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Efeitos do resveratrol e do sulforafano sobre cultura de astrócitos hipotalâmicos ao longo do processo de envelhecimento

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"*Mas o que salva a humanidade*
É que não há quem cure a curiosidade."
(Tom Zé)

SUMÁRIO

PARTE I

RESUMO

O envelhecimento é caracterizado como um declínio na integridade fisiológica dos organismos, com mudanças no metabolismo celular, na resposta inflamatória e na homeostase redox, sendo considerado um fator de risco para diversas doenças, incluindo as do sistema nervoso central. Neste sentido, o hipotálamo é uma região cerebral diretamente relacionada a tais funções, sendo então uma estrutura chave na caracterização de alterações ao longo do processo de envelhecimento. Além disso, ele regula atividades essenciais à vida e que conectam o sistema nervoso central à periferia do corpo. Os astrócitos são células responsáveis por diversas funções essenciais à manutenção da homeostasia cerebral. Em particular, astrócitos hipotalâmicos são fundamentais para detectar e responder a mudanças ambientais, pois possuem receptores e transportadores para hormônios e moléculas envolvidos em uma variedade de funções celulares. Além disso, a remodelação destas funções astrocitárias pode acelerar ou atenuar o processo de envelhecimento. Portanto, moléculas glioprotetoras, que modulam parâmetros astrogliais podem contribuir para prevenir e/ou evitar eventos precoces associados ao envelhecimento, mantendo assim a funcionalidade hipotalâmica e, consequentemente, a homeostase do sistema nervoso central. A partir do exposto acima, o objetivo da presente tese foi verificar os potenciais efeitos glioprotetores do resveratrol em culturas de astrócitos hipotalâmicos de ratos Wistar de 2, 90, 180 e 365 dias, bem do sulforafano em culturas de astrócitos hipotalâmicos e em suspensão celular da mesma região cerebral de ratos Wistar de 24 meses. Nossos resultados demonstram que o resveratrol e o sulforafano apresentam efeitos glioprotetores, modulando importantes vias de sinalização celular e resposta inflamatória. Particularmente em relação ao sulforafano, nossos resultados também apontam a modulação da expressão de genes relacionados à inflamação, defesa antioxidante e fatores neurotróficos. Em suma, nosso estudo destaca os potenciais benefícios do resveratrol e do sulforafano na promoção da glioproteção, podendo representar promissoras intervenções terapêuticas contra disfunções cerebrais relacionadas ao processo de envelhecimento.

Palavras-chave: astrócitos hipotalâmicos, envelhecimento, glioproteção, resveratrol, sulforafano.

ABSTRACT

The aging process is characterized by a decline in the physiological integrity of organisms, with changes in cellular metabolism, inflammatory response, and redox homeostasis, making it a risk factor for several diseases, including those of the central nervous system. In this context, the hypothalamus is a brain region directly related to these functions and is considered a key structure in characterizing changes during the aging process. Additionally, it regulates essential life activities and connect the central nervous system to the body periphery. Astrocytes are cells responsible for various essential functions in maintaining cerebral homeostasis. Particularly, hypothalamic astrocytes are crucial in detecting and responding to environmental changes, as they possess receptors and transporters for hormones and molecules involved in a variety of cellular functions. Moreover, the remodeling of these astrocytic functions can either accelerate or attenuate the aging process. Therefore, glioprotective molecules that modulate astroglial parameters may contribute to preventing and/or avoiding early events associated with aging, thus maintaining hypothalamic functionality and consequently, the central nervous system's homeostasis. Based on the above, the objective of this thesis was to verify the glioprotective effects of resveratrol on hypothalamic astrocyte cultures from 2, 90, 180, and 365-days-old of Wistar rats, as well as the potential effect of sulforaphane on hypothalamic astrocyte cultures and cell suspension from the same brain region of 24-month-old Wistar rats. Our results demonstrate that resveratrol and sulforaphane have glioprotective properties, modulating important cellular signaling pathways and inflammatory response. Regarding to sulforaphane, our results also indicate the modulation of gene expression related to inflammation, antioxidant defense, and neurotrophic factors. In summary, our study highlights the potential benefits of resveratrol and sulforaphane in promoting glioprotection, representing promising therapeutic interventions against brain dysfunctions related to the aging process.

Keywords: aging, glioprotection, hypothalamic astrocytes, resveratrol, sulforaphane.

LISTA DE ABREVIATURAS

ALDH1L1	Aldeído desidrogenase 1, membro L1
AMPK	Proteína cinase ativada por monofosfato de adenosina
AQP4	Aquaporina 4
BDNF	Fator neurotrófico derivado do encéfalo
COX-2	Ciclo-oxigenase-2
ERO	Espécies reativas de oxigênio
ERN	Espécies reativas de nitrogênio
GCL	Glutamato-cisteína ligase
GDNF	Fator neurotrófico derivado de células da glia
GFAP	Proteína glial fibrilar ácida
GLAST	Transportador glutamato-aspartato
GLT-1	Transportador de glutamato 1
GS	Glutamina sintetase
GSH	Glutationa
HO-1	Heme oxigenase-1
IL	Interleucina
iNOS	Óxido nítrico sintase induzível
NFκB	Fator nuclear kappa B
Nrf2	Fator nuclear eritróide 2 relacionado ao fator 2
PGC1-α	Coativador-1 alfa do receptor ativado por proliferadores de peroxissoma gama
SIRT1	Sirtuína 1
SNC	Sistema nervoso central
SOD	Superóxido dismutase

SOX10	Fator de transcrição *SRY-Box 10*
TNF-α	Fator de necrose tumoral alfa
Trk	Receptor de tirosina cinase
VEGF	Fator de crescimento endotelial vascular

INTRODUÇÃO

1. Hipotálamo

O hipotálamo é uma região cerebral heterogênea, composta por aproximadamente vinte núcleos, localizada na base do cérebro, evolutivamente conservada, que regula atividades essenciais à vida, tais como: balanço energético, termorregulação, ciclo circadiano e reprodução. Além disso, também está envolvido em comportamentos sociais e emoções bem como memória espacial e episódica, entre outros (Burdakov & Peleg-Raibstein, 2020; Rizzi et al., 2021; Saper & Lowell, 2014).

Essa estrutura cerebral faz parte do sistema límbico, com interconexões com o hipocampo, amígdala e córtex pré-frontal, e é responsável por integrar várias informações para tomar decisões importantes sobre funções vitais básicas (Burdakov & Peleg-Raibstein, 2020). O hipotálamo compara as informações recebidas – como temperatura corporal, glicose e hormônios – com os níveis ideais dos mesmos para, em seguida, ativar respostas autônomas, endócrinas e comportamentais que visam manter a homeostase corporal (Saper & Lowell, 2014). Esta estrutura conecta o sistema nervoso central (SNC) à periferia do corpo, e alterações hipotalâmicas e/ou periféricas relacionadas à idade podem resultar em diversas alterações fisiológicas (Kim & Choe, 2019; Zhang et al., 2013).

Assim, durante o processo de envelhecimento, frequentemente, ocorre um declínio das funções metabólicas, devido alterações hormonais, bem como um impacto no ritmo circadiano e no processo de cognição. Todas essas funções são moduladas de alguma forma pelo hipotálamo, sendo o estudo da funcionalidade hipotalâmica de extrema importância para elucidar os mecanismos fisiopatológicos associados ao envelhecimento (Kim & Choe, 2019). Neste sentido, particularmente em termos celulares, neurônios e células gliais desta região estão sendo cada vez mais estudados.

2. Glia

O SNC é composto por dois principais tipos celulares: os neurônios e as células da glia. Os neurônios são responsáveis pela transmissão sináptica/processamento de informações e, no passado, acreditava-se que a função cerebral resultava exclusivamente de sua atividade, e que as células da glia serviam somente como elementos de suporte para eles (Losada-Perez, 2018). No entanto, essa ideia tem sido refutada pelo acúmulo de evidências que suportam que a funcionalidade cerebral surge da atividade conjunta desses dois grupos celulares (Perea et al., 2009; Pérez-Alvarez & Araque, 2013). Durante a evolução, a proporção entre neurônios e células gliais tem se alterado, aumentando o número destas à medida que a complexidade do sistema nervoso também aumenta. A porcentagem estimada de células gliais em diferentes organismos é de 16% em nematódeos, 20% em moscas, 50% em camundongos e 90% em algumas estruturas do cérebro humano (Losada-Perez, 2018).

As células da glia desempenham funções indispensáveis tanto no desenvolvimento quanto na manutenção da homeostasia do SNC e, além disso, também estão envolvidas na resposta do mesmo à doenças e traumas (Jessen, 2004). A glia é composta por diversos tipos celulares: a microglia, que são células fagocíticas envolvidas na resposta inflamatória; os oligodendrócitos, que são as células responsáveis pela síntese da bainha de mielina; as células ependimais, que revestem os ventrículos cerebrais; e os astrócitos, que terão suas propriedades detalhadas a seguir (Barres, 2008; Quincozes-Santos et al., 2021).

2.1 Astrócitos

Os astrócitos são células que desempenham um papel fundamental em diversas funções essenciais para a manutenção da homeostasia do SNC como, por exemplo: regulam a plasticidade sináptica, o metabolismo de neurotransmissores e o equilíbrio iônico; fornecem suporte metabólico aos neurônios através do metabolismo da glicose, glicogênio e ciclo

glutamato-glutamina; produzem e liberam fatores de crescimento, mediadores inflamatórios e antioxidantes; bem como participam da formação e manutenção da barreira hematoencefálica (Argente-Arizón et al., 2017; Benarroch, 2016; Perea et al., 2009; Pérez-Alvarez & Araque, 2013; Quincozes-Santos et al., 2021; Santos et al., 2018a).

Os astrócitos mantém suas projeções em contato tanto com os neurônios quanto com os vasos sanguíneos, possibilitando o monitoramento do ambiente ao seu entorno e, também, permitindo-os responder dinamicamente a alterações tanto do SNC, quanto periféricas (Teschemacher et al., 2015). As projeções astrocitárias que interagem com terminais pré e pós-sinápticos formam a sinapse tripartite e, dessa forma, são capazes de regular a atividade neuronal (Perea et al., 2009). Além disso, os astrócitos estão interconectados por meio de junções comunicantes que permitem a formação de sincícios compostos por centenas de células (Benarroch, 2016; Perea et al., 2009).

Alterações hormonais e/ou metabólicas provocadas, por exemplo, pelo envelhecimento, são capazes de modificar a morfologia e também as funções astrocíticas, incluindo a produção de citocinas e outros fatores, bem como o transporte de nutrientes e neurotransmissores (Argente-Arizón et al., 2017). Devido à importância dos astrócitos para a manutenção da homeostasia do SNC, o estudo dessas células é fundamental para auxiliar na compreensão dos mecanismos fisiopatológicos e na descoberta de intervenções para amenizar certas condições.

2.1.1 Proteínas do citoesqueleto astrocitário

A rede de filamentos intermediários do citoesqueleto confere integridade e resiliência às células, sendo talvez a função mais conhecida desta rede a de fornecer suporte mecânico para a membrana (Middeldorp & Hol, 2011). Dois desses filamentos são encontrados nos astrócitos, tanto *in vivo* quanto *in vitro*: a proteína glial fibrilar ácida (GFAP) e a vimentina

(Middeldorp & Hol, 2011; Ridge et al., 2022). Ao longo do processo de diferenciação celular, a vimentina é gradualmente substituída pela GFAP, entretanto, as duas proteínas continuam sendo coexpressas, inclusive em células em cultivo (Middeldorp & Hol, 2011; Santos et al., 2018a). Em cérebros maduros, os papeis exatos delas continuam sendo estudados porém, elas parecem ser necessárias para a polimerização de outras proteínas do citoesqueleto, sendo, portanto, fundamentais na manutenção: da forma celular, da citoarquitetura do SNC e da estabilidade mecânica (Hol & Pekny, 2015). Em casos de injúria ou de doenças neurodegenerativas, o aumento da expressão de GFAP e de vimentina é decisivo para a progressão da astrogliose reativa, o que contribui para a formação da cicatriz glial (Sofroniew, 2009).

Além da GFAP e da vimentina, os astrócitos também expressam a actina. Ela também determina a morfologia celular e está envolvida em processos como motilidade, migração e adesão celular. A polimerização inadequada da actina altera funções reguladas pelos astrócitos como, por exemplo, o crescimento celular e a captação de glutamato. Em cultura primária de astrócitos, a actina se apresenta em um arranjo clássico com fibras organizadas paralelamente (Perez et al., 2005; Santos et al., 2018a; Souza et al., 2013).

2.1.2 Astrócitos hipotalâmicos

Em particular, os astrócitos hipotalâmicos são fundamentais para detectar e responder a mudanças ambientais, uma vez que possuem receptores e transportadores para hormônios e moléculas envolvidos em uma variedade de funções homeostáticas (Leloup et al., 2016; Santos et al., 2018b; Teschemacher et al., 2015). Além disso, no hipotálamo a barreira hematoencefálica possui capilares fenestrados, permitindo que os astrócitos recebam e processem mais informações do que nas demais regiões cerebrais (Leloup et al., 2016; Miller & Spencer, 2014).

Sob condições de estresse ou ao longo do processo de envelhecimento, o hipotálamo pode desenvolver uma inflamação crônica (Cai & Khor, 2021). Esse processo inflamatório associado aos astrócitos hipotalâmicos parece ter um papel fundamental na obesidade, bem como no início e progressão de doenças relacionadas à idade (Argente-Arizón et al., 2017; Miller & Spencer, 2014). Estudos têm relacionado essas condições à ativação de vias pró-inflamatórias no hipotálamo, o que leva à desregulação neuronal, à astrogliose, à microgliose e à perda de células-tronco/progenitoras neurais (Cai & Khor, 2021).

Embora este processo de inflamação hipotalâmica associada aos astrócitos seja um processo complexo e multifacetado, foram realizados trabalhos que revelaram como ela contribui para a síndrome metabólica e o envelhecimento. Estudos que inibiram o processo inflamatório nessa região cerebral mostraram efeitos benéficos contra tais condições (Cai & Khor, 2021). Portanto, estudar mecanismos capazes de modular a inflamação nos astrócitos hipotalâmicos é de extrema importância.

2.1.3 Astrócitos no processo de envelhecimento

De acordo com a organização Mundial da Saúde (WHO, 2021), todos os países do mundo estão experimentando o crescimento tanto no tamanho quanto na proporção de pessoas mais velhas na população. Em 2030, 1 em cada 6 pessoas terá 60 anos ou mais. Até 2050, a população mundial de pessoas com 60 anos ou mais dobrará (2,1 bilhões). O número de pessoas com 80 anos ou mais deve triplicar entre 2020 e 2050, atingindo 426 milhões. Embora essa mudança na distribuição da população de um país em direção a idades mais avançadas tenha começado em países de alta renda, agora são os países de baixa e média renda que estão experimentando a maior mudança. Até 2050, dois terços da população mundial com mais de 60 anos viverão em países de baixa e média renda (WHO, 2021). À

medida que a população mundial envelhece, as mudanças encefálicas durante o envelhecimento apresentam desafios para nossa saúde e sociedade.

Pesquisas sobre envelhecimento cerebral identificaram a senescência celular como um fator de risco crítico para a neurodegeneração e o declínio cognitivo. Este processo é desencadeado por meio de uma interação complexa de mecanismos, incluindo neuroinflamação, disfunção mitocondrial, sobrecarga de estresse oxidativo e comprometimento da integridade nuclear e da barreira hematoencefálica (Sahu et al., 2022; Yankner et al., 2008).

Os astrócitos são capazes de sintetizar e liberar uma ampla gama de fatores tróficos, incluindo o fator neurotrófico derivado do encéfalo (BDNF), fator neurotrófico derivado de células da glia (GDNF), fator de crescimento endotelial vascular (VEGF), proteína S100B, entre outros, que promovem a sobrevivência celular, bem como a plasticidade sináptica (Matias et al., 2019). Estes fatores tróficos também podem ter como alvo outras células da glia e/ou células da barreira hematoencefálica, participando assim da diferenciação, ativação e metabolismo dessas regiões (Farina et al., 2007).

Entretanto, durante o processo de envelhecimento, os astrócitos podem adotar um fenótipo ativado, com aumento da secreção de citocinas pró-inflamatórias, produção excessiva de espécies reativas de oxigênio/nitrogênio (ERO/ERN), eventuais alterações na expressão da GFAP e diminuição da síntese e liberação e fatores tróficos, que podem desencadear os processos de neurotoxicidade e neuroinflamação (Jurga et al., 2021; Santos et al., 2018a; Zhang et al., 2013). Portanto, defesas contra esses danos são fundamentais para manter a homeostasia do SNC.

Para lidar com isso, os astrócitos possuem enzimas que participam de sistemas antioxidantes como, por exemplo, a superóxido dismutase (SOD), a glutamato-cisteína ligase (GCL), a óxido nítrico sintase induzível (iNOS) e o coativador-1 alfa do receptor ativado por

proliferadores de peroxissoma gama (PGC1-α) (Quincozes-Santos et al., 2021). Outra estratégia utilizada pelas células para prevenir o estresse oxidativo e a inflamação é a ativação de vias citoprotetoras, como por exemplo, a da heme oxigenasse 1 (HO-1) e do fator nuclear eritróide 2 relacionado ao fator 2 (Nrf2), e a inibição de vias como a do fator nuclear kappa B (NFκB) (Quincozes-Santos et al., 2021). A ativação do NFκB ocorre em resposta à inflamação, resultando na síntese e liberação de diversos mediadores inflamatórios tais como: fator de necrose tumoral alfa (TNF-α), interleucinas (IL), quimiocinas e prostaglandinas (Jensen et al., 2013).

