UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:

BIOQUÍMICA

A CULTURA DE ASTRÓCITOS ADULTOS COMO FERRAMENTA DE ESTUDOS PARA COMPREENSÃO DA FUNCIONALIDADE CEREBRAL

Débora Guerini de Souza

Orientador: André Quincozes dos Santos

Co-orientador: Diogo Onofre Gomes Souza

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Dedico esta tese a todos os professores que já tive.

“Le véritablevoyage de découverte ne consiste pas à chercher de nouveaux paysages, mais à avoir de nouveaux yeux”

“A verdadeira viagem de descoberta não consiste em procurar novas paisagens, mas em ter novos olhos”

Marcel Proust

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APRESENTAÇÃO

Esta tese apresenta os seguintes tópicos: Introdução, Objetivos, Capítulos, Discussão, Conclusões, Perspectivas e Bibliografia.

A Introdução apresenta o embasamento teórico que nos levou a formular as propostas apresentadas nos artigos. O Objetivo Geral e os Objetivos Específicos são apresentados em seguida. Os resultados desta tese são apresentados na forma de Capítulos, os quais contêm os artigos científicos que foram elaborados de maneira a contemplar os objetivos propostos. Os experimentos foram desenvolvidos no Departamento de Bioquímica – ICBS – UFRGS.

Na Discussão desta tese apresentamos uma interpretação geral dos resultados obtidos nos diferentes trabalhos. Os tópicos Conclusões e Perspectivas abordam objetivamente as conclusões desta tese, bem como possibilidades de futuros experimentos a partir dos resultados descritos, respectivamente.

No tópico Referências consta a lista de trabalhos científicos citados na Introdução e Discussão, sendo a lista de referencias citadas em cada artigo apresentadas no final de cada capítulo.

SUMÁRIO

PARTE I

RESUMO

Astrócitos são células gliais com fundamental importância no sistema nervoso central (SNC), tanto em condições fisiológicas quanto patológicas. Estas células são essenciais na plasticidade neural, no metabolismo de neurotransmissores, na defesa antioxidante, na regulação do metabolismo energético, na homeostase iônica, na resposta inflamatória, na manutenção da barreira sangue-cérebro, na migração neuronal e na estabilização da comunicação entre as células. Assim, alterações em funções astrocitárias (como as que ocorrem no envelhecimento) estão relacionadas a importantes alterações na funcionalidade cerebral. Desta forma, esta tese teve por objetivo demonstrar que a cultura de astrócitos derivada de ratos Wistar adultos, desenvolvida e caracterizada pelo nosso grupo de pesquisa, pode ser um modelo de estudo fidedigno e versátil das propriedades celulares astrocitárias. Nossos resultados apontam que esta metodologia pode ser utilizada para elucidar o perfil de aminoácidos e gliotransmissores assim como da atividade enzimática glial e gerenciamento de neurotransmissores. Também demonstramos que a cultura adulta não é derivada de progenitores neurais e que parâmetros mitocondriais observados no cérebro adulto foram reproduzidos *in vitro*. A análise de respostas a estímulos demonstrou ser variável, dependendo da idade dos animais. Da mesma forma, o uso de culturas de diferentes idades revelou o efeito antienvelhecimento da guanosina, sugerindo sua atividade glioprotetora. Finalmente, demonstramos que culturas preparadas a partir de animais neonatos submetidas a um modelo de senescência *in vitro* apresentam respostas diferentes das apresentadas por culturas preparadas a partir de animais adultos e/ou envelhecidos, demonstrando que o modelo mais adequado para elucidar propriedades astrocitárias do cérebro maduro é o derivado de animais adultos. Portanto, demonstramos com este estudo a importância da disponibilidade de uma ferramenta como a cultura de astrócitos adultos e elucidamos características bioquímicas, celulares e moleculares desta ferramenta, evidenciando algumas de suas diferenças em comparação à cultura preparada a partir de ratos neonatos. Assim, ampliamos a compreensão das propriedades e funções celulares desta ferramenta, fornecendo respostas mais aproximadas às respostas fornecidas por astrócitos do cérebro maduro *in vivo*, especialmente no estudo do envelhecimento e das doenças neurodegenerativas.

Palavras-chave: astrócitos adultos, funcionalidade glial, glioproteção, neurotoxicidade, guanosina.

ABSTRACT

Astrocytes are glial cells of pivotal importance in the central nervous system (CNS), both in physiological and pathological conditions. Some of their roles include neural plasticity, neurotransmitter metabolism, antioxidant defenses, control of energy metabolism, ionic homeostasis, inflammatory response, formation and maintenance of blood-brain barrier, neuronal migration and cellular communication. Thus, changes in astrocytic function (such as occurs in aging) are related to changes in brain function. The aim of this thesis was to demonstrate that astrocyte cultures from adult Wistar rats (developed and characterized by our research group) might be a reliable and versatile tool for studying astrocytic cellular properties. Our results suggest that this culture model is suitable to study the amino acids content and gliotransmitters, as well as glial enzymatic activity and neurotransmitter management. Next, we showed that the astrocyte cultures are not derived from neural progenitors and tissue mitochondrial parameters were reproduced in *in vitro* cultures. Responses to stimulus were variable, depending on the animals' age. Accordingly, guanosine presented an anti-aging effect, indicating its glioprotective activity. Finally, we showed that cultures prepared from newborn rats submitted to an *in vitro* senescence model presented different responses when compared with mature animals, indicating that our culture model is the most suitable model to represent astrocytic properties in the mature brain. Therefore, this study demonstrates the relevance of this tool to understanding the biochemical, cellular and molecular properties of adult astrocytes, showing some differences related to the culture prepared from newborn animals. Thus, we amplify the comprehension about cellular functions of this tool, providing closer responses related to mature brain *in vivo*, especially regarding studies about aging and neurodegenerative diseases.

Key-words: adult astrocytes, glial functionality, glioprotection, neurotoxicity, guanosine.

LISTA DE ABREVIATURAS

Ach	Acetilcolina
AchE	Acetilcolinesterase
AD	Adulto
AG	Envelhecido (do inglês “aged”)
AIV	Envelhecido *in vitro* (do inglês “aged *in vitro*”)
ATP	Adenosina trifosfato
BSC	Barreira sangue-cérebro
CAT	Catalase
CLAE	Cromatografia Líquida de Alta Eficiência
D-Asp	D-Aspartato
ERO/ERN	Espécies reativas de oxigênio/nitrogênio
GABA	Ácido γ-aminobutírico
GAT1	Transportador de GABA 1 (do inglês “GABA transporter 1”)
GDNF	Fator neurotrófico derivado da glia (do inglês “Glial derived neurotrophic fator”)
GFAP	Proteína glial fibrilar ácida (do inglês “Glial fibrillary acidic protein”)
GLAST	Transportador glutamato-aspartato (do inglês “Glutamate-aspartate transporter”)
GLT1	Transportador de glutamato tipo 1 (do inglês “Glutamate transporter 1”)
GLUT1	Transportador de glicose 1 (do inglês “Glucose transporter 1”)
GPx	Glutationa peroxidase
GS	Glutamina sintetase
GSH	Glutationa
GSSG	Glutationa oxidada
HO1	Heme oxigenase 1
IGF	Fator de crescimento semelhante à insulina (do inglês “Insulin-like growth fator”)
IL1β	Interleucina 1β
iNOS	Óxido nítrico sintase induzível (do inglês “inducible Nitric oxide synthase”)

kDa	Kilodalton
LPS	Lipopolissacarídeo
MAPK	Proteínas cinases ativadas por mitógenos (do inglês “Mitogen-activated protein kinase”)
MCT	Transportador de monocarboxilatos (do inglês “Monocarboxilate transporter”)
Na^+/K^+-ATPase	Enzima sódio/potássio ATPase
NADPH	Nicotinamida adenina dinucleotídeo fosfato
NB	Neonato (do inglês “Newborn”)
NFκB	Fator nuclear κB (do inglês “Nuclear factor κ-light-chain-enhancer of activated B cells”)
NO	Óxido nítrico
Nrf2	Fator nuclear eritróide 2 (do inglês “Nuclear factor erythroid-2 related factor-2”)
PCRq	PCR quantitativo
RNAm	RNA mensageiro
SNC	Sistema nervoso central
SOD	Superóxido dismutase
TNFα	Fator de necrose tumoral α (do inglês “Tumor necrosis factor α”)
VEGF	Fator de crescimento do endotélio vascular (do inglês “Vascular endothelial growth factor”)

INTRODUÇÃO

1. Sistema nervoso central

O sistema nervoso central (SNC) é composto pelo encéfalo e medula espinhal. O encéfalo é formado por cerebelo, tronco encefálico e cérebro, sendo este último, a maior e mais complexa porção do encéfalo. O córtex, a porção cerebral mais externa, controla atividades como comportamento, planejamento, fala, movimento, visão, audição, consciência e dor (Skeide & Friederici 2016, Plakke & Romanski 2015, Ji & Neugebauer 2012). No córtex cerebral de roedores adultos estão presentes vários tipos celulares distintos (Zeisel et al. 2015), sendo um destes tipos o objeto de estudo desta tese, que teve como enfoque astrócitos de ratos Wistar adultos.

Entre os principais tipos celulares constituintes do córtex estão os neurônios, responsáveis pela transmissão, armazenamento e processamento de informações através de comunicações sinápticas, que ocorrem devido à propagação de sinais elétricos e químicos (Brady et al, 2012; Purves, 2008). Nenhuma das diversas atividades neuronais ocorreria de maneira eficiente se estas células não interagissem intimamente com as células da glia, pois, de maneira cooperativa, os tipos celulares presentes no SNC garantem a adequada funcionalidade e a homeostase cerebral (Barres, 2008).

A glia, maior grupo celular do SNC, foi inicialmente descrita como uma espécie de “cola” que conectava os neurônios entre si e ao tecido conjuntivo (Parpura et al. 2012). À medida que as técnicas de estudo do SNC foram se tornando mais aprofundadas e precisas, foram sendo demonstradas as inúmeras funções deste grupo celular. As células gliais compreendem a

microglia, células fagocíticas envolvidas na resposta inflamatória; os oligodendrócitos, responsáveis pela síntese da bainha de mielina; as células ependimais, que revestem os ventrículos cerebrais e os astrócitos, maior subgrupo glial, que terão suas propriedades detalhadas a seguir (Young 1991, Vallejo et al. 2010).

Existem em torno de três neurônios para cada célula glial no córtex cerebral de roedores (Schildge et al. 2013, Nedergaard et al. 2003) e esta relação tende a ficar mais equivalente e até ser invertida em cérebros mais desenvolvidos, como, por exemplo, no cérebro humano (Herculano-Houzel 2014). A relação direta entre o aumento do número de células gliais e o aumento da complexidade cerebral dá indícios da importância deste grupo celular no refinamento motor, cognitivo e intelectual de uma determinada espécie (Herculano-Houzel 2014, Barres 2008).

1.1. Astrócitos

Os astrócitos são células extremamente dinâmicas e versáteis, e estão envolvidos em praticamente todos os aspectos da funcionalidade cerebral (Allen & Barres 2009, Hertz & Zielke 2004, De Pitta et al. 2015, Bacci et al. 1999). Eles são divididos em dois tipos: os protoplasmáticos, na substância cinzenta (frequentemente ramificados e com largas expansões) e os fibrosos, na substância branca (com menos ramificações, cilíndricas e longas) (Young 1991). Para estudos mais específicos destas células através do uso de culturas primárias, são geralmente utilizados astrócitos da substância cinzenta (Lange et al, 2012).

Entre as principais funções astrocitárias podemos citar: a) síntese e secreção de gliotransmissores e de fatores tróficos, que modulam a atividade neuronal e das outras células vicinais (Araque et al. 2014); b) controle extracelular de neurotransmissores, especialmente na sinalização exercida por glutamato, ácido γ-aminobutírico (GABA) e acetilcolina (Danbolt 2001, Schousboe et al. 2013); c) controle da disponibilidade de substratos energéticos, pela possibilidade de produzir e estocar glicogênio, pelo seu fenótipo altamente glicolítico e pelo seu papel único no ciclo glutamato-glutamina (Wang & Bordey 2008, McKenna 2007, Pellerin 2005); d) defesa contra estresse oxidativo, especialmente considerando a produção e exportação de glutationa (GSH), uma das principais defesas antioxidantes não-enzimáticas do SNC (Dringen 2000); e) produção e liberação de citocinas, que sinalizam e amplificam a resposta inflamatória (Wang & Bordey 2008); f) envolvimento na homeostase iônica, especialmente em relação aos íons potássio e controle do volume extracelular (Wang & Bordey 2008); g) formação e manutenção da barreira sangue-cérebro (BSC) e suporte estrutural de neurônios através do citoesqueleto, especialmente pela expressão da proteína glial fibrilar ácida (GFAP) (Middeldorp & Hol 2011). Alguns destes tópicos serão explorados em detalhes nas sessões seguintes.

Todas as funções supracitadas – dentre outras exercidas por astrócitos – devem ser executadas com precisão e eficiência para garantir a funcionalidade do SNC. Entretanto, apesar da homeostase cerebral ser altamente controlada, sabe-se que muitas funções celulares são modificadas ou perdidas ao longo do tempo, o que pode ser um dos fatores iniciais no desenvolvimento e/ou progressão de quadros patológicos (Lee et al, 2000).

Desta maneira, para compreender a complexidade cerebral ao longo do desenvolvimento e do envelhecimento é necessário compreender as funções astrocitárias e como elas se modificam em diferentes momentos da vida. Uma maneira de estudar funções e propriedades celulares é analisar respostas bioquímicas, celulares e moleculares de maneira isolada, através de culturas de células *in vitro*. Desde que astrócitos começaram a ser estudados desta forma, muitas de suas funções, características e propriedades foram esclarecidas (Hertz et al. 1998, Lange et al. 2012).

1.1.1. Culturas primárias de astrócitos

Existem muitas vantagens em estudar uma população celular a partir do ponto de vista da cultura primária. A ausência de outros tipos celulares permite o estudo de funções astrocitárias detalhadamente, excluindo-se possíveis interferências. O cultivo desenvolve-se em condições controladas e a obtenção de respostas alvo-específicas é facilitada pela ausência de respostas de outros tipos celulares. Entretanto, as condições de cultivo podem afetar a expressão gênica e o funcionamento de receptores, influenciando a interpretação dos dados obtidos (Lange et al. 2012).

Os primeiros estudos utilizando esta ferramenta datam da década de 1970 e, desde então, diversos protocolos vêm sendo aplicados por grupos de pesquisa ao redor do mundo para obter o cultivo da maneira mais adequada para seu propósito de estudo. Cabe ressaltar que estudos com cultivo astrocitário primário derivado de cérebros de roedores em período perinatal são muito mais comuns e abundantes na literatura do que estudos com cultivos derivados de animais adultos (Lange et al. 2012).

O tecido cerebral do animal neonato encontra-se num cenário de menor organização tecidual, visto que a conectividade intra e intergrupos celulares está recém sendo estabelecida ou ainda nem ocorreu (Herculano-Houzel 2014). Este cenário possui diversos fatores de crescimento e de diferenciação para que o tecido obtenha sua especialização e complexidade necessárias ao bom funcionamento, ou seja, as células tendem a ter uma maior plasticidade visto que precisam se adaptar aos estímulos e estabelecer as conexões entre elas (Bower, 1990).

Por outro lado, o tecido adulto apresenta alta interatividade entre as células (especialmente conexões sinápticas), e relações, papéis e funções celulares bem designadas. Assim, células provenientes do tecido maduro tendem a ter um maior grau de diferenciação do que as provenientes do tecido neonato (Herculano-Houzel 2014, Souza et al. 2013). É importante considerar todas estas características para entender como a cultura proveniente de animais neonatais pode ser tão diferente da cultura proveniente de animais adultos e, desta forma, optar pelo cultivo que permitirá chegar à conclusão mais representativa do que ocorre *in vivo*.

O cultivo celular derivado do cérebro maduro – adulto ou envelhecido – é uma ferramenta que pode fornecer respostas mais realistas e confiáveis dependendo do tema de estudo, por se tratar de células provenientes do tecido que está mais propício a sofrer alterações associadas ao envelhecimento e vir a desenvolver alguma patologia (Souza et al. 2013). Conforme demonstrado pelo nosso grupo de pesquisa, a cultura de astrócitos de animais adultos é uma ferramenta adequada e robusta para o estudo das

propriedades e funções astrocitárias em diferentes condições experimentais (Bellaver et al. 2016, Souza et al. 2013, Bellaver et al. 2014).

1.1.2. Marcadores astrocitários

1.1.2.1. GFAP

A GFAP é uma proteína constituinte do citoesqueleto de astrócitos, da classe dos filamentos intermediários do tipo III. Assim como outras proteínas de filamentos intermediários, os monômeros de GFAP se convertem em filamentos pela agregação em dímeros paralelos, a seguir pela agregação antiparalela em tetrâmeros, octâmeros e assim por diante (Yang & Wang 2015). A GFAP é a principal constituinte do citoesqueleto astrocitário, responsável pela estrutura celular, manutenção da força mecânica, suporte estrutural a neurônios e à BSC (Eng et al. 2000).

Esta proteína, de peso molecular aparente de 50 kDa em roedores, é amplamente utilizada como marcador astrocítico e pode indicar tanto o estado basal quanto o estado reativo dos astrócitos, sendo de grande importância no processo de astrogliose (ou gliose) reativa, condição caracterizada por hipertrofia e proliferação celular (Sofroniew 2009). Em condições de injúria cerebral, como trauma crânio-encefálico, é aceito que um baixo nível de gliose reativa pode ser benéfico para o processo de recuperação, enquanto que gliose excessiva, e as respostas inflamatórias associadas à ela, terão um impacto negativo na estrutura cerebral e na recuperação funcional. Este segundo cenário é o que é observado, por exemplo, nas Doenças de Parkinson e de Alzheimer (Czlonkowska & Kurkowska-Jastrzebska 2011). Evidências clínicas vêm sugerindo que a

GFAP é um promissor biomarcador de dano no SNC, o que indica que propriedades astrocitárias podem contribuir para o diagnóstico e o prognóstico de doenças cerebrais (Yang & Wang 2015).

1.1.2.2. Vimentina

Apesar de a GFAP ser a proteína de citoesqueleto mais característica de astrócitos, cabe ressaltar a importância da vimentina, um filamento intermediário fundamental no citoesqueleto de astrócitos no início de seu desenvolvimento (Bignami & Dahl 1989, Eliasson et al, 1999). A vimentina é incapaz de formar filamentos isoladamente, ao contrário da GFAP, necessitando estar associada obrigatoriamente à outra proteína de citoesqueleto. Durante o processo de diferenciação celular, a vimentina vai deixando de ser a principal proteína astrocitária para dar lugar à GFAP como filamento intermediário astrocitário mais importante (Eliasson et al, 1999, Bignami & Dahl 1989, Dahl et al. 1981); entretanto, ela segue sendo expressa e pode estar presente em quantidades significativas no cérebro envelhecido (Bignami & Dahl, 1989).

Em cérebros adultos, os papéis exatos da GFAP e vimentina continuam sendo estudados, mas, aparentemente, estas proteínas parecem ser necessárias para a polimerização das outras proteínas de citoesqueleto, sendo, portanto, decisivas na manutenção da forma, citoarquitetura do SNC e estabilidade mecânica (Hol & Pekny 2015). Em caso de injúria ou no curso de doenças neurodegenerativas, o aumento na expressão de GFAP e de vimentina é decisivo para a astrogliose reativa, contribuindo para a cicatriz

glial, local em que a proliferação e hipertrofia astrocitárias são mais severas (Sofroniew 2009).

1.2. Envelhecimento cerebral

O envelhecimento é um processo natural capaz de induzir diversas mudanças na funcionalidade cerebral, como alterações na eficiência sináptica e na comunicação neurônio-glia, com consequente prejuízo nas atividades dos dois tipos celulares (Jiang & Cadenas 2014). Classicamente, espécies reativas de oxigênio/nitrogênio (ERO/ERN) e mediadores pró-inflamatórios estão aumentados no envelhecimento cerebral (Frank et al. 2006, Halliwell 2006a). Considerando que a expectativa de vida da população mundial vem aumentando nas últimas décadas, compreender e controlar estas alterações pode ser uma importante estratégia para aumentar a qualidade de vida e talvez até aumentar a expectativa de vida, pela redução da morbimortalidade relacionada às doenças neurodegenerativas (Jiang & Cadenas, 2014).

Por isso, é de suma importância o uso de ferramentas que permitam o estudo do cérebro envelhecido, pois é nesta etapa da vida que a maioria das doenças neurodegenerativas – como Doença de Alzheimer, de Parkinson, de Huntington, esclerose múltipla, esclerose lateral amiotrófica, acidente vascular encefálico (especialmente relacionados à alterações metabólicas) e depressão grave – ocorrem. Considerando que os astrócitos apresentam inúmeras funções essenciais para o funcionamento cerebral, é compreensível que a perda de funcionalidade nestas células devido ao envelhecimento leve à disfunções severas na homeostase cerebral (Lee et al, 2010, Parpura et al, 2012). Algumas das principais funções astrocitárias são discutidas a seguir.

2. Funções astrocitárias

2.1. Síntese e secreção de gliotransmissores e de fatores tróficos

Os astrócitos são capazes de monitorar a comunicação neuronal, o que é evidente pela expressão de diversos receptores de neurotransmissores, transportadores e enzimas em suas membranas (Belanger & Magistretti 2009). Em resposta aos estímulos recebidos do meio, eles produzem e liberam gliotransmissores, como, por exemplo, o glutamato, a D-serina e o trifosfato de adenosina (ATP), que interagem com receptores pré- e pós-sinápticos na sinapse tripartite (Harada et al. 2016, Araque et al. 2014). Eles também liberam fatores vasoativos que modulam o fluxo sanguíneo em momentos de alteração da demanda energética (Takano et al. 2006). Além disso, astrócitos secretam moléculas capazes de controlar a maturação, sobrevivência e diferenciação celulares, entre os quais podemos citar o Fator Neurotrófico Derivado da Glia (GDNF); o Fator de Crescimento do Endotélio Vascular (VEGF); o Fator de Crescimento Semelhante à Insulina (IGF) e a proteína S100B (Donato 2001).

2.2. Regulação dos níveis de neurotransmissores

2.2.1. Glutamato

O glutamato é uma molécula ímpar na complexidade cerebral (Danbolt 2001). Este aminoácido não-essencial está envolvido na maior parte das funções cerebrais, sendo o mais importante neurotransmissor excitatório do SNC (Danbolt 2001, Anderson & Swanson 2000); substrato para síntese de proteínas, da GSH e da glutamina (Dringen 2000, McKenna 2007); podendo

também atuar como substrato energético (Swanson et al. 1990) ou como gliotransmissor na sinalização astrocitária (Harada et al. 2016). Ele está envolvido em diversos processos cerebrais como aprendizado, memória, cognição e comportamento (Danbolt 2001).

Como neurotransmissor, o glutamato ativa receptores ionotrópicos e metabotrópicos durante a sinapse excitatória, sendo seu efeito modulado e/ou terminado pelos transportadores de alta afinidade presentes predominantemente nos astrócitos (Anderson & Swanson 2000, Quincozes-Santos & Gottfried 2011). Além da captação do glutamato na fenda sináptica, os transportadores também são capazes de modificar a extensão, padrão e tempo de ativação dos receptores sinápticos e extra-sinápticos. Os principais transportadores de glutamato do cérebro adulto de roedores são o Transportador de Glutamato-Aspartato (GLAST) e o Transportador de Glutamato tipo 1 (GLT1) (Benarroch 2010, Halassa & Haydon 2010), que utilizam energia do gradiente eletroquímico para realizar a transferência do neurotransmissor do meio extracelular para o meio intracelular, sendo que, com cada glutamato transportado, ocorre o co-transporte de três íons sódio (Na^+) e um íon hidrogênio (H^+), em troca de um íon potássio (K^+) (Danbolt 2001). Neste sentido, é importante considerar que o transporte do glutamato é fundamentalmente dependente do correto funcionamento da enzima Na^+/K^+-ATPase que retira os íons sódio do meio intracelular, mantendo o gradiente eletroquímico favorável (Benarroch 2010). De uma maneira geral, GLT1 é o principal transportador do cérebro adulto, mas GLAST é predominante em algumas regiões, como, por exemplo, no cerebelo (Regan et al. 2007).

Uma vez captado pelos astrócitos, o glutamato também pode gerar energia pela entrada como α-cetoglutarato no ciclo dos ácidos tricarboxílicos (Swanson et al. 1990). Além disso, o glutamato tem um papel chave no ciclo glutamato-glutamina, em que o glutamato captado é convertido em glutamina através da atividade da enzima exclusivamente astrocitária glutamina sintetase (GS), e pode retornar para neurônios para repor os níveis de glutamato ou GABA, ou para ser utilizada como substrato energético (Bak et al. 2006, Schousboe et al. 2013).

2.2.2. GABA

O GABA é o principal neurotransmissor inibitório do SNC (Schousboe et al. 2013). Astrócitos são responsáveis por cerca de 30% da captação do GABA presente no espaço extracelular e, ao contrário de como ocorre com o glutamato, a maior parte do GABA extracelular é captada diretamente por neurônios e utilizada para repor os estoques do neurotransmissor. Quando captado por astrócitos, o GABA é completamente oxidado na mitocôndria para gerar energia, entrando no ciclo dos ácidos tricarboxílicos na forma de α-cetoglutarato. Os transportadores GAT1 e GAT3 são expressos por astrócitos, sendo GAT1 o principal transportador gabaérgico astrocitário (Minelli et al. 1995).

2.2.3. Acetilcolina

A acetilcolina (Ach) foi o primeiro neurotransmissor estudado e, no SNC, está envolvida em adesão celular, proliferação e crescimento de neuritos em diversos tipos de neurônios, incluindo os não-colinérgicos

(Koenigsberger et al. 1997). Diferentemente do glutamato e GABA, a acetilcolina é hidrolisada extracelularmente, não captada por transportadores específicos. Isso se deve a atividade da enzima acetilcolinesterase (AchE) (Thullbery et al. 2005), que rapidamente hidrolisa Ach em ácido acético e colina, terminando, assim, a sinalização colinérgica. Os principais produtores de AchE são os neurônios, mas foi demonstrado que culturas primárias de astrócitos produzem AchE funcional (Thullbery et al. 2005).

2.3. Regulação dos níveis de substratos energéticos

Conforme citado anteriormente, os astrócitos são as únicas células no SNC que expressam a enzima GS, portanto são os responsáveis pelo ciclo glutamato-glutamina, que é uma importante fonte energética neuronal (Bak et al. 2006). Entretanto, sabe-se que a glicose é o substrato mais utilizado para suprir as demandas energéticas cerebrais (Mergenthaler 2013). Por isso, é importante considerar que os pés astrocíticos (nome dado aos prolongamentos celulares astrocitários) cobrem em torno de 99% dos capilares sanguíneos cerebrais e, assim, em ação coordenada com as células endoteliais, os astrócitos constituem a BSC, portanto, praticamente todas as substâncias que entram no cérebro o fazem via astrócitos (Wang & Bordey 2008). Atualmente, sabe-se que os astrócitos são conectados entre si formando um sincício, e assim, as substâncias captadas dos vasos sanguíneos se difundem rapidamente e são distribuídas para as células circundantes via transportadores específicos (Pellerin & Magistretti 2012).

O principal transportador de glicose astrocitário é o GLUT1, que possui K_m de 1 mM. Sendo o valor normal de glicemia 5,5 mM de glicose, é possível

concluir que o GLUT1 está constantemente operando em velocidade máxima na captação de glicose para suprimento da demanda energética cerebral (Benarroch 2014). Uma vez captada e distribuída, a glicose tem dois destinos importantes nos astrócitos: síntese de glicogênio e via glicolítica. A síntese e o depósito de glicogênio cerebral se dão exclusivamente em astrócitos e, apesar da baixa concentração comparada ao fígado ou músculo, estudos demonstram que ele é fundamental para mobilização rápida de glicose em momentos que a oferta não acompanha a demanda (Dienel & Cruz 2014).

A via glicolítica é predominantemente ativa em astrócitos comparado com neurônios, que possuem baixa atividade da enzima 6-fosfofruto-2-cinase/frutose-2,6-bifosfatase, a enzima produtora de frutose-2,6-bisfosfato, um regulador essencial da fosfofrutocinase 1, a enzima marca-passo da via glicolítica (Bolanos 2016). Estima-se que nestas células uma parte considerável da glicose seja desviada para a via das pentoses-fosfato, para geração de nicotinamida adenina dinucleotídeo fosfato (NADPH), requerida para defesa antioxidante, especialmente para a redução da glutationa oxidada (GSSG) em GSH, o principal antioxidante não-enzimático do SNC. O produto da glicólise – piruvato – pode ser utilizado pelos astrócitos para abastecer o ciclo dos ácidos tricarboxílicos e gerar energia para suas próprias demandas, ou pode ser convertido em lactato e ser exportado através de transportadores específicos para uso pelas células circundantes. Os astrócitos possuem a enzima piruvato desidrogenase – responsável pela entrada do piruvato no ciclo dos ácidos tricarboxílicos – parcialmente inibida, desviando a maior parte do piruvato para síntese de lactato e exportação, o

que sugere que há um intenso acoplamento energético entre neurônios e astrócitos (Pellerin et al. 1998).

De acordo com a hipótese da lançadeira de lactato astrócito-neurônio, proposta por Pellerin e Magistretti, os astrócitos, altamente glicolíticos, transferem o lactato gerado pela glicólise através de transportadores de monocarboxilatos (MCTs) para os neurônios, altamente oxidativos, utilizarem diretamente como substrato energético (Pellerin et al. 1998, Magistretti 2006). Desta forma, tornou-se conhecido o importante papel dos MCTs na homeostase cerebral, visto que a disponibilidade de substratos energéticos deve ser muito bem controlada, especialmente em um tecido com alta demanda energética como o cérebro (Pellerin, 2005).

2.4. Respostas celulares e vias de sinalização

2.4.1. Espécies reativas de oxigênio/nitrogênio e defesas antioxidantes

Todo sistema biológico que utiliza oxigênio na respiração celular tem o potencial de gerar Espécies Reativas de Oxigênio e Nitrogênio (ERO/ERN), ou radicais livres, especialmente durante o funcionamento da cadeia de transporte de elétrons na mitocôndria (Halliwell 2006b). As mitocôndrias são organelas extremamente dinâmicas, que coordenam diversos processos celulares, percebendo o status metabólico/redox e adaptando suas funções para regular a produção energética, termogênese e morte celular (Wai & Langer 2016). A principal função mitocondrial (ver a seguir) ocorre devido ao gradiente eletroquímico dependente da manutenção do potencial de membrana ($\Delta\psi m$), o qual é fundamental para o funcionamento da organela, sendo o estresse oxidativo um evento que desencadeia disfunção

mitocondrial por alterar esta propriedade (Waldbaum & Patel 2010). Um dos eventos mais primordiais para o funcionamento celular ocorre nesta organela: a geração de ATP para custear as reações químicas necessárias às funções celulares; entretanto esse processo é dependente da cadeia de transporte de elétrons, sendo, portanto, um possível gerador de radicais livres (Halliwell, 2001).

Radical livre é um termo que se refere a qualquer espécie capaz de existência independente que contém um ou mais elétrons desemparelhados, ou seja, um elétron que ocupa um orbital atômico ou molecular sozinho, o que confere instabilidade e reatividade à espécie (Droge 2002). Atualmente sabe-se que radicais livres atuam fisiologicamente na sinalização celular, como por exemplo, na regulação do tônus vascular; na detecção e regulação de funções relacionadas à tensão de oxigênio; no aprimoramento da transdução de sinal de vários receptores de membrana e em respostas oxidativas que garantem a manutenção da homeostase redox (Droge, 2002).

Entretanto, os radicais livres foram descobertos devido a seus efeitos deletérios nas biomoléculas e, inicialmente, postulou-se que eles teriam apenas efeitos patológicos, levando à perda das funções celulares e/ou mutagênese. Após a descoberta da enzima superóxido dismutase (SOD), que dismuta o ânion superóxido em peróxido de hidrogênio, os pesquisadores começaram a ter ideia da importância fisiológica dos radicais livres. Entre os principais radicais livres podemos citar o ânion superóxido e o radical hidroxil (Halliwell 2007).

Desta maneira, quando a produção de radicais livres é exacerbada em relação às defesas antioxidantes, ocorre o estresse oxidativo. As principais

defesas antioxidantes não enzimáticas do cérebro são a GSH e o ascorbato (Dringen, 2000). A GSH é um tripeptídeo constituído por glutamato, cisteína e glicina, sintetizado em dois passos enzimáticos (Hertz & Zielke 2004). A enzima γ-glutamil-cisteinil sintetase catalisa a ligação entre glutamato e cisteína para formar o dipeptídeo γ-glutamil-cisteinil, o qual pode seguir na via ou ser exportado dos astrócitos e ser utilizado como substrato para a síntese de GSH neuronal. A enzima GSH sintetase adiciona glicina ao dipeptídeo, tendo como produto a GSH. O efeito antioxidante da GSH se dá pela reação direta (de maneira não-enzimática) com ERO/ERN, ou pela atuação como substrato da enzima glutationa peroxidase (GPx) (Dringen, 2000).

A GPx atua detoxificando ERO/ERN, gerando glutationa oxidada a partir da GSH. Posteriormente, a glutationa oxidada será reduzida através da ação da glutationa redutase às custas de NADPH. Assim, a glutationa redutase, a GPx e a SOD, em conjunto com a catalase (que catalisa a formação de água a partir de peróxido), constituem as principais defesas antioxidantes enzimáticas do SNC (Droge 2002).

2.4.2. Resposta inflamatória

Inflamação é uma condição presente em diversos eventos neuropatológicos. A resposta mais comum do tecido cerebral a um estímulo inflamatório é a ativação de microglia e astrócitos, através da liberação de citocinas e alteração das funções fisiológicas, como perda da permeabilidade celular (Farina 2007). A resposta inflamatória é primariamente deflagrada no SNC pelas células microgliais, mas os astrócitos são essenciais no monitoramento da homeostase cerebral e, portanto, são capazes de

reconhecer os eventos iniciais do processo inflamatório, responder e amplificar a sinalização microglial (Wang & Bordey 2008).

Células ativadas durante o processo inflamatório rapidamente produzem e secretam mediadores inflamatórios, de modo a sinalizar e responder ao evento adverso. As principais citocinas pró-inflamatórias produzidas e secretadas por astrócitos são a interleucina 1β (IL1β) e o fator de necrose tumoral α (TNFα) (Gosselin & Rivest 2007). Estas citocinas são conhecidas como pleiotrópicas, pois dependendo da concentração em que se encontram podem exercer efeitos fisiológicos e necessários para o funcionamento tecidual, mas induzem danos se presentes em altas concentrações (Gosselin & Rivest, 2007).

Foi demonstrado que a IL1β em baixas concentrações tem função de neuromodulador da comunicação sináptica, atuando no processo de aprendizado e na regulação do sono. Em altas concentrações, a IL1β demonstrou contribuir para a epileptogênese, para danos no aprendizado e memória e para o estabelecimento da cascata de eventos que levam ao estabelecimento dos danos observados na Doença de Alzheimer (Gosselin & Rivest, 2007). Similarmente, em baixas concentrações, o TNFα desempenha um papel crítico na gliotransmissão glutamatérgica e é requerido para a estabilização das redes neuronais em resposta a mudanças prolongadas na atividade neuronal (Santello et al. 2011). Em altas concentrações, o TNFα é citotóxico para oligodendrócitos e causa danos à bainha de mielina. Desta forma, é extremamente importante que a produção destas citocinas seja bem controlada, o que depende da ativação de vias de sinalização, especialmente a via do Fator Nuclear κB (NFκB) (Gosselin & Rivest 2007, Shih et al. 2015).

O NFκB é um regulador pleiotrópico de várias vias de sinalização, relacionado a diversas respostas de sobrevivência e homeostase celular (Shih et al. 2015, Kim et al. 2006). Este fator tem um papel importante em vários processos fisiológicos como plasticidade sináptica, aprendizado e memória; proteção contra excitotoxicidade; regulação da permeabilidade celular e transporte intracelular relacionado ao papel dos astrócitos na BSC. Sob seu comando está a transcrição de genes que codificam quimiocinas, citocinas, enzimas pró-inflamatórias (como a óxido nítrico sintase induzível – iNOS, por exemplo), moléculas de adesão e outros fatores que determinam a resposta celular. Portanto, a modulação da atividade do NFκB em condições inflamatórias/oxidativas pode ser benéfica para evitar a geração de uma quantidade exacerbada de mediadores químicos e ERO/ERN que podem piorar o quadro de dano celular (Kaltschmidt & Kaltschmidt 2009).

Diretamente relacionada à atividade do NFκB e ao processo inflamatório está a via de sinalização das proteínas cinases ativadas por mitógenos (MAPK), envolvida no processo de astrogliose reativa, infiltração de células imunes e resposta imune adaptativa (Kaminska et al. 2009). Sendo a resposta inflamatória um evento de extrema complexidade, composto por diversas etapas, é natural que seja controlado em vários níveis e por vários elementos celulares ativados independentemente.

2.4.3. Via de sinalização da heme oxigenase 1

A proteína heme oxigenase, em sua forma induzível, 1 (HO1), é fundamental para ativar mecanismos de defesa celulares frente à uma alteração oxidativa/inflamatória. A HO1 é a principal responsável pela

conversão do grupamento heme em monóxido de carbono e os produtos antioxidantes biliverdina e bilirrubina, sendo apontada como um possível alvo terapêutico no processo de envelhecimento e/ou em doenças neurodegenerativas (Cuadrado & Rojo 2008). Aumento da atividade da HO1 é relacionado à proteção contra condições estressoras, tais como hipóxia/isquemia, estresse oxidativo e neuroinflamação. A HO1 regula a transcrição do NFκB, agindo de maneira contrária a este. Ela e seu regulador, o fator de transcrição Nrf2 (do inglês “nuclear factor erythroid-2 related factor-2”), atuam ativando mecanismos de defesa antioxidantes, promovendo a atividade da GPx e aumento do conteúdo de GSH intracelular (Quincozes-Santos et al. 2013b).

Desta forma, através de processos bioquímicos, celulares e moleculares, astrócitos atuam ativamente na funcionalidade cerebral, modulando a atividade dos outros tipos celulares e promovendo a homeostase necessária ao bom funcionamento do SNC (Hertz & Zielke, 2004).

2.4.4. Efeito glioprotetor da guanosina

A guanosina é um nucleosídeo derivado da guanina que atua como molécula de comunicação extracelular, podendo ser liberada por astrócitos (Bettio et al, 2016). Diversos protocolos experimentais empregaram a guanosina como agente neuro/glioprotetor e ela demonstrou exercer um efeito benéfico em modelos de convulsão, hipóxia, hipoglicemia e neuroinflamação (Bellaver et al. 2015, Schmidt et al. 2000, Quincozes-Santos et al. 2014, Dal-Cim et al. 2013, Bettio et al. 2016). Estudos prévios do nosso

grupo de pesquisa demonstraram que a guanosina possui propriedades antioxidantes, que podem ser relacionadas tanto da sua habilidade de neutralizar diretamente ERO/ERN (atuando como um *scavenger*), quanto da sua habilidade de ativar vias celulares envolvidas nas defesas antioxidantes (Bellaver et al, 2015; Quincozes-Santos et al, 2014). A via da HO1 foi apontada como sendo uma das vias responsáveis pela atuação benéfica da guanosina e de outros compostos neuro-/glioprotetores (Quincozes-Santos et al. 2014).

OBJETIVOS

Objetivo geral

Caracterizar respostas e funções da cultura de astrócitos adultos, visando estabelecer seu uso como um modelo de estudo fidedigno e versátil das propriedades celulares astrocitárias, elucidando o papel destas células em condições fisiológicas e patológicas.

Objetivos específicos

1. Caracterizar propriedades da cultura adulta (90 dias), estabelecendo seu perfil intra e extracelular de aminoácidos e purinas, bem como atividade enzimática e controle de neurotransmissores;
2. Demonstrar que a cultura adulta é proveniente de células adultas e não de precursores celulares; analisar comparativamente a funcionalidade mitocondrial de culturas preparadas a partir de animais neonatos, adultos e envelhecidos;
3. Analisar as respostas idade-dependente em culturas preparadas a partir de animais neonatos, adultos e envelhecidos frente à exposição ao glutamato, em uma concentração não-tóxica;
4. Analisar o efeito antienvelhecimento da guanosina em culturas preparadas a partir de animais neonatos, adultos e envelhecidos;
5. Analisar propriedades gliais em culturas preparadas a partir de animais neonatos, adultos e envelhecidos em um modelo de senescência.

PARTE II

CAPÍTULO 1

Artigo aceito para publicação no periódico Neurochemical Research

Characterization of Amino Acid Profile and Enzymatic Activity in Adult Rat Astrocyte Cultures

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ORIGINAL PAPER

Characterization of Amino Acid Profile and Enzymatic Activity in Adult Rat Astrocyte Cultures

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Abstract Astrocytes are multitasking players in brain complexity, possessing several receptors and mechanisms to detect, participate and modulate neuronal communication. The functionality of astrocytes has been mainly unraveled through the study of primary astrocyte cultures, and recently our research group characterized a model of astrocyte cultures derived from adult Wistar rats. We, herein, aim to characterize other basal functions of these cells to explore the potential of this model for studying the adult brain. To characterize the astrocytic phenotype, we determined the presence of GFAP, GLAST and GLT 1 proteins in cells by immunofluorescence. Next, we determined the concentrations of thirteen amino acids, ATP, ADP, adenosine and calcium in astrocyte cultures, as well as the activities of Na^+/K^+-ATPase and acetylcholine esterase. Furthermore, we assessed the presence of the GABA transporter 1 (GAT 1) and cannabinoid receptor 1 (CB 1) in the astrocytes. Cells demonstrated the presence of glutamine, consistent with their role in the glutamate–glutamine cycle, as well as glutamate and D-serine, amino acids classically known to act as gliotransmitters. ATP was produced and released by the cells and ADP was consumed. Calcium levels were in agreement with those reported in the literature, as were the enzymatic activities measured. The presence of GAT 1 was detected, but the presence of CB 1 was not, suggesting a decreased neuroprotective capacity in adult astrocytes under in vitro conditions. Taken together, our results show cellular functionality regarding the astrocytic role in gliotransmission and neurotransmitter management since they are able to produce and release gliotransmitters and to modulate the cholinergic and GABAergic systems.

Keywords Adult astrocytes · Gliotransmitters · Amino acids · Na^+/K^+-ATPase · AChE

Introduction

Astrocytes are multitasking players in brain complexity, acting as secretory cells of the central nervous system (CNS) releasing neurotransmitters, neuromodulators and trophic factors [1–3]. They sense neural communication, as is evident by the expression of numerous neurotransmitter receptors, transporters and enzymes on their membranes [4]. These cells also release gliotransmitters, such as glutamate, D-serine and ATP, which interact with pre- and post-synaptic receptors in the tripartite synapse [5–7]. Astrocytes are also primary homeostatic cells of the brain, and most of their functionality has been unraveled through the study of primary astrocyte cultures, especially those related to glutamate and GABA metabolism, their antioxidant defense and energy capabilities [8–11].

Recent studies have employed astrocyte cultures derived from adult rats, which are unsurprisingly different to those derived from newborn animals, since their connections on the tissue that they are inserted in are far more complex. As such, we previously published a routine astrocyte culture protocol from adult Wistar rats; these cells present classical astrocytic markers, take up glutamate, and actively participate in antioxidant and inflammatory responses [12]. Additionally, these cells presented age-related glial

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responses, including under neurotoxic and neuroprotective stimuli [13]. However, the presence of gliotransmitters in adult astrocyte cultures remains unclear.

Na^+/K^+-ATPase (EC 3.6.1.37) is a crucial enzyme that is responsible for the generation of membrane potential, through the active transport of Na^+ and K^+ ions in the CNS, consuming about 60 % of the ATP generated in this tissue [14]. As astrocytes are involved in the regulation of ionic homeostasis and the glutamate transporter (highly dependent on Na^+ ion), there is a close relationship between Na^+/K^+-ATPase and astrocyte functionality [15, 16]. In addition, as well as the gliotransmitters, acetylcholine serves as an extracellular signaling substance in the neural cells. Acetylcholine (ACh) is specifically hydrolyzed by acetylcholinesterase (AChE, EC 3.1.1.7) and has also been associated with astrocytic activity [17].

The amino acid profile, enzymatic activity and neurotransmitter management in cultivated cells are useful for indicating the culture functionality, which is pivotal for the routine use of a model as a reliable tool when studying neurochemical properties of the adult brain. Thus, the aim of this study was to determine some aspects of our adult astrocyte cultures under basal conditions, such as the concentration of thirteen amino acids, ATP, ADP and adenosine (ADO) in the intra and extracellular medium, as well as the activities of Na^+/K^+-ATPase and acetylcholinesterase (AChE). Additionally, to study GABAergic and cannabinoid transmission, the presence of GABA transporter 1 (GAT 1) and cannabinoid receptor 1 (CB 1) was assessed in the cells. Morphological analysis of the glial fibrillary acidic protein (GFAP) and of the excitatory amino acid transporters, GLAST (EAAT1) and GLT 1 (EAAT2), were performed to ensure the astrocytic phenotype of the cultured cells.

Experimental Procedures

Reagents

Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) and other materials for cell culture were purchased from Gibco/Invitrogen (California, USA). Papain was acquired from Merck (Darmstadt, Germany). DNase, cysteine, albumin and monoclonal anti-GAPDH were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitrocellulose membrane and an ECL kit were purchased from Amersham (Buckinghamshire, UK). Polyclonal anti-GLAST and anti-GLT 1 were from Alpha Diagnostics (Texas, USA), polyclonal anti-GFAP was from Dako (Glostrup, Denmark), polyclonal anti-GAT 1 was from Merck Millipore (Darmstadt, Germany) and polyclonal anti-CB 1 was from Santa Cruz Biotechnology (California, USA). All other chemicals were from common commercial suppliers.

Animals

Male Wistar rats (90 days old) were obtained from our breeding colony (Department of Biochemistry, UFRGS, Brazil) and maintained under a controlled environment (12 h light/12 h dark cycle; 22 ± 1 °C; ad libitum access to food and water). All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Federal University of Rio Grande do Sul Animal Care and Use Committee (process number 24419).

Cell Culture Preparation and Maintenance

Male Wistar rats (90 days old) were sacrificed by decapitation, had their cerebral cortices aseptically dissected and meninges removed. The tissue was digested using trypsin and papain at 37 °C, as previously described [12]. After mechanical dissociation and centrifugation, the cells were resuspended in DMEM/F12 [10 % fetal bovine serum (FBS), 15 mM HEPES, 14.3 mM $NaHCO_3$, 1 % Fungizone® and 0.04 % gentamicin], plated on 6- or 24-well plates pre-coated with poly-L-lysine and cultured at 37 °C in a 5 % CO_2 incubator. The cells were seeded at a density of 3–5 × 10^5 cells/cm^2. Twenty-four hours later, the culture medium was exchanged; during the 1st week, the medium was replaced once every 2 days and from the 2nd week on, once every four days. From the 3rd week on, the astrocytes received medium supplemented with 20 % FBS until they reached confluence (at approximately the 4th week). No dibutyryl cAMP was added to the culture medium in order to observe the naive response of the cells. Specific proteins of neurons and microglia were tested in order to determine the purity of the astrocyte culture, which was found to be around 95 % (data not shown).

Immunofluorescence

Immunocytochemistry was performed as described previously [12]. Briefly, cell cultures were fixed with 4 % paraformaldehyde for 20 min and permeabilized with 0.1 % Triton X-100 in PBS for 5 min at room temperature. After blocking overnight with 4 % albumin, the cells were incubated overnight with anti-GFAP (1:400), anti-GLAST (1:500) or anti-GLT 1 (1:500) at 4 °C, followed by PBS washes and incubation with a specific secondary antibody conjugated with Alexa Fluor® 594 or 488 for 1 h at room temperature. Cell nuclei were stained with 0.2 mg/mL of 4′,6′-diamidino-2-phenylindole (DAPI). The cells were

visualized with a Nikon inverted microscope and the images were transferred to a computer with a digital camera (Sound Vision Inc.).

High-Performance Liquid Chromatography (HPLC) Procedure—Amino Acids

The assay was performed with intra and extracellular samples to quantify thirteen amino acids [alanine (Ala), aspartate (Asp), glutamine (Gln), glutamate (Glu), glycine (Gly), isoleucine (Ile), leucine (Leu), lysine (Lys), ornitine (Orn), phenylalanine (Phe), D-serine (Ser), tyrosine (Tyr) and valine (Val), according to [18]]. Briefly, cells were homogenized in 7 % trifluoroacetic acid (TFA) and centrifuged. The supernatant was collected, neutralized with 1.5 M potassium bicarbonate and filtered (0.22 μm pore). Samples were derivatized with o-phthalaldehyde and separation was carried out with a reverse phase column (Supelcosil LC-18, 250 mm × 4.6 mm, Supelco) in a Shimadzu Class-VP chromatography system. The mobile phase flowed at a rate of 1.4 mL/min and column temperature was 24 °C. Buffer composition was A: 0.04 mol/L sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 20 % of methanol; B: 0.01 mol/L sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 80 % of methanol. The gradient profile was modified according to the content of buffer B in the mobile phase: 0 % at 0.00 min, 100 % at 55 min, 0 % at 55–60.00 min. Absorbance was read at 360 nm and 455 nm, excitation and emission respectively, with a Shimadzu fluorescence detector. For glutamine determination, samples were diluted 10×. Samples of 100 μL were used and concentration was expressed as mean ± SEM, in nmol/mg protein for intracellular content and μmol/dL for extracellular medium. The amino acids appear in the chromatogram in the following order: Asp; Glu; Ser; Gln; Gly; Ala; Tyr; Val; Phe; Ile; Leu; Orn and Lys.

HPLC Procedure—Purines

The assay was performed in culture supernatant in order to measure the ATP, ADP and ADO concentration, according to Domanski and colleagues [19]. Analyses were performed with a Shimadzu LC-20A series chromatography system, consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, auto injector and UV detector. Separations were achieved on a reverse-phase column (Supelcosil LC-18, 250 mm × 4.6 mm, Supelco). The mobile phase flowed at a rate of 1.2 mL/min and the column temperature was 24 °C. Buffer composition was: (a) 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride and (b) 15 % acetonitrile in buffer A. The gradient profile underwent the following changes of buffer B in the mobile phase: 0 % at 0.00 min, 2 % at 0.05 min, 7 % at 2.45 min, 50 % at 10.00 min, 100 % at 11.00 min, and 0 % at 12.40 min. Samples of 20 μL were injected. Absorbance was read at 254 nm. Concentrations of purines are expressed as mean ± SEM in μmol/dL.

AChE Activity Assay

For the AChE assay, the cells were homogenized in ten volumes of 0.1 mM potassium phosphate buffer, pH 7.4, and centrifuged for 10 min at 1000×*g*. The supernatants were used for the enzymatic AChE analyses. AChE activity was determined according to the method of Ellman and colleagues [20], with some modifications [21]. Hydrolysis rates were measured at ACh concentration of 0.8 mM in 300 μL assay solution with 30 mM phosphate buffer, pH 7.5, and 1.0 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) at 25 °C. About 15 μL of cell homogenate supernatant was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). Samples were run in triplicate, data are expressed as mean ± SEM, in μmol/h/mg protein.

Na^+/K^+-ATPase Activity Assay

The reaction mixture for the Na^+/K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 170 μL. The reaction was initiated by the addition of ATP. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. The activity was calculated by the difference between the two assays, as previously described [22]. Released inorganic phosphate (Pi) was measured by the method of Chan and colleagues [23]. Samples were run in triplicate. Specific activity of the enzyme was expressed as mean ± SEM, in nmol Pi/min/mg protein.

Western Blot Analyses

Cells were solubilized in lyses solution containing 4 % SDS, 2 mM EDTA, 50 mM Tris–HCl (pH 6.8). Protein content was measured, the samples were standardized in sample buffer [62.5 mM Tris–HCl (pH 6.8), 4 % (v/v) glycerol, 0.002 % (w/v) bromophenol blue] and boiled at 95 °C for 5 min. Samples were separated by SDS/PAGE (45 mg protein per sample), and transferred to nitrocellulose membranes, as previously described [12]. Adequate

loading of each sample was confirmed using Ponceau S staining. Membranes were incubated overnight (4 °C) with anti-GAT 1 (1:100) or anti-CB 1 (1:100). The membranes were then washed and incubated with a peroxidase-conjugated anti-rabbit immunoglobulin in a dilution of 1:3000 for 2 h. Chemiluminescence signals were detected in an Image Quant LAS4000 system (GE Healthcare) using ECL kit.

Calcium Measurement

Calcium intracellular levels were evaluated in 40 µL of cells homogenate, using the Calcium colorimetric assay kit from Sigma Aldrich; results are expressed as mean ± SEM for six experimental determinations performed in duplicate.

Protein Assay

Protein content was measured using Bicinchoninic Acid method with bovine serum albumin as a standard [24].

Results

Astrocytic Characterization

The morphology of the cultured astrocytes was verified by immunofluorescence for GFAP, an intermediary filament of cytoskeleton that is one of the principal markers of astrocytes in in vivo and in vitro conditions (Fig. 1a). Additionally, to insure that cells expressed an astrocytic phenotype, we determined the presence of the two major glutamate transporters, GLAST (Fig. 1b) and GLT 1 (Fig. 1c).

Amino Acids and Purine Profile

With regard to gliotransmission, we determined the concentration of amino acids in the intra and extracellular medium of the astrocyte cultures. Figure 2a shows the chromatogram for the thirteen amino acids measured in the intracellular content. Figure 2b shows their quantification in nmol/mg protein: Ala 206.5 ± 22.0; Asp 15.7 ± 1.2; Gln 12016.2 ± 1114.3; Glu 157.3 ± 17.1; Gly 484.0 ± 18.1; Ile 143.7 ± 9.4; Leu 197.5 ± 11.4; Lys 292.0 ± 14.5; Orn 68.7 ± 4.3; Phe 112.9 ± 7.2; Ser 122.8 ± 3.7; Tyr 97.3 ± 6.7 and Val 202.4 ± 11.0. Figure 3a shows the chromatogram for the thirteen amino acids measured in the extracellular medium. Figure 3b shows their quantification in µmol/dL: Ala 34.3 ± 3.5; Asp 1.07 ± 0.28; Gln 4795.7 ± 427.5; Glu 9.3 ± 2.1; Gly 70.2 ± 12.1; Ile 23.1 ± 5.1; Leu 29.6 ± 6.8; Lys 42.4 ± 8.5; Orn 10.3 ± 2.1; Phe 15.8 ± 2.7; Ser 19.9 ± 3.7; Tyr 14.6 ± 2.4 and Val 29.9 ± 5.5.

Figure 4 depicts the extracellular concentrations of ATP, ADP and ADO. The values obtained for ATP, ADP and ADO in extracellular medium were, respectively, 646.5 ± 22.7; 126.28 ± 32.1 and 56.2 ± 5.68 nmol/dL.

Calcium and Enzymatic Measurement

Enzymatic activity was measured in the adult astrocytes as depicted in Table 1. AchE can end acetylcholine signaling; therefore, the findings that adult astrocytes presented an AchE enzymatic activity of 2.50 ± 0.15 µmol/h/mg protein indicates that these cells may modulate the cholinergic system. Na^+/K^+-ATPase maintains the sodium and potassium transmembrane gradients needed for the functionality of the nervous tissue, and its basal value in astrocyte

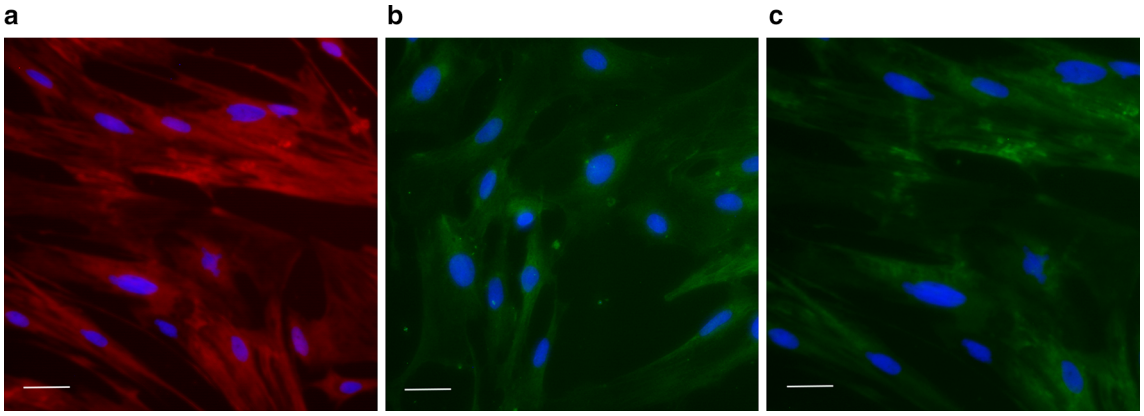

Fig. 1 Astrocyte characterization. Under normal conditions, primary adult astrocytes present a polygonal-to-fusiform and flat morphology. **a** Astrocytes present intense immunolabeling for GFAP. The cells express (**b**) GLAST and (**c**) GLT 1. Nuclei were counterstained with DAPI (*blue*). *Scale bar* 50 µm (Color figure online)

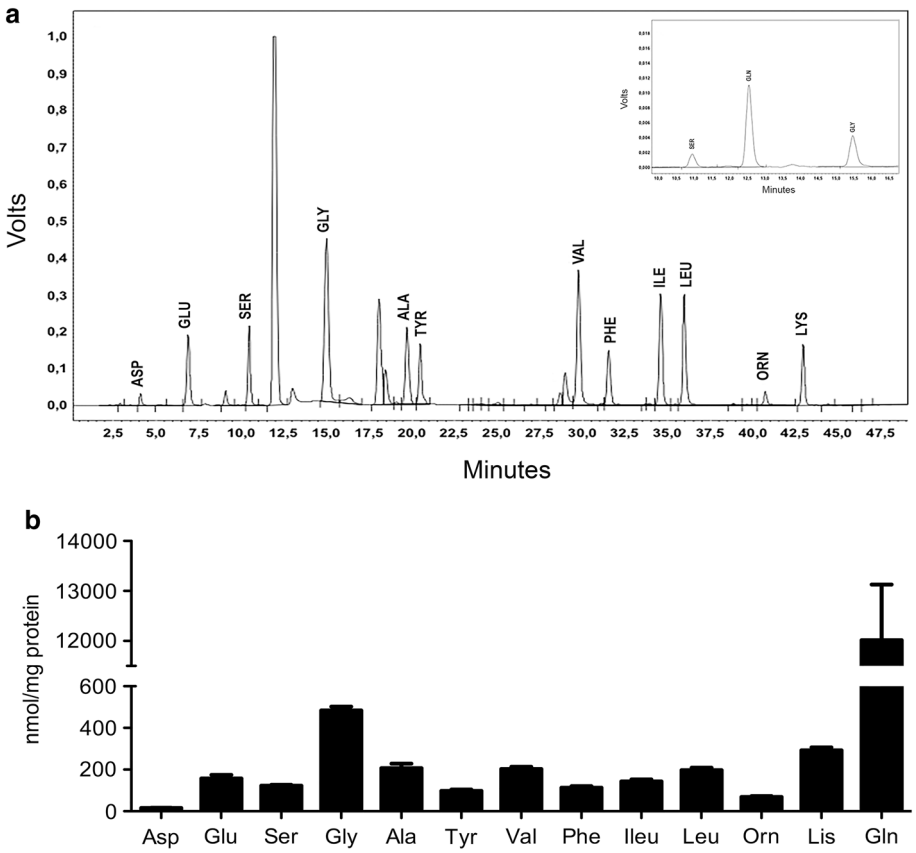

Fig. 2 Amino acid separation of intracellular medium of adult astrocyte cultures by HPLC. **a** Representative chromatogram showing the amino acids separation (*inset* shows glutamine dosage, which was performed by dilution). **b** Concentration of each amino acid in the intracellular medium (nmol/mg protein). Data are presented as the means + SEM, n = 5

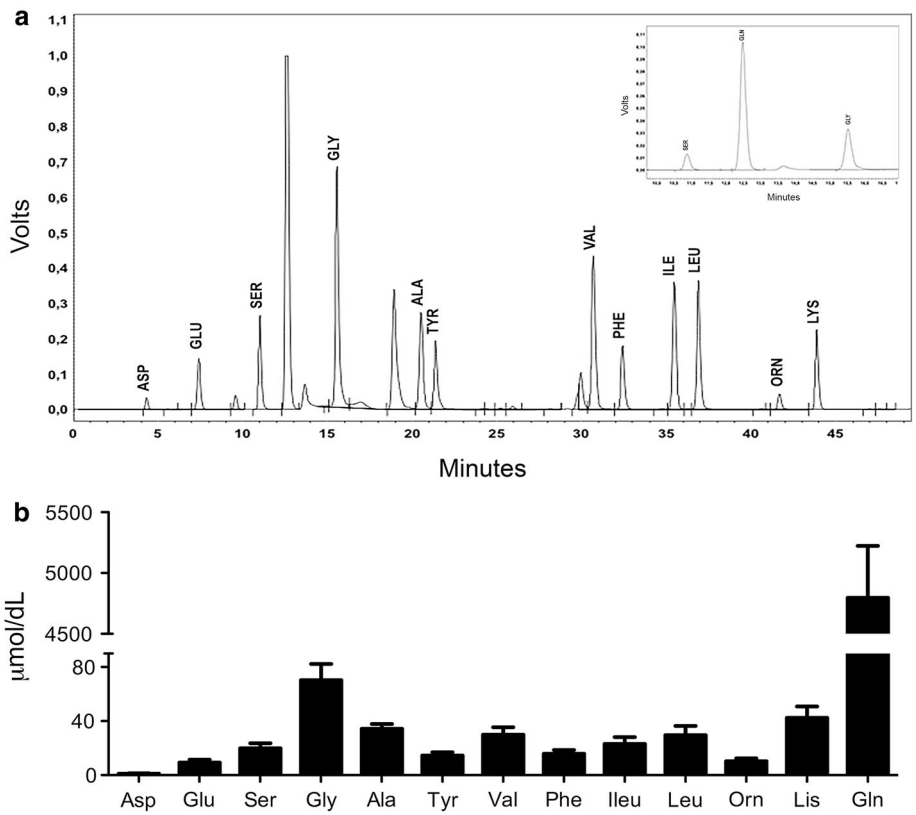

Fig. 3 HPLC procedure for amino acid separation in the extracellular medium of adult astrocyte cultures. **a** Representative chromatogram showing the amino acid separation (*inset* shows glutamine dosage, which was performed by dilution). **b** Concentration of each amino acid in the extracellular medium (µmol/dL). Data are presented as the means + SEM, n = 5

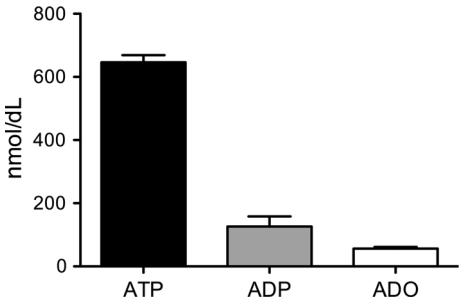

Fig. 4 Measurement of purines in the extracellular medium of adult astrocyte cultures. After cells reached confluence, the extracellular ATP, ADP and ADO concentrations in extracellular medium were determined: 646.5 ± 22.7; 126.28 ± 32.1 and 56.2 ± 5.7 nmol/dL, respectively, whereas basal values for these purines in DMEM/F12 20 % FBS are, 543.5 ± 52.3; 217.3 ± 41.5 and 52.3 ± 0.4 nmol/dL, respectively. Data are presented as the mean + SEM, n = 5

cultures was found to be 42.33 ± 4.33 nmol PI/min/mg protein. The concentration of intracellular calcium, fundamental for glial functionality as well as gliotransmission in adult astrocytes, was 125.54 ± 1.14 nM (Table 1).

Neurotransmitter Receptor Expression

To investigate GABAergic transmission in adult astrocytes functionality, we determined the expressions of the GAT 1 transporter and of the CB 1 receptor by immunoblotting. Figure 5a shows that adult astrocytes expressed the GAT 1 transporter, the classical GABA transporter described for astrocytes. However, adult astrocyte cultures did not express the CB 1 receptor, which controls cannabinoid actions in astrocytes (Fig. 5b).

Discussion

Numerous studies, conducted in primary cultures, have facilitated the progress of astrocytic biology comprehension, both under physiological and pathological conditions. We, herein, investigated some parameters related to cellular functionality in astrocyte cultures prepared from adult rats to determine their amino acid and purine profiles, neurotransmitter management and enzymatic activity. We firstly determined the presence of important astrocytic

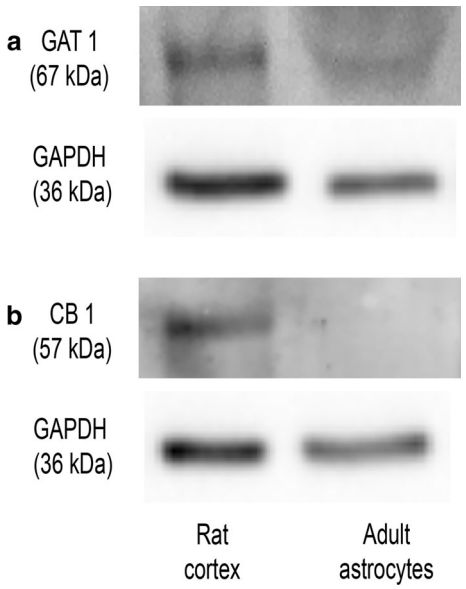

Fig. 5 Neurotransmitter transporters in adult astrocyte cultures. Representative immunoblot band for **a** GAT 1 and **b** CB 1. The *left band* represents rat cortex homogenate and the *right band* represents adult astrocyte culture. Adult astrocytes express the GABA transporter 1 (GAT 1), however they do not express the cannabinoid receptor 1 (CB 1); n = 5

markers in the cells (Fig. 1a). GFAP staining shows that the adult cultures present typical polygonal-to-fusiform and flat morphology. The presence of the glutamatergic transporters also confirmed the astrocytic character of the cultures (Fig. 1b, c).

In addition to its pivotal role in neurotransmitter management, astrocytes also manage gliotransmitters. Several molecules are known to act as gliotransmitters, and these may be exchanged through GAP junctions for communication between astrocytes and/or for communication between astrocytes and other types of cells. Our data indicate the presence intra and extracellular aspartate, glutamate, D-serine and glycine in the cell cultures (Fig. 2, 3). Furthermore, it has been reported that astrocytes discharge gliotransmitters to modulate or respond to neuronal activity; these gliotransmitters activate receptors located at several sites of surrounding cells, leading to alterations in synaptic transmission and plasticity, therefore, turning astrocytes into active elements in brain functionality [2].

Table 1 Cellular parameters in adult astrocyte cultures

Cellular parameter	Adult astrocytes
AchE activity (μmol/h/mg protein)	2.50 ± 0.15
Na^+/K^+ ATPase activity (nmol PI/min/mg protein)	42.33 ± 4.33
Intracellular calcium (nM)	125.54 ± 1.14

AchE and Na^+/K^+ ATPase activities were measured as described in the experimental procedures section. Intracellular calcium was measured by colorimetric assay. Data are presented as the mean ± SEM, n = 5

Glutamine is the major amino acid present in the intracellular and extracellular medium of the cultures, consistent with its role in glutamate–glutamine cycle (closely associated to glutamine synthetase activity [26]) and its ability to act as ammonia carrier. Glycine, the second most abundant amino acid, besides being a gliotransmitter, is a glutathione precursor and, therefore, essential for astrocytic function, as glutathione is an important antioxidant in the brain. Glutamate, aspartate and D-serine, some of the principal gliotransmitters, are present in low concentrations, as are the branched-chain amino acids isoleucine, leucine and valine.

In addition to its classical energetic role, ATP has also been demonstrated to act as a gliotransmitter, and when released from astrocytes may act in a bidirectional fashion, regulating neuronal excitability depending on concentration. In the astrocyte cytosol, ATP is produced via glycolysis and oxidative phosphorylation and reaches high cytosolic concentrations, thus establishing a concentration gradient favoring its exit from the cell [25], as also observed in the present study, under in vitro conditions. Some studies have shown that, once released, ATP is rapidly converted into ADO; however, the quantity of ATP measured was higher than that of ADO. As such, this signaling might be lost in astrocytes when in vitro (Fig. 4). Moreover, all synapses in the CNS are highly dependent on ATP and approximately 60 % of ATP produced in the CNS is consumed by Na^+/K^+-ATPase. For this reason, we measured Na^+/K^+-ATPase activity, under basal conditions, due to its vital role in cellular physiology, especially considering that astrocytes interact with neurons to promote the clearance of synaptically-released glutamate, in association with the co-transport of Na^+ ions. Together with Ca^{2+}, Na^+ regulates the communication between astrocytes and neurons, since the intracellular levels of these ions rise when stimulation occurs at the tripartite synapse. After the entry of Na^+ ions into the cytosol, the basal concentration of Na^+ must be restored; the Na^+/K^+-ATPase acts as the major transporter of Na^+ ions in astrocytes, and the activity of this enzyme is essential for the function of glutamate transporters. Since the clearance of glutamate from the synaptic cleft is a function of astrocytes, Na^+/K^+-ATPase might be impaired in aging and in neuropathological conditions, where excitotoxicity is an ordinary feature.

Previous studies have shown that astrocytes take up glutamate and GABA in physiological conditions; moreover, astrocytes play a pivotal role in the synthesis of these neurotransmitters, since they express glutamine synthetase, which provides the precursor for both glutamate and GABA [26]. Once taken up by astrocytes, GABA is completely metabolized to yield energy, whereas glutamate may, besides being metabolized via the tricarboxilic acids cycle, also replenish neurotransmitter pools via the glutamate–glutamine cycle or be used as a substrate for glutathione synthesis. We, herein, showed that astrocyte cultures express the GAT 1 protein, the major astrocytic GABA transporter (Fig. 5).

In addition to controlling the glutamatergic and GABAergic systems, astrocytes may also control the cholinergic system, since they present AchE activity, as shown in Table 1. The brain cholinergic system may modulate several important functions such as learning, memory, control over cerebral blood flow and inflammatory response; thus, our findings indicate that, even after weeks in culture, astrocyte cultures from adult animals continue to play roles that are important for neurotransmitter management. As such, our data indicate that these astrocytes are functional and suitable to better comprehend adult and aged brain.

Astroglial cannabinoid signaling plays a role in the modulation of synaptic plasticity, Ca^{2+} signaling and in the communication between astrocytes and neurons, through the release of gliotransmitters, via the cannabinoid receptors CB 1 and CB 2. Previous studies have shown that astrocytes from different brain regions may present either CB 1 or CB 2, where the CB 1 receptor is involved in the exocytosis of gliotransmitters, and, hence, in synaptic plasticity [27]. Herein, we have not found the presence of CB 1 in adult astrocyte cultures under basal conditions; this lack of receptor expression may compromise some astrocytic functions, particularly those related to neuroprotection. In fact, adult astrocytes culture and astrocytes aged in vitro have already demonstrated less capacity of fulfilling neuroprotective roles and perhaps the loss of CB 1 through the process of adaptation to the in vitro condition may be related to that constraint [13, 28].

Calcium signaling constitutes a primitive pathway of cellular communication that is conserved in most living organisms [29]. Several events are able to transiently elevate cytosolic Ca^{2+} in the astrocyte intracellular medium, including synaptically released neurotransmitters and neuromodulators and retrograde messengers such as endocannabinoids [1]. In order maintain a low Ca^{2+} threshold to permit $[Ca^{2+}]$ changes in the cytosol, Ca^{2+} concentrations must be kept low, with the extracellular concentration being $\sim$10,000–20,000 times higher than the intracellular concentration. Accordingly, herein we showed that adult astrocytes cultures maintain their intracellular levels of Ca^{2+} at around 125 nm, in line with previous reports [29]. This large difference between intra and extracellular medium $[Ca^{2+}]$ allows the rapid progradient diffusion of Ca^{2+} ions into the cells, to induce intracellular signaling.

Figure 6 summarizes the findings of our research group concerning the main properties of cultures of astrocytes

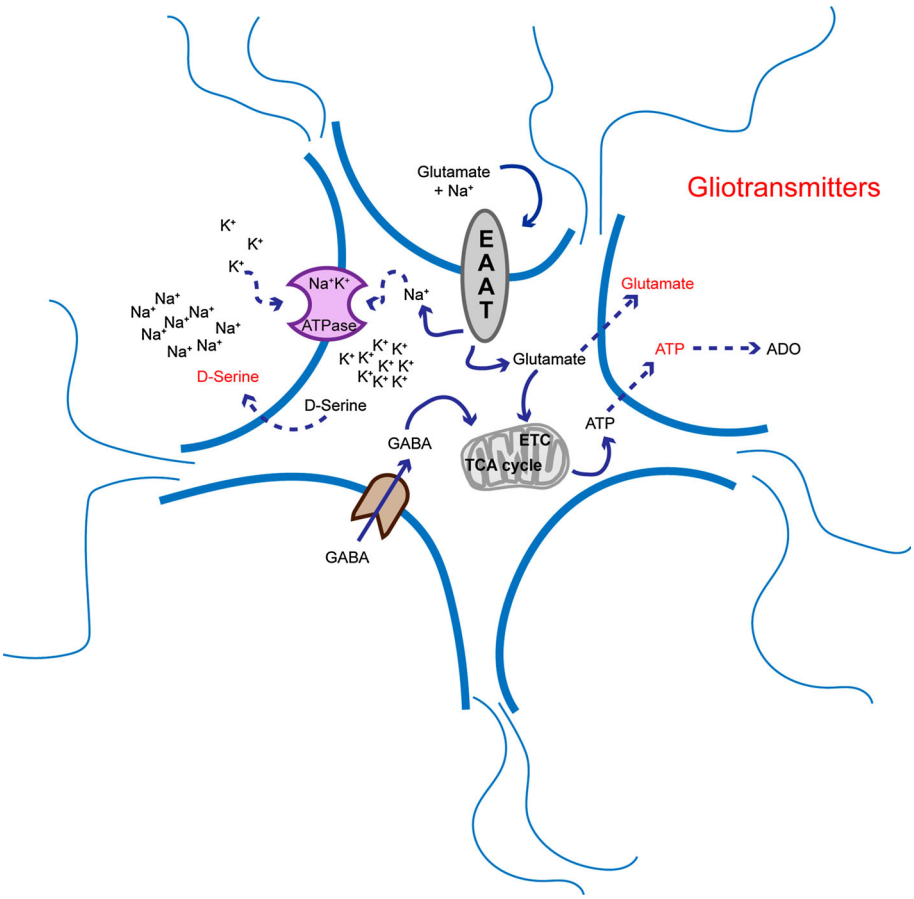

Fig. 6 Functionality of adult astrocyte cultures. Figure 6 summarizes some of our findings regarding adult astrocyte cultures. These cells are able to take up glutamate through high-affinity transporters, in association with the co-transport of sodium ions (Na^+). The Na^+ ions can be released from the cell by Na^+/K^+ ATPase, while glutamate may have several fates within the cell. Herein we show that glutamate may enter the mitochondria for energy yielding or be released from astrocytes as a gliotransmitter, similarly to D-serine. GABA may enter the astrocyte through GAT 1 and generate ATP in the mitochondria, via the TCA cycle and ETC. ATP may either be used for cellular energetic requirements or be released as a gliotransmitter. *EAAT* excitatory amino acid transporter, *TCA* tricarboxylic acids cycle, *ETC* electron transport chain

from adult rats. Since we showed here that adult astrocytes possess and release gliotransmitters and are able to affect synaptic communication, not only at glutamatergic sites, we provide some evidence that this methodology is suitable for the routine study of the physiology and pathology of astrocytes. These cultures are an innovative and functional tool to study the cellular, biochemical and molecular properties of adult and aged brains; and further studies may employ these cultures for investigations of astrocytic biology, to characterize the roles of these cells in aging and senescence.

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Compliance with Ethical Standards

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

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CAPÍTULO 2

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In vitro astrocytes derived from mature cells: implications for study of aging.

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***In vitro* astrocytes derived from mature cells: implications for aging studies**

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Abstract

Astrocytes are versatile cells involved in synaptic information processing, energy metabolism, redox homeostasis, inflammatory response and structural support of the brain. Astrocyte primary cultures have been extensively used to characterize the biochemical, pharmacological and morphological properties of the central nervous system and most of these studies were performed in newborn astrocytes. Recently, we established a routine protocol of cultured astrocytes derived from adult and aged Wistar rats, which present several different responses compared with newborn astrocytes. Thus, the aim of this study was to confirm that our *in vitro* astrocytes were derived from mature cells, since another protocols have suggested that these cultures are resulting of the immature glial stem cells pool. Therefore, we evaluated cytoskeleton proteins, such as glial fibrillary acidic protein and vimentin, as well as Sox10, an essential marker of immature glial cells, in *ex vivo* tissue and *in vitro* astrocytes of the same animals of 1, 90 and 180 days old. In addition, we examined the mitochondrial functionality by the measurement of mitochondrial membrane potential, reactive oxygen/nitrogen species generation, and enzymatic activity of cellular energy homeostasis and antioxidant defenses. Our results suggest that adult and aged astrocytes are derived from mature cells and changes in mitochondrial dynamics in *ex vivo* tissue were reproduced in *in vitro* astrocytes. Thus, with this tool we expect contribute to elucidate the role of astrocytes in brain aging and better understand comparison between *in vivo* and *in vitro* interventions.

Key words: adult/aged astrocytes; Sox10; mitochondrial function; aging

Introduction

Astrocytes, the most versatile cells of the central nervous system (CNS), are crucial for the metabolic and redox homeostasis, inflammatory response and structural support of the brain (Hertz & Zielke 2004, Bolanos 2016, Khakh & Sofroniew 2015). Much of the understanding of astrocytes has been derived from studies conducted with primary culture of these cells in physiological and pathological conditions (Lange *et al.* 2012). In this sense, cultured astrocytes are most often derived from newborn mice or rats, when gliogenesis is not complete, thus the immature astrocytes continue to divide *in vitro* until confluence (Lange et al. 2012, Hertz 1998).

There are important differences between the immature and mature brain, with significant implications in brain functionality (Herculano-Houzel 2014, Sun *et al.* 2013). Astrocytes derived from mature animals contain well-established connections, more organized than immature tissue, which is more plastic and labile to stimuli. Thus, mature astrocytes may respond more reliably and help to elucidate the role of astrocytes in brain aging (Souza *et al*. 2013, Bellaver et al. 2016). In line with this, we recently established a routine protocol of mature astrocytes derived from adult and aged Wistar rats to study biochemical, pharmacological and morphological properties of the CNS associated to aging and/or age-related neurological diseases (Souza *et al.* 2013). However, previous report suggested that astrocyte cultures from adult animals do not result in mature astrocytes in culture, because these astrocytes appear to be derived from

a small population of immature glial stem cells that remain in brain throughout life (Norton, 1989).

We have previously demonstrated that adult/aged astrocytes present different responses compared with newborn cells; therefore, the aim of this study was to verify if our cortical astrocyte cultures from adult and aged Wistar rats are derived from mature or immature cells. For this purpose, we evaluated the classical astrocytic cytoskeleton markers, glial fibrillary acidic protein (GFAP) and vimentin (Yang & Wang 2015, Hol & Pekny 2015), as well as Sox10, an essential marker of immature glial cells (Kim *et al.* 2003, Kuhlbrodt *et al.* 1998), in *ex vivo* tissue and *in vitro* astrocytes from 1, 90 and 180 days old rats, respectively, newborn, adult and aged rats. Additionally, we assessed the redox parameters, particularly mitochondrial membrane potential ($\Delta\Psi_m$), superoxide levels, NADPH oxidase (NOX), creatine kinase (CK) and antioxidant enzymatic defenses comparing *ex vivo* tissue and *in vitro* astrocytes.

Materials and Methods

Animals

Male Wistar rats were obtained from our breeding colony (Department of Biochemistry, UFRGS, Porto Alegre, Brazil), maintained under controlled environment (12 h light/12 h dark cycle; 22 ± 1 °C; *ad libitum* access to food and water). All animal experiments were performed in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals and Brazilian Society for Neuroscience and Behavior recommendations for animal

care. The experimental protocols were approved by the Federal University of Rio Grande do Sul Animal Care and Use Committee (process number 24419).

Primary astrocyte cultures

This procedure was carried out as previously described (Souza *et al.* 2013). Briefly, the cerebral cortex of male Wistar rats (1, 90 and 180 days old) was digested using trypsin only – for newborn tissue – or trypsin and papain – for mature tissue – at 37ºC. After mechanical dissociation and centrifugation, the cells were resuspended in DMEM/F12 [10% fetal bovine serum (FBS), 15 mM HEPES, 14.3 mM $NaHCO_3$, 1% Fungizone® and 0.04% gentamicin], and either used for flow cytometry determination (*ex vivo* samples) or plated on 6- or 24-well plates pre-coated with poly-L-lysine and cultured at 37°C in a 5% CO_2 incubator. From the 3rd week on, the astrocytes received medium supplemented with 20% FBS until they reached confluence (at approximately the 4th week).

Flow cytometry

The protocol was carried out in the dark, under agitation and at room temperature. Tissue suspension (*ex vivo*) was assayed immediately after the dissection procedure was finished. Cell suspension (*in vitro*) was obtained from cultures at confluence, removed from the plates with 0.5% trypsin for 15 min at 37°C. Tissue or cell suspensions, around 10^6 cells, were incubated in 0.05% (v/v) Triton X-100 phosphate-buffered saline (PBS) for 10 min. After centrifugation, cells were resuspended in PBS containing the primary antibody 1:50 [Polyclonal

anti-GFAP (Dako); Polyclonal anti-vimentin (Sigma-Aldrich) or Polyclonal anti-Sox10 (Millipore)] and incubated for 30 min. After a PBS wash and another centrifugation, the cells were incubated with Alexa 488-anti-rabbit IgG 1:100 for 30 min. Control samples omitting the primary and the secondary antibody were carried out in parallel and considered as blank. Data from 10,000 events (intact cells) were acquired. The number of positive GFAP, vimentin and Sox10 were determined in a FACSCalibur flow cytometer. The analysis was carried out as previously described (Hansel *et al.* 2014). Results are expressed as percentage of positive cells.

Immunofluorescence

Immunofluorescence was performed as previously described (Souza et al. 2013). Briefