2).

3.2. Plasma cholesterol levels and body weight measures

As shown in Fig. 2A, the high cholesterol diet exposure induced a significant increase in plasma cholesterol levels in mice. On the other hand, GNPs treatment did not modify the level of cholesterol in the plasma of animals fed both normal diet or high cholesterol diet (Fig. 2A).

Additionally, animals fed a high cholesterol diet presented decreased food consumption compared to those fed a regular diet. GNPs administration also caused a reduction in food intake in normal-diet-fed mice, but not in mice fed a high cholesterol diet (Fig. 2B). Mice exposed to a hypercholesterolemic diet exhibited increased calorie intake, which was not prevented by GNPs. Moreover, GNPs administration was associated with a diminished calorie intake in mice fed a standard diet (Fig. 2C).

At the end of the experimental period, animals fed a high cholesterol diet displayed a significant increase in body weight, which was not modified by GNPs treatment (Fig. 2D). Finally, subtracting the final weight of animals by their respective initial weight, we observed that hypercholesterolemic mice presented increased weight gain when compared with those animals that received a healthy diet. This parameter has not changed with GNPs exposure (Fig. 2E).

3.3. GNPs treatment prevented the behavioral alterations induced by hypercholesterolemia

The effects of high cholesterol diet and GNPs treatment on mice's performance in the behavioral tests are shown in Fig. 3. Firstly, the high cholesterol diet exposure and treatment with GNPs did not significantly change the number of crossings in the open field arena (Fig. 3A). In the sequence, the recognition memory of mice was accessed by the object recognition test. The high cholesterol diet consumption reduced the recognition index of the animals, i.e., induced memory impairment. Notably, GNPs treatment prevented this cognitive decline in mice fed a hypercholesterolemic diet (Fig. 3B).

In addition, hypercholesterolemic mice displayed an increase in the immobility time in the tail suspension test, featuring a depressive-like behavior. The GNPs treatment ameliorated the depressive-like behavior in the animals fed a hypercholesterolemic diet (Fig. 3C). High cholesterol diet exposure significantly increased the cataleptic posture in mice in comparison to a normal diet exposure. Importantly, GNPs treatment was able to reduce this cataleptic behavior induced by hypercholesterolemia in mice (Fig. 3D).

Table 2
Toxicological study in the liver and plasma of mice exposed to GNPs treatment.

Parameters	Normal diet + vehicle	High cholesterol diet + vehicle	Normal diet + GNP	High cholesterol diet + GNP
Liver MTT (^{A}570/g tissue)	16.67 ± 1.15	18.55 ± 1.48	15.23 ± 1.12	17.84 ± 2.49
Liver total protein (mg/mL)	15.43 ± 0.36	14.87 ± 0.48	15.91 ± 0.51	15.28 ± 0.47
Plasma ALT (U/L)	4.63 ± 1.30	7.15 ± 1.64	3.23 ± 0.44	6.25 ± 2.07
Plasma γ GT (U/L)	3.39 ± 0.38	3.89 ± 0.30	3.72 ± 0.31	2.70 ± 0.68
Plasma creatinine (mg/dL)	1.34 ± 0.34	1.32 ± 0.17	1.39 ± 0.38	1.24 ± 0.09

Results are expressed as mean ± SEM (n = 5 per group). Data were analyzed by two-way ANOVA followed by Duncan test. *Abbreviations:* GNP = gold nanoparticles, ALT = alanine aminotransferase, γ-GT = gamma-glutamyl transferase.

3.4. GNPs reduced the levels of inflammatory cytokine in the liver in hypercholesterolemic mice

To evaluate the inflammation in the plasma and liver, we performed the ELISA assay for TNF-α, a pro-inflammatory cytokine. Hypercholesterolemic diet intake significantly increased TNF-α levels in the liver (Fig. 4B), and the GNPs treatment restored the TNF-α content to control levels in the liver of hypercholesterolemic mice (Fig. 4B). Plasma TNF-α content was not changed by GNPs or hypercholesterolemic diet (Fig. 4A).

3.5. GNPs ameliorated blood-brain barrier disruption induced by hypercholesterolemia

The sodium fluorescein assay evaluated the BBB permeability in the hippocampus, prefrontal cortex, striatum, and olfactory bulb. As shown in Fig. 5, hypercholesterolemia increased sodium fluorescein fluorescence in the hippocampus (Fig. 5A) and olfactory bulb (Fig. 5D), but not in the striatum (Fig. 5C), and prefrontal cortex (Fig. 5B) of mice. Treatment with GNPs, on the other hand, restored the BBB permeability to sodium fluorescein in the hippocampus ($p = 0.062$; Fig. 5A) and olfactory bulb ($p = 0.076$ – Fig. 5D), improving the BBB integrity in these brain structures.

3.6. GNPs treatment enhanced mitochondrial respiratory chain complex I in mice's prefrontal cortex

To assess the mitochondrial respiratory chain function, mitochondrial complex I activity was analyzed in the hippocampus, prefrontal cortex, and striatum of the animals. As shown in Fig. 6B, GNPs treatment *per se* increased the complex I activity in the prefrontal cortex of animals, regardless of the diet (normal or high cholesterol diet). Mitochondrial complex I activity was not modified by GNPs or the high cholesterol diet in the hippocampus and striatum of animals (Fig. 6A, C).

4. Discussion

Dementia is a growing health concern. Nowadays, approximately 50 million people live with dementia worldwide, which is projected to increase to 152 million by 2050 due to the increment in life expectancy [41]. The neurodegenerative diseases, such as AD and PD, are the primary forms of dementia [41,42]. Epidemiological studies have reported an association between vascular risk factors and the development of AD and PD [5,6,43–45]. For instance, hypercholesterolemia has been strongly associated with cognitive impairments and cerebral changes characteristic of neurodegenerative diseases [8–10,46,47]. Specifically, the BBB disruption and coexistent neuroinflammation have been considered the main events connecting hypercholesterolemia to cognitive decline occurrence [48,49]. Importantly, GNPs, due to their significant anti-inflammatory properties [25], may be a potential therapeutic strategy to mitigate the pathophysiological features associated with hypercholesterolemia. In this regard, we investigated the effects of GNPs treatment on hypercholesterolemia-induced metabolic, inflammatory, and brain dysfunction.

To induce an experimental hypercholesterolemia model, we fed three-month-old Swiss mice with a high cholesterol diet (1.25 % cholesterol plus 20 % fat) for eight weeks. According to a previous study that used the same experimental protocol [9], we observed that mice fed a hypercholesterolemic diet showed a 30 % increase in the plasma cholesterol levels. The exposure of rodents (e.g., mice and rats) to a cholesterol-enriched diet is widely used as an experimental model of hypercholesterolemia with significant effects on plasma cholesterol levels [9,10,37,50,51]. Also, in our study, the animals fed a high

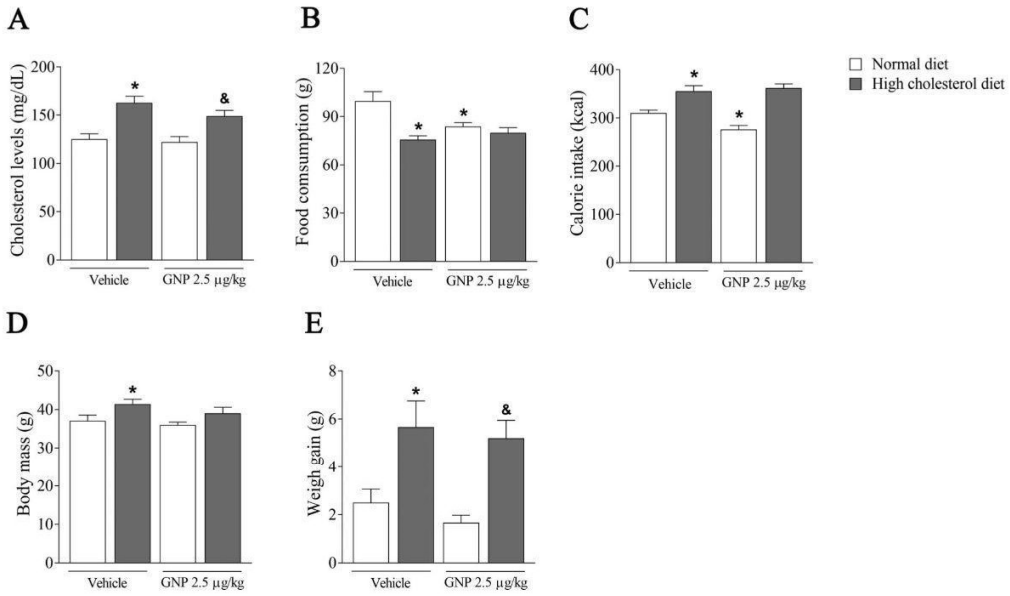

Fig. 2. Effects of a high cholesterol diet and gold nanoparticles (GNPs) treatment on plasma cholesterol levels, food consumption, calorie intake, and body mass parameters in mice. (A) Plasma cholesterol levels. (B) Food consumption. (C) Calorie intake. (D) Body mass at the end of the experimental period. (E) Weight gain. Data are expressed as mean ± SEM (n = 8 per group). * $P < 0.05$ compared to normal diet and vehicle; & $P < 0.05$ compared to normal diet and GNP treatment (Two-way ANOVA followed by Duncan test).

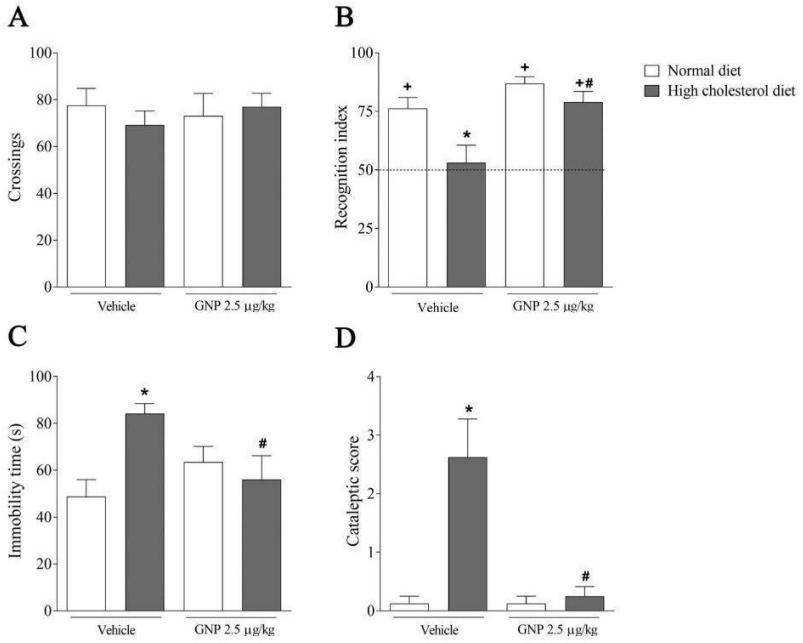

Fig. 3. Effects of a high cholesterol diet and gold nanoparticles (GNPs) treatment on behavioral parameters in mice. (A) Crossings number (open field test). (B) Recognition index (object recognition test). (C) Immobility time (tail suspension test). (D) Catalepsy test. Data are expressed as mean ± SEM (n = 8 per group). * $P < 0.05$ compared to normal diet and vehicle; # $P < 0.05$ compared to high cholesterol diet and vehicle (Two-way ANOVA followed by Duncan test and unpaired *t*-test).

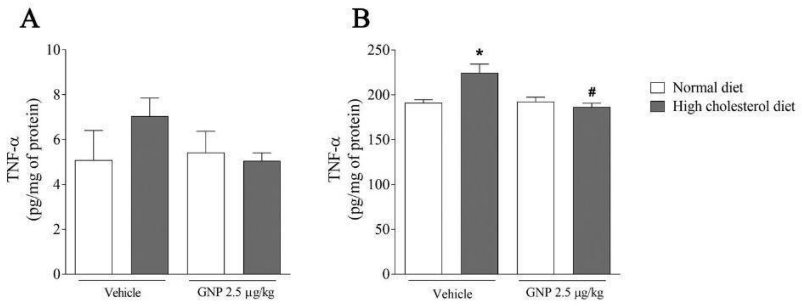

Fig. 4. Effects of a high cholesterol diet and gold nanoparticles (GNPs) treatment on TNF-α levels in the plasma and liver of animals. (A) TNF-α levels in the plasma. (B) TNF-α levels in the liver. Data are expressed as mean ± SEM (n = 5 per group). * $P < 0.05$ compared to normal diet and vehicle; # $P < 0.05$ compared to high cholesterol diet and vehicle (Two-way ANOVA followed by Duncan test).

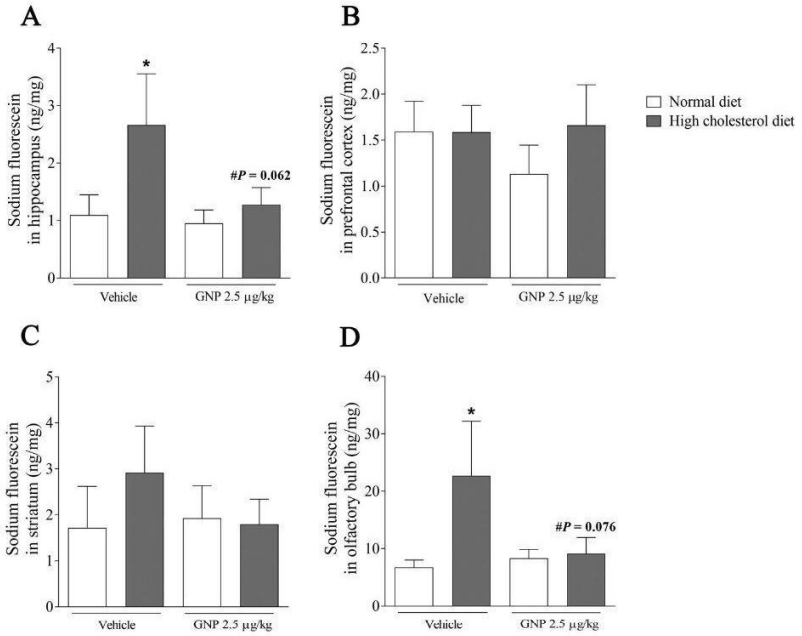

Fig. 5. Effects of a high cholesterol diet and gold nanoparticles (GNPs) treatment on blood-brain barrier permeability to sodium fluorescein. (A) Sodium fluorescein concentration in the hippocampus, (B) prefrontal cortex, (C) striatum, and (D) olfactory bulb. Data are expressed as mean ± SEM (n = 8 per group). * $P < 0.05$ compared to normal diet and vehicle; # $P < 0.05$ compared to high cholesterol diet and vehicle (Two-way ANOVA followed by Duncan test).

cholesterol diet gained more weight than animals fed a regular diet, despite having eaten less food. These results agree with studies that used high fat cholesterol-enriched and high fat diets to induce hypercholesterolemia and diabetes in mice, respectively [51,52]. The differences in food consumption, weight gain and body mass observed between animals fed a hypercholesterolemic diet and animals fed a normal diet, could be explained by the discrepancies between the energy values of each diet. This high cholesterol diet has a caloric value of 4.7 kcal/g, while the regular diet for rodents has a caloric value of 3.3 kcal/g. Looking at the calorie values of these diets, we can see that hypercholesterolemic animals ingested less food and gained more weight due to the increased caloric intake associated with the high cholesterol diet consumption.

Additionally, to induce hypercholesterolemia and cause changes in body weight, exposure to a high cholesterol diet-induced short-term recognition memory impairment in mice, which is in agreement with a previous study conducted by De Souza et al. [51]. In addition, it was reported that Swiss mice exposed to the hypercholesterolemic diet presented a cognitive decline in the object location task, another hippocampus-dependent task [9]. We also observed that hypercholesterolemic mice exhibited a high cataleptic score, a movement alteration characteristic of PD. In line with this, recently, Paul and collaborators [10] demonstrated that mice exposed to a hypercholesterolemic diet (5% of cholesterol) for 12 weeks displayed akinesia and catalepsy posture. Of note, our results showed that even using a lower concentration of hypercholesterolemic diet (1.25 %), a posture impairment could be observed in mice. Another behavioral alteration found in mice fed a high cholesterol diet was depressive like-behavior. The hypercholesterolemic mice presented an increased immobility time in the suspension tail test. Importantly, depression is a non-motor symptom

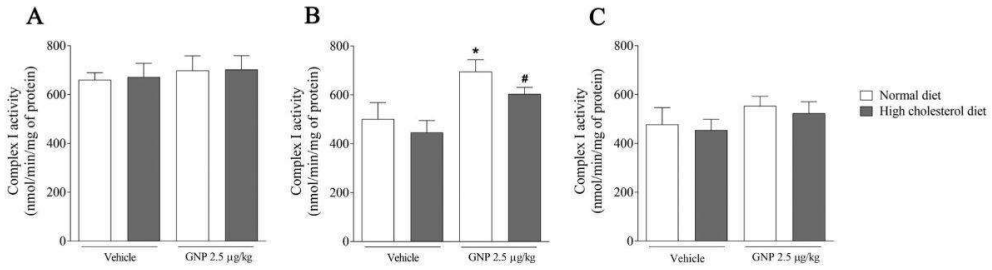

Fig. 6. Effects of a high cholesterol diet and gold nanoparticles (GNPs) treatment on the activity of mitochondrial complex I in mice's brain structures. Mitochondrial respiratory chain complex I activity in the hippocampus (A), prefrontal cortex (B), and striatum (C). Data are expressed as mean ± SEM (n = 5 per group). * $P < 0.05$ compared to normal diet and vehicle; # $P < 0.05$ compared to high cholesterol diet and vehicle (Two-way ANOVA followed by Duncan test).

commonly present in patients with PD and is also an AD comorbid [53, 54]. By contrast, Moreira and collaborators [9] did not observe depressive-like behavior when hypercholesterolemic Swiss mice were submitted to the forced swim test. On the other hand, C57BL/6 J mice fed a high cholesterol diet (0.2 % cholesterol) for three weeks displayed a depressive-like state observed in the forced swim and tail suspension tests [16]. Paul and collaborators [10] also reported that Swiss mice fed a 5% cholesterol diet exhibited depressive like-behavior in the forced swim test.

An important question is which mechanisms are involved in the connection between hypercholesterolemia and cognitive dysfunction development. A possible underpinning mechanism is the hypercholesterolemia-induced inflammatory disruption of BBB [55,56] since hypercholesterolemia is associated with systemic inflammation [57]. Cholesterol excess can accumulate in arteries and the liver, contributing to the increase of the inflammatory process in these peripheral tissues [58]. In order to evaluate the impact of hypercholesterolemia in systemic and hepatic inflammation, we performed a measure of TNF-α cytokine. Hypercholesterolemia did not alter serum TNF-α levels. In this context, an analysis of plasma levels of TNF-α in patients with familial hypercholesterolemia revealed that high serum cholesterol levels might not be associated with increased plasma TNF-α content [59]. By contrast, exposure of rats to a high-fat cholesterol-enriched diet (5% of cholesterol) for two weeks already induced an increase in the plasmatic TNF-α levels, which worsened with more prolonged exposure periods (8 and 12 weeks) [60].

Moreover, here we observed that hypercholesterolemic mice have higher hepatic levels of TNF-α than those fed a regular diet. Similar results were found when mice were fed a high cholesterol diets (0.2 % and 1.25 % of cholesterol) for twelve weeks [61]. As the liver is a central organ of lipid metabolism, the accumulation of cholesterol in hepatic tissue leads to an exacerbation of inflammatory processes and contributes to hepatic disease development, such as non-alcoholic fatty liver disease [58].

Importantly, BBB integrity is vulnerable to systemic inflammation. During peripheral inflammatory processes, changes in the integrity and, consequently, dysfunction of the BBB have been described [62–64]. The BBB separates the brain from the periphery and is critical to neuronal homeostasis. This barrier allows the brain to be an "immune privileged" place. Herein, we observed that Swiss mice fed a high cholesterol diet presented increased leakage of BBB in the hippocampus and olfactory bulb. In this sense, Ullrich and collaborators [50] reported an increased cortical BBB leakage to IgG in rats exposed to a high cholesterol diet. These authors also observed an enhanced microglial immunoreactivity in the cerebral cortex of hypercholesterolemic rats. We have previously observed that hypercholesterolemic mice presented hippocampal BBB dysfunction, associated with astrogliosis [49]. Hypercholesterolemic mice also presented increased astrocyte density in the striatum and substantia nigra [47]. The leaked BBB could cause brain inflammation and, ultimately, neurodegeneration. Of note, BBB dysfunction was detected in patients with dementia [65,66]. Evidence has pointed BBB disruption as a possible triggering event for AD [67,68]. Regarding PD, neuronal death regions coincide with increased BBB permeability [69]. In a recent study, we observed that in a mouse model of familial hypercholesterolemia, the same brain regions previously presenting BBB increased permeability [49] also had an exacerbation of neuronal apoptosis [70].

In this regard, anti-inflammatory molecules could be an attractive therapeutic strategy to manage brain damage caused by hypercholesterolemia, and ultimately prevent neuropathologies. One example of these promising compounds are the GNPs [25]. Of particular interest, GNPs can easily cross the BBB and be found in significant concentrations in the brain [71]. Herein, GNPs treatment attenuated the BBB dysfunction induced by hypercholesterolemia in the mice's hippocampus and olfactory bulb. Additionally, GNPs prevented the recognition memory impairment, posture alterations, and depressive-like behavior associated with hypercholesterolemia. Our findings are in agreement with recent studies from Muller et al. [26] and Dos Santos Tramontin et al. [28], which showed that GNPs (2.5 mg/kg) modulated the neuroinflammatory response and ameliorated cognition functions in animal models of AD. Muller et al. [26] attributed these beneficial effects of GNPs to a decrease in NF-kB expression and subsequent inflammatory reactions. Moreover, Hu et al. [72] treating MPTP-induced PD mice with GNPs (2 mg/kg) observed reduced apoptosis of dopaminergic neurons in the substantia nigra. Attenuation of neuroinflammation, oxidative stress, and better motor coordination are other effects associated with GNPs treatment in PD experimental model [73]. In line with this, recently, Córneo and collaborators [74] indicated that GNPs treatment for five consecutive days reverted the behavioral alterations (such as motor alterations) and oxidative stress in the brain structures in a mice model of PD. Although the effects of GNP on depressive behavioral changes are still unknown, there is evidence indicating that the treatment with GNPs causes changes in the dopamine and serotonin concentrations in rats [75], demonstrating a possible antidepressant action of GNPs through a modulation in the levels of excitatory neurotransmitters. The improvement in the behavioral performance of hypercholesterolemic mice treated with GNPs appeared to be related to the impact of these molecules in the BBB function through anti-inflammatory actions. It is worth mentioning that, in our study, the treatment with GNPs in a lower dose (2.5 µg/kg), i.e., thousand times smaller than the dose used in previous works, has already demonstrated significant neuroprotective effects, which is essential to prevent possible toxicological effects. In fact, it has been previously demonstrated that chronic treatment with GNPs 2.5 mg/kg of 20 nm, besides effective, is safe [27]. In line with this evidence, we did not observe any toxicological effect caused by GNP administration at the dose of 2.5 µg/kg as evidenced by no changes in plasma activities of ALT and γ – GT (blood markers of liver damage), as well as no alteration in plasma creatinine

levels (a marker of kidney function). The mitochondrial tissue integrity measured by MTT and protein levels in the liver were also not changed by the GNPs treatment, regardless of the diet.

Neuroinflammation and neurodegeneration can be amplified through the production of mitochondrial reactive oxygen species (ROS) [76]. Of particular interest, impaired mitochondrial chain respiratory complex I is the primary ROS source in the brain [77]. Notably, our data indicated that mice treated with GNPs, regardless of the diet, had an upregulation of complex I activity in the prefrontal cortex. In this regard, previously Muller and collaborators [27] observed that GNPs administered every 48 h for 21 days induced an increase in the activity of complex I of rats' brain mitochondria, which was associated with decreased levels of ROS. In this sense, the GNPs treatment associated with an antioxidant enhanced the complex I activity and reduced oxidative stress in the brains of rats submitted to an experimental model of sepsis, an inflammatory condition [78]. Although herein, the moderate hypercholesterolemia was not associated with alterations in the complex I function, others and we have pointed out the effects of high cholesterol levels on cerebral mitochondrial respiratory complex activities [47,79].

We also investigated the effects of GNPs exposure on inflammatory response in the liver and plasma of hypercholesterolemic mice. Our results demonstrated that GNPs administration reduced inflammatory cytokine (TNF-α) in the liver of hypercholesterolemic mice. In line with this, the treatment of obese mice with 0.785 μg/g of GNPs restored the hepatic TNF-α expression, which was upregulated due to the exposure to a high-fat diet [29]. Also, Chen and coauthors [29] observed a reduction in the gene expression of toll-like receptor 4 (TLR-4) in the liver of mice fed a high-fat diet. TLR-4-mediated inflammation is also involved in chronic diseases, such as metabolic disorders [80]. On the other hand, we did not observe GNPs effects in the plasma TNF-α content. We could speculate that the absence of anti-inflammatory effects of GNPs in the blood and the presence of such effects in the liver were due to a large amount of GNPs present in the hepatic tissue. Their pharmacokinetic properties could explain this specific anti-inflammatory response of GNPs. GNPs can penetrate the cells membrane, including in the liver. The first organs to receive GNPs in large amounts are the liver and spleen, thanks to their characteristic reticuloendothelial transport systems [81]. Thereby, the neuroprotective effects of GNPs were associated with an attenuation of liver inflammation in hypercholesterolemic mice.

The treatment of hypercholesterolemic animals with GNPs did not significantly affect the plasma cholesterol levels, body mass, and weight gain. Contrasting with our findings, GNP's daily administration at a dose of 0.785 μg/g promoted corporal weight loss in obese mice [29]. One possible explanation for this is the different doses used, i.e., we administered a lower dose of GNPs and the different periods of administration of these nanoparticles. While in our study, GNPs were administered on alternate days for eight weeks, Chen et al. [29] performed daily administrations of GNP for nine weeks. Other works also observe that GNPs did not alter cholesterol levels of animals [27]. Therefore, the neuroprotective and anti-inflammatory actions of GNPs were not associated with effects in the plasma cholesterol levels.

5. Conclusion

Our data showed that hypercholesterolemia in mice was associated with cognitive deficits, depressive-like behavior, and cataleptic posture. These cognitive and behavioral impairments in hypercholesterolemic mice were associated with changes in body weight, liver inflammation, and BBB dysfunction. We reinforced BBB disruption as a crucial event in the brain alterations induced by hypercholesterolemia. Of particular importance, GNPs treatment attenuated inflammatory and brain alterations, mainly those associated with neurodegenerative diseases and depression, induced by hypercholesterolemia. GNPs peripheral anti-inflammatory effects and consequent improvements of BBB function seemed to be involved in the attenuation of behavioral alterations related to hypercholesterolemia. However, in further studies, the mechanisms involved with neuroprotective actions of GNPs should be better investigated.

CRediT authorship contribution statement

Matheus Scarpatto Rodrigues: Writing - original draft, Writing - review & editing, Methodology, Investigation. **Gabriela Cristina de Paula:** Writing - review & editing, Investigation. **Mariane Bernardo Duarte:** Investigation. **Victoria Linden de Rezende:** Investigation. **Jonathann Correa Possato:** Investigation. **Hemelin Resende Farias:** Investigation. **Eduarda Behenck Medeiros:** Investigation. **Paulo Emilio Feuser:** Investigation. **Emilio Luiz Streck:** Writing - review & editing. **Ricardo Andrez Machado de Ávila:** Writing - review & editing. **Rachel Krolow Santos Silva Bast:** Writing - review & editing. **Josiane Budni:** Writing - review & editing. **Andreza Fabro de Bem:** Writing - review & editing. **Paulo César Lock Silveira:** Writing - review & editing. **Jade de Oliveira:** Methodology, Writing - original draft, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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REVIEW ARTICLE

Gold nanoparticles application to the treatment of brain dysfunctions related to metabolic diseases: evidence from experimental studies

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Abstract

Nanotechnology is an emerging and expanding technology worldwide. The manipulation of materials on a nanometric scale generates new products with unique properties called nanomaterials. Due to its significant expansion, nanotechnology has been applied in several fields of study, including developing materials for biomedical applications, i.e., nanomedicine. The use of nanomaterials, including nanoparticles, in nanomedicine, is promising and has been associated with pharmacokinetics, bioavailability, and therapeutic advantages. In this regard, it is worth mentioning the Gold Nanoparticles (AuNPs). AuNPs' biomedical application is extensively investigated due to its high biocompatibility, simple preparation, catalytic, and redox properties. Experimental studies have pointed out critical therapeutic actions related to AuNPs in different pathophysiological contexts, mainly due to their anti-inflammatory and antioxidant effects. Thus, in this review, we will discuss the main experimental findings related to the therapeutic properties of AuNPs in metabolic, neurodegenerative diseases, and ultimately brain dysfunctions related to metabolic diseases.

Keywords Nanotechnology · Nanomedicine · Gold nanoparticles · Neuroinflammation · Brain oxidative damage · Metabolic diseases · Neurodegenerative diseases

Introduction

Nanotechnology can be defined as the manipulation of molecules and structures at a nanometer scale (Jain et al. 2012). It is an emerging technology that has grown on a global proportion. Notably, nanotechnology has been referred to as the fifth technology revolution, after the steam (the 1700s), electricity (1800s), chemicals and mass production (the 1900s), and computers (the 2000s millennium) revolutions (Gyles 2012). Nanotechnology has been used to develop new materials, devices, and systems and will likely affect every aspect of our living in the future, helping us solve common problems in multiple fields (Roco 2011). In nanomedicine, the nano molecules' application arouses tremendous interest and optimism due to the advances that its use provides in diagnosing and treating pathologies (Sajja et al. 2009; Ventola 2012a).

Nanomedicine is the science that uses nanomaterials for biomedical purposes, including diagnosis, drug delivery, and treatment of diseases (Tinkle et al. 2014). The employ of these materials is associated with a reduction of dose and toxicity, an increase in the efficacy (i.e., increase of the bioavailability), providing drug targeting, and improving the distribution of drugs within the body and across biological barriers (Chan 2006; Zhang X et al. 2008; Siddique and Chow 2020; Moore and Chow 2021). Due to the numerous beneficial pharmacokinetic properties, nanotherapeutics can revolutionize the treatment of many diseases. In fact, nanomedicines have been introduced in clinical practice for therapeutic indications, e.g., treatment of iron deficiency, neoplasms, multiple sclerosis, psoriasis, hepatitis B, and other pathophysiological disorders (Soares et al. 2018). The most popular application of nanomaterials to medicine is drug delivery (Wagner et al. 2006). Specifically, incorporating compounds in nanoparticles can increase intracellular concentration and improve the effectiveness, selectivity, and

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therapeutic action of its active ingredient (Wilczewska et al. 2012; Xin et al. 2017).

Nanoparticles are nanomaterials with a size range from 10 nm to less than 1000 nm, which offer many different sizes, shapes, compositions, and functionalities (Wang EC and Wang AZ 2014). The ideal size of nanoparticles for therapeutic and biomedical purposes is estimated to be below 200 nm (Biswas et al. 2014). In this regard, one of the first biomedical applications of metal nanoparticles is from 1970 using colloidal gold nanoparticles (AuNPs), which were applied for labeling antibodies, forming an immunogold-labeling complex for biological staining by electron microscopy (Faulk and Taylor 1971). AuNPs are gold-based products widely studied as a therapeutic agent in experimental models (Khlebtsov and Dykman 2011). These nanoparticles' applications are extensively investigated because they are biocompatible, have simple preparation, easy conjugation with biomolecules, catalytic and redox properties (Daniel and Astruc 2004). The experimental utilization of AuNPs has shown promising results. The beneficial effects of AuNPs are mainly related to their anti-inflammatory and antioxidant actions (Silveira et al. 2014; Muller et al. 2017b; dos Santos Haupenthal et al. 2020b; Petronilho et al. 2020). In line with this, experimentally, the therapeutic use of AuNPs has proved to be efficient under many pathophysiological conditions, such as neurodegenerative and metabolic diseases (Muller et al. 2017b; Chen et al. 2018a, b; Rodrigues et al. 2021).

Neurodegenerative diseases are characterized by a marked and progressive loss of neuronal functions, the consequences of which include memory loss, motor, and psychiatric disorders (Erkkinen et al. 2018). Given their complexity and progressive nature, the study of better prevention strategies for these pathologies is of great importance, as their prevalence tends to grow with the increase in the population's life expectancy (Wyss-Coray 2016). Besides that, there are a number of additional risk factors that contribute to an increased risk of developing neurodegenerative diseases, including metabolic disorders (Mayeux and Stern 2012). These diseases are characterized by interrelated metabolic changes, including insulin resistance, chronic low-grade inflammation, hypertension, hyperglycemia, and atherogenic dyslipidemia (O'Neill and O'Driscoll 2015). In fact, metabolic disorders act as significant risk factors for neuropathologies development, such as Alzheimer's disease (AD) (Kivipelto et al. 2005; Cai et al. 2012; Bharadwaj et al. 2017). Since neurodegenerative diseases still do not have a genuinely effective treatment (Noorbakhsh et al. 2009), the management of risk factors associated with their development, such as metabolic disorders, could be a possible solution to mitigate the deleterious impact of this disease on the population. As described above, the use of AuNPs in experimental studies has shown promising therapeutic actions against the consequences of metabolic and neurodegenerative diseases, either by improving cognition (Muller et al. 2017b; Sanati et al. 2019; dos Santos Tramontin et al. 2020) or contributing to the reestablishment of metabolic homeostasis (Chen et al. 2018a, b).

Considering that pathology of both neurodegenerative and metabolic diseases can be modified by AuNPs' treatment, in this review, we aimed to strengthen the potential therapeutic effect of AuNPs administration to mitigate the negative effects of metabolic diseases in the brain especially those related to neurodegenerative diseases. The development of AuNP-based therapies for the treatment of neurodegenerative disorders presents interesting perspectives for pharmacological delivery strategies due to limitations imposed by the blood-brain barrier and also due to active pro-oxidant and pro-inflammatory signaling of affected areas. In this context, our focus is to identify and summarize the potential advantages presented by the use of AuNPs that have been demonstrated so far. For this, we used Pubmed and Google Scholar databases. The search terms used were: gold nanoparticles synthesis and biodistribution; gold nanoparticles and inflammation; gold nanoparticles and oxidative stress; gold nanoparticles and neurodegenerative diseases; gold nanoparticles and metabolic diseases.

Gold nanoparticles: synthesis and pharmacokinetics

AuNPs can be synthesized by physical, chemical, photochemical, thermal, and biological methods (Freitas de Freitas et al. 2018; Siddique and Chow 2020; Chow 2021; Moore and Chow 2021). Physical synthesis is based on the transfer of energy from irradiation to a specific material that is compartmentalized and reduced at a nanometric scale when irradiated. Examples of this method include gold processing by ionizing radiation (Abedini et al. 2013), microwave radiation (Gangapuram et al. 2018), and photochemical process (Wang et al. 2008). An ecological approach to the synthesis of AuNPs is based on its reduction and processing by bacteria and fungi, a process called the biological synthesis of AuNPs (Menon et al. 2017). In fact, the most used method to produce AuNPs is chemical synthesis, also known as the colloidal method (Siddique and Chow 2020). Chemical ways of AuNPs synthesis are basically composed of a metallic precursor, a strong or mild reducing, and a stabilized agent. In 1951, Turkevich and collaborators introduced the most classical chemical method to AuNPs production, in which the metallic precursor tetrachloroauric acid ($HAuCl_4$) is reduced by sodium citrate. This method provides nanoparticles with 15 nm diameter, but methodological changes

allow synthesizing nanoparticles up to 150 nm in diameter (Turkevich et al. 1951).

Regardless of the synthesis method used, knowing the pharmacokinetics of AuNPs is essential to offer safer administration. In this context, the distribution, toxicity, and therapeutic efficacy of AuNPs can be regulated for innumerable pharmacological properties, including the route of administration (Zhang et al. 2010; Chenthamara et al. 2019). It was shown that AuNPs administration when made by oral route, only a small amount was absorbed by the tissues. On the other hand, when this administration was made intravenously, a greater concentration of AuNPs was present in tissues, mainly in the liver, after feces and urine (Bednarski et al. 2015). Once in the bloodstream, the half-life of AuNPs may vary according to their size. In general terms, AuNPs with a bigger size (more than 40 nm) have a short plasma half-life, whereas AuNPs with less than 40 nm circulate in the body for a longer time (Hoshyar et al. 2016). In this sense, it is worth mentioning that smaller AuNPs need to form conjugates to increase their thermodynamic forces, which facilitates their cellular uptake (Moore and Chow 2021). Since AuNPs are initially seen as a foreign element for the body, their uptake by blood cells prevents them from being transported out of the bloodstream through the reticuloendothelial system and increases their plasma half-life (Zhang et al. 2009). Organs belonging to the reticuloendothelial system (liver and spleen) are the first affected by the accumulation of AuNPs, and their biodistribution is more irregular with smaller particle sizes (Khlebtsov and Dykman 2011). Despite having a longer plasma half-life, smaller AuNPs penetrate tissues and organs more easily than AuNPs with a bigger size (Sonavane et al. 2008).

Another critical aspect involved in the AuNPs biodistribution, as well as in its toxicity, is the administered dose. Administering AuNPs of the same size but in different doses (40, 100 and 400 ug/Kg/day for eight days) in mice, Lasagna-Reeves et (Lasagna-Reeves et al. 2010) showed that different doses affect the AuNPs biodistribution but do not influence their toxicity. The AuNPs were more accumulated in the spleen, followed by liver, kidney, lungs, and brain. In these organs, the gold concentration increased according to the dose administered (Lasagna-Reeves et al. 2010). On the other hand, mice exposed to AuNPs in a high dose (1.000 mg/kg) developed liver damage and increased serum pro-inflammatory cytokines. In addition, rats treated with high doses of AuNPs exhibited a higher accumulation of these nanoparticles in the spleen and increased fecal excretion. Also, the administration of AuNPs caused the death of rats but not of mice (Bahamonde et al. 2018). In other words, AuNPs toxicity can also vary among species. Regarding their excretion, significant amounts of AuNPs are found primarily in urine and after feces, which indicates AuNPs could be excreted by both routes (Schleh et al. 2012; Bahamonde et al. 2018).

Given that the biodistribution and toxicity of AuNPs are relative, the development of protocols for the safe administration of these nanomaterials in biological systems is very relevant. In this sense, Muller et al. (2017b) developed a safe protocol for the administration of AuNPs in rats. The authors administered AuNPs intraperitoneally at the dose of 2.5 mg/kg for 21 days every 24 or 48 h. Toxicity analyses revealed low toxicity of AuNPs when administered every 48 h for 21 days, which reveals a therapeutic potential in the biomedical use of these nanoparticles at a dose of 2.5 mg/kg (Muller et al. 2017a). Therefore, regardless of the synthesis method used, many variables (e.g., size, route of administration, and dose administered) contribute to the biodistribution and bioavailability of AuNPs, which directly affects their toxicity and beneficial effects. Among the mechanisms related to its therapeutic properties are the modulation of the inflammatory response and the reduction of oxidative stress.

AuNPs: important anti-inflammatory and antioxidant agent

Inflammation is an immune system response to cell damage or pathogens to remove the injurious stimulus and reestablish homeostasis (Medzhitov 2010). The inflammatory response can be divided into two distinct biological phases: acute or chronic inflammation. Acute inflammation starts rapidly, and its symptoms usually last a few days. This response is characterized by the exudation of proteins, angiogenesis, migration of neutrophils and other leukocytes to the damaged tissue, and an increase in the production of pro-inflammatory cytokines and reactive oxygen species (ROS), to favor phagocytosis (Freire and van Dyke 2013; Eming et al. 2017). If this pro-inflammatory signaling persists, an acute inflammatory response can progress to persistent chronic inflammation. It is a slow and long-term inflammatory response, lasting from several months to years, depending on the triggering stimuli (Pahwa et al. 2018). On the other hand, when the stimuli of the inflammatory response are eliminated, the leukocytes, mainly M2 macrophage, begin to secrete anti-inflammatory cytokines and growth factors to complete tissue repair and promote their homeostasis (Lawrence and Gilroy 2007).

As mentioned before, redox reactions play roles in inflammatory responses since the generation of ROS and its "respiratory burst" helps eliminate inflammatory response triggers and reestablish cell homeostasis (Lugrin et al. 2014). Given the importance of ROS in the inflammatory response, some studies treat ROS as a second messenger for leukocytes activation and inflammatory mediators'

production (Cruz et al. 2007; Forman et al. 2010; Lee and Yang 2012). However, the increase of ROS production leads to negative consequences, such as oxidative stress. Oxidative stress is a condition characterized by an imbalance between ROS production and its respective neutralization by antioxidant molecules in a movement that favors cell and tissue damage, e.g., oxidative damage (Burton and Jauniaux 2011). Increased patterns of oxidative stress biomarkers can be easily observed in metabolic(Ceriello 2006) inflammatory (Kooy et al. 1997; Karbach et al. 2014), cardiovascular and neurodegenerative diseases (Griendling and FitzGerald 2003; Kim et al. 2015).

Due to the importance of inflammatory processes and oxidative stress in several diseases' development, substances capable of modulating these biological responses are essential to secure homeostasis when the body cannot respond adequately to stressors agents. In this regard, experimental findings support that AuNPs can modulate the inflammatory and oxidative stress responses. To better investigate the anti-inflammatory effect of AuNPs, Sumbayev et al.(Sumbayev et al. 2013) administered interleukin 1 beta (IL1-β) intraperitoneally in mice and concomitantly performed the treatment with AuNPs. *In vivo* treatment with AuNPs has been shown to reduce the intracellular expression of PI3K protein and cytokine TNFα. Interestingly, the authors showed by transmission microscopy that AuNPs bind extracellularly to IL1 molecules, thus reducing their respective interaction with IL-1 cell receptors (Sumbayev et al. 2013). Decreasing of the TLR-4-NFkB inflammatory signaling cascade (Pereira et al. 2012; Zhu et al. 2018; Gao et al. 2019; Vyas and Goswami 2019), reduction of pro-inflammatory cytokines (Gao et al. 2019; dos Santos Haupenthal et al. 2020b), inhibition of angiogenesis (Mukherjee et al. 2005), acceleration of macrophage phenotypic changes(Taratummarat et al. 2018) and improve the therapeutic effect of anti-inflammatory drugs(dos Santos Haupenthal et al. 2020c, a) are among the mechanisms behind the anti-inflammatory property of AuNPs.

The antioxidant effect of AuNPs is also notable. Studying the antioxidant effect of AuNPs in cell culture, Markus et al. (Markus et al. 2016) showed that AuNPs have important scavenger activity against free radical molecules in a dose-dependent manner, but even more remarkable than their precursor salt. This evidence helps to explain some of AuNPs' antioxidant activities found in experimental studies. First, the treatment of rodents with AuNPs showed that nanoparticles could reduce ROS production (Sul et al. 2010; Pereira et al. 2012) and reactive nitrogen species (Mukherjee et al. 2005; Rizwan et al. 2017). Interestingly, AuNPs treatment also increased the activity of antioxidant enzymes (Leonavičienė et al. 2012; Kirdaite et al. 2019) by a mechanism not yet fully understood. Consequently, AuNPs helps to reduce oxidative damage to lipids, proteins, and DNA in the context of many pathologies (Victor et al. 2012; Abdelmegid et al. 2019; Haupenthal et al. 2020), including metabolic (Chen et al. 2018b, a) and neurodegenerative diseases (dos Santos Tramontin et al. 2020).

Although many mechanisms were proposed to explain how AuNPs modulate the inflammatory response and oxidative stress, their anti-inflammatory and antioxidant properties are not yet fully understood. However, these therapeutic actions of AuNPs in different pathological contexts reinforce the potential of these molecules in the management and treatment of complex pathologies, such as metabolic disorders and neuropathologies.

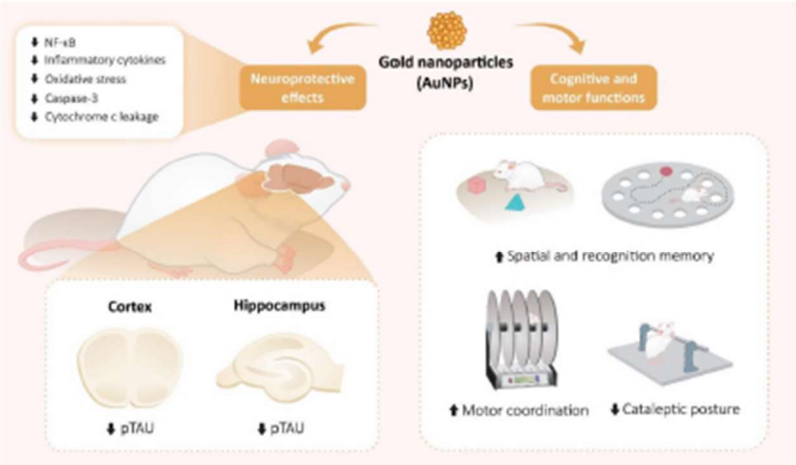

Fig. 1 Effects of AuNPs administration in neurodegenerative diseases and its consequences. The AuNPs administration reduces the oxidative stress and neuroinflammation in animals' brain. In addition, AuNPs could reduce pTAU expression in the cortex and hippocampus in an Alzheimer disease animal model. Still, AuNPs prevents cognitive deficits and motor alterations associated with neurodegenerative diseases. NFkβ = factor nuclear kappa B

Effects of AuNPs on neuropathologies

Inflammation and oxidative stress play important roles in neuropathologies, especially in neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (Chen et al. 2016; de Oliveira et al. 2021). In this sense, the study of therapeutic strategies capable of attenuating these pathophysiological changes is potentially relevant. As pointed out by Silveira et al. (Silveira et al. 2021), AuNPs, due to their well-known antioxidant and anti-inflammatory properties, are better discussed in the previous topic could potentially be used to treat neuropathologies. In fact, the use of AuNPs in experimental studies exhibited interesting effects for the management of neuropathologies. Some of AuNPs' beneficial effects in brain diseases are summarized in Fig. 1 and best discussed in this topic.

AD is a progressive and irreversible neurodegenerative disease and the most frequent cause of dementia worldwide (Apostolova 2016). The prevalence of AD is approximately 24 million, a number that can quadruple up to 2050 (Reitz and Mayeux 2014). Sporadic Alzheimer's disease is a multifactorial neurodegenerative disorder responsible for more than 95% of AD cases in the world population; the other 5% includes cases of genetic origin (Reitz et al. 2011). In an experimental model of sporadic AD induced by streptozotocin, AuNPs treatment prevented neuroinflammation, oxidative stress and improved the brain mitochondrial function. Improvements in spatial and recognition memories have also been observed in the AD animal model after AuNPs treatment (Muller et al. 2017b; dos Santos Tramontin et al. 2020). Different proteins play a central role in AD pathogenesis, including β- amyloid peptide (Aβ) and TAU protein. In this sense, negatively charged AuNPs significantly inhibit the Aβ aggregation into fibrils (Liao et al. 2012). Likewise, Kogan et al(Kogan et al. 2006) demonstrate that AuNPs selectively bind to Aβ fibrils, preventing its aggregation and neurotoxic effects *in vitro*. Studying the AuNPs' effects against the toxicity of Aβ *in vivo*, Sanati et al(Sanati et al. 2019) showed that AuNPs treatment improves the acquisition and retention of spatial memory damaged by Aβ accumulation on the brain. Moreover, AuNPs also increased the expression of proteins involved with neural survival in this model of AD induced by Aβ (Sanati et al. 2019). Regarding TAU protein, intraperitoneal AuNPs administration helped maintain normal TAU phosphorylation levels and prevented the exacerbation of oxidative stress response in the hippocampus and cerebral cortex in an animal model of AD (dos Santos Tramontin et al. 2020).

Parkinson's disease, in its turn, is characterized by progressive loss of dopaminergic neurons in the substantia nigra, which leads to a decrease in brain dopamine resulting in deficits of voluntary movements – including bradykinesia, rigidity, and tremor at rest (Dickson 2012; Mazzoni et al. 2012). Interestingly, the AuNPs synthesized using the root extract of *Paeonia montan* alleviated the neuroinflammatory response (i.e., reduced levels of IL-6, IL-1b, and PGE2), reduced the oxidative stress parameters in the substantia nigra, and ameliorated the motor coordination in a mice model of Parkinson's disease (Xue et al. 2019). Similar effects of AuNPs treatment were described by da Silva Córneo et al. (da Silva Córneo et al. 2020), where AuNPs significantly reduced oxidative stress, improved neurotrophic factors levels in brain structures and ameliorated the latency of motor stimulus and the animal's catalepsy score. Moreover, AuNPs composites significantly inhibited the apoptosis of dopaminergic neurons in the substantia nigra in an animal model of PD induced by MPTP (Hu et al. 2018).

Taken together, the studies have shown that treatment with AuNPs offers neuroprotective actions through modulation of neuroinflammation, oxidative stress, and reduction of neurotoxic protein aggregates. Thereby, AuNPs also ameliorate the cognitive deficits and motor coordination characteristics of neurodegenerative diseases, which reinforce their neuroprotective potential. Moreover, risk factors for developing neuropathologies, such as metabolic diseases and their consequences, can also be treated with AuNPs. In fact, promising effects of AuNPs administration in the context of metabolic disorders have been described in the literature, which will be better addressed and discussed in the next section.

Effects of AuNPs on metabolic diseases

Proper functioning of the metabolic system is vital for maintaining body homeostasis (Hotamisligil 2006). Metabolism is understood as a set of biochemical reactions that occur in order to generate (catabolic process) or to consume energy (anabolic process) (DeBerardinis and Thompson 2012). Therefore, metabolic dysfunctions are related to diseases, such as obesity. Obesity is a multifactorial and complex disease characterized by excess body weight and a body mass index greater than 30 kg/m^2 (World Health Organization, 2016). The increase of body mass also increases the risk for obese people to develop several associated diseases such as type 2 diabetes mellitus (Al-Goblan et al. 2014), cardiovascular diseases (Czernichow et al. 2011), musculoskeletal disorders (Anandacoomarasamy et al. 2009), types of cancers (Lauby-Secretan et al. 2016) and neurodegenerative diseases (Mazon et al. 2017). It is important to say that obese individuals generally have a condition of low-grade chronic inflammation triggered by several factors, such

as adipocyte hypertrophy (Monteiro and Azevedo 2010; Longo et al. 2019).

The administration of AuNPs shows promising therapeutic effects in the context of metabolic diseases (Fig. 2). In obese mice, AuNPs treatment ameliorated hyperlipidemia, improved glucose tolerance, and reduced the production of TNF-α in adipose and hepatic tissue of obese mice (Chen et al. 2018b). In mice with long-term obesity, daily AuNPs administration for five weeks could promote weight loss, better glycemic control, and serum lipid profile. Negative regulation of inflammatory markers in the liver has also been observed (Chen et al. 2018a). Lean mice who received AuNPs show smaller fat mass and significant reduction of TNF α and IL-6 mRNA levels in abdominal fat 72 h after AuNPs administration (Chen et al. 2013). In mature adipocytes (3T3L1 cells), AuNPs suppress adipogenesis through downregulation of transcription factors PPARγ and CEBPα, reinforcing the anti-obesity activity of AuNPs (Simu et al. 2019).

One of the obesity outcomes is insulin resistance, a clinical condition characterized by low interaction of insulin with its cellular receptors and, consequently, by low cell uptake of glucose (Wilcox 2005). In order to improve this clinical condition, some studies have used AuNPs as a carrier to improve insulin action. Insulin-coated AuNPs significantly reduced the postprandial hyperglycemia (Joshi et al. 2006) and decreased blood glucose levels by up to three times compared to free insulin due to decreased degradation by enzymes in diabetic mice (Shilo et al. 2015). In this context, the AuNPs administration has demonstrated several beneficial effects against hyperglycemia (Fig. 2). The prolonged exposure of cells to hyperglycemia enhanced the production of reactive oxygen species and the release of pro-inflammatory factors from cells (Oguntibeju 2019). Thus, AuNPs improve the diabetic condition by reducing the inflammatory process, suppressing oxidative stress, and increasing antioxidant defense (Shaheen et al. 2016; Sengani 2017). Diabetic mice treated with AuNPs showed improved antioxidant defense, inhibition of lipid peroxidation and ROS generation in the liver, as well as better glycemic control compared to untreated animals (BarathManiKanth et al. 2010). In a mice model of diabetes induced by alloxan monohydrate, AuNPs reduce plasmatic glucose, cholesterol, and triglyceride levels. In this same study, AuNPs increased plasma insulin significantly, showing a potential antidiabetic effect of AuNPs (Venkatachalam et al. 2013).

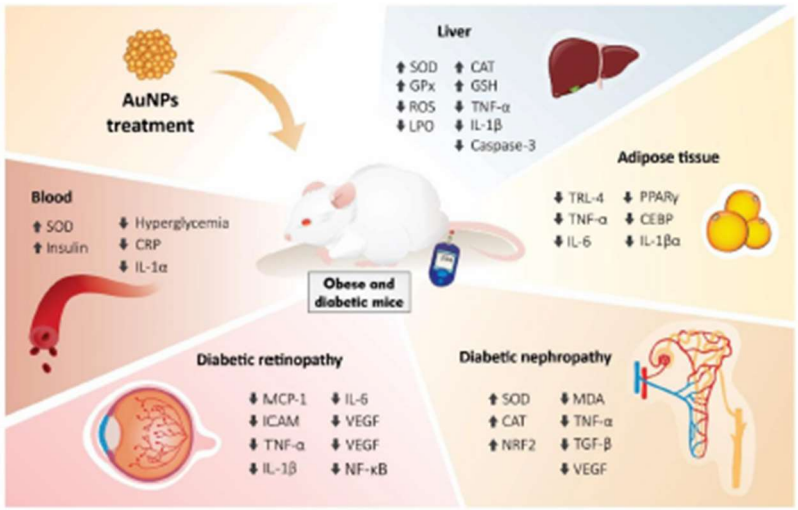

Fig. 2 Effects of AuNPs administration on metabolic diseases and its complications. The AuNPs administration exercise beneficially effects on diabetes and associated pathologies. Among the possible mechanisms involved with this therapeutic action are the modulation of oxidative stress, inflammatory and cell death pathways. Hypoglycemic effect and improved insulin sensitivity are also among the beneficial effects related to AuNPs administration. CAT = catalase; CEBPα = CCAAT-Enhancer-Binding Protein-alpha; CRP = C Reactive Protein; ERK = Extracellular Signal-regulated Kinases; GPx = Glutathione Peroxidase; GSH = Reduced Glutathione; ICAM = Intercellular Adhesion Molecule; IL-1α = Interleukin 1 alpha; IL-1β = Interleukin 1 beta; IL-6 = Interleukin 6; LPO = Lipoperoxidase; MDA = Malondialdehyde; MCP-1 = Monocyte Chemotactic Proteins-1; NFkβ = Factor Nuclear kappa B; NRF2 = Nuclear factor (erythroid-derived 2)-like 2; PPAR-γ = Peroxisome Proliferator-activated Receptor gamma; ROS = Reactive Oxygen Species; SOD = Superoxide Dismutase; TLR4 = Toll like Receptor 4; TNF α = Tumor Necrosis Factor alpha; TGF-β = Transforming Growth Factor beta; VEGF = Vascular Endothelial Growth Factor

Biochemical parameters like better lipid profile, liver damage, and inflammatory markers reinforce the therapeutic effects of AuNPs on diabetes and its complications (Ansari et al. 2019).

Diabetes leads to a series of secondary complications, including microvascular changes such as retinopathy, nephropathy, neuropathy, and atherosclerosis (Long and Dagogo-Jack 2011). Thus, treatment of diabetic rats with resveratrol coated-AuNPs marked reduced VEGF-1, TNF-α, IL-6, IL-1B, MCP-1, and ICAM mRNA expressions on retinal vessels (Dong et al. 2019). In addition, AuNPs modulated the ERK and NFkB signaling pathways, which contributes to a better prognostic of diabetic retinopathy (Dong et al. 2019). A downregulation in renal protein expression of TGF-β, TNF-α, and VEGF are associated with AuNPs treatment on diabetic nephropathy experimental model. Furthermore, AuNPs restored redox homeostasis, improving the activity of antioxidant enzymes, and reducing MDA levels in the kidney tissue of diabetic rats (Alomari et al. 2020). The activation of NRF2 and its antioxidant activity also appears to be a beneficial effect linked to AuNPs in diabetic nephropathy (Manna et al. 2019). In addition, exposure of macrophages to high glucose levels increases inflammation, apoptosis, and oxidative-nitrosative stress. On the other hand, administration of AuNPs to macrophage cells reduces these oxidative profile effects promoted by high glucose conditions through a modulation on the mTOR/NFkB pathway (Rizwan et al. 2017).

As summarized in Fig. 2, the administration of AuNPs exerts therapeutic effects against metabolic disorders and their complications. Modulation of the inflammatory response, oxidative stress, and increased insulin bioavailability in the periphery are among the main effects linked to treatment with AuNPs in these physiopathological conditions. Thus, based on the evidence described in this topic and Sect. 4 of this review, it seems promising that AuNPs can exert therapeutic actions against brain dysfunctions related to metabolic diseases. This hypothesis will be better explored in the following topic.

Effects of AuNPs on brain dysfunctions linked to metabolic diseases

Our brain plays a central role in the regulation of body metabolism. Its regulatory activity involves releasing central and peripheral signals through specialized neuronal networks (Roh and Kim 2016). On the other hand, dysregulation of metabolism can be harmful to the brain and increases the risk of developing neuropathologies, including neurodegenerative diseases (Procaccini et al. 2016). Midlife obesity is considered a risk factor for dementia in later life

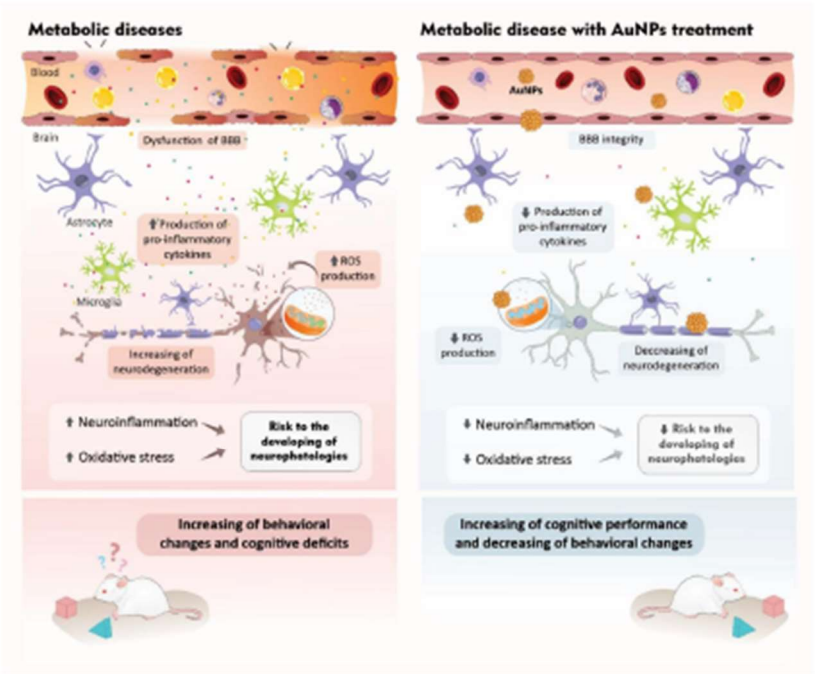

Fig. 3 Role of AuNPs in the treatment of brain disorders related to metabolic diseases. Metabolic diseases, such as diabetes and hypercholesterolemia, increases the dysfunction of BBB and the crossing of peripheral cells through BBB. As a consequence, metabolic dysfunctions contribute to the enhancement of neuroinflammatory response, oxidative stress and neurodegeneration on the brain. On the other hand, the AuNPs treatment ameliorates the BBB dysfunction related to metabolic disorders. Also, AuNPs decrease neuroinflammatory and oxidative stress parameters on brain, helping to the decrease of neurodegeneration as well as the cognitive deficits related to metabolic diseases. AuNPs = Gold Nanoparticles; BBB = Blood-brain-barrier; ROS = Reactive Oxygen Species

(Whitmer et al. 2008). In this sense, diabetic patients show a higher risk of developing AD than non-hyperglycemic subjects (Ott et al. 1999), in a process that occurs regardless of age (Kloppenborg et al. 2008). Hypercholesterolemia also contributes to the development of brain diseases. Epidemiological evidence shows that people diagnosed with hypercholesterolemia in "middle-aged" exhibited an increased risk of cognitive impairment and dementia later in life (Kivipelto et al. 2001; Tolppanen et al. 2012). In addition, early exposure to elevated cholesterol levels is associated with a greater incidence of mild cognitive impairment, a prodromal stage of AD (Zambón et al. 2010).

In fact, metabolic disorders can exert a wide range of effects on the brain. An interesting review showed that metabolic diseases, such as obesity and diabetes, enhance vascular inflammation causing dysfunction of the BBB (van Dyken and Lacoste 2018). Furthermore, the resulting neuroinflammation and oxidative stress in the brain contribute to the neurodegeneration and cognitive decline observed in many neuropathologies (Thirumangalakudi et al. 2008; Craft 2009; Procaccini et al. 2016; Arshad et al. 2018). As discussed in the previous topics, AuNPs exhibited significant therapeutic effects on treating metabolic and neurodegenerative diseases, mainly because of their antioxidant and anti-inflammatory effects. Therefore, in this section, we will discuss findings of the effects of AuNPs on brain dysfunctions related to metabolic diseases (Fig. 3).

In addition to causing important changes in the periphery, obesity also has negative effects on the brain. Increased BBB disruption, neuroinflammation, mitochondrial impairment, oxidative stress, and alterations in synaptic plasticity are among the mechanisms modulated by obesity in this organ (Tucsek et al. 2014; Cavaliere et al. 2019; Mullins et al. 2020). Evaluating the effect of AuNPs on the brain consequences of obesity, Prá et al. (Prá et al. 2021) observed that obese mice treated with AuNPs showed a significant reduction in inflammatory and oxidative stress parameters in the prefrontal cortex and hippocampus. Similar effects were observed in non-obese animals, in which AuNPs treatment reduced the cerebral levels of TNF α, oxidative DNA damage, pro-apoptotic markers, and mitochondrial dysfunction on the brain of animals with central and systemic inflammation (Pedersen et al. 2009; Petronilho et al. 2020). Since brain regions such as the hippocampus and prefrontal cortex are involved with memory formation, the administration of AuNPs might help manage the adverse effects of obesity in the CNS.

Diabetes, an obesity complication, and insulin resistance can also negatively affect the brain. Studies have shown that brain insulin resistance contributes to AD-like neurodegeneration by many mechanisms, including the accumulation of Aβ peptide in the brain (de la Monte and Wands 2008; M de la Monte 2012). Also, the aggregation of insulin in amyloid fibrils causes memory impairment and increases amyloid plaques formation in the rat's brain (Kheirbakhsh et al. 2015), which inhibition of their protein aggregation of great relevance. In this sense, AuNPs significantly reduced insulin amyloid fibrillation *in vitro* (Hsieh et al. 2013). Likewise, AuNPs reduced the toxicity of human islet amyloid polypeptide (IAPP), a protein released by pancreatic B-cells, which was shown to impair BBB permeability and contributes to diabetes-induced dementia (Raimundo et al. 2020), and promotes its immunogenic response by human T cells *in vitro* (Javed et al. 2017). Although the effects of AuNPs on the diabetic brain are not yet investigated, this evidence suggests that AuNPs treatment could be effective against toxic products related to diabetes-induced cognitive deficits.

Hypercholesterolemia, another vital cardiovascular risk factor, also causes relevant consequences to the brain. Experimental studies reported that this metabolic disorder enhances the BBB permeability, neuronal apoptosis, impairs adult hippocampal neurogenesis and cognition (Moreira et al. 2014; Engel et al. 2019; de Oliveira et al. 2020a, b). Because of that, an important relationship between high blood cholesterol levels and the development of AD and PD has been reported in the literature (Zambón et al. 2010; Paul et al. 2017). Notably, treating hypercholesterolemic mice with AuNPs reduces the BBB permeability in the olfactory bulb and hippocampus. Interestingly, AuNPs treatment improved hippocampal-dependent memory and reduced depressive-like behavior, as well as cataleptic posture in these animals (Rodrigues et al. 2021). The neuroprotective effects of AuNPs in hypercholesterolemic mice were associated with a reduction in peripheral inflammation, i.e., decreased levels of liver TNF α.

The neuroprotective effects related to the administration of AuNPs mentioned above and better summarized in Fig. 3, support the hypothesis that these nanoparticles are important allies against brain dysfunctions related to metabolic disorders, either reducing the risk of developing neuropathologies or improving cognitive performance. However, further studies are needed to better elucidate the mechanisms behind the therapeutic effects of AuNPs in this context.

Limitations on the use of AuNPs for biomedical applications

The manipulation of materials in a nanometer-scale generates materials with different properties and characteristics of their respective precursor material on a normal scale (Uskokovic 2009). The exact process occurs with AuNPs in

relation to macroscopic gold (Giljohann et al. 2010). Thus, there are several obstacles in developing a nanotherapeutic product and for these products to "reach the shelves", which hampers the translation from experimental therapeutic use to the pharmacological use of this new therapy by the general population (Ventola 2012b). These obstacles include extensive preclinical and clinical testing to better explore these nanomaterials' pharmacological, toxicological, and immunological properties (Resnik and Tinkle 2007). Still, issues such as physicochemical characterization, biocompatibility, process control and scale reproducibility also impact its production and, later, its translation for biomedical applications (Soares et al. 2018). Regarding AuNPs, the application of those nanoparticles in humans remains uncertain since their safety is not yet well established (Hornos Carneiro and Barbosa Jr 2016).

As we demonstrated earlier, several experimental findings report therapeutic effects associated with AuNPs to treat metabolic and brain dysfunctions related to metabolic diseases. However, it is important to emphasize that AuNPs are not biodegradable. Thus, its application in biological systems must be carefully studied to avoid associated toxic effects. In fact, the major limitation of using AuNPs for biomedical purposes is their toxicity (Hornos Carneiro and Barbosa Jr 2016). Factors such as the shape, size, concentration, surface chemistry, and surface charge affect the toxicity of AuNPs, which makes their toxicity very relative (Chithrani et al. 2006; Chithrani and Chan 2007). Despite this, AuNPs also have important drug delivery properties. AuNPs can improve the hypoglycemic action of insulin in diabetic mice (Joshi et al. 2006), improve drug carrier and radiation dose absorption by the tumor cells (Jain et al. 2012; Haume et al. 2016). Due to its excellent biocompatibility and ability of AuNPs to easily bind with a broad range of materials, the use of AuNPs for drug delivery ends up being harmed due to its inability to direct the drug to a specific target area (Anderson et al. 2019). Lastly, it is important to say that nanomaterials, including AuNPs, still have largely unknown properties, which should be better studied for their safe biomedical application in the future.

Closing remarks and future perspectives

The use of nanoparticles in medicine provides a wide range of possibilities and the development of new treatments to create new therapies or ameliorate the efficacy of the ones already available. In particular, neurological and metabolic diseases are complex in etiology and treatment, complicating the search for new therapeutic strategies. The exacerbation of inflammatory response, as well as oxidative stress, are among the mechanisms that are underpinning the progression of these pathologies and also with the brain dysfunctions related to metabolic diseases. Although the use of nano compounds in the treatment of metabolic and brain diseases is not yet well explored, the experimental findings discussed in this review point out that AuNPs, inorganic nanoparticles, are molecules that can exert critical therapeutic effects in this context. Concerning their effects in metabolic diseases, our literature review showed that reducing the inflammatory response, oxidative stress, increased bioavailability of insulin, and reduction of hyperglycemia are the main beneficial effects of AuNPs in animal models. Likewise, anti-inflammatory and antioxidant actions are also associated with the therapeutic effects of AuNPs in the context of neuropathologies. Other important actions such as reducing neurotoxic compounds in the brain, e.g. Aβ peptide and pTAU, and improving cognitive performance in animal models of neurodegenerative diseases support the neuroprotective action of AuNPs.

Additionally, experimental findings have pointed towards a neuroprotective action of AuNPs against the brain dysfunctions caused by metabolic diseases. Mechanisms such as the prevention of neurodegeneration, reduction of the BBB integrity, and improvement of cognitive performance in animal models of metabolic diseases are the mechanisms by which AuNPs exert beneficial effects in the cognition in experimental models of metabolic disorders. Moreover, as AuNPs have high compatibility and interact with pharmaceuticals, the drug delivery properties of AuNPs offer unique potential to improve the therapeutic efficacy of drugs already approved for the treatment of both neurological and metabolic diseases. Nevertheless, some questions still need to be clarified in further studies, such as mechanisms associated with its toxicity, biodistribution, neuroprotective properties, and its application in improving drug delivery to the target area and increasing its effectiveness. Finally, the therapeutic application and the safety of AuNPs need to be better studied in the future for therapeutic aid in brain disorders associated with metabolism.

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Declarations

Declaration of Competing Interest The authors report that they have no declarations of interest.

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Review

Inflammatory Cascade in Alzheimer's Disease Pathogenesis: A Review of Experimental Findings

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Abstract: Alzheimer's disease (AD) is the leading cause of dementia worldwide. Most AD patients develop the disease in late life, named late onset AD (LOAD). Currently, the most recognized explanation for AD pathology is the amyloid cascade hypothesis. It is assumed that amyloid beta (Aβ) aggregation and deposition are critical pathogenic processes in AD, leading to the formation of amyloid plaques, as well as neurofibrillary tangles, neuronal cell death, synaptic degeneration, and dementia. In LOAD, the causes of Aβ accumulation and neuronal loss are not completely clear. Importantly, the blood–brain barrier (BBB) disruption seems to present an essential role in the induction of neuroinflammation and consequent AD development. In addition, we propose that the systemic inflammation triggered by conditions like metabolic diseases or infections are causative factors of BBB disruption, coexistent inflammatory cascade and, ultimately, the neurodegeneration observed in AD. In this regard, the use of anti-inflammatory molecules could be an interesting strategy to treat, delay or even halt AD onset and progression. Herein, we review the inflammatory cascade and underlying mechanisms involved in AD pathogenesis and revise the anti-inflammatory effects of compounds as emerging therapeutic drugs against AD.

Keywords: Alzheimer's disease; neurodegenerative disease; dementia; neuroinflammation; inflammatory cascade; systemic inflammation; BBB disruption; anti-inflammatory effects; therapy

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1. Introduction

An estimated 50 million people worldwide have dementia, a number projected to double every 20 years until 2050 [1,2]. The majority of demented individuals develop Alzheimer's disease (AD). Thus, AD represents a tremendous healthcare challenge of the 21st century [3,4]. The typical clinical presentation of this age-related neurodegenerative disease is a gradual and progressive decline in different cognitive domains, most commonly involving episodic memory and executive functions that cause social and occupational deficits [5].

AD is characterized by pathological hallmarks such as extracellular amyloid plaques, formed by the deposition of amyloid β-peptide (Aβ), the appearance of intracellular neurofibrillary tangles, composed of hyperphosphorylated tau and extensive synaptic and neuronal loss in the cerebral cortex and hippocampus [6]. In addition, it has been proposed that neuroinflammation is an early feature of AD [7,8]. Thus far, the amyloid cascade hypothesis is the main influential model to explain the progression of AD pathogenesis [9,10]. However, the field is gradually moving away from the simplistic assumption of linear causality proposed in the original amyloid hypothesis [11,12]. Importantly, it has been shown that tau pathogenesis could be associated with neurodegeneration and neuroinflammation regardless of Aβ pathology [13,14]. Moreover, APOE4 is the strongest genetic risk factor for LOAD. The presence of APOE4 is associated with increased Aβ deposition, but also tau pathology [15,16]. In fact, APOE is involved in tau pathogenesis through neuroinflammation processes [17].

AD can be divided into early-onset AD (EOAD) and late-onset AD (LOAD). EOAD is rare, accounting for less than 5% of the cases [18,19] and its onset occurs before 65 years. Mutations in three genes, which encode amyloid protein precursor (APP), presenilin-1 (PSEN1), and presenilin-2 (PSEN2), are known to cause a proportion of autosomal dominant inherent EOAD, or autosomal dominant AD (ADAD). In the genetic form of AD, Aβ accumulation is due to a significant higher peptide production [20]. The most common form, LOAD, is assumed to be a multifactorial and polygenic disease and, therefore, the etiology of Aβ deposition and neurodegeneration in these cases is unknown [19].

Considering the high epidemiological impact of AD, it is fundamental to understand the mechanisms involved in its pathological onset and advancement. In this regard, inflammation seems to play an essential role in disease development and progression. On one hand, clinical and preclinical studies analyzing brains from individuals with AD or experimental models of AD provide evidence for the activation of inflammatory pathways. On the other hand, anti-inflammatory compounds are associated with a reduced risk of developing and disease progression [21–23]. During decades, inflammatory processes have been explored in an effort to identify alternative therapeutic targets, alone or in combination with other drugs. In this review, we discuss the most relevant evidences that point out neuroinflammation as a crucial event in AD pathophysiology and its potential as an innovative target to treat AD.

2. Pathogenic Mechanisms of Alzheimer's Disease

Until now, amyloid hypothesis has been the most stablished model of AD pathogenesis. It proposes that the deposition of misfolded and aggregated Aβ is a critical and the initial pathological event in AD, triggering synaptic dysfunction, neuronal loss, and cognitive impairment [10,24].

During the amyloidogenic pathway, Aβ, a peptide of 36–43 amino acids, is generated by cleavage of the transmembrane amyloid precursor protein (APP) through sequential proteolytic processing. In this via, APP is first cleaved by β-secretase (beta-site amyloid precursor protein-cleaving enzyme 1, BACE-1), producing an extracellular soluble fragment (i.e., sβAPP) and an intracellular C-terminal portion termed C99. Subsequently, the resulting cell-associated C-terminal fragments are subjected to intramembrane proteolysis mediated by γ-secretase, which generates a spectrum of Aβ peptides of varied lengths. Concurrently, the non-amyloidogenic APP proteolysis involves cleavage by α-

and γ-secretases, resulting in the generation of a long-secreted form of APP (sAPPα) and C-terminal fragments (CTF 83, p3 and AICD50). The APP non-amyloidogenic processing produces non-toxic fragments. The cleavage site for α-secretase in APP lies within the Aβ sequence and thus precludes Aβ formation. Usually, about 90% of APP enters the non-amyloidogenic pathway, while the rest follows the amyloidogenic via [25,26].

The total Aβ burden is regulated by synthesis and clearance rates. In fact, Aβ clearance or degradation, rather than its synthesis, has been considered critical in Aβ accumulation. The clearance of the peptide by transport into the cerebrospinal fluid (CSF), the blood across the blood–brain barrier (BBB), and the removal by macrophages have been suggested as the responsible mechanisms for controlling the brain Aβ levels [27]. Furthermore, CSF clearance seems to be impaired in AD, contributing to increase Aβ burden and disease progression [28]. Some proteases, such as some cathepsins and insulin-degrading enzyme (IDE), play essential roles by cleaving Aβ into shorter soluble fragments without neurotoxic effect [29]. The central receptors for Aβ transport across the BBB from the brain to the bloodstream and from the blood to the brain are low-density lipoprotein receptor (e.g., LRP-1) and the receptor for advanced glycation end products (RAGE), respectively [30]. A chronic imbalance between Aβ production and clearance may result in the agglomeration of intracellular and extracellular aggregates in the brain.

Aβ peptides spontaneously aggregate into soluble oligomers, fibrils, and deposit as senile plaques. These events cause toxicity through several mechanisms, including oxidative injury, microglial and astrocytic activation, as well as altering kinase/phosphatase activity, eventually leading to synaptic damage and neuronal death. It is important to mention that Aβ oligomers are the most neurotoxic form [24,31].

Besides the strong evidence about the relation between Aβ and neurodegeneration, there is a continuous debate about the Aβ hypothesis [10,32]. This is mainly due to the constant failure of developing disease-modifying drugs targeting Aβ, preventing neither its aggregation, accumulation, nor clearance. Nowadays, the Aducanumab efficiency has been extensively discussed, a human IgG1 anti-Aβ monoclonal antibody specific for Aβ oligomers and fibrils [33]. Other reasons are the difficulty correlating the Aβ deposits and AD pathology and the disconnection between Aβ and phosphorylated tau deposition. In fact, tau pathology (tauopathy) has been related to neurodegeneration and neuroinflammation independently of Aβ. In addition, there are substantial differences between familial and sporadic diseases. Importantly, peripheral inflammatory diseases have been considered risk factors for AD development, which may not be directly associated with the Aβ dyshomeostasis [13,32]. This is a discussion that is far from over. Then, new insights into AD pathophysiology are driving the development of drugs towards novel therapeutic targets [34,35]. In this scenario, the inflammatory process has been evaluated as an important component of AD pathogenesis. It is well known that Aβ causes neuroinflammation, and many studies have demonstrated the role of inflammation in the early stages of AD development [36,37].

Moreover, the neurofibrillary tangles (NFT), which Alois Alzheimer first described, are another crucial hallmark of AD pathogenesis [38–40]. In this case, the tau protein is aberrantly misfolded and abnormally hyperphosphorylated [40,41]. Tau protein regulates the assembly and stabilization of microtubules. It can be expressed in neurons and oligodendrocytes [42–46].

In AD, the NFT could appear after Aβ accumulation. Considering that plaque-associated dystrophic neurites are not associated with tau, it is probably true that Aβ-mediated neuritic dystrophy occurs first, and the tau accumulation is a consequence of this [47–49]. Furthermore, a study conducted by Hurtado et al. [50] showed that Aβ accelerated NFT formation and enhanced tau amyloidosis. Thus, it seems in AD that Aβ plaque deposition drives cortical tau pathology and tau-mediated neurodegeneration [51,52]. In AD neurodegeneration, Aβ diffuse deposits, non-neuritic plaques occur first. Then, the microglia are activated by Aβ deposits, inducing dystrophic neurites that lack tau. This leads to the aggregation of tau hyperphosphorylated facilitating the spread of tau from the

limbic system to the cerebral neocortex. The tau hyperphosphorylated distributes from the plaque-associated dystrophic neurites forming NFT throughout the neuron, causing neuronal damage and dementia [53–57].

These events showed the relationship between Aβ and tau, mediated by microglia, causing neurodegeneration and the consequent development of dementia [56].

3. Inflammatory Cascade in Alzheimer's Disease

The innate immune system is the first line of defense against pathogens [58]. The microglia have a fundamental role in early response to central nervous system (CNS) alterations such as damage or infection, development, and homeostasis [59]. Microglia activation is an important event during aging and in neurodegenerative diseases. These cells participate in neuroinflammatory events directly via phagocytosis and cytokine production, as shown by identifying disease-specific microglia, or indirectly responding to cues from the adaptive immune system [60,61].

Neuroinflammation plays a crucial role in the pathogenesis of AD. Several studies have reported the presence of inflammatory markers in the brain of patients, including elevated levels of cytokines/chemokines in serum and CSF, along to microgliosis [62–66]. The increase in these molecules is positively correlated to the cognitive impairment at different stages of AD as well as in individuals with mild cognitive impairment (MCI) [67–69].

Recent genome-wide association studies have shown that most polymorphisms recently found in AD patients are involved in the immune response and microglial function. For instance, complement receptor 1 (CR1), CD33, membrane-spanning 4A (MS4A), clusterin (CLU), ATP-binding cassette sub-family A member 7 (ABCA7), sortilin-related receptor 1 (SORL1), inositol polyphosphate-5-phosphatase D (INPP5D), and triggering receptor expressed on myeloid cells 1 and 2 (TREM1, TREM2) [70,71]. Among them, the most prominent polymorphism was found in TREM2, which is associated with phagocytosis [72].

Several studies have shown that Aβ can activate microglia and inflammatory cytokines production [73–75]. Even in the prodromal stages of AD, Aβ soluble oligomers can impair synaptic plasticity, inhibit long-term potentiation, and activate microglia [10]. The intracerebral administration of Aβ causes neuroinflammation and memory impairment even in normal adult rodents [74,76,77]. In addition, neuroinflammation and proinflammatory cytokines increase tau phosphorylation and decrease synaptophysin levels, which leads to cytoskeletal instability and neuronal death [78].

The activation of the immune system in the brain occurs through microglial activation of pattern recognition receptors (PRRs), which identify potentially harmful molecules, activating the innate immune system [79]. Several studies have shown that Aβ species can activate PRRs, consequently triggering an immune response [74,80–83].

Aβ activates several microglial receptors, such as CD36, a class B scavenger receptor, causing secretion of cytokines, chemokines, and reactive oxygen species [84]. Its binding to RAGE induces inflammatory pathways and increases expression of proinflammatory cytokines, such as TNFα and IL-6 [85]. However, the best described pathway is through the activation of Toll-like receptors (TLRs), including TLR2, TLR4, and TLR6 and TREM2 [86,87]. The TLR pathway is responsible for the maturation and release of IL-1β, one of the main pro-inflammatory cytokines involved in the pathophysiology of AD. In fact, IL-1β polymorphism is correlated with the age at AD onset in humans, whereas inhibition of its receptor recues cognitive impairment in animal models [88,89]. IL-1β is produced as a precursor, pro-IL-1β, and requires cleavage to become biologically active. To this end, the pro-IL-1β is cleaved by a complex of intracellular proteins that form the nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3, known as the NLRP3 inflammasome [90,91]. For NLRP3 assembly, two signals are needed. The first one is the activation of TLRs by Aβ or another potentially harmful molecule. After TLR activation, the signal is transduced through myeloid primary response protein 88 (MyD88), activating nuclear factor kappa B (NF-kB), which leads to transcription of NLRP3 components and proinflammatory cytokines, mainly IL-1β. The second signal is triggered

by damage-associated molecular patterns (DAMPs), such as heat shock proteins or ATP, released after cell death. It was proposed by Heneka and col. that this second signal also occurs through phagocytosis of Aβ fragments via TREM2 [90].

The Aβ overload leads to a deficient lysosome degradation of the Aβ fragments, leading to lysosome disruption, cathepsin B release, and the induction of NLRP3 inflammasome assembly [90,92,93]. The inflammasome complex assembly includes the NLRP3 protein, the adapter apoptosis-associated speck-like protein containing a CARD (ASC), and the effector caspase-1. After the NLRP3 complex assembles, caspase-1 cleaves the pro-IL-1β and pro-IL-18, generating the mature form of these cytokines, which activate neutrophils, macrophages, and other microglial cells, amplifying the response inflammatory [94], as represented in Figure 1. This inflammatory pathway has been increasingly described in different neurodegenerative diseases [75,90,91,95,96].

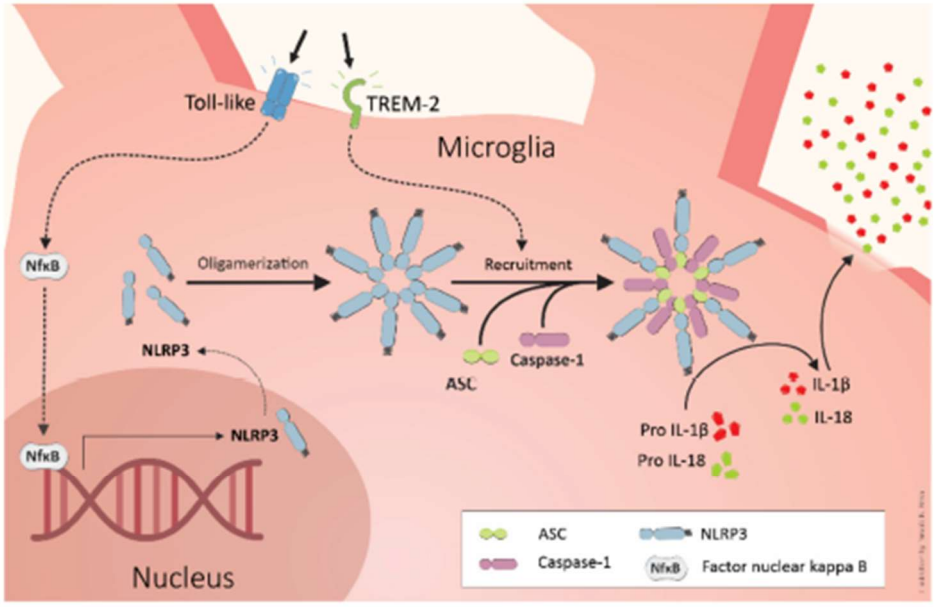

Figure 1. The activation of Toll-like receptors by ligands such as LPS or Aβ species can trigger the first signal for assembly of the NLRP3 inflammasome through nuclear factor kappa B (NF-kB). The second signal can be triggered after Aβ-mediated phagocytosis by TREM2. The Aβ overload causes lysosome disruption, releasing cathepsin B, which induces the signal for assembly of NLRP3. The inflammasome contains caspase-1 that cleaves pro-IL-1β and pro-IL-18, generating the mature forms of these inflammatory cytokines. IL-1β and IL-18, in turn, activate neutrophils, macrophages, and other microglial cells, amplifying the inflammatory response.

The inflammasome activation finally causes cell death by apoptosis and pyroptosis. Pyroptosis is a form of cell death less organized than apoptosis, triggered by inflammation, as Gasdermin-D-mediated pore formation occurs in the membrane and osmotic lysis, with extravasation of intracellular content [97]. When the NLRP3 inflammasome disassembles, its ASC particles are released from the protein complex, which can activate neighboring microglia, perpetuating the immune response. These particles can also bind to Aβ, contributing to its aggregation [98]. Thus, the efficient degradation of the inflammasome components is a critical step to limit the inflammatory response.

The mechanisms of microglial activation by Aβ depositions have remained not fully elucidated. However, the mechanism known is illustrated in Figure 1. Moreover, Aβ induces the secretion of a variety of additional inflammatory molecules. These molecules include members of the minor compounds of the COX metabolism (prostaglandins), short-lived molecules like nitric oxide (NO), and chemokines [90,99–103].

4. TREM2 and Alzheimer's Pathogenesis

The interest in TREM2 increased after the identification of TREM2 variants as risk factors for AD [104,105]. Individuals bearing the heterozygous mutation (rs75932628) in exon 2 of TREM2, a single nucleotide polymorphism that changes arginine to histidine at position 47 (R47H), produce a four-fold increase in the risk of developing AD [105]. This polymorphism has been validated in neuropathology-confirmed cases and has been shown to increase the risk of sporadic AD as significantly as the ApoE ε4 allele [106]. TREM2 polymorphism causes structural changes in the receptor, leading to a partial loss of its function. However, the role of TREM2 in neurodegeneration and AD remains unclear [107].

TREM2 is a single-pass transmembrane protein whose ligand-binding domain includes an extracellular immunoglobulin-like domain, anchoring the protein to the membrane and contains the intramembranous lysine residue necessary for association with its intracellular membrane adaptor, DAP12. The binding of agonists to TREM2 through the DAP12 protein recruits the cytosolic spleen tyrosine kinase (Syk), which, in turn, activates signaling components including, phosphatidylinositol 3-kinase (PI3K), Akt, mitogen-activated protein kinases (MAPK), and increases intracellular calcium levels. Thus, its activation exerts functions such as cell maturation, survival, proliferation, phagocytosis, and inflammatory regulation [108]. TREM2 ligands include bacteria, bacterial cell components such as lipopolysaccharide (LPS), lipoproteins, such as apolipoprotein A (ApoA), ApoB, ApoE, low-density lipoprotein (LDL), DNA, HSP60 chaperone protein, and Aβ [109].

TREM2 expressed on the cell surface can also undergo proteolysis by α-secretase and γ-secretase [110] to generate soluble TREM2 (sTREM2). The catalytically active components of the γ-secretase complex are PSEN 1 and 2, the same proteins mutated in familial AD and responsible for Aβ processing. Inhibition of γ-secretase leads to accumulation of TREM2 c-terminal fragments (CTFs) on the cell surface, impairing signaling and interfering with normal receptor function [111]. This relationship between TREM and PSEN provides evidence about a functional connection between genetic factors found in AD patients.

One of the main mechanisms of TREM2 is undoubtedly its phagocytic activity. In addition, TREM2 expression increases myeloid cell number in response to inflammation or disease [45], besides regulating the inflammatory responses and the clearance of apoptotic neurons and Aβ. However, it may depend on the activating by ligand and the availability of the TREM2 signaling machinery [112]. The AD-associated TREM2 variant, R47H, reduces receptor binding capacity and, consequently, decreases Aβ phagocytosis [113].

In AD patients, microglial cells have decreased phagocytic capacity and a pro-inflammatory phenotype and morphology [114,115]. Therefore, amyloidosis levels can be altered since phagocytosis is one of the principal mechanisms for Aβ clearance. The increase in the microglial TREM2 expression reduces Aβ1-42 soluble and insoluble forms, the formation of senile plaques, and improves cognitive impairment in AD transgenic mice [116]. Conversely, TREM2 deficiency seems to interrupt the formation of the neuroprotective barrier composed of microglia around the amyloid plaques, responsible for their isolation, increasing neuronal toxicity [117]. However, Aβ overload can cause phagocytosis disruption, cathepsin B release, and NLRP3 assembly, which leads to the amplification of the inflammatory response.

Thus, it is controversial whether phagocytosis and inflammation in AD are beneficial or harmful. Perhaps, in the early stages of the disease, the activation of the immune system and induction of phagocytosis can contribute to Aβ clearance, preventing its toxicity and formation of amyloid deposits. However, chronic inflammation can become detrimental because, if the amyloid load cannot be resolved, it can contribute to the progression of AD [118]. This possible dual role of inflammation in AD progression can be related to microglial function. The microglia are important to scavenging duties. However, it produces reactive oxygen species, secretion of proinflammatory cytokines, or degradation of neuroprotective retinoids when activated. In this case, it may thus unnecessarily put surrounding healthy neurons in danger [119]. Then, the microglia are essential during development and homeostasis, performing critical roles in synaptogenesis and synaptic plasticity. However, in aging and AD, the microglial function is altered, leading the detrimental inflammatory environment [120].

5. BBB as a Target of Systemic Inflammation: Importance to Alzheimer's Disease Development

In the brain, BBB blood vessels present particular properties. BBB essentially regulates the passage of substances and cells between blood and the CNS [121]. Several cell types interact to form and support the BBB, which is now referred to as the "neurovascular unit (NVU)" and is composed of the cerebral endothelial cells, basal lamina, astrocytic foot processes, microglia, and pericytes [122].

BBB disruption is closely associated with several neurological diseases [123]. Evidence has pointed out the alteration of BBB as a trigger to AD pathology [124–127]. Previously, Ujiie and col. observed that BBB permeability is higher in a 10-month-old transgenic mouse model of AD than in age-matched non-transgenic animals [128]. In fact, the impairment in the BBB is already evident in the AD mouse model at 4 months of age. The BBB leakage seems to occur even before the Aβ deposition and the appearance of other pathological hallmarks of the disease [128]. Corroborating the experimental data, the increased BBB permeability has been demonstrated in early AD individuals [129]. Plasma proteins such as immunoglobulin G (IgG), fibrinogen, and albumin, normally unable to pass the BBB, have been detected around senile plaques in the brain of AD patients [130–132]. The presence of peptides derived from hemoglobin and prothrombin in AD brains has been associated with increased leakage of blood. Prothrombin is not produced by the normal brain but shows increased levels in AD brains consistent with leakage across a disrupted BBB [133].

Another important point is that BBB is vital to regulate the brain Aβ metabolism and load, and Aβ deposition could result from an inefficient clearance through BBB [132,134]. Firstly, Shibata et al. [135] and other authors pointed out that brain Aβ is mainly cleared across BBB via LRP-1. Importantly, the LRP-1 content is down-regulated in AD brain [132,135–137]. After that, the function of other transporters in the Aβ clearance and AD pathogenesis have been studied, such as RAGE [132,134,138]. On the other hand, Aβ deposition appears to cause BBB damage but is not well evidenced [134]. In addition, it has been demonstrated that BBB endothelial cells respond to truncated tau fragments, ultimately resulting in BBB disruption [139,140]

In parallel, other research groups demonstrated the presence and accumulation of peripheral immune cells and increase in pro-inflammatory cytokines, such as IL-1β, in AD patient's brain [21,141–144]. The constituents of innate immunity seem to participate in many processes of the underlying pathological cascade in AD. In addition, compiling studies show that innate immunity is involved in the etiology of LOAD [145,146]. In this regard, increased peripheral inflammation levels can be detected in the early stages of AD [147]. A meta-analysis showed that the blood concentrations of several pro-inflammatory mediators, such as IL-6 and IL-1β, are increased in AD patients [62,148]. In line with this, previous studies showed that inflammatory mediators' levels are enhanced in the plasma of AD patients 5 years before the clinical onset of dementia compared with age-matched individuals [149,150]. However, it is not well established yet whether brain inflammation in AD subjects is a cause or a consequence of the disease. Although it was previously

thought that the CNS was an immune-privileged site, it is now admitted that inflammatory processes occur in response to an injury, infection, or disease and the peripheral immune system can infiltrate into the brain to mediate this response [151,152]. Indeed, the systemic inflammation seems to be the causative factor of BBB disruption and, consequently, neurodegeneration and cognitive dysfunction [153–156]. Although still debatable, evidence suggests that early or lifelong systemic inflammation triggers long-lasting modulation of CNS immune responses leading to AD development in late life [157].

6. Systemic Inflammatory Diseases and the Connection with Alzheimer's Disease Development

The etiology of sporadic AD is complex and associated with several genetic and behavioral risk factors, in addition to aging [19]. Some of these risk factors related to AD are peripheral diseases, for example, metabolic disorders, such as hypercholesterolemia, diabetes, obesity, and hypertension [158–160]. One possible common event between systemic and brain diseases is chronic systemic inflammation [161]. In line with this, an essential feature of the metabolic disorder's physiopathology is the increased production of pro-inflammatory cytokines [162].

The inflammatory response of the peripheral adipose tissue is an important event of diabetes and obesity [163]. In obese individuals, adipocytes, and resident immune cells of adipose tissue, especially lymphocytes and macrophages, contribute to the increased levels of circulating cytokines, such as TNF-α, IL-6, and IL-1β, as well as C reactive protein (CRP) [164]. Nowadays, white adipose tissue (WAT) is known to be a secretory tissue, which seems crucial in brain dysfunction development [165]. In hyperglycæmia conditions, the NF-κB—a transcription factor that regulates the induction of several inflammatory genes—is rapidly and strongly activated in vascular cells, resulting in enhanced leukocytes adhesion and pro-inflammatory cytokines transcription [166]. Hyperlipidemias, primarily hypercholesterolemia and hypertriglycæridemia, are also related to systemic inflammation. For instance, the study of Lohmann et al. [167] demonstrated that mice fed with a high cholesterol diet presented generalized inflammation, characterized by increased in T lymphocytes and macrophage recruitment in adipose tissue, inducing cytokine production [167].

Most of these metabolic risk factors are associated with BBB leakage and neuroinflammation. An epidemiological study showed that overweight or obesity in middle-aged individuals is associated with loss of BBB integrity several years later [168]. Diabetes is usually associated with macro- and microvascular complications, including CNS alterations that result, at least in part, in BBB damage. Impairment of the cerebral microvascular structure, characterized by reduction in capillary density and tight junctions damage, is a relevant mechanism of BBB dysfunction induced by diabetes [169]. Our group have recently demonstrated that hypercholesterolemic mice present high BBB permeability in the hippocampus, associated with intense astrogliosis [170,171].

According to experimental and clinical studies, the BBB is altered in hypercholesterolemia, resulting in the infiltration of immune cells in the brain parenchyma and consequently the production of inflammatory mediators [172–174]. On one hand, this inflammatory response, associated with persistent activation of glial cells, induces neuronal damage and, ultimately, leads to cognitive dysfunction and dementia [170,171,175]. On the other hand, when the BBB is damaged, the transport of Aβ is defective. RAGE is overexpressed and the expression of LRP's decreases, leading to the accumulation of Aβ in the brain [174,176].

In obese individuals, there is a chronic systemic inflammation that induces production of pro-inflammatory cytokines, such as IL-6, and adipokines (such as TNF-α), mainly produced by WAT. These inflammatory molecules could cause alterations in BBB permeability and, consequently, brain inflammation and neurodegeneration. Deregulation of these molecules could link obesity and AD development [165]. The effects of saturated fatty acids (e.g., palmitic acid) on microglia and astrocyte activation have also been demonstrated. For instance, these fatty acids promote the pro-inflammatory phenotype of microglia, resulting in activation of NF-κB pathway, TLR-4 receptors, interferon-γ (IFN-γ), and TNF-α production [177].

Metabolic diseases are not the only example of systemic inflammatory pathologies associated with BBB disruption and neurodegeneration. Sepsis is another relevant condition that has been implicated in dysfunction and loss of neurons [178,179]. A possible consequence of sepsis is the septic encephalopathy, which occurs in 8–70% of septic patients. It is related to BBB disruption, leucocyte infiltration, up-regulation of aquaporin-4, activation of microglia, astrocytosis, and neuronal death [178]. This relationship between sepsis and brain alterations is an opportunity to better understand the effects of systemic inflammation in cerebral function [179–181]. In fact, it has been reported that RAGE may be involved in sepsis-mediated increase in amyloidogenic proteins and cognitive impairment [182].

In this context, viral infections associated with intense systemic inflammation have been a concern in the neuroscience field. For instance, COVID-19 could lead to neurodegeneration and increase the risk of AD due to an intensive brain inflammatory process as a result of systemic inflammation. Furthermore, SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) is potentially neuroinvasive, suggesting that neurological consequences could occur after direct brain infection [183,184]. Recent studies have reported neurological complications in COVID-19 patients [185–188].

However, it is important to mention that although, growing evidence indicated the relationship between infections and AD, more studies are needed to elucidate better the mechanisms involved. Notably, chronic viral, bacterial, and other infections might be causative factors for the BBB breakdown and coexistent brain inflammatory pathway activation and consequently neurodegeneration [189,190].

Additionally, gut microbiota alterations can cause neuroinflammation and consequently interfere in AD development. Several reports have pointed out the gut microbiota as a modulator of the neuroinflammatory process in AD [191,192]. Importantly, an imbalance in the gut microbiome is related to systemic inflammation and peripheral conditions like diabetes. Moreover, dietary changes may induce a loss of microbiota ecosystem homeostasis [193,194]. Chronic dysbiosis appears to cause BBB leakage and release of pro-inflammatory molecules, endotoxins, ultimately leading to microglia and astrocytes activation [195]. This scenario also increases intestinal permeability, promoting translocation of bacteria and endotoxins across the epithelial barrier and activation of both enteric neurons and glial cells [191,196]. Moreover, the oral microbiota are also related to AD [197,198]. For instance, periodontitis, the most common chronic oral bacterial infection in adults, is generally caused by *Porphyromonas gingivalis* [199]. This bacterium has been detected in the brains of AD patients [197], indicating a strong association between periodontal pathogens and AD [198]. Additionally, the reduction in GSK3β activation may help delay the periodontitis-promoted pathological progression of AD [200]. Figure 2 represents some events connecting systemic inflammatory conditions and AD development.

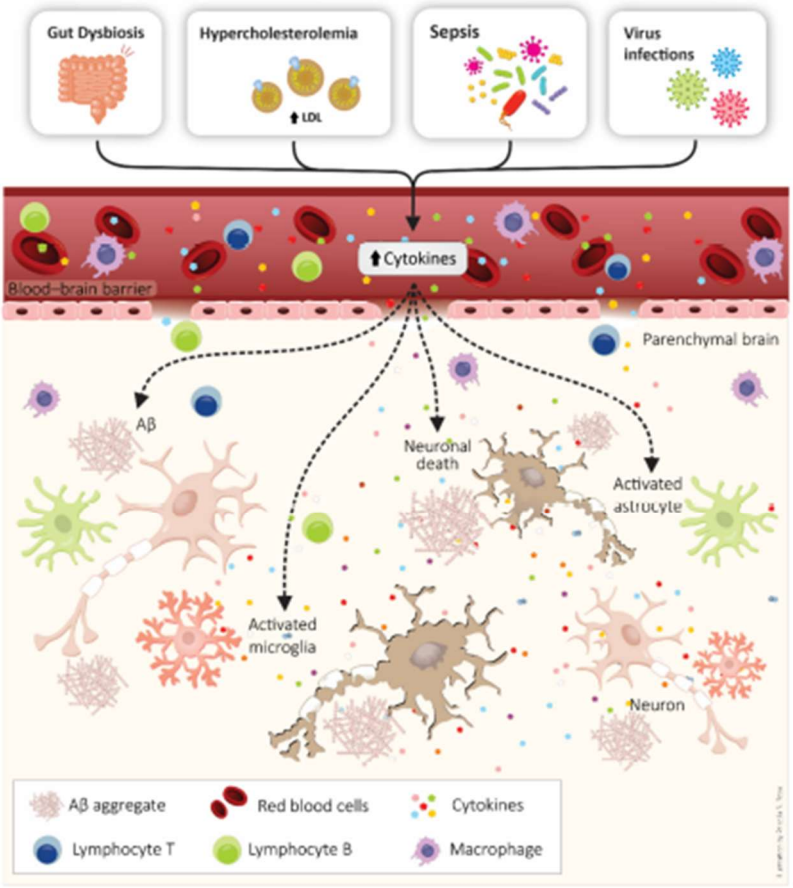

Figure 2. Peripheral diseases are a risk factor for β-amyloid peptide (Aβ) peptide accumulation, neurodegeneration, and Alzheimer's disease development. Systemic inflammatory conditions, such as metabolic disease, sepsis, virus infections, and dysbiosis, are associated with blood–brain barrier (BBB) disruption and coexistent neuroinflammation. Neuroinflammation is characterized by the presence of the peripheral immune system, activation of glial cells (astrocytes and microglia), and increased production of pro-inflammatory molecules (e.g., cytokines).

7. Anti-Inflammatory Approaches in the Alzheimer's Disease

Current approaches for AD management, based on neurotransmission dysfunctions, are not sufficient. These therapies, such as acetylcholinesterase inhibitors and memantine, do not modify the natural course and outcome of the disease. Available treatments are palliative rather than curative or disease-modifying therapies [201]. In this regard, a series of anti-inflammatory drugs have been pointed out as therapeutic strategies to control AD progression [202].

Epidemiological and experimental studies suggest a positive effect of the treatment with non-steroidal anti-inflammatory drugs (NSAIDs) in AD [203–208]. Earlier experimental works indicated that BACE1 expression (mRNA and protein) is stimulated by pro-inflammatory mediators and inhibited by NSAIDs [209,210]. Other studies indicated that treatment with certain NSAIDs decreased brain Aβ accumulation in animal models of

AD, which was related to anti-inflammatory mechanisms [211–213]. In line with this, in a preclinical study, Medeiros and col. [214] reported that aspirin, the most commonly used NSAID, decreased activation of NF-κB and generation of pro-inflammatory molecules in Tg2576 mice. These anti-inflammatory effects caused the activation of phagocytic microglia, resulting in Aβ clearance and improvement of memory. However, these compounds, considered classic anti-inflammatory molecules, have not convincingly shown any beneficial effects during clinical trials in AD patients [215]. In fact, the actions of NSAIDs in dementia depend on the stage of disease progression. Lichtenstein and col. [216] affirmed that the motto for NSAID therapeutics in AD should be "the earlier, the better".

Other studies have examined different anti-inflammatory drugs, e.g., glucocorticoids. Minocycline, for instance, reduces inflammatory parameters in the brain and serum and reverses memory impairment in a mouse model of AD [73]. Specifically, minocycline administration reduces the production of IL-1β, TNF-α, IL-4, and IL-10 induced by Aβ42 oligomer inoculation in the hippocampus and cortex of mice, which was associated with an improvement in spatial memory. Moreover, the anti-inflammatory effects of minocycline exposure involve TLR2 receptors and NLRP3 [75].

Natural products (e.g., nicotine, vitamin D, vitamin E, melatonin, and resveratrol) also present promising effects in AD [217,218]. Experimental evidence demonstrates anti-inflammatory effects and decreased Aβ levels induced by natural compounds [219–223]. For instance, high vitamin D3 diet caused a decrease in amyloid plaques in the brain of APP transgenic mice. The effect of the vitamin D3 supplementation was correlated with diminished levels of TNF-α in the brains of the AD transgenic mouse model [220]. Zhao and col. [221] showed that resveratrol administration in an experimental model of AD (female rats ovariectomized treated with galactose) decreased the NF-κBp65 and RAGE expression and increased the expression of claudin-5 in the hippocampus. The control induced by resveratrol exposure on neuroinflammation, and BBB permeability reduced the content of insoluble Aβ1–42 in the hippocampus of the rats. Omega-3 fatty acids (e.g., α-linolenic acid) can regulate the microglial activation and control brain inflammation, which seems to prevent AD [177,224,225]. Supplementation with oil fish, containing eicosapentaenoic acid and docosahexaenoic acid, decreased neuroinflammation and improved cognitive impairment in septic aged rats [226].

However, when some anti-inflammatory agents are tested in AD patients, the results are not satisfactory. For instance, Nivaldipine that showed potential anti-inflammatory effects in animals, was not beneficial in treating mild to moderate AD [227]. For cognitively intact individuals, low-dose naproxen does not significantly reduce the progression of presymptomatic AD [228]. A clinical trial showed that minocycline did not delay cognitive or functional impairment progress in patients with mild AD over 2 years [229]. Other clinical trials performed with aspirin showed no evidence of reducing the risk of dementia, MCI, or cognitive decline [230].

Nanotechnology has also been tested as an anti-inflammatory strategy to treat neuropathologies, particularly in AD [231,232]. Gold nanoparticles treatment prevented neuroinflammation and cognitive impairment in a rat model of sporadic dementia [231]. Rats exposed to streptozotocin, a sporadic AD model, that present memory impairment, increased levels of IL-1β and NF-κB, and showed to improve after gold nanoparticle treatment. Additionally, gold nanoparticle administration ameliorated BBB disruption and brain dysfunction in hypercholesterolemic mice by improving peripheral inflammation [233]. However, more studies are needed, especially to better establish the safety of gold nanoparticle administration. Figure 3 summarizes the main mechanisms of anti-inflammatory drugs and compounds that display anti-inflammatory effects in AD brains.

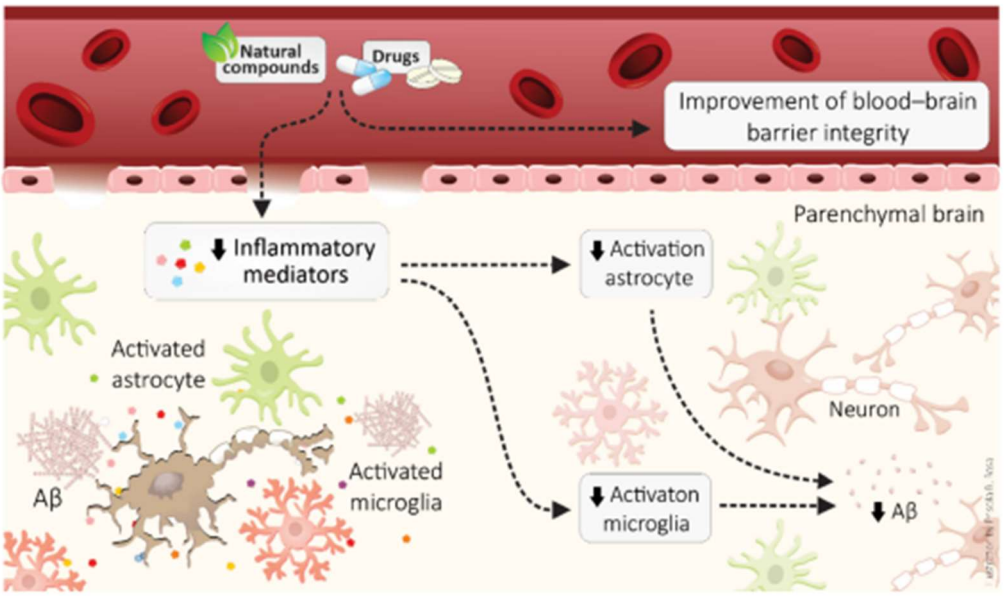

Figure 3. Anti-inflammatory approaches, such as drugs and natural compounds, in Alzheimer's disease. Anti-inflammatory therapeutic strategies improve the blood–brain barrier and neuroinflammation, decreasing activation of astrocytes and microglia as well as generation of pro-inflammatory molecules.

8. Conclusions

AD is a complex, multifactorial, heterogeneous, and severe neurodegenerative disease. It initiates many years before symptoms, as illustrated in Figure 4. Many risk factors are responsible for the development of AD, including genetics, aging, bacteria, diabetes, gut dysbiosis, hypercholesterolemia, obesity, and virus. These risk factors induced systemic inflammation (1) and BBB disruption (2). Thus, Aβ aggregation, tau hyperphosphorylation and, neuroinflammation occur as a possible consequence (3). The neuroinflammation involves the glial activation and release of inflammatory mediators such as NO, IL1- β, Il-18, TNF-α, prostaglandins, and chemokines such as fractalkine (CX3CL1), MIP-1α (CCI3), IP10 (CXCL10) and, MCP-1 (CCL2). These events lead to neuronal death (4) that culminates in memory loss and changes in mood or personality, featuring dementia-like DA (5). Therefore, anti-inflammatory drugs and compounds that display anti-inflammatory effects in AD brains can be an interesting strategy for AD. Finally, it is essential to mention that due to the existence of many failed pathways involved with AD pathogenesis, the success of anti-inflammatory therapy to treat or prevent AD is still impaired. This phenomenon is exemplified in clinical trials with anti-inflammatory molecules. Therefore, just one anti-inflammatory agent does not have benefit enough in the disease. A combination of therapeutic agents may be needed to have the most significant potential to prevent or treat AD development and/or progression.

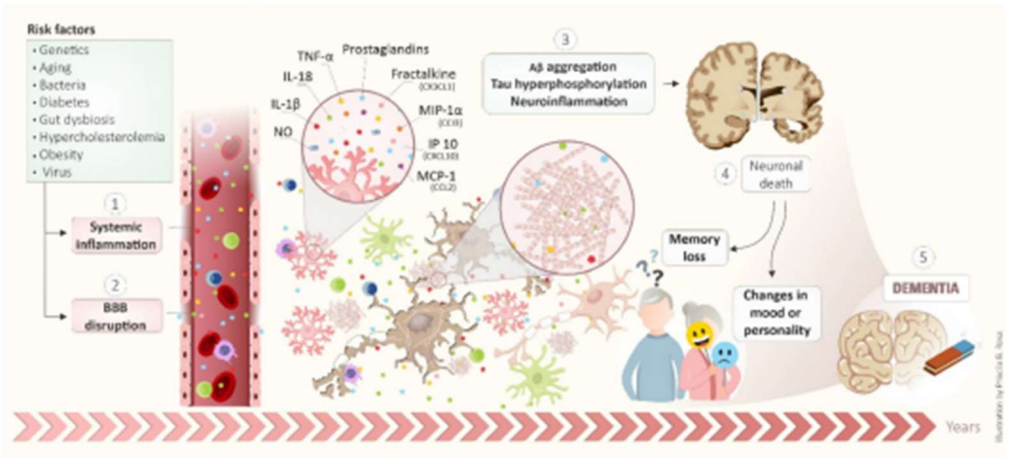

Figure 4. Schematic integrated view of mechanisms involved in development and progression of AD.

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