Por sua vez, o sistema adenosinérgico também está relacionado ao processo inflamatório e seus efeitos são mediados por seus receptores A_1, A_{2A}, A_{2B} e A_3. A ativação desse sistema está associada ao aumento da comunicação entre os neurônios e a glia e à redução da inflamação no SNC (Boison et al., 2010).

2.1.4 Cultura primária de astrócitos

As culturas de astrócitos começaram a ser utilizadas por volta dos anos 70 para auxiliar na caracterização de propriedades bioquímicas, farmacológicas e morfológicas relacionadas ao SNC, e muito do conhecimento sobre a funcionalidade astrocitária foi descoberto com a utilização de cultura de células de animais neonatos (Lange et al., 2012). Como característica do encéfalo em maturação, animais neonatos apresentam células com maior capacidade plástica e menor grau de diferenciação em comparação às células de animais adultos e, dessa forma, quando submetidas a estímulos (protetores e/ou tóxicos) podem responder de forma menos fidedigna (Santos et al., 2018a; Santos et al., 2018b). Portanto, a utilização da cultura de astrócitos de animais adultos, que já apresentam diferenciação/especialização celular e conexões sinápticas bem definidas, pode possibilitar o melhor entendimento da fisiopatologia do cérebro maduro. Neste sentido, nosso grupo de

pesquisa estabeleceu protocolos de cultivo de astrócitos de diferentes estruturas cerebrais, como córtex, hipocampo e hipotálamo provenientes de ratos Wistar adultos e envelhecidos (Bellaver et al., 2017; Santos, et al., 2018a; Souza et al., 2013).

Astrócitos de diferentes regiões cerebrais diferem em sua expressão de glicoproteínas de membrana, canais iônicos, receptores de neurotransmissores, transportadores, moléculas de sinalização, entre outros (Yeh et al., 2009). Particularmente em relação ao hipotálamo, foram demonstradas importantes alterações neuroquímicas em comparação com outras regiões cerebrais, como o hipocampo. Por exemplo, em comparação com os astrócitos hipocampais de animais de mesma idade, o perfil dos astrócitos hipotalâmicos diferiu: na expressão dos transportadores GLAST e GLT-1, na atividade da enzima glutamina sintetase (GS), na expressão do Nrf2 e da HO-1 – inclusive mostrando uma melhora nos animais envelhecidos em comparação aos animais adultos, o que não ocorreu nos astrócitos hipocampais –, na expressão da iNOS, nos níveis de glutationa (GSH) e nos níveis de IL-10, entre outros (Bellaver et al., 2017; Santos et al., 2018a).

3. Glioproteção

Como exposto anteriormente, as células gliais são fundamentais para a homeostasia do SNC e, sabe-se que mudanças celulares, moleculares e neuroquímicas nessas células, podem resultar em efeitos tóxicos ou levar a uma diminuição da sua capacidade de proteger neurônios e/ou outras células da glia, causando gliotoxicidade (Bernaus et al., 2020; Patel et al., 2019). Assim, situações gliotóxicas tais como, o estresse metabólico, o estresse oxidativo, a inflamação e a excitotoxicidade, estão diretamente associadas à patogênese de doenças neurológicas (Quincozes-Santos et al., 2021).

Por estarem em constante comunicação entre si e com os neurônios, além de serem responsáveis pelas diversas funções já citadas, alterações gliais são críticas para todo o SNC.

Portanto, esta classe de células representa um importante alvo terapêutico para doenças neurológicas.

Por sua vez, a glioproteção está associada a respostas gliais nas quais tais células possam se proteger e, também, proteger os neurônios, causando uma melhora funcional do SNC em condições fisiopatológicas. A glioproteção pode ser alcançada através das próprias funções protetivas das células gliais, bem como através da utilização de moléculas exógenas que auxiliam na modulação da funcionalidade glial (Quincozes-Santos et al., 2021). A seguir, serão elucidados os papeis de duas dessas moléculas, o resveratrol e o sulforafano.

3.1 Resveratrol

O resveratrol (3,4',5-trihidroxiestilbeno) é um composto polifenólico natural encontrado em uvas, frutas vermelhas, amendoim, vinho tinto e outras fontes alimentares, capaz de aumentar a resistência ao estresse e estender a expectativa de vida de diferentes organismos, desde leveduras até vertebrados (Bhullar & Hubbard, 2015).

Os efeitos benéficos proporcionados pelo resveratrol têm sido associados às suas propriedades: antioxidante, anti-inflamatória, imunomodulatória, hipotensora, hipolipidêmica e antitumoral. Tais efeitos protegem os indivíduos contra doenças cardiovasculares, neurodegenerativas e metabólicas. Além disso, estudos destacam sua importância no processo de envelhecimento por meio da diminuição do estresse oxidativo e da resposta inflamatória, da melhoria da função mitocondrial e da modulação do processo de apoptose (Zhou et al., 2021).

Diversos estudos demonstram que o resveratrol regula funções astrocitárias, como a resposta inflamatória, a liberação de fatores tróficos, as defesas antioxidantes e o metabolismo do glutamato (Bellaver et al., 2014; dos Santos et al., 2006; Zhang et al., 2012). Esses efeitos promovidos pelo resveratrol estão relacionados a diferentes mecanismos, incluindo proteínas

e fatores de transcrição, como: proteína cinase ativada por monofosfato de adenosina (AMPK), HO-1, NFκB, Nrf2, sirtuína 1 (SIRT1), entre outros (Quincozes-Santos et al., 2021). No entanto, os efeitos do resveratrol nos astrócitos hipotalâmicos durante o processo de envelhecimento, ainda não foram esclarecidos.

3.2 Sulforafano

O sulforafano (4-metil-sulfonil-butil isotiocianato) também é um composto natural, encontrado em vegetais crucíferos, como brócolis, couve e couve de bruxelas, como um sistema de defesa contra ataques de patógenos (Bricker et al., 2014).

Os efeitos benéficos proporcionados pelo sulforafano têm sido associados às suas propriedades: antioxidantes, anti-inflamatórias, antitumorais e cardioprotetoras (Angeloni et al., 2009; Quincozes-Santos et al., 2021). Essa molécula é capaz de atravessar a barreira hematoencefálica e chegar ao SNC para exercer efeitos neuroprotetores em condições patológicas como isquemia, lesão cerebral traumática e doenças neurodegenerativas (Benedict et al., 2012; Carrasco-Pozo et al., 2015; Tarozzi et al., 2013).

O sulforafano é capaz de modular uma ampla gama de funções astrogliais, incluindo: a captação de glutamato, a atividade da GS, o metabolismo da GSH, a liberação de fatores tróficos e a resposta inflamatória (Bobermin et al., 2020).

Os efeitos protetores promovidos pelo sulforafano são mediados tanto pela inibição do NFκB quanto pela ativação do Nrf2. Por sua vez, a ativação do Nrf2 é responsável pela indução da expressão gênica de importantes enzimas antioxidantes e de desintoxicação, incluindo a HO1 (Benedict et al., 2012; Quincozes-Santos et al., 2021). No entanto, até o momento não há trabalhos descrevendo os efeitos do sulforafano em astrócitos hipotalâmicos provenientes de animais envelhecidos.

3.3 Mecanismos de glioproteção do resveratrol e do sulforafano

Os efeitos anti-inflamatórios promovidos tanto pelo resveratrol quanto pelo sulforafano são mediados, pelo menos em parte, pela inibição do NFκB, resultando numa diminuição na expressão de fatores pró-inflamatórios como iNOS, ciclo-oxigenase-2 (COX-2), TNF-α e várias IL (Bellaver et al., 2016; Benedict et al., 2012).

Por sua vez, a ativação do Nrf2 por essas moléculas, promove a glioproteção através de seus efeitos antioxidantes e, também, anti-inflamatórios. Após sua ativação, o Nrf2 é translocado para o núcleo, onde controla: a expressão de genes que codificam enzimas antioxidantes como, por exemplo, a SOD; e também estimula a produção de enzimas que contribuem para a síntese de GSH como, por exemplo, a GCL (Quincozes-Santos et al., 2021; Wakabayashi et al., 2010).

A HO-1 é regulada pelo Nrf2 e está associada à propriedade antioxidante tanto do resveratrol quanto do sulforafano. Esta enzima catalisa a degradação do grupo heme em biliverdina, bilirrubina, monóxido de carbono e ferro livre. Tais produtos medeiam efeitos protetores por possuírem propriedades antioxidantes e anti-inflamatórias ao inibirem a atividade da iNOS e a ativação do NFκB (Loboda et al., 2016; Quincozes-Santos et al., 2021). Assim sendo, a HO-1 é um elo crucial na ligação entre as vias do Nrf2 e do NFκB, bem como um potencial mecanismo associado a glioproteção.

JUSTIFICATIVA

A partir do exposto acima, considerando a importância dos astrócitos hipotalâmicos tanto para a manutenção da homeostasia do SNC quanto da periferia corporal, torna-se fundamental o entendimento dos efeitos de moléculas com potencial ação glioprotetora sobre os mecanismos bioquímicos, celulares e moleculares associados ao processo de envelhecimento nessa estrutura cerebral. Além disso, o resveratrol e o sulforafano são moléculas que atravessam a barreira hematoencefálica, possuindo potencial de modular a funcionalidade glial. Cabe destacar, também, que estas moléculas regulam importantes vias de sinalização como, por exemplo, o sistema Nrf2/HO-1, que é fundamental para o SNC e expresso predominantemente em astrócitos. Assim, o melhor entendimento dos mecanismos de ação destes compostos pode representar novas estratégias preventivo-terapêuticas, bem como, contribuir para a melhor caracterização das células gliais como alvos terapêuticos no SNC.

OBJETIVOS

Objetivo Geral

Investigar alterações astrocitárias em cultura de astrócitos hipotalâmicos de ratos Wistar durante o processo de envelhecimento e, também, avaliar o potencial glioprotetor do resveratrol e do sulforafano.

Objetivos Específicos

Objetivo 1: Avaliar os efeitos dependentes da idade do resveratrol em culturas primárias de astrócitos hipotalâmicos de ratos Wistar recém-nascidos (2 dias), adultos (90 dias) e envelhecidos (180 e 365 dias). Para isso, foram avaliados a viabilidade celular, a liberação de lactato, a morfologia dos astrócitos, a liberação de fatores tróficos e citocinas inflamatórias, bem como os níveis das proteínas Nrf2 e HO-1, dois mecanismos clássicos pelos quais o resveratrol medeia a glioproteção.

Objetivo 2: Caracterizar os efeitos glioprotetores do sulforafano em cultura de astrócitos hipotalâmicos de ratos Wistar envelhecidos (24 meses). Para isso, foram avaliados: o conteúdo extracelular de proteínas e/ou expressão de mRNA de genes relacionados à inflamação, senescência, respostas citoprotetoras, marcadores astrocitários, fatores tróficos e homeostase redox. Além disso, para confirmar o efeito predominante do sulforafano sobre as células gliais, avaliamos a expressão de genes cruciais relacionados às funções astrocitárias em suspensão celular proveniente do tecido hipotalâmico de ratos da mesma idade.

PARTE II

CAPÍTULO I

Age-dependent effects of resveratrol in hypothalamic astrocyte cultures

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Age-dependent effects of resveratrol in hypothalamic astrocyte cultures

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Objectives

The hypothalamus plays critical roles in maintaining brain homeostasis and increasing evidence has highlighted astrocytes orchestrating several of hypothalamic functions. However, it remains unclear how hypothalamic astrocytes participate in neurochemical mechanisms associated with aging process, as well as whether these cells can be a target for antiaging strategies. In this sense, the aim of this study is to evaluate the age-dependent effects of resveratrol, a well-characterized neuroprotective compound, in primary astrocyte cultures derived from the hypothalamus of newborn, adult, and aged rats.

Methods

Male Wistar rats (2, 90, 180, and 365 days old) were used in this study. Cultured astrocytes from different ages were treated with 10 and 100 μM resveratrol and cellular viability, metabolic activity, astrocyte morphology, release of glial cell line-derived neurotrophic factor (GDNF), transforming growth factor β (TGF-β), tumor necrosis factor α (TNF-α), interleukins (IL-1β, IL-6, and IL-10), as well as the protein levels of Nrf2 and HO-1 were evaluated.

Results

In vitro astrocytes derived from neonatal, adults, and aged animals changed metabolic activity and the release of trophic factors (GDNF and TGF-β), as well as the inflammatory mediators (TNF-α, IL-1β, IL-6, and IL-10). Resveratrol prevented these alterations. In addition, resveratrol changed the immunocontent of Nrf2 and HO-1. The results indicated that the effects of resveratrol seem to have a dose- and age-associated glioprotective role.

Conclusion

These findings demonstrate for the first time that resveratrol prevents the age-dependent underlying functional reprogramming of *in vitro* hypothalamic astrocytes, reinforcing its antiaging activity, and consequently, its glioprotective role.

Keywords: aging, astrocytes, hypothalamus, resveratrol

Introduction

The hypothalamus is a crucial brain region involved in different functions, including regulation of energy balance, maintenance of the temperature, circadian rhythm, reproduction, and emotions [1,2]. In addition, it connects the central nervous system (CNS) to the periphery of the body, receiving information, processing it, and generating a response to the initial input, thus representing an important regulator in the aging process [3].

Astrocytes are glial cells that perform different functions to maintain brain homeostasis, such as synaptic plasticity, metabolism of neurotransmitters, metabolic support, production and release of trophic factors, and inflammatory mediators [4–6]. Particularly, hypothalamic astrocytes can act as a metabolic sensor since they express receptors and transporters for hormones and other molecules [7,8]. In line with this, hypothalamic astrocytes participate in the cellular and neurochemical mechanisms associated with aging and can be a target for the development of antiaging strategies.

Resveratrol is a natural polyphenol compound found in grapes, berries, peanuts, red wine, and other dietary sources, capable to enhance stress resistance and to extend the lifespan of different organisms from yeast to vertebrates [9]. Several studies demonstrate that this molecule regulates astrocyte functions, such as inflammatory response, trophic factor release, antioxidant defenses, and glutamate homeostasis [10–12]. These effects promoted by resveratrol are related to different mechanisms including nuclear factor erythroid-derived 2-like 2/heme oxygenase-1 (Nrf2/HO-1), sirtuin 1, adenosine monophosphate (AMP)-activated protein kinase, nuclear factor kappa B, among others [5]. However, the effects of resveratrol in hypothalamic astrocytes remains unclear.

In this sense, the aim of this study was to evaluate the age-dependent effects of resveratrol in primary astrocyte cultures derived from the hypothalamus of newborn (2 days old), adult (90 days old), and aged (180 and 365 days old) Wistar rats. For this, it was

evaluated cellular viability, metabolic activity, astrocyte morphology, the release of trophic factors and inflammatory cytokines, as well as the protein levels of Nrf2 and HO-1, two classical mechanisms by which resveratrol mediates glioprotection.

Materials and methods

Animals

Male Wistar rats were obtained from Federal University of Rio Grande do Sul (UFRGS) and maintained under a controlled environment (12 h-light/12 h-dark cycle, 22 ± 1 °C; ad libitum access to food and water). Animal experiments were performed in accordance with the National Institute of Health guide for the care and use of laboratory animals and were approved by the UFRGS animal care and use committee (process number 35387).

Hypothalamic primary astrocyte cultures

The protocol was previously described by Santos et al. [6]. Wistar rats at 2, 90, 180, and 365 days old were euthanized by decapitation. They subsequently had their hypothalamus dissected, and the meninges removed. The tissue was enzymatically digested in Hank's balanced salt solution (HBSS) containing 0.05% trypsin at 37 °C for 7 min. The tissue was then mechanically dissociated for 7 min and centrifuged at 100 g for 5 min. The pellet was resuspended in HBSS and again mechanically dissociated until complete homogenization, and then centrifuged at 100 g for 5 min. Then, cells were resuspended in Dulbecco's modified Eagle's medium/F12 (DMEM/F12), supplemented with 10% fetal bovine serum (FBS), 15 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, 14.3 mM $NaHCO^3$, 1% Fungizone, and 0.04% gentamicin. Cells were seeded (approximately 2–4 × 105 cells/cm^2) into 6- or 24-well plates pre-coated with poly-l-lysine and cultured at 37 °C in a 5% CO^2 incubator. After 24 h, the culture medium was exchanged; during the first week, the medium

was replaced once every 2 days, and from the second week on, once every 4 days. From the second week on, the astrocytes received medium supplemented with 20% FBS until they reached confluence (at approximately the fourth week). No dibutyryl-cAMP was added to the culture medium. To determine whether the culture contained microglia or neurons after reaching confluence, we used anti-ß-tubulin III, anti-NeuN, and anti-CD11.

Cellular treatments

After confluence, the culture medium was exchanged with serum-free DMEM/F12, and the astrocytes were incubated in the absence or presence of resveratrol (0.1, 1, 10, and 100 μM) for 24 h. Cells were maintained at an atmosphere with 5% CO^2 and 37 °C.

MTT reduction assay

5-diphenyltetrazolium bromide (MTT) was added to the culture medium at a concentration of 50 μg/ml and cells were incubated for 3 h at 37 °C in an atmosphere of 5% CO^2. Subsequently, the medium was removed and the MTT crystals were dissolved in dimethylsulfoxide. Absorbance values were measured at 560 and 650 nm.

PI incorporation assay

Cells were incubated with 7.5 μM propidium iodide (PI) for 1 h after the treatments. The optical density of fluorescent nuclei (labeled with PI) was determined with Optiquant version 5.0 software (Packard Instrument Company).

Immunofluorescence analysis

Immunofluorescence was performed as described previously [6]. Cells were fixed and permeabilized. After blocking overnight with 4% albumin, the cells were incubated with anti-

glial fibrillary acidic protein (GFAP; 1 : 400) or anti-vimentin (1 : 800), followed by incubation with secondary antibody conjugated with Alexa Fluor. For actin staining, cells were incubated with 10 μg/ml rhodamine-labeled phalloidin. Anti-ß-tubulin III (1 : 500), anti-NeuN (1 : 50), and anti-CD11 (1 : 400) were also used.

Extracellular lactate levels

Lactate levels in the extracellular medium were quantified using a commercial UV assay kit. Results are expressed in mmol/l.

Trophic factor and inflammatory cytokine measurements

Glial cell line-derived neurotrophic factor (GDNF) and transforming growth factor β (TGF-β) levels, as well as tumor necrosis factor α (TNF-α) and interleukins (IL-1β, IL-6, and IL-10) were measured in the extracellular medium using commercial ELISA kits. Results are expressed in ng/ml or pg/ml.

Western blot analysis

Astrocytes were homogenized in lysis solution and equal amounts (10 μg) of protein from each sample were placed on polyacrylamide gels and transferred to nitrocellulose membranes, which were blocked overnight and then incubated with anti-Nrf2 (1 : 1000), anti-HO-1 (1 : 1000), and anti-horseradish peroxidase conjugated actin (1 : 20 000). Subsequently, membranes were incubated for 1 h with anti-rabbit IgG diluted 1 : 10 000. Immunoblots were quantified by optical densities. Results are expressed as protein/actin ratio.

Protein assay

Protein content was measured using Lowry's method [13].

Statistical analyses

Data were statistically analyzed using two-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. P values <0.05 were considered significant. The data represent the mean ± SEM of at least six independent experiments.

Results

Effects of resveratrol on cellular viability and integrity, metabolic activity, and morphology in hypothalamic astrocyte cultures First, we tested different doses of resveratrol on cell viability (MTT assay) and membrane integrity (PI incorporation). Resveratrol (0.1, 1, 10, and 100 μM for 24 h) did not change cellular viability or integrity in any age (data not shown). Therefore, based on previous studies of our group, we choose 10 and 100 μM of resveratrol for 24 h to perform the experiments [10,14,15]. After, we measured the levels of lactate, a product of glycolysis, which were decreased in aged (180 and 365 days) astrocyte cultures compared to control newborn cultures (Fig. 1). While resveratrol did not change lactate levels in astrocyte cultures from newborn and 90 days rats, 100 μM resveratrol increased lactate in cultures from animals with 365 days, restoring the levels near to newborn cultured astrocytes (control conditions).

Next, we performed morphology and immunofluorescence analyses to evaluate the effects of resveratrol on hypothalamic astrocyte cultures from different ages (Supplementary Figure 1, Supplemental digital content 1, http://links.lww.com/WNR/A696). Astrocyte cultures showed polygonal to fusiform and flat morphology, as well as parallel arrangement of the stress fibers (actin staining), in accordance with our previous publication [6]. In addition, immunofluorescence for GFAP and vimentin, classical astrocytic cytoskeleton markers, were not altered by age or resveratrol (Supplementary Figure 1, Supplemental digital content 1, http://links.lww.com/WNR/A696). Moreover, in accordance with previous data [6],

less than 5% of cells were labeled to specific proteins of microglia (CD11) and neurons (ß-tubulin III and NeuN) (data not shown).

Age-dependent effects of resveratrol on trophic factors and inflammatory mediators

As shown in Fig. 2a, aging decreased GDNF release. In addition, 100 μM resveratrol increased GDNF levels in all ages, preventing the age-dependent decline of this trophic factor. However, 10 μM resveratrol increased the levels of GDNF compared to the respective untreated controls only in aged astrocytes. Aging also increased TGF-β levels (Fig. 2b), which were prevented by 100 μM resveratrol only in cultured astrocytes from animals with 365 days old.

Regarding pro-inflammatory cytokines, as expected, aging enhanced their release (Fig. 2c–e). In addition, 100 μM resveratrol was able to decrease TNF-α release in astrocytes from adult and aged animals (Fig. 2c), as well as IL-1β from aged cultures, compared to their respective untreated controls (Fig. 2d). However, 10 μM resveratrol was able to decrease TNF-α and IL-1β in astrocytes only from aged animals. For IL-6, 100 μM resveratrol decreased this cytokine only in astrocytes derived from animals of 365 days old (Fig. 2e). In contrast, anti-inflammatory cytokine IL-10 decreased along the aging process (Fig. 2f), being its decrease prevented by 10 and 100 μM resveratrol in astrocytes from animals with 365 days old.

Potential mechanisms associated with glioprotective effects of resveratrol

Mature hypothalamic astrocyte cultures presented lower protein levels of Nrf2 than newborn cultured astrocytes — control conditions (Fig. 3a and b, respectively). Regarding Nrf2, resveratrol further decreased its content in astrocytes from rats of 2, 90, and 180 days old, while increased Nrf2 immunocontent compared to its untreated condition in cultured

astrocytes from rats of 365 days. In addition, cultured astrocytes from animals of 90 and 365 days old showed a decrease in HO-1 protein levels compared to control conditions (newborn cultures). Interestingly, cultures from animals of 180 days old did not change the levels of HO-1 compared to newborn. Resveratrol decreased the levels of HO-1 compared to respective basal condition in astrocyte cultures from animals of 2, 90, and 180 days old. Moreover, there was a significant difference between resveratrol concentrations (10 and 100 μM) in adult astrocytes (90 days old). Resveratrol did not present effect compared to respective untreated control in cultured astrocytes from 365 days old.

Discussion

Aging is characterized as a decline in the physiological integrity of the organisms with significant changes in metabolism, inflammatory response, and redox homeostasis, leading to increased risk factor for several diseases, including in the CNS [16]. In line with this, hypothalamus has been hypothesized to regulate these functions, being associated with aging and the progression of neurodegenerative diseases [15]. In addition, evidence suggests that remodeling of astrocyte functions can accelerate or attenuate the aging process. Therefore, glioprotective molecules that modulate astrocytic parameters, such as resveratrol, can contribute to maintain hypothalamic functionality, and consequently, CNS homeostasis. Here, we observed significant changes in metabolic activity, release of inflammatory and trophic factor mediators, and signaling pathways related to cellular responses in an *in vitro* experimental model of hypothalamic astrocytes derived from differently aged rats, as well as the effects of resveratrol, which appears to have a dose- and age-associated glioprotective role.

Our previous publication showed that hypothalamic astrocyte cultures from mature Wistar rats (90 and 180 days old) reproduce changes in glial functionality observed in the

aging brain, including glutamatergic homeostasis, glucose metabolism, synthesis and release of antioxidant defenses, trophic support, inflammatory response, and signaling pathways related to mechanisms associated with cellular alterations [6]. However, in the present study, we used animals of 365 days old to better characterize the aging process. In this sense, phase contrast images showed the typical polygonal to fusiform and flat morphology, and the parallel arrangement of actin fibers in cultured astrocytes from animals of 365 days old, in accordance with our previous publications and those of other authors who also described routine protocols of adult and aged animals to better characterize biochemical and physiological properties of astrocytes, and consequently, a useful cell model of astroglial studies of mature brain [6,8,17,18]. In addition, our findings are in accordance with previous publications of our group on cortical and hippocampal astrocytes, and reinforce the protective role of resveratrol on hypothalamic astrocytes from aged rats [5]. Interestingly, while other brain regions seem to have a single pattern of increase or decrease in their functions during aging, age-related differences in some functions of hypothalamic astrocytes appear like a U-curve (a decrease followed by a recovery) [6], and here, we demonstrated the ability of resveratrol in modulating glial parameters even with this difference.

Intermediate filaments, such as GFAP and vimentin, regulate cell development and differentiation, as well as synaptic function [17], and the staining of these proteins was not altered in astrocytes from different ages, which is in accordance with other studies that have demonstrated coexpression of these proteins in in vitro astrocytes [6]. Moreover, resveratrol did not change these glial markers, probably due to the acute treatment (24 h). It is noteworthy that a long-term resveratrol treatment downregulated the mRNA expression of GFAP in aged hypothalamic astrocytes [15]. In addition, we observed a decrease in lactate release in aged astrocyte cultures. Astrocytes have a critical role related to the CNS metabolism, because these cells take up glucose from blood vessels and can transform it into

lactate, which is then transported to neurons to be used as an energy source [19]. Classically, alterations in glucose metabolism are associated with aging and pathomechanisms of neurodegenerative diseases. In agreement with that, we observed a decrease in lactate levels in aged astrocytes (180 and 365 days), with potential impacts in brain homeostasis [6]. Resveratrol prevented the decrease in lactate levels in astrocyte cultures from animals of 365 days old, indicating a protective effect that can be related to maintenance of metabolic activity and ATP production, as well as to oxidative stress management and synthesis of neurotransmitters, functions strongly impaired during aging process [19].

Beyond the metabolic role, astrocytes synthesize and release trophic factors, such as GDNF and TGF-β. In accordance with previous publication, we observed an age-dependent decrease in the levels of GDNF, which is essential for neuronal survival and synaptic plasticity [6]. However, resveratrol increased GDNF levels in all ages and, interestingly, the lower dose of resveratrol (10 μM) acted only in aged astrocytes. This data reinforces the antiaging effect of resveratrol, since reduction in GDNF levels strongly impacts neural networks, which can be compromised in the aging process. We also observed an increase in TGF-β levels with aging, while resveratrol prevented this effect only in astrocytes obtained from animals of 365 days old. TGF-β can induce hypothalamic inflammation that is frequently found in the pathogenesis of neurodegenerative diseases [20]. On the other hand, it is important to note that TGF-β is critical to cell growth, differentiation, and transformation, due to its role in neurological development or synapse function, therefore normal TGF-β levels is required for CNS homeostasis [20]. Consistent with this idea, the effect of resveratrol on this trophic factor is important to glioprotection/neuroprotection.

Hypothalamic inflammation is a common feature of the aging process, and several studies indicate the role of astrocytes in this event [3]. Astrocytes participate in the inflammatory response by releasing mediators such as TNF-α, IL-1β, IL-6, and IL-10 [21]. In

line with this, aging increased TNF-α and IL-1β, and decreased IL-10, a classical event in neuroinflammation [6]. Regarding IL-10, this anti-inflammatory cytokine can attenuate inflammatory response and inflammation-induced cytotoxicity [22]. Interestingly, resveratrol may counteract the pro-inflammatory state, since both concentrations of resveratrol were able to avoid the release of pro-inflammatory mediators in aged astrocytes, indicating an age-dependent anti-inflammatory activity of this compound. Therefore, these findings suggest that hypothalamic astrocyte cultures acquire a pro-inflammatory state during aging, and recent literature has increasingly uncovered the role of hypothalamic inflammation in the pathogenesis of age-related neurodegenerative diseases. Thus, resveratrol can attenuate or delay this aging phenotype due to its glioprotective properties.

Metabolic, trophic, and inflammatory functions can be driven by a wide range of signaling pathways. Particularly related to resveratrol, the transcriptional factor Nrf2 and the cytoprotective enzyme HO-1 are the most promising pathways [5]. In this context, Nrf2 and HO-1 protein levels were modulated in an age and resveratrol dose dependent manner. Surprisingly, resveratrol potentiates the Nrf2 decrease in astrocyte cultures of 90 and 180 days

old, while an opposite activity was observed in aged astrocytes from 365 days old. It is important to note that Nrf2 is a master regulator of genes that encode mediators of redox and inflammation, including HO-1, and it's up-or down-regulation can impact cellular function [23]. In the CNS, astrocytes may be the predominant cell type of Nrf2 activation, and the effect of resveratrol in aged astrocytes (365 days) may be associated with cellular compensatory mechanisms to avoid aging-induced gliotoxicity. These data are in agreement with an upregulation of Nrf2 and HO-1 genes in aged hypothalamic astrocytes upon a long-term resveratrol treatment [15]. However, it is important to note that neonatal and adult hypothalamic astrocytes may have different abilities to promote protective cellular responses. Particularly regarding to resveratrol, our group have reported the involvement of Nrf2 and

HO-1 in the glioprotective effects of resveratrol treatment for 24 h in neonate, adult, and aged hippocampal astrocytes, indicating a region-dependent functional and molecular heterogeneity of astrocyte responses [10,14]. Therefore, due to the functions of hypothalamic astrocytes, Nrf2 and HO-1 may not be the main targets of resveratrol in these cells, at least at 24 h incubation.

Finally, our findings demonstrate the glioprotective effects of resveratrol in in vitro hypothalamic astrocytes from neonatal to aged rats. Resveratrol is a well-recognized anti-inflammatory, antioxidant, and antiaging polyphenol, but it is poorly studied in hypothalamus, a crucial brain region that orchestrate several biological processes, in both physiological and pathological conditions. Two possible limitations of this study should be mentioned. First, although our results about cellular reprograming involve an in vitro experimental model, they reinforce the glioprotective role of resveratrol in hypothalamic astrocytes. Second, the effects of resveratrol were evaluated in primary cultures from neonatal to aged rats. In this regard, data from *in vivo* experimental models of aging, particularly after resveratrol exposure, will confirm and expand the knowledge about the effects of resveratrol on hypothalamus during aging process. It is important to note that cultured astrocytes have significant differences when compared to brain-aged astrocytes, including on intermediate filaments expression [17,18,24], however, cultures of hypothalamic astrocytes from the mature brains are poorly studied and could represent an important tool for understanding age-related glial functions in this brain region. Thus, our findings reinforce the age-dependent effects of resveratrol on hypothalamic astrocyte cultures, which can induce an underlying reprogramming in these cells, providing insights for this compound as a potential future treatment for aging or neurodegenerative diseases.

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Conflicts of interest

There are no conflicts of interest.

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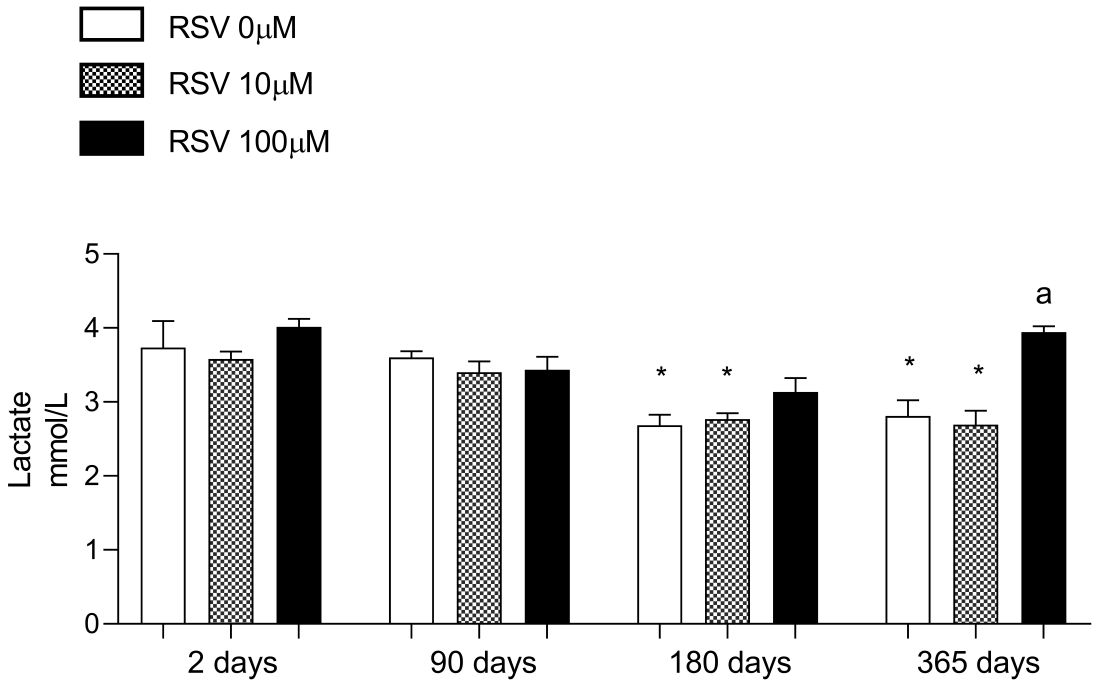
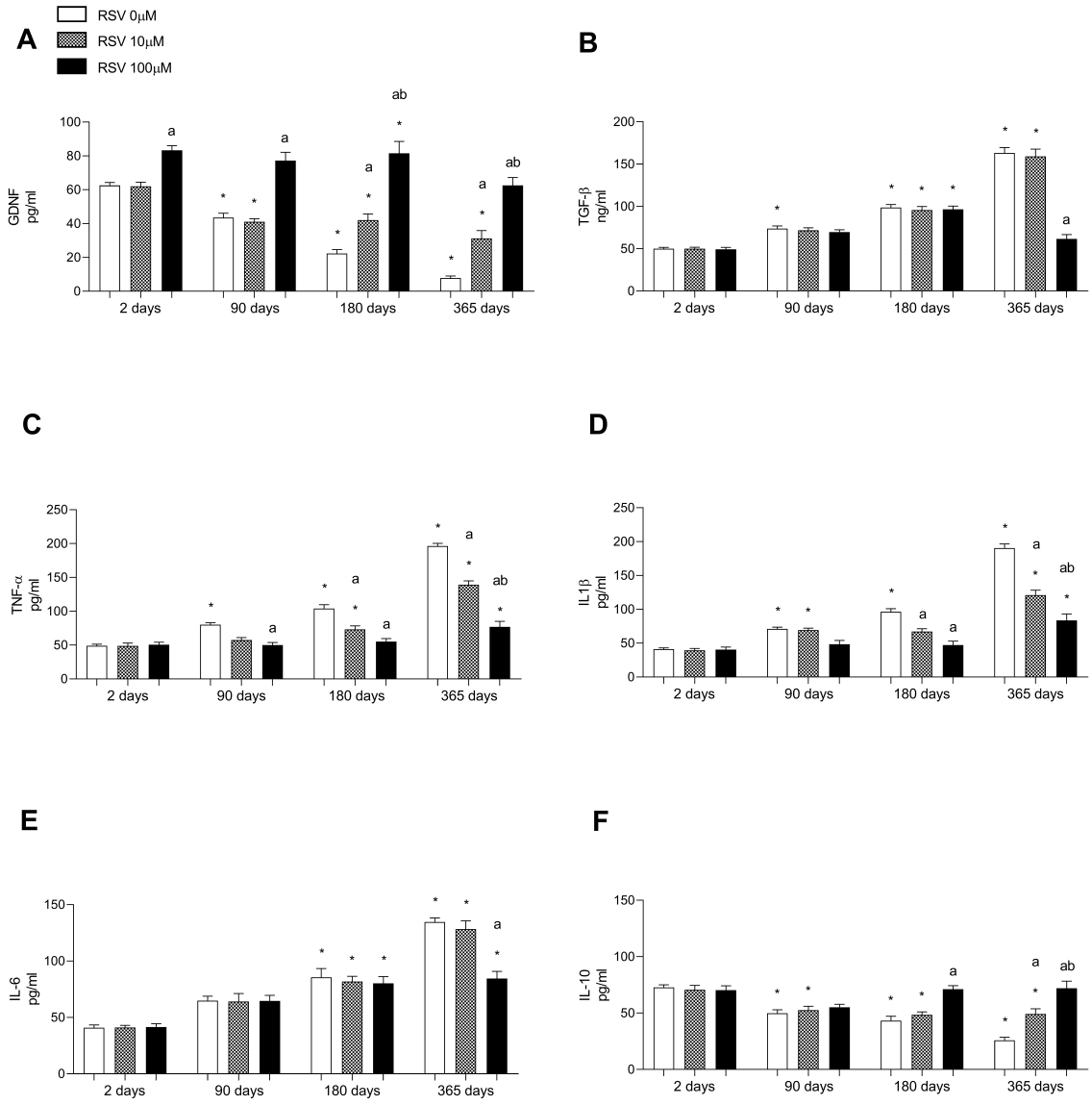
Figure legends

Figure 1. Lactate release in hypothalamic astrocyte cultures from different ages in the absence or presence of resveratrol (10 or 100 μM) for 24 h. The data represent the mean ± SEM of at least six independent experiments, performed in triplicate and statistically analyzed by two-way ANOVA followed by Tukey's test. * refers to statistically significant differences from newborn untreated cells; 'a' refers to statistically significant differences from untreated cells of the same age group. ANOVA, analysis of variance.

Figure 2. Trophic and inflammatory mediators of hypothalamic astrocyte cultures from different ages in the absence or presence of resveratrol (10 or 100 μM) for 24 h. GDNF (a), TGF-β (b), TNF-α (c), IL-1β (d), IL-6 (e), and IL-10 (F). The data represent the mean ± SEM of at least six independent experiments, performed in triplicate and statistically analyzed by two-way ANOVA followed by Tukey's test. * refers to statistically significant differences from newborn untreated cells; 'a' refers to statistically significant differences from untreated cells of the same age group and 'b' refers to statistically significant differences between resveratrol concentrations in the same age group. ANOVA, analysis of variance; GDNF, glial cell line-derived neurotrophic factor; IL, interleukin; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α.

Figure 3. Western blotting analysis of Nrf2 (a) and HO-1 (b). The data represent the mean ± SEM of at least six independent experiments, performed in triplicate and statistically analyzed by two-way ANOVA followed by Tukey's test. * refers to statistically significant differences from newborn untreated cells; 'a' refers to statistically significant differences from untreated cells of the same age group and 'b' refers to statistically significant differences between resveratrol concentrations in the same age group. ANOVA, analysis of variance.


RSV 0μM
RSV 10μM
RSV 100μM
Lactate
mmol/L
5
4
3
2
1
0
*
*
*
*
a
2 days
90 days
180 days
365 days


A
RSV 0μM
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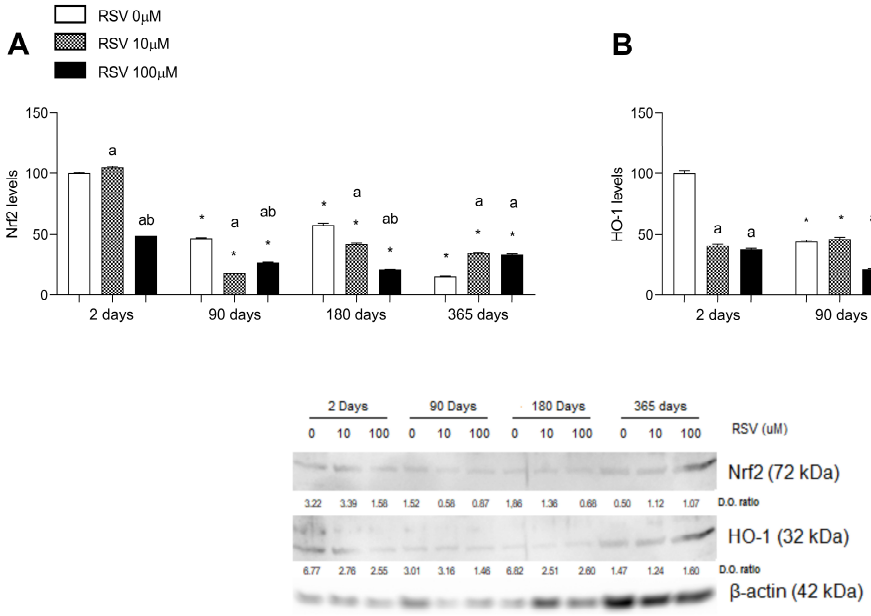
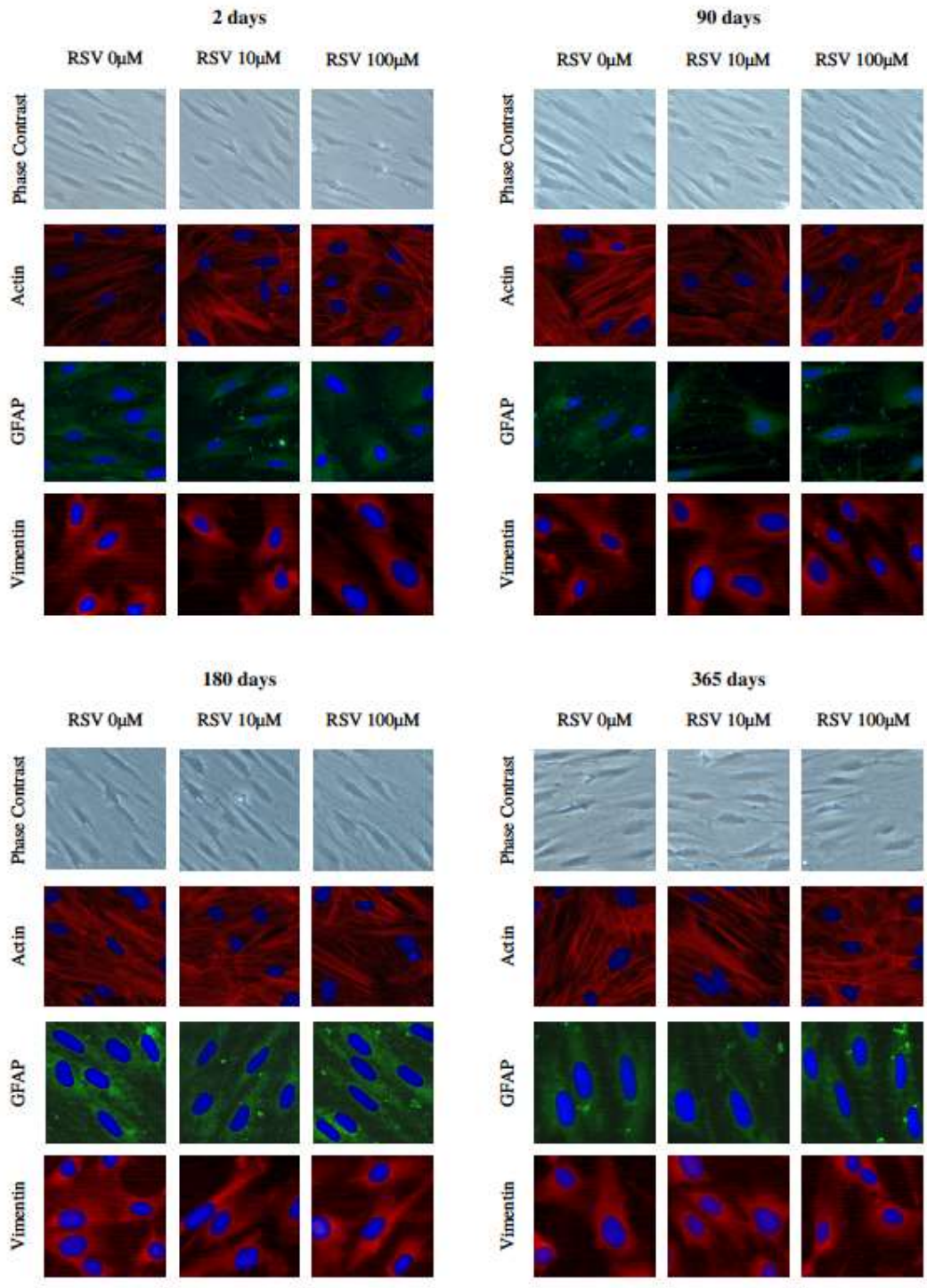
GDNF pg/ml
2 days
90 days
180 days
365 days
B
TGF-β ng/ml
C
TNF-α pg/ml
D
IL1β pg/ml
E
IL-6 pg/ml
F
IL-10 pg/ml


A
RSV 0μM
RSV 10μM
RSV 100μM
Nrf2 levels
150
100
50
0
2 days
90 days
180 days
365 days
B
HO-1 levels
150
100
50
0
2 days
90 days
180 days
365 days

2 Days
90 Days
180 Days
365 days
0 10 100 0 10 100 0 10 100 0 10 100
RSV (uM)
Nrf2 (72 kDa)
3.22 3.39 1.58 1.52 0.58 0.87 1.86 1.36 0.68 0.50 1.12 1.07
D.O. ratio
HO-1 (32 kDa)
6.77 2.76 2.55 3.01 3.16 1.46 6.82 2.51 2.60 1.47 1.24 1.60
D.O. ratio
β-actin (42 kDa)



Supplementary figure Cellular morphology of primary astrocyte cultures obtained from 2, 90, 180 and 365 days old Wistar rats. Phase contrast microscopy images of primary astrocyte cultures, actin staining, and immunofluorescence analysis for GFAP and vimentin are presented. All images are representative fields from at least three independent experiments

CAPÍTULO II

Glioprotective effects of sulforaphane in hypothalamic astrocyte cultures: focus on the aging brain

Camila Leite Santos, Fernanda Becker Weber, Adriane Belló-Klein, Larissa Daniele Bobermin, André Quincozes-Santos

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PARTE III

DISCUSSÃO

O envelhecimento é caracterizado como um declínio na integridade fisiológica dos organismos, com mudanças significativas no metabolismo, resposta inflamatória e homeostase redox, sendo considerado um importante fator de risco para várias doenças, incluindo as que acometem o SNC (Partridge et al., 2018). Neste sentido, sabe-se que o hipotálamo regula tais funções, especificamente metabólicas e inflamatórias, estando assim associado ao envelhecimento e à progressão de doenças neurodegenerativas (Sovrani et al., 2023). Além disso, evidências sugerem que a remodelação das funções dos astrócitos pode acelerar ou atenuar o processo de envelhecimento. Portanto, moléculas glioprotetoras que modulam parâmetros astrogliais, podem contribuir para prevenir e/ou evitar eventos associados ao envelhecimento, mantendo a funcionalidade hipotalâmica e, consequentemente, a homeostase do SNC. Neste contexto, esta tese avaliou importantes parâmetros funcionais em astrócitos hipotalâmicos de diferentes idades (neonatos, adultos e envelhecidos), bem como o potencial papel glioprotetor do resveratrol e do sulforafano.

Como descrito anteriormente, o hipotálamo é uma região fundamental para a manutenção da homeostasia corporal e astrócitos hipotalâmicos participam ativamente deste papel (Argente-Arizón et al., 2017; Benarroch, 2016; Perea et al., 2009; Pérez-Alvarez & Araque, 2013; Quincozes-Santos et al., 2021; Santos et al., 2018a; Santos et al., 2018b). Os astrócitos desempenham importantes funções para manutenção da homeostase do SNC, participando do metabolismo de neurotransmissores/gliotransmissores, da síntese e liberação de mediadores inflamatórios, fornecendo defesas antioxidantes, contribuindo para a formação e manutenção da barreira hematoencefálica, assim como realizando a liberação de fatores tróficos (Quincozes-Santos et al., 2021). Adicionalmente, astrócitos hipotalâmicos geram importantes respostas neuroquímicas, através de reguladores chaves do processo metabólico,

como por exemplo, via sinalização da leptina. Assim, inúmeros estudos têm demonstrado que alterações periféricas, impactam fortemente o SNC, e uma das áreas mais afetadas é o hipotálamo, por isso esta região cerebral tem ganhado significativo destaque no campo da Neurociência nos últimos anos (Yan et al., 2014). Dessa forma, devido a versatilidade das células astrogliais, particularmente na geração de respostas dinâmicas em diferentes fases da vida, tem se buscado moléculas que possam regular a funcionalidade destas células, processo denominado como glioproteção (Quincozes-Santos et al., 2021). É importante destacar que moléculas glioprotetoras podem contribuir para atenuar perdas funcionais relacionadas ao processo de envelhecimento.

Estudos demonstram que o resveratrol e o sulforafano, moléculas previamente descritas como glioprotetoras por nosso grupo de pesquisa, regulam funções astrocitárias, incluindo: defesas antioxidantes, resposta inflamatória, liberação de fatores tróficos e metabolismo do glutamato (Quincozes-Santos et al., 2021). Além disso, elas são capazes de prevenir alterações relacionadas ao envelhecimento e esses efeitos estão associados a diferentes vias de sinalização, como Nrf2/HO-1, SIRT1, PI3K/Akt, AMPK, receptores de adenosina e NFκB (Bastianetto et al., 2015; Bellaver et al., 2016; Bobermin et al., 2020; Quincozes-Santos et al., 2013). Diversos trabalhos buscam desenvolver glioterapias para o tratamento de doenças do SNC, e as células gliais parecem ter um papel crucial para a compreensão, prevenção e tratamento de algumas condições (Bernstein et al., 2015; Valori et al., 2019). Portanto, caracterizar o papel das células gliais na fisiopatologia do SNC, bem como identificar alvos glioterapêuticos, é de extrema importância para auxiliar a aplicação de futuras glioterapias (Möller & Boddeke, 2016).

O resveratrol é um composto muito estudado por sua propriedade antienvelhecimento, sendo capaz de prolongar a expectativa de vida em diversos modelos animais – tanto *in vivo* quanto *in vitro* – principalmente pela indução da autofagia, redução do estresse oxidativo e da

inflamação, e pela promoção da neuroproteção (Bhullar & Hubbard, 2015; Zhou et al., 2021). Para avaliar os efeitos do resveratrol em astrócitos hipotalâmicos ao longo de diferentes idades, utilizamos culturas primárias de ratos Wistar de 2, 90, 180 e 365 dias. É importante ressaltar que as células expostas ao veículo utilizado para a administração do resveratrol, o etanol, não diferiram daquelas mantidas em condições basais, o que está de acordo com publicações anteriores (Bobermin et al., 2012, 2022; dos Santos et al., 2006; Quincozes-Santos et al., 2013). Duas concentrações de resveratrol foram utilizadas (10 e 100 μM) e não foram observadas alterações na viabilidade celular, o que está de acordo com trabalhos anteriores (Bellaver et al., 2015; Bobermin et al., 2022; Lu et al., 2010; Sovrani et al., 2023).

Em seguida, foi analisada a morfologia – através do contraste de fase – e a marcação de actina, GFAP e vimentina – por imunocitoquímica –, na ausência e na presença de resveratrol nas quatro idades estudadas. Os astrócitos, em todas as idades e concentrações de resveratrol estudadas, apresentaram a típica morfologia poligonal a fusiforme e plana e o arranjo paralelo das fibras de actina, além da tradicional coexpressão de GFAP e vimentina observada em astrócitos em cultivo (Santos, et al., 2018a). Além disso, o resveratrol não alterou esses marcadores gliais, provavelmente devido ao tratamento agudo, por 24 horas, já que um tratamento a longo prazo foi capaz de diminuir a expressão de GFAP em astrócitos hipotalâmicos de animais envelhecidos (Sovrani et al., 2023). Astrócitos envelhecidos costumam apresentar uma expressão aumentada de GFAP, o que pode estar associado à reatividade astrocitária, e tal diminuição observada no estudo citado, sugere que o tratamento crônico com resveratrol pode exercer um efeito protetor, atenuando a ativação dos astrócitos causada pelo processo de envelhecimento. No entanto, a exposição aguda ao resveratrol não alterou os principais marcadores de citoesqueleto astrocitários.

Os astrócitos têm um papel fundamental em relação ao metabolismo do SNC, pois possuem transportadores capazes de captar a glicose dos vasos sanguíneos e são capazes de

convertê-la em lactato, que é posteriormente enviado aos neurônios e utilizado como fonte energética por estas células (Benarroch, 2014; Magistretti, 2006; Pellerin et al., 1998). Alterações no metabolismo da glicose estão associadas ao processo de envelhecimento e a doenças neurodegenerativas e, neste trabalho, observamos uma diminuição na liberação de lactato em culturas de astrócitos obtidos de animais de 180 e 365 dias. Por sua vez, o resveratrol preveniu tal diminuição em culturas de astrócitos provenientes de animais de 365 dias, indicando um potencial efeito glioprotetor em células envelhecidas. Vale destacar que o encéfalo adulto representa aproximadamente 2% do peso corporal e consome cerca de 20% da glicose no corpo. A glicose é seu principal substrato energético, e os astrócitos – que estão em contato direto com os vasos sanguíneos – possuem receptores capazes de captá-la (Benarroch, 2014). Ainda, os astrócitos possuem a enzima piruvato desidrogenase, responsável pela entrada do piruvato no ciclo de Krebs, parcialmente inibida, resultando em uma maior conversão de piruvato em lactato, que pode ser posteriormente exportado para os neurônios (Magistretti, 2006; Pellerin et al., 1998). Dessa forma, a partir dos nossos resultados podemos inferir que o resveratrol pode prevenir importantes alterações metabólicas que comprometem a manutenção energética das células neurais, bem como a regulação da excitabilidade neuronal. Adicionalmente, o lactato tem sido descrito como uma importante molécula sinalizadora no SNC devido a seu relevante papel em diferentes funções homeostáticas e adaptativas, funções fortemente afetadas durante o processo de envelhecimento (Magistretti & Allaman, 2018).

Os astrócitos também são responsáveis pela produção e liberação de fatores tróficos, tais como o GDNF e TGF-β. De acordo com nossa publicação anterior, observamos uma diminuição dependente da idade nos níveis de GDNF, um fator trófico essencial para a sobrevivência neuronal e para a plasticidade sináptica (Santos et al., 2018a). No estudo atual, verificamos que o resveratrol foi capaz de aumentar os níveis de GDNF em todas as idades e,

curiosamente, a menor concentração de resveratrol (10 μM) atuou apenas em astrócitos envelhecidos, reforçando o efeito antienvelhecimento desta molécula. Também observamos um aumento nos níveis de TGF-β ao longo das idades estudadas, sendo que o resveratrol foi capaz de impedir esse efeito apenas em astrócitos obtidos de animais de 365 dias. É importante ressaltar que o TGF-β possui uma ação dual dependendo da sua concentração, podendo ser tanto um fator trófico quanto um fator tóxico. Tal fator pode induzir à uma inflamação hipotalâmica, que é uma condição frequentemente associada a doenças neurodegenerativas, por outro lado, o TGF-β é crítico para o crescimento, diferenciação e transformação celular, devido ao seu papel no desenvolvimento neurológico e na função sináptica (Yan et al., 2014). Portanto, o efeito do resveratrol sobre este fator trófico é importante para a glioproteção/neuroproteção.

A inflamação hipotalâmica é uma característica comum do processo de envelhecimento, e os astrócitos participam da resposta inflamatória através da produção e da liberação de mediadores, tais como TNF-α, IL-1β, IL-6 e IL-10 (Jiang & Cadenas, 2014; Kim & Choe, 2019). No nosso estudo avaliamos os níveis dos mediadores citados acima, e verificamos que o envelhecimento causou um aumento nos níveis dos fatores pró-inflamatórios TNF-α e IL-1β enquanto diminuiu os níveis da citocina anti-inflamatória IL-10, um evento clássico na neuroinflamação (Santos et al., 2018a). O tratamento com ambas concentrações de resveratrol foi capaz de atenuar a liberação de mediadores pró-inflamatórios em astrócitos de animais envelhecidos, indicando um efeito anti-inflamatório dependente da idade. Assim, o resveratrol pode atenuar ou retardar esse processo inflamatório desencadeado pelo envelhecimento devido à sua propriedade anti-inflamatória, levando à uma consequente glioproteção.

As funções metabólicas, tróficas e inflamatórias, podem ser conduzidas por uma ampla gama de vias de sinalização e, particularmente relacionado ao resveratrol, o fator de

transcrição Nrf2 e a enzima citoprotetora HO-1 são as vias mais promissoras (Quincozes-Santos et al., 2021). Portanto, os níveis destas proteínas foram analisados tanto na presença quanto na ausência de resveratrol nas diferentes idades estudadas. Surpreendentemente, o resveratrol potencializou a diminuição do Nrf2 em culturas de astrócitos de 90 e 180 dias de idade, enquanto uma atividade oposta foi observada em astrócitos de 365 dias de idade.

É importante observar que o Nrf2 regula a expressão de genes que codificam mediadores relacionados aos estados redox e inflamatório, incluindo a HO-1, sendo que sua regulação positiva ou negativa é capaz de afetar a funcionalidade das células (Liddell, 2017). No SNC, os astrócitos podem ser o tipo celular predominantemente responsável pela ativação do Nrf2, e o efeito do resveratrol em astrócitos de 365 dias pode estar associado a mecanismos celulares compensatórios para evitar a gliotoxicidade induzida pelo envelhecimento. Esses dados estão de acordo com uma regulação positiva dos genes Nrf2 e HO-1 em astrócitos hipotalâmicos envelhecidos após tratamento prolongado com resveratrol (Sovrani et al., 2023). No entanto, é importante notar que astrócitos hipotalâmicos de animais neonatos e adultos podem ter habilidades diferentes para promover respostas celulares protetoras. Particularmente com relação ao resveratrol, nosso grupo relatou o envolvimento do Nrf2 e da HO-1 nos efeitos glioprotetores do tratamento com esta molécula por 24 horas em astrócitos hipocampais de animais neonatos, adultos e envelhecidos, indicando uma heterogeneidade funcional e molecular das respostas astrocitárias que é dependente da região cerebral (Bellaver et al., 2014; Bobermin et al., 2022). Portanto, devido às funções dos astrócitos hipotalâmicos, Nrf2 e HO-1 podem não ser os principais alvos do resveratrol nessas células, pelo menos em 24 horas de tratamento.

Em suma, nossos achados demonstram os efeitos glioprotetores do resveratrol em astrócitos hipotalâmicos *in vitro* de ratos Wistar de diferentes idades. O resveratrol é um polifenol anti-inflamatório, antioxidante e antienvelhecimento, mas é pouco estudado no

hipotálamo, uma região cerebral crucial que orquestra vários processos biológicos, em condições fisiológicas e patológicas. Estudos *in vivo* são fundamentais para uma melhor caracterização da atividade glioprotetora do resveratrol, mas vale destacar que este foi o primeiro estudo a avaliar os efeitos dos resveratrol nesta região cerebral, em idades representativas das diferentes fases do envelhecimento.

Neste trabalho, também avaliamos os efeitos de outro composto glioprotetor, o sulforafano, uma molécula com propriedades antioxidantes, anti-inflamatórias, antitumorais e cardioprotetoras (Angeloni et al., 2009; Quincozes-Santos et al., 2021). Recentemente, publicamos um estudo mostrando a capacidade protetora do sulforafano em células astrogliais C6, frente a insulto com lipopolissacarídeo (Bobermin et al., 2020), no entanto, o efeito antienvelhecimento do sulforafano em astrócitos ainda permanece desconhecido. Assim, verificamos que o sulforafano foi capaz de modular a expressão de vários biomarcadores ligados à inflamação, senescência celular e estresse oxidativo, em culturas de astrócitos hipotalâmicos e em suspensão de tecido, de ratos Wistar provenientes de animais de 24 meses.

Ao longo do processo de envelhecimento, marcadores de senescência, como o p21, podem ter sua expressão aumentada, uma vez que esta proteína em particular causa a interrupção da proliferação celular e acelera o processo inflamatório, promovendo efeitos deletérios para os mais diferentes tipos celulares (Papismadov et al., 2017). Devido a estas características, o p21 tem sido amplamente utilizado em trabalhos sobre envelhecimento celular, mas pouco explorado em relação ao hipotálamo. Neste trabalho, observamos que o sulforafano foi capaz de diminuir a expressão da p21, sugerindo uma abordagem potencial para o tratamento de condições relacionadas à idade, bem como reforçando o efeito antienvelhecimento deste composto.

Como já citado anteriormente, o processo de envelhecimento também é caracterizado por um aumento na resposta inflamatória. Uma gama de citocinas pró-inflamatórias é liberada através da ativação da via do NFκB e, em nosso trabalho anterior, esse fator de transcrição mostrou-se elevado de uma maneira dependente da idade (Santos et al., 2018a). Por sua vez, a regulação negativa de NFκB tem sido associada a uma melhoria das condições inflamatórias, e o sulforafano foi capaz de diminuir não só a expressão do NFκB, mas também de TNF-α, IL-1β e IL-6. Vale destacar, também, que o sulforafano reduziu o conteúdo extracelular destes medicadores inflamatórios, bem como aumentou a expressão e a liberação de IL-10, uma importante citocina relacionada a atividade anti-inflamatória. Adicionalmente, verificamos que o sulforafano diminuiu a expressão do RNAm da COX-2, enzima envolvida na síntese de mediadores inflamatórios, e que pode ser um alvo transcricional do NFκB (Poligone & Baldwin, 2001). Além disso, nossos resultados mostraram um aumento na expressão do RNAm dos receptores de adenosina A_1 e A_{2A}, conhecidos por seus efeitos anti-inflamatórios nas células gliais (Németh et al., 2005; Pasquini et al., 2021). Em relação a estes resultados, é importante destacar que evidências sugerem que insultos microinflamatórios alteram a funcionalidade hipotalâmica, resultando em desequilíbrio metabólico e progressão do envelhecimento (Cai & Khor, 2021); portanto, o efeito do sulforafano sobre a resposta inflamatória corrobora fortemente seus efeitos neuroprotetores.

Classicamente, o sulforafano é um ativador do Nrf2, um fator capaz de aumentar a expressão de enzimas citoprotetoras e de outros fatores capazes de controlar o equilíbrio redox e o processo inflamatório, como GCL, NFκB, HO-1, entre outros (Niture et al., 2014; Wakabayashi et al., 2010). A HO-1 é responsável por produzir respostas celulares contra condições estressoras e tem sido relacionada aos efeitos protetores do resveratrol (Quincozes-Santos et al., 2013; Sovrani et al., 2023). Observamos que o sulforafano aumentou a expressão do RNAm tanto do Nrf2 quanto da HO-1, enquanto p65 NFκB apresentou um

efeito oposto. Vale ressaltar que Nrf2 e HO-1 exercem um controle a montante de NFκB, modulando negativamente sua ativação. Juntos, Nrf2 e NFκB são considerados os principais fatores de transcrição para induzir respostas celulares, incluindo respostas metabólicas. Em consonância com isso, a sinalização da AMPK também está intimamente relacionada ao balanço energético, além de regular a função mitocondrial, a desintoxicação e as defesas antioxidantes, promovendo a homeostase celular e, consequentemente, o envelhecimento saudável (Petsouki et al., 2022).

Embora a ativação da AMPK tenha sido relacionada aos efeitos do sulforafano nos tecidos adiposo e hepático (Masuda et al., 2022; Zhang et al., 2022), observamos uma regulação negativa na expressão da AMPK em astrócitos hipotalâmicos. É digno de nota que há um "crosstalk" entre a sinalização do Nrf2 e da AMPK, na qual a ativação do Nrf2 pode suprimir os níveis de RNAm da AMPK (Masuda et al., 2022), o que pode explicar a regulação negativa induzida pelo sulforafano. O Nrf2 também regula a transcrição da enzima GCL, que participa da síntese da glutationa, um antioxidante crucial que protege as células do estresse oxidativo produzida principalmente pelos astrócitos no SNC (Dahal et al., 2023). O PGC-1α também pode controlar a expressão de várias enzimas envolvidas na eliminação de espécies reativas de oxigênio e, neste trabalho, o sulforafano foi capaz de regular positivamente tanto a GCL quanto o PGC-1α. Portanto, este fator de transcrição também pode representar um importante alvo molecular em relação aos efeitos glioprotetores do sulforafano. Neste sentido, é bem descrito na literatura que o PGC-1α é modulado pelo sulforafano em outros tipos celulares (Fernandes et al., 2015; Tian et al., 2022), mas este trabalho descreve pela primeira vez sua ação sobre astrócitos de animais envelhecidos. No entanto, alvos da atividade transcricional do PGC-1α, como SOD1 e SOD2, não foram alterados pelo sulforafano. A expressão da iNOS pode ser desencadeada por diferentes estímulos, incluindo inflamação, citocinas e estresse oxidativo. Nossas observações indicaram

que o sulforafano reduz a expressão do RNAm dessa enzima, que está a jusante da via Nrf2/HO-1, sendo um alvo relevante em processos patológicos associados à inflamação e desequilíbrio redox. Portanto, o sulforafano foi eficaz em nosso modelo experimental na modulação de diferentes vias de sinalização que podem prevenir alterações funcionais dependentes da idade em astrócitos. Assim, nosso trabalho está alinhado a recentes estudos sobre a ações protetoras do sulforafano (Bai et al., 2015; Benedict et al., 2012; Bobermin et al., 2020; Carrasco-Pozo et al., 2015; Fernandes et al., 2015; Tarozzi et al., 2013; Tian et al., 2022).

Além de regular a resposta inflamatória e homeostase redox em astrócitos cultivados, o sulforafano foi capaz de modular a expressão de marcadores astrogliais. Embora esta molécula não tenha alterado a expressão de marcadores astrocíticos clássicos, como GFAP e GS, modulou os níveis de RNAm da aldeído desidrogenase 1, membro L1 (ALDH1L1), do fator de transcrição *SRY-Box 10* (SOX10), da aquaporina 4 (AQP4) e do VEGF. ALDH1L1 é uma enzima altamente expressa em astrócitos e o sulforafano foi capaz de aumentar sua expressão. Essa enzima participa do metabolismo do folato, influenciando a biossíntese de nucleotídeos e, consequentemente, as respostas de neurônios e células gliais, com impactos potenciais no processo regenerativo do encéfalo (Dahal et al., 2023). Em nosso estudo, também observamos que o sulforafano aumentou a expressão de RNAm de SOX10. Este fator de transcrição está envolvido no desenvolvimento e manutenção de vários tipos celulares no sistema nervoso, incluindo astrócitos (Bhattarai et al., 2022). É importante notar que SOX10 desempenha muitas funções essenciais, incluindo a remodelação da plasticidade neural, e nossos dados reforçam os efeitos do sulforafano contra os déficits observados no envelhecimento cerebral.

O canal de água AQP4, uma proteína expressa pelos astrócitos, é um importante parâmetro funcional astrocítico (Salman et al., 2022). Além de seu papel na manutenção da

homeostase hídrica, essa proteína também tem sido associada a processos de neuroinflamação e neurodegeneração (Fukuda & Badaut, 2012; Yang et al., 2017). Trabalhos anteriores do nosso grupo mostraram que astrócitos derivados de culturas de animais adultos expressam mais AQP4 do que animais neonatos e, além disso, também demonstramos que compostos como o resveratrol são capazes de diminuir a expressão dessa proteína nessas condições (Bobermin et al., 2020; Sovrani et al., 2023). Este dado corrobora o resultado encontrado no presente estudo, uma vez que o sulforafano reduziu a expressão de AQP4, possivelmente para manter a homeostase astrocítica. Por sua vez, o VEGF desempenha um papel crucial na regulação da formação e manutenção da unidade neurovascular, promovendo a formação de novos vasos sanguíneos em resposta a lesões ou doenças, o que pode restaurar o fluxo sanguíneo para áreas danificadas do cérebro (Wuestefeld et al., 2012). Ademais, o VEGF derivado de astrócitos pode mediar a inflamação e há uma interação entre VEGF e Nrf2 (Li et al., 2016), ambos regulados positivamente pelo sulforafano.

Os fatores tróficos podem atuar por meio de receptores de superfície celular de alta afinidade como o TrkA e o TrkB, garantindo a sobrevivência e a plasticidade das células neurais e prevenindo a morte celular após lesões (Skaper, 2018). Em nosso estudo observamos que o sulforafano foi capaz de aumentar a expressão do RNAm e os níveis extracelulares de GDNF e NGF, além de aumentar a liberação de BDNF. Além disso, o sulforafano aumentou a expressão do RNAm de TrkA, receptor bastante associado a funcionalidade astrocitária (Hutton et al., 1992). Foi relatado que a diminuição de BDNF, GDNF e NGF pode estar associada à fisiopatologia da neurodegeneração, enquanto seus níveis aumentados podem proteger as células neurais contra insultos tóxicos. Adicionalmente, a expressão dos fatores tróficos pode ser modulada pela via Nrf2/HO-1, que também se mostrou aumentada pelo sulforafano (Quincozes-Santos et al., 2021).

Uma das limitações do nosso estudo é o fato de culturas de astrócitos de animais envelhecidos gerarem menor número de células, possivelmente pelo cérebro maduro possuir conexões sinápticas bem estabelecidas e menor taxa proliferativa das células em condições *in vitro* quando comparado às culturas derivadas de animais neonatos. Isto foi observado em trabalhos anteriores de padronização das culturas de células de animais adultos realizados por nosso grupo de pesquisa (Bellaver et al., 2017; Santos et al., 2018; Souza et al., 2013). Dessa forma, optamos por realizar a avaliação do RNAm de diversos genes associados a importantes funções neuroquímicas astrocitárias e caracterizamos pela primeira vez, pelo menos no nosso entendimento, o efeito glioprotetor do sulforafano sobre astrócitos provenientes de animais envelhecidos *in vitro*. No entanto, também testamos se o sulforafano exercia atividade protetora em suspensão celular proveniente de tecido hipotalâmico, uma preparação *ex vivo* contendo além dos astrócitos, outras células gliais e neuronais. Neste sentido, verificamos que o tratamento agudo com sulforafano (1 hora) também modulou diversos genes associados a resposta inflamatória, bem como fatores tróficos e de transcrição. Porém, observamos respostas distintas para TNF-α, IL-1β e IL-6, indicando que os efeitos deste composto podem estar diretamente associados aos astrócitos, o que corroboraria o seu papel glioprotetor. Os diferentes resultados podem ser relacionados a outras populações celulares e não necessariamente indicam prejuízo na sua capacidade protetiva, bem pelo contrário, aumentam a possibilidade de ação sobre diferentes vias de sinalização, de maneira célula específica. Em relação a vias de sinalização, o sulforafano não diminuiu o RNAm do NFκB, embora tenha aumentado na suspensão celular o RNAm da SIRT1. Esta sinalização é fortemente associada a efeitos antienvelhecimento, particularmente porque regula processos metabólicos, de sobrevivência celular, resposta ao estresse oxidativo e controle sobre funções de reparo ao DNA (Ng, 2015). Além disso, o sulforafano em outros modelos experimentais foi capaz de modular a atividade da SIRT (Chen et al., 2021; Li et al., 2016). Para o fator de transcrição

Nrf2 a resposta observada foi a mesma. A similaridade de resposta para o RNAm dos fatores tróficos BDNF, GDNF e NGF mostra a relevância dos astrócitos na modulação da atividade neural em condições tanto fisiológicas quanto patológicas. Interessantemente, no modelo experimental *ex vivo*, o sulforafano foi capaz de modular além do receptor TrkA, o receptor TrkB. Por fim, corroborando as ações do sulforafano sobre astrócitos, este composto não foi capaz de aumentar o RNAm da enzima ALDH1L1 na suspensão hipotalâmica, sendo que esta enzima é um dos mais bem caracterizados marcadores astrocitários (Cahoy et al., 2008). O fator de transcrição SOX10 também apresentou resultados distintos nos dois modelos experimentais, indicando mais uma vez a heterogeneidade das populações celulares observadas neste trabalho.

Nosso grupo de pesquisa tem trabalhado com a padronização e a caracterização de culturas primárias de células de diferentes regiões cerebrais, principalmente culturas de astrócitos, de animais adultos e envelhecidos (Bellaver et al., 2017; Santos et al., 2018a; Souza et al., 2013). Além disso, tais protocolos têm sido utilizados no intuito de desvendar os intricados processos relacionados ao processo de envelhecimento cerebral e, também, as culturas vêm sendo utilizadas como uma importante ferramenta para avaliar possíveis moléculas glioprotetoras e/ou gliotóxicas e seus mecanismos de ação (Bellaver et al., 2014, 2015, 2016; Bobermin et al., 2012, 2020, 2022; Santos et al., 2018b; Sovrani et al., 2023). Particularmente, com relação às culturas de astrócitos hipotalâmicos de animais adultos e envelhecidos, verificamos que tais células desta região cerebral sofreram remodelação neuroquímica ao longo das diferentes idades estudadas quanto à regulação da homeostase glutamatérgica, defesas antioxidantes, metabolismo de aminoácidos e glicose, suporte trófico, resposta inflamatória, sensibilidade à leptina e vias de sinalização (Santos et al., 2018a). Coletivamente, nossas descobertas indicaram que a capacidade dos astrócitos em manter a homeostase no hipotálamo muda com a idade e isso pode estar relacionado com o

aparecimento de distúrbios. Posteriormente, utilizamos as culturas de astrócitos hipotalâmicos já padronizadas e caracterizadas com o intuito de avaliar os efeitos da leptina sobre estas células de animais de 1-2, 90 e 180 dias (Santos et al., 2018b). Em tal estudo, nossos dados indicaram que as células quando expostas à leptina, aumentaram a produção de citocinas, o que pode levar à uma neuroinflamação e à resistência à leptina. Este evento pode ser induzido tanto pela obesidade quanto pelo envelhecimento sem qualquer aumento anterior na massa corporal, indicando que a idade por si só pode induzir alterações na sinalização da leptina e consequentemente desencadear diversas alterações metabólica. Assim, nossas observações suportam a evidência de que a leptina pode contribuir para o estado pró-inflamatório observado no hipotálamo durante essas condições. Por fim, nossos resultados reforçam o envolvimento dos astrócitos na neuroinflamação, que é uma característica do cérebro envelhecido. Recentemente, utilizamos as culturas de astrócitos hipotalâmicos para avaliar possíveis alterações nos receptores de adenosina, IGF1 e HIF1α durante o envelhecimento, o que pode impactar na funcionalidade dos astrócitos hipotalâmicos e, consequentemente, no desenvolvimento e na progressão de disfunções cerebrais metabólicas dependentes da idade (Anexo 1). Nossos achados reforçam/complementam o conhecimento de nosso relatório anterior, no qual descobrimos que astrócitos hipotalâmicos cultivados sofrem remodelação neuroquímica com a idade (Santos et al., 2018a).

Em suma, os resultados encontrados neste trabalho demonstram o efeito glioprotetor tanto do resveratrol quanto do sulforafano em astrócitos hipotalâmicos *in vitro* – e/ou em suspensão de células provenientes do tecido da mesma região cerebral – de ratos recém-nascidos a idosos, modulando a expressão de genes relacionados à inflamação, defesa antioxidante e fatores tróficos, bem como as vias de sinalização que regulam esses efeitos. Tais moléculas estudadas aqui possuem propriedades bem estabelecidas como anti-inflamatórias, antioxidantes e antienvelhecimento, mas são pouco estudadas no hipotálamo,

uma região cerebral crucial que orquestra diversos processos biológicos, em condições fisiológicas e patológicas. Além disso, culturas de astrócitos hipotalâmicos de cérebros maduros são pouco estudadas e podem representar uma ferramenta importante para a compreensão das funções gliais. Embora mais estudos sejam necessários para entender completamente os mecanismos de ação do resveratrol e do sulforafano no SNC, nossos achados reforçam o potencial glioprotetor destas moléculas em astrócitos hipotalâmicos. Tais efeitos podem induzir à uma reprogramação subjacente nestas células, o que pode representar uma intervenção terapêutica experimental promissora contra disfunções cerebrais relacionadas ao envelhecimento e contribuir para um envelhecimento cerebral mais saudável.

CONCLUSÕES

- Nosso estudo demonstra os efeitos glioprotetores do resveratrol em culturas de astrócitos hipotalâmicos de ratos Wistar de 2, 90, 180 e 365 dias;
- A morfologia dos astrócitos em cultura não foi alterada pelas diferentes idades estudadas, nem pelo tratamento com resveratrol por 24 horas;
- O resveratrol modulou a expressão proteica de Nrf2 e HO-1, diminuiu a liberação de fatores pró-inflamatórios enquanto aumentou a liberação de fatores anti-inflamatórios e, também, de fatores tróficos nas diferentes idades estudadas;
- O sulforafano apresenta efeitos glioprotetores tanto em cultura de astrócitos quanto em suspensão celular da região hipotalâmica de ratos Wistar de 24 meses, modulando negativamente a expressão de genes relacionados ao processo pró-inflamatório e modulando positivamente a expressão de genes relacionados à resposta anti-inflamatória, à defesa antioxidante e fatores tróficos; bem como as vias de sinalização que regulam esses efeitos;
- Nossos achados reforçam os efeitos glioprotetores do resveratrol e do sulforafano, que podem induzir uma remodelação em astrócitos ao longo do processo de envelhecimento, fornecendo *insights* sobre estes compostos como potenciais tratamentos futuros para o envelhecimento e/ou doenças neurodegenerativas

PERSPECTIVAS

- Utilizar as culturas primárias de astrócitos hipotalâmicos tratadas com leptina (Santos et al., 2018b) e avaliar os potenciais efeitos anti-inflamatórios do resveratrol e do sulforafano através da liberação de diferentes fatores pró e anti-inflamatórios e da expressão de genes envolvidos com a resposta inflamatória;
- Avaliar os efeitos de tratamentos agudos e crônicos do resveratrol e do sulforafano sobre a funcionalidade astrocitária – avaliando a morfologia celular, o metabolismo da glicose e do glutamato, produção e liberação de fatores tróficos e inflamatórios, entre outros - *in vivo* e *in vitro* com foco no processo de envelhecimento;
- Utilizar inibidores para diferentes etapas das vias Nrf2 e HO-1 para elucidar os mecanismos utilizados pelo resveratrol e pelo sulforafano em culturas de astrócitos hipotalâmicos de diferentes idades.

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ANEXOS

Anexo 1

Artigo submetido ao periódico Aging Brain

Aging changes the expression of adenosine receptors, insulin-like growth factor 1 (IGF1), and hypoxia-inducible factor 1α (HIF1α) in hypothalamic astrocyte cultures

Camila Leite Santos, Larissa Daniele Bobermin, André Quincozes-Santos

Anexo 2

Artigo de revisão publicado no periódico Molecular Neurobiology

Gliotoxicity and Glioprotection: the Dual Role of Glial Cells

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Gliotoxicity and Glioprotection: the Dual Role of Glial Cells

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Abstract

Glial cells (astrocytes, oligodendrocytes and microglia) are critical for the central nervous system (CNS) in both physiological and pathological conditions. With this in mind, several studies have indicated that glial cells play key roles in the development and progression of CNS diseases. In this sense, gliotoxicity can be referred as the cellular, molecular, and neurochemical changes that can mediate toxic effects or ultimately lead to impairment of the ability of glial cells to protect neurons and/or other glial cells. On the other hand, glioprotection is associated with specific responses of glial cells, by which they can protect themselves as well as neurons, resulting in an overall improvement of the CNS functioning. In addition, gliotoxic events, including metabolic stresses, inflammation, excitotoxicity, and oxidative stress, as well as their related mechanisms, are strongly associated with the pathogenesis of neurological, psychiatric and infectious diseases. However, glioprotective molecules can prevent or improve these glial dysfunctions, representing glial cells-targeting therapies. Therefore, this review will provide a brief summary of types and functions of glial cells and point out cellular and molecular mechanisms associated with gliotoxicity and glioprotection, potential glioprotective molecules and their mechanisms, as well as gliotherapy. In summary, we expect to address the relevance of gliotoxicity and glioprotection in the CNS homeostasis and diseases.

Keywords Glial cells · Gliotoxicity · Glioprotection · Glioprotective molecules

Introduction

Rudolf Virchow first described neuroglia as a connective tissue surrounding neurons in the nineteenth century [1]. Afterwards, different types of cells that constitute the neuroglia, also known as glial cells, were identified and referred as macroglia, composed by astrocytes and oligodendrocytes, and microglia. Each of these cells display several versatile functions necessary to maintain and support neuronal networks [2].

Glial cells are critical for the central nervous system (CNS) homeostasis, in both physiological and pathological conditions. In fact, it has been increasingly reported that these cells can be involved in the development and progression of neurological diseases [3]. Since each type of glial cell is able to directly affect the functionality of the others, dysfunctions in any of them can generate self-amplifying detrimental processes and synergistically impair the neuronglia communication and/or neuronal function [4, 5]. In this sense, glial cells may represent important cellular therapeutic targets for CNS disorders. With this regard, cellular, molecular, and neurochemical changes in these cells, which enable them to mediate toxic effects or ultimately lead to an impairment of their ability to protect neurons and/or other glial cells, can be referred as gliotoxicity. On the other hand glioprotection is associated with specific responses of glial cells, both in physiological and pathological conditions, by which they can protect themselves as well as neuronal cells, resulting in an overall improvement of the CNS functioning [6].

Considering that our research group has studied the role of glial cells on toxic and protective outcomes, this review will provide a brief summary of types and functions of glial cells and thus address molecular and cellular mechanisms associated with gliotoxicity and glioprotection, potential glioprotective molecules, and perspectives on gliotherapies (e.g., therapies for CNS pathologies focused on glial cells).

Types and Functions of Glial Cells

Astrocytes

Astrocytes are the most abundant glial cells and are recognized by a variety of homeostatic functions in the CNS. They have a refined cytoarchitecture with numerous stellate processes that allows their interaction with blood vessels, neurons, and other cell populations [7]. These morphological features of astrocytes make them important elements in the connection between peripheral and central systems, besides being associated with several of their functional roles, such as formation and maintenance of the blood–brain barrier (BBB); supply of oxygen and nutrients to the brain; regulation of synaptic transmission; and plasticity at the tripartite synapse [8–10]. The close contacts of astrocytes with synapses and blood vessels ensure their metabolic support to neurons through the coupling between synaptic activity and glucose utilization (neurometabolic coupling) [11].

Astrocytes participate in ionic homeostasis and metabolism of neurotransmitters, particularly of the glutamate [12]. They also regulate the diffusion and response to circulating

factors, such as peripheral hormones, metabolites, and inflammatory mediators [13–16], in addition to participating in the defense against oxidative stress and in the detoxification of harmful molecules [17]. Moreover, astrocytes act as important secretory cells, releasing a wide range of signaling molecules (trophic and growth factors, gliotransmitters, peptides, and proteins) that will impact the functions of the surrounding glial, neuronal, and endothelial cells [18]. Inflammatory and immune responses are also important functional properties of astrocytes [19]. Moreover, astrocytes can respond to abnormal events in the CNS through a morphological, physiological, metabolic, biochemical, and transcriptional remodeling, which can impair their homeostatic functions and has been collectively called as reactive astrogliosis [20].

Oligodendrocytes

The major function of oligodendrocytes is synthesis of myelin sheath, a lipid-enriched specialized and compacted structure, which is extended in spirals around the axons of many neurons [21]. In an adult CNS, oligodendrocyte progenitor cells (OPCs) persist and can continuously proliferate and differentiate into mature and myelinating oligodendrocytes [22]. Both differentiation and myelination processes require a series of sequential steps and are orchestrated by several molecular and cellular signals, including those from neurons and other glial cells [23]. The rate of myelinating oligodendrocyte generation decreases throughout life, as the loss of myelin may result in cognitive disabilities and altered sensory and motor functions [21].

Recently, oligodendrocytes have been reported to provide metabolic support by transferring energy metabolites (particularly lactate and pyruvate) to neurons through monocarboxylate transporters (MCT) and cytoplasmic channel [24]. In addition, oligodendrocytes have been shown to perform immune functions by expressing both immune-related receptors and immunomodulatory molecules, which probably display pleiotropic roles in oligodendrocytes and other glial cells [25].

Microglia

Microglia are the resident immune cells of the CNS, distributed over the entire parenchyma and playing important roles to maintain brain homeostasis [26]. In response to brain damage or infections, they are usually the first cells to be activated to perform several well-established functions, among these, pathogen recognition, inflammatory responses, and phagocytosis [27, 28].

Under physiological conditions, microglia possess a specialized morphology with a small soma containing elongated, branched, and highly dynamic processes, which allows

scanning the surrounding area and surveillance of the CNS [29, 30]. Upon CNS disorders, microglia quickly responds and can assume different activation patterns, which are usually associated with morphological changes. Microglial polarization may result in the neurotoxic M1-type or neuroprotective M2-type, based on the expression and release of cytokines, chemokines, and/or trophic factors [31, 32]. However, accumulating evidence has suggested that activation of microglia is heterogeneous and involves different but functionally overlapping phenotypes [33].

Besides their classical immune functions, microglia are implicated in several homeostatic processes, such as the release of trophic factors; promotion of neuronal survival, as well as the generation and maintenance of other neural cells; generation, maturation, regulation, and plasticity of synapses; synaptic pruning that redefines synapses and circuits; clearance of cells and debris; regulation of myelination; and memory formation and learning [26, 32].

Neurochemical Changes and Molecular Mechanisms Associated with Gliotoxicity

Considering the versatile and dynamic roles played by the different types of glial cells described in the previous section, it is not surprising that dysfunctions of these cells may be related to several pathological conditions. Interestingly, brain diseases may share many neurochemical, cellular, and molecular mechanisms related to gliotoxicity (Fig. 1), which is addressed in this section.

Metabolic Stresses

Glucose is the essential energy substrate for the CNS; therefore, pathological conditions associated with altered availability of glucose and/or oxygen (hypoxia/ischemia, hypoglycemia and hyperglycemia), as well as impairments in the metabolic machinery of

cells, can largely impact glial functioning, inducing gliotoxicity. Hypoglycemia/glucose deprivation, hyperglycemia, and/or fluctuations in glucose concentration (hyperglycemia followed glucose deprivation) have been associated with several glial dysfunctions. In astroglial cells, metabolic stress alters glutamate metabolism, mitochondrial activity/redox balance, inflammatory response, release of trophic factors, and different signaling pathways [34–36]. Glucose-related metabolic stress also affects microglia and oligodendrocytes/OPCs, promoting microglial activation and contributing to inflammatory and oxidative injuries to neurons [37, 38]. Hypoglycemia inhibits oligodendrocyte development and differentiation, in addition to trigger apoptosis in OPCs [39], while glucose-oxygen deprivation causes intracellular lactate accumulation and acidosis [40]. However, hyperglycemia increased the expression of the pre-oligodendrocyte marker O4 without affecting the expression of NG2, a marker of OPC that is downregulated during the process of cell differentiation. Although hyperglycemia can improve the differentiation rate of OPCs, the mechanisms underlying this effect and its impacts are largely unknown [41].

Another important gliotoxicity condition associated with metabolic stress is caused by ammonia. Brain detoxification of ammonia occurs mainly via glutamine synthetase (GS) [42], a specific astrocytic enzyme, but hyperammonemia can exceed the metabolic capacity of cells. Ammonia-induced gliotoxicity is associated with cellular edema, energy depletion, oxidative stress, impairment in glutamate clearance and inflammatory response [43–45]. Moreover, ammonia can upregulate the senescence marker p21, thus potentially causing a glial-inflammaging process [14]. Although astrocytes are the primary targets of ammonia toxicity, microglia may also be affected. In a co-culture model of astrocyte and microglia, ammonia decreased cellular viability and promoted microglial activation, with an increase in the percentage of phagocytic type of microglia [46]. Additionally, ammonia induced oxidative stress and up-regulated the microglial activation marker ionized calcium-binding adaptor

molecule-1 (Iba-1) in cultured microglia and in *post mortem* brain tissue from patients with hepatic encephalopathy, a neuropsychiatric syndrome associated with hyperammonemia [47]. Our group also have demonstrated that azide, an inhibitor of cytochrome c oxidase, induces gliotoxicity, promoting alterations in redox homeostasis, inflammatory response, and glutamate metabolism [6, 48].

Oxidative Stress, Inflammatory Response, and Excitotoxicity

Reactive oxygen/nitrogen species (ROS/RNS) can play neuromodulatory roles at physiological concentrations, including the regulation of neuronal polarity and neurite outgrowth, differentiation, cytoskeletal changes, synaptic plasticity, and activation of a wide range of signaling pathways and gene expression [49–51]. ROS provide a rapid and reversible mechanism for alter protein function by modulating the redox state of amino acid residues, particularly of cysteine, thus modifying the function of signaling proteins, ion channels, transporters, and transcription factors [52, 53]. On the contrary, excessive production of ROS/RNS by activated astrocytes and microglia can induce gliotoxicity, contributing to the pathomechanisms of neuropathological conditions [54, 55]. Oxidative/nitrosative stress in the brain can also be associated with unsaturated lipid enrichment, the presence of redox active transition metals, the neurotransmitter auto-oxidation and metabolism, the excessive glutamatergic signaling, as well as the increased expression and/or activity of NADPH oxidase and inducible nitric oxide synthase (iNOS) [56].

Although brain cells are equipped with enzymatic antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), the non-enzymatic antioxidant defense glutathione (GSH) plays a crucial role in maintaining brain redox homeostasis [54]. Furthermore, GSH depletion in glial cells is associated with oxidative stress, and the pathophysiology of brain disorders [57] and both this

depletion and oxidative stress are closely related to inflammatory response in glial cells [58, 59]. Under pathological conditions, increased ROS production triggers inflammatory responses that, in turn, exacerbate the pro-oxidant status [53], thus establishing a self-amplifying cycle that contribute to gliotoxicity. This interplay between oxidative stress and inflammation is importantly mediated by the nuclear factor kappa B (NFκB) signaling [53].

Among glial cells, inflammatory and immune responses are primarily associated with microglia and astrocytes [60], although oligodendrocytes can be also involved to a lesser extent [25]. A wide range of extracellular stimuli can elicit inflammatory responses in glial cells, including pathogenassociated molecular pattern (PAMPs) and damage-associated molecular pattern (DAMPs), cytokines, and other molecular insults [19, 27]. PAMPs are small-molecule motifs present in pathogenic bacteria, protozoa, and viruses; DAMPs are molecular motifs associated with cellular injury and tissue damage (e.g., misfolded and aggregated proteins, miss-localized nucleic acids and other alarmins originated from damaged cells). Both molecular motif types can be recognized by specific pattern recognition receptors, in particular Toll-like receptors (TLRs), which are expressed by glial cells and can trigger innate immune responses [19, 26]. Mainly under the control of NFκB, the master regulator of inflammation, microglia, and astrocytes becomes an important source of several inflammatory mediators, including tumor necrosis factor alpha (TNF-α), interleukins (IL-1β, IL-6, IL-18), chemokines, and prostaglandins, in addition to nitric oxide (NO) and ROS [32, 61]. This plethora of inflammatory mediators will impact the surrounding environment, importantly participating in the inflammatory activation of the other glial cells and in the recruitment of peripheral immune cells [32, 62]. Excessive and chronic inflammatory responses lead to neuronal death and are involved in several CNS disorders [63].

Glutamate is the predominant excitatory neurotransmitter in the CNS and can be neurotoxic when inappropriately remaining at high levels in the synaptic cleft, a phenomenon

referred as excitotoxicity [64]. Glial cells, particularly astrocytes, are responsible for the rapid removal of glutamate from synaptic cleft through excitatory amino acid transporters, EAAT1 (or glutamate-aspartate transporter, GLAST), and EAAT2 (or glutamate transporter 1, GLT-1) [65]. With this regard, downregulation and/or hypofunction of glutamate transporters may be associated with pathological conditions [64, 66], and their activity can be impaired as consequence of oxidative stress and/or inflammatory responses. It is well documented that glutamate transporters are highly susceptible to oxidation, which impairs their ability to take up extracellular glutamate [67]. Moreover, TNF-α has inhibitory effect on glutamate transport [68], probably associated with TNF-α-induced oxidative stress. Therefore, excitotoxicity, oxidative stress, and inflammation are processes closely related and represent important gliotoxicity mechanisms.

Gliotoxicity and CNS Diseases

Neurochemical changes and processes related to glial cells previously discussed represent, individually or collectively, crucial points in the pathogenesis of several brain diseases, including Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis (ALS), multiple sclerosis, psychiatric disorders, stroke, diabetes mellitus, hepatic encephalopathy, the aging process, and infectious diseases.

Neuropsychiatric Disorders and Aging Process

Alzheimer's disease, Parkinson's disease, and ALS are neurodegenerative diseases that present the accumulation and aggregation of proteins, such as β-amyloid and tau, α-synuclein, and TAR DNA-binding protein 43 (TDP-43), respectively, as a common hallmark [69]. The pathophysiology of psychiatric disorders including major depressive disorder, bipolar disorder, and schizophrenia pointed toward monoamine disturbances and

glutamatergic hypothesis [70]. However, the mechanisms of cognitive dysfunctions, neuronal degeneration, onset, and progression of these neuropsychiatric disorders have not yet been clearly elucidated. Interestingly, although they affect different CNS areas, producing different outcomes and symptoms, neuropsychiatric disorders share deleterious processes that may involve glial cells, including neuroinflammation, glutamate excitotoxicity, oxidative stress, and metabolic/trophic support [71–74]. Moreover, astrocyte reactivity can contribute to the pathomechanisms of the above-referred diseases. By definition, astrocyte reactivity involves morphological, molecular, and functional changes in response to pathological situations in surrounding tissue, such as CNS disease or injury, which may be reversible or chronic. When astrocytes undergo a reactive state, loss of homeostatic functions and gain of detrimental functions may occur, including some involved in glutamate and ionic homeostasis, glucose metabolism, production of inflammatory mediators and ROS/RNS, proliferation, BBB integrity, and Ca^{2+} signaling [20]. These astrocyte dysfunctions potentially lead to a transition from physiologic to pathologic roles that, without being the sole or primary initiators of pathology, may affect disease outcomes/progression [20]. With this regard, glial cells are the basis of many biomarkers of CNS diseases and, consequently, have emerged as important therapeutic targets for these pathological conditions. In addition, changes in several signaling pathways in glial cells corroborate their role in the pathomechanisms of neuropsychiatric disorders and neurodegenerative diseases [75–77].

Aging is a complex process characterized by an intrinsic physiological and functional decline of an organism. Although brain aging increases the risk of neurodegenerative diseases, it is not pathological and may be related to adaptive mechanisms of cell physiology over time [78]. Aged human brains display only mild and heterogeneous changes in astrocyte morphology or GFAP levels [79]. However, other cellular and molecular hallmarks of aging have been studied in glial cells. Aging has been associated with decreased glucose and

glycogen metabolism, as well as with decreased ATP production, decreasing astrocytic fuel provision of neurons [80–82]. Glutamate transporter downregulation and/or hypofunction has been also observed with aging [81, 83], which impair glutamate uptake and favor excitotoxicity. In addition, aged astrocytes and microglia have been shown to accumulate ROS and produce increased amounts of pro-inflammatory mediators, which may be exacerbated in response to harmful stimuli [84, 85]. Considering both senescence and inflammation in particular, the phenomenon of inflammaging has emerged as a mechanism shared by age-related diseases [86], notably involving astrocytes and microglia.

Neurological Dysfunctions Associated with Infectious Diseases

Despite the protective barriers, such as BBB, CNS can be directly and/or indirectly affected by bacterial and viral infections. In the context of bacterial infections, lipopolysaccharide (LPS), a toxin present in the outer membrane of gram-negative bacteria, has been widely used as a model of peripheral and central inflammatory responses and their related cognitive decline [87]. A large body of evidence has demonstrated that LPS peripherally administrated is able to induce inflammatory responses within CNS [88, 89]. Due to the position of astrocytes, they can serve as a bridge between systemic inflammation and neuroinflammation [15]. Although LPS classically activates microglia and astrocytes, oligodendroglial cells can also respond to this bacterial inflammogen [90]. Of note, glial cells can be major responsible for LPS-induced neuronal damage [91, 92]. Therefore, LPS has exhaustively used as an in vivo and in vitro experimental model of brain diseases, such as Alzheimer's disease and schizophrenia, among others.

With regard to viral infections, glial cells can be primary targets of neurotropic viruses, such as the human immunodeficiency virus type 1 (HIV-1) and zika virus (ZIKV).

Microglia and astrocytes constitute CNS reservoirs of HIV-1 [93, 94], promoting neuroinflammation, which can explain neuronal damage and neurocognitive disorders in a number of patients, considering the relative incapacity of HIV-1 to directly infect neurons [95]. Due to the presence of AXL receptor, astrocytes and microglia are potentially the primary cells targeted by ZIKV in the CNS [96]. In both astrocytes and microglia, ZIKV elicited classical inflammatory responses [96], while, for astrocytes, it induced oxidative stress, mitochondrial failure, and DNA damage in astrocytes [97]. Our group have demonstrated that an acute hippocampus exposure to ZIKV is also able to induce neuroinflammation and oxidative stress, affecting neuron-glia communication [98].

COVID-19 has been also recently associated with neurological dysfunctions, yet it is unclear whether they are consequence of direct CNS infection by SARS-CoV-2 or whether they result from peripheral cytokine storm and metabolic dysfunctions, although investigators have found that neurons and astrocytes are susceptible to SARS-CoV-2 infection [99, 100]. Anosmia and ageusia are common neurologic symptoms in COVID-19 patients, which can be associated with dysfunction in the olfactory bulb [101]. In addition, this brain structure can mediate direct viral invasion [102]. Interestingly, olfactory impairment is a common and early (preclinical) sign of neurodegenerative diseases, including Alzheimer's and Parkinson's diseases, in addition to be associated with depression and other neuropsychiatric disorders [103, 104]. The precise mechanisms that connect these diseases with olfactory loss are also still unclear but potentially involve neuroinflammation [103, 104]. Therefore, infectious diseases mainly target glial cells and might generate long-term consequences including cognitive deficits, neurodegenerative diseases, psychiatric disorders, and others that are currently unknown.

Mechanisms Underlying Glioprotection

Glioprotection can be achieved by endogenous homeostatic and protective functions of glial cells, which in turn may be positively modulated by a wide range of exogenous molecules, named as glioprotective molecules. They can promote protection to the CNS by improving glial functions and avoiding gliotoxicity. This section will discuss the main points associated with glioprotection (Fig. 1).

Metabolic Support

Astrocytes are recognized as energy substrate suppliers, since they are responsible for glucose uptake and its distribution to other neural cells, besides being able to store it as glycogen [9]. Moreover, astrocytes largely metabolize glucose glycolytically to produce ATP, generating lactate, which can be later transferred to neurons to be fully oxidized under conditions of high energy demands or when glucose supply is low [9, 105]. More recently, it has been demonstrated a metabolic coupling between oligodendrocytes and neurons, in which lactate derived from the glucose metabolism of these glial cells can also be transferred to the axon, contributing to ATP synthesis in neurons [106]. Of note, besides its metabolic function, signaling roles of extracellular lactate have been also recently investigated particularly in neurons, associated with neuronal excitability, synaptic plasticity, memory consolidation, and expression of trophic factors. Such signaling effects can be mediated either by the G protein-coupled receptor GPR81, extracellular acidification changes in redox state, or depolarization of target cells [107, 108]. In addition to lactate, astrocytes can also transfer healthy mitochondria to neurons, replacing damaged organelles of these cells and thus providing a protection against neuronal mitochondrial dysfunction [109].

Another important metabolic cooperation between astrocytes and neurons comprises the glutamate-glutamine cycle [11]. Once taken up by astrocytes, glutamate can be converted into glutamine by the enzyme GS, which participates both in glutamate metabolism and in

ammonia detoxification [42]. Glutamine is then exported to neurons, allowing recycling of glutamate. Since glutamate is also the precursor of gamma aminobutyric acid (GABA), the glutamateglutamine cycle is crucial for maintaining glutamate and GABA-based neurotransmission [110]. Moreover, de novo synthesis of glutamate in the brain occurs in astrocytes via the pyruvate carboxylase pathway and thus also depends on glucose [111].

Concerning the lipid metabolism, astrocytes are an important cholesterol source to mature neurons, since these glial cells express the enzymes for cholesterol synthesis and the apolipoproteins necessary to export it [112]. Moreover, although oligodendrocytes are able to synthesize cholesterol, a critical component of the myelin structure, they also depend on the supply from astrocytes [112]. In addition, there is a metabolic coupling between astrocytes and neurons regarding detoxification of neuronal-derived toxic fatty acids, which are transferred to astrocytes and metabolizedvia mitochondrial β-oxidation [113].

Trophic Support

Synthesis and release of a wide range of trophic factors by glial cells, especially by astrocytes, constitute another important mechanism of glioprotection. These trophic factors include brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), S100B, transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF- 1), nerve growth factor (NGF), and neurotrophins 3 and 4. These multifunctional molecules can mostly act on nearby neurons, regulating neuronal survival, differentiation, function, plasticity and regeneration, as well as neurogenesis [32, 114, 115]. In addition, trophic factors can also target other glial and even endothelial cells, regulating several processes, such as oligodendrocyte differentiation, survival and remyelination; microglial activation; astrocyte proliferation, function and metabolism; angiogenesis; and BBB integrity [116–118].

Modulation of Triad Oxidative Stress, Excitotoxicity, and Inflammatory Response

Glial cells display efficient antioxidant defense mechanisms that allow their functions in the defense and repair of the brain. In particular, astrocytes are able to maintain their protective roles even after surviving intense oxidative stress, thus playing a crucial role for providing antioxidant support to neurons [119]. In light of this, neuron-astrocyte interactions mediate an essential mechanism for recycling of ascorbic acid, an important neuronal antioxidant defense [120]. In addition, GSH, a tripeptide consisting of glutamate, cysteine, and glycine that is synthesized by the enzymes γ-glutamyl cysteine ligase (GCL) and GSH synthase [121], is an important antioxidant molecule able to react with free radicals or participate in enzymatic reactions, such as those catalyzed by GPx and glutathione-S-transferase (GST). While microglia and oligodendrocytes synthetize GSH for their self-protection, astrocytes are also able to readily release it [121, 122]. This is particularly important because neurons are dependent on astrocytic GSH for providing extracellular cysteine for their synthesis of GSH, since they are less capable of importing cystine [122, 123]. Extracellular GSH, in turn, can be protected from oxidation by other "guardian" molecules, including the SOD, secreted by astrocytes [124].

GSH metabolism and glutamatergic neurotransmission/homeostasis are processes closely interconnected in several ways. Besides astrocytes, microglia and oligodendrocytes also express glutamate transporters and uptake glutamate, but they are probably associated with the GSH demands of these cells [125, 126]. Thus, glial glutamate transporters can provide intracellular glutamate for GSH synthesis, as well as for Cys-Glu exchanger (system xc−) operation. This transporter, present in glial cells, plays a crucial role for GSH synthesis, since it mediates the uptake of cystine, the bioavailable form of cysteine, in exchange for glutamate [127]. Maintenance of adequate GSH levels, therefore, is important to protect

glutamate transporters from oxidation and avoid excitotoxicity. Moreover, and interestingly, it has been recently hypothesized that GSH is a relevant glutamate reservoir and could supply it for synaptic transmission when the glutamate-glutamine cycle is impaired [128].

Additionally, both microglia and astrocytes can be involved in suppression of inflammation and immune responses [129, 130]. Alternative activation patterns adopted by these glial cells are related to production and release of several anti-inflammatory molecules, such as IL-4, IL-10, IL-11, and IL-27, as well as TGF-β, that function mainly by suppressing the pro-inflammatory milieu [131, 132]. Thus, they establish a bidirectional crosstalk for a reciprocal anti-inflammatory modulation of microglia and astrocytes. To illustrate this relationship, activated M2-like microglia produce anti-inflammatory cytokine IL-10 that stimulate astrocytes to secrete TGF-β, which in turn reduces microglial pro-inflammatory activation, ultimately preserving neuronal and oligodendroglial functioning [132].

Signaling Pathways associated with Glioprotection

The nuclear factor erythroid-derived 2-like 2 (Nrf2) is a stress-responsive transcription factor that acts as a key regulator of redox, metabolic, and inflammatory homeostasis [133–135]. Upon activation, Nrf2 is translocated into the nucleus and controls the expression of genes that encode antioxidant enzymes, including SOD, GPx, and GST [133, 135]. It also stimulates the expression of proteins that contribute to GSH biosynthesis and homeostasis, such as system xc−, GCL, GSH synthase, and the NADPH-generating enzyme glucose-6-phosphate dehydrogenase [133]. Moreover, Nrf2 may directly or indirectly influence intermediary metabolism and mitochondrial function. It directly regulates the expression of important enzymatic steps of metabolic pathways related to synthesis of carbohydrates, nucleic acids, lipids, and amino acids. Indirectly, Nrf2 can affect its own expression [136] and the other transcription factors [e.g., peroxisome proliferator-activated receptor γ (PPARγ) and

retinoid X receptor α (RXRα)] [137] that in turn regulate metabolic genes, in addition to influence the activity of metabolic enzymes that are susceptible to thiol modifications [e.g., pyruvate dehydrogenase kinase 2, pyruvate kinase, AMP-activated protein kinase (AMPK)], since Nrf2-mediated expression of antioxidant genes can prevent or reverse oxidation of cysteine residues [133].

Heme oxygenase 1 (HO-1) is one of the classical genes regulated by Nrf2, which is associated with responses against oxidative challenges. This enzyme catalyzes the degradation of heme into biliverdin, bilirubin, carbon monoxide, and free iron. Products of HO-1, in particular bilirubin an CO, mediate protective effects since they have antioxidant and anti-inflammatory properties [138]. Of note, they can inhibit iNOS activity and NFκB activation [135]; thus HO-1 is an important element in the connection between Nrf2 and NFκB signaling pathways. In fact, Nrf2 signaling negatively regulates NFκB-driven inflammatory and oxidative stress responses [135]. In the context of glioprotection, although microglia and oligodendrocytes exhibit functional Nrf2/HO-1 signaling, astrocytes may be the predominant neural cell type for activation of Nrf2 [139].

Other signaling pathways that act as key regulators of cell survival, responses to stressful conditions, and metabolic effectors can mediate glioprotective effects, including sirtuin 1 (SIRT1), AMPK, phosphoinositide3-kinase (PI3K)/Akt, and protein kinase C (PKC) [35, 140, 141].

Glioprotective Molecules

A wide range of molecules has been investigated as candidates to mediate protective effects on the CNS by targeting glial cells (Table 1). Resveratrol, a polyphenol stilbene found in grapes and wine, is one of these promising molecules. Several studies have shown that resveratrol regulates diverse astroglial functions, including antioxidant defenses,

inflammatory response, trophic factor release, and glutamate homeostasis, both at basal conditions and against harmful stimuli [43, 48, 140, 142–147]. Additionally, resveratrol is able to prevent age-related functional alterations of astrocytes [148]. These effects are associated with different signaling pathways, including Nrf2/HO-1, SIRT1, PI3K/Akt, AMPK, adenosine receptors, and NFκB [48, 140, 144, 149]. Moreover, resveratrol also exhibits glioprotective effects on microglial and oligodendroglial cells [90, 145, 150] and in different in vivo experimental models [151–153].

Besides resveratrol, other naturally occurring molecules of plant origin can promote glioprotection, such as curcumin (polyphenolic compound found in the rhizome of *Curcuma longa Linn*) [154–163], isoflavones (flavonoid polyphenols present in leguminous plants) [164–174], and sulforaphane (isothiocyanate found in cruciferous vegetables) [175–179]. Endogenous mammalian compounds including lipoic acid (an essential cofactor for different mitochondrial enzymes) [141, 180–185] and guanosine (a guanine-based purine) have been also investigated as potential glioprotective agents [6, 35, 83, 186–190]. The mechanisms underlying the protective effects of these molecules in glial cells involve antioxidant and anti-inflammatory activities, improvement of mitochondrial function, Nrf2/HO-1 activation and NFκB inhibition, glutamate clearance and metabolism, regulation of microglial activation, survival of oligodendrocytes, and delay of demyelination (Table 1).

Perspectives on Gliotherapies

A wide variety of medications currently used to treat psychiatric disorders and neurodegenerative diseases are shown to have beneficial effects on glial cells, which may participate in their therapeutic effects. Antipsychotics, such as risperidone, haloperidol, clozapine and quetiapine are able to regulate inflammatory responses in astrocytes and/or microglia [191–193]. Risperidone, in particular, modulate glutamate uptake, GS activity,

GSH content, and S100B release in astroglial cells [191, 194, 195]. Major antidepressants (serotonin-specific reuptake inhibitors, tricyclic antidepressants) also demonstrate anti-inflammatory properties [196], as well as improving the release of trophic factors by glial cells [196, 197]. In addition, riluzole, the only drug approved for ALS, mainly target glutamate excitotoxicity, at least in part, by improving astroglial glutamate uptake [198], and it may also increase synthesis of trophic factors and induce Nrf2/HO-1 signaling [199].

In line with this, many studies strive to develop specific gliotherapies for treatment of neurological diseases [200, 201], demonstrating that glial cells can represent a novel basis for understanding, preventing, and treating these conditions, such as Alzheimer's disease and schizophrenia. Moreover, characterizing the role of glial cells in the pathophysiology of CNS diseases as well as identifying gliotherapeutic targets can improve future gliotherapies [202].

Concluding Remarks

The last 25 years have brought significant progress in the understanding of glial functionality, since these cells play a critical role in CNS homeostasis, as well as in pathogenesis and progression of CNS diseases. With these concept changes, it is believed that we will be able to make rapid progress in the findings, as well as in a broader and more efficient way to demonstrate that glial cells can be targets to drug development. Currently, it is well established that under oxidative and inflammatory challenges, glial cells can switch from having a protective role to a harmful phenotype. In addition, triad oxidative stress, neuroinflammation, and excitotoxicity are strongly associated with several neurological and psychiatric disorders. Considering the relevance of glial cells for physio/pathological processes, our Lab has studied these cells in different models of gliotoxicity to propose glioprotective strategies in the future, as well as to characterize the mechanisms of glioprotection. By understanding gliotoxicity, glial-based preventive/therapeutic strategies

might emerge to delay and to prevent the development of CNS diseases and their consequences.

Finally, this review represents an overview of gliotoxicity and glioprotection and was wrote by researchers from the Neurotoxicity and Glioprotection Lab of Federal University of Rio Grande do Sul, as a remote activity during COVID-19 pandemic.

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Authors' contributions

AQS and LDB conceptualized the review. All authors written the original draft of the manuscript, revised, edited, and approved the manuscript.

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Figure legends

Figure 1. Gliotoxicity and glioprotection-associated mechanisms. Gliotoxicity may be linked to several detrimental processes, including metabolic and oxidative stresses, inflammation, and excitotoxicity. On the other hand, antioxidant defenses, metabolic and trophic support, anti-inflammatory response, and glutamate homeostasis are mechanisms associated with glioprotection. Changes in several signaling pathways in glial cells may result in both gliotoxic and glioprotective effects. The cells on the left represent reactive (dysfunctional) glial cells (astrocyte is represented in blue, microglia is represented in yellow, and oligodendrocyte is represented in purple); while the cells on the right represent functional glial cells (ramified astrocyte is represented in blue, ramified microglia is represented in yellow, and oligodendrocyte is represented in blue)

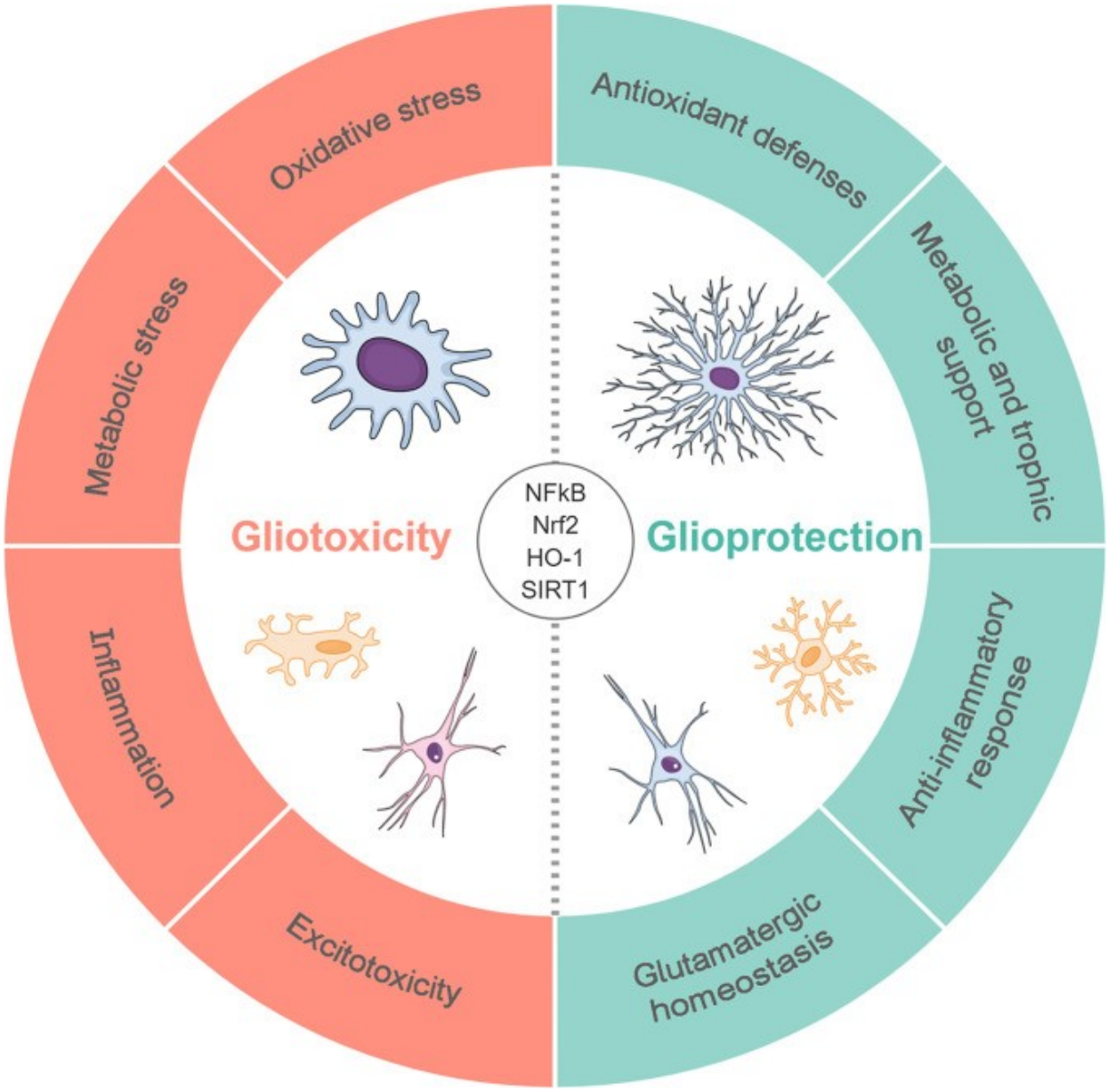
Oxidative stress
Antioxidant defenses
Metabolic stress
Metabolic and trophic support
Gliotoxicity
NFkB
Nrf2
HO-1
SIRT1
Glioprotection
Inflammation
Anti-inflammatory response
Excitotoxicity
Glutamatergic homeostasis

Table 1. Potential glioprotective molecules and their effects.

Molecule	Glioprotective functions	References
Curcumin	Improvement of mitochondrial functioning in astrocytes	[142, 143]
	Antioxidant and anti-inflammatory effects through Nrf2 and HO-1 expressions in microglial cells	[141, 144]
	Anti-inflammatory effects in microglia through downregulation of TLR4, NFκB, p38 MAPK, and PI3K/Akt signaling pathways	[145, 146]
	Reduction of NO and PGE2 production by inhibiting iNOS and COX-2 expression in microglial cells	[147]
	Inhibition of cytoskeletal disruption in astroglial cells	[143]
	Reduction of apoptosis, glial activation, and glial scar formation	[148, 149]
	Promotion of OPC differentiation	[140]
Guanosine	Regulation of astroglial oxidative and inflammatory responses through HO-1	[6, 175]
	Modulation of glutamatergic parameters and oxidative/nitrosative damages in astroglial cells with involvement of adenosine receptors, PI3K, MEK, and PKC pathways	[35, 176]
	Antiapoptotic effect in astrocytes through PI3K/Akt pathway	[177]
	Anti-aging effects in astrocytes in an HO-1 dependent manner	[172]
	Prevention of oxidative stress and excitotoxicity in focal ischemia	[173]
	Promotion of myelinogenesis and remyelination	[174]
Isoflavones	Anti-inflammatory effects in astrocytes through suppression of NFκB and increase of PPARγ expression	[150, 151]
	Induction of synthesis and secretion of neurotrophic	[154]

	factors in astrocytes	
	Anti-inflammatory and immunomodulatory properties in microglia by inhibiting TLR4/NFκB signaling and expression of COX-2, iNOS, TNF-α, IL-1β and IL-6	[152, 155, 156, 157]
	Regulation of glutamate uptake in rat brain	[158]
	Increased glial cell migration	[159]
	Prevention of oxidative stress and decreased monoamine oxidase enzyme activity in brain tissue	[160]
	Alleviation of demyelination in mouse hippocampus	[153]
Lipoic acid	Antioxidant and anti-inflammatory effects in astroglial cells through HO-1	[127, 166, 168]
	Regulation of glutamate uptake, glutamate transporter expression, GS activity, and GSH content in astroglial cells	[127, 166, 169]
	Reduction of hyperammonemia-induced damage by regulating ERK and HO-1 pathways	[127, 168]
	Prevention of inflammation and dysfunction caused by TLR3 and PKR in viral pathologies in glial cells	[169]
	Inhibition of GSK-3β with anti-inflammatory effects in microglial cells	[170]
	Induction of M2 phenotype in microglia, reduction of ROS and NFκB signaling, improved cell survival, autophagy, and inhibition of apoptosis	[171]
	Prevention of demyelination via oligodendrocyte survival and promotion of regenerative mechanisms	[167]
Resveratrol	Improved glutamate uptake, GS activity, S100B secretion, and GSH system in astroglial cells	[56, 128]
	Antioxidant, anti-inflammatory, and genoprotective effects in astroglial cells	[48, 130, 133]
	Prevention of ammonia toxicity in astroglial cells by modulating glutamate metabolism, redox status, and inflammatory response	[43, 44, 45]
	Anti-inflammatory effects in astrocytes and microglia	[126, 129, 131]

	through NFκB, HO-1, adenosine receptors, ERK, and p38 MAPK	
	Enhancement of astroglia-derived trophic factor release	[126, 132]
	Increased antioxidant defenses and decreased pro-inflammatory cytokines in astrocytes during aging	[134]
	Regulation of microglia M1/M2 polarization via PGC-1α	[136]
	Modulation of inflammation, oxidative stress, and release of trophic factors in OPC through Nrf2/HO-1 pathway.	[84]
Sulforaphane	Modulation of inflammatory response, antioxidant defenses, glutamatergic system, and trophic factor release in astroglial cells challenged with LPS	[161]
	Prevention of oxidative stress associated with oxygen and glucose deprivation by Nrf2 induction	[162]
	Anti-inflammatory effect through inhibition of JNK/AP-1/NFκB and activation of Nrf2/HO-1 in activated microglia	[164, 165]
	Activation of microglial processes via Akt signaling	[163]

Abbreviations: AP-1, activator protein-1; COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinases; GS, glutamine synthetase; GSH, glutathione; GSK-3β, glycogen synthase kinase-3 beta; HO-1, heme-oxigenase 1; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinases; LPS, lipopolysaccharide; MEK, mitogen-activated protein kinase kinase; NFκB, nuclear factor kappa B; NO, nitric oxide; Nrf2, nuclear factor erythroid-derived 2-like 2; OPC, oligodendrocyte precursor cells; p38 MAPK, p38 mitogen-activated protein kinases; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PGE2, Prostaglandin E2; PI3K, phosphoinositide3-kinase; PKC, protein kinase C; PKR, protein kinase R; PPARγ, peroxisome proliferator-activated receptor gamma; ROS, reactive oxygen species; TLR3, toll-like receptor 3; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor alpha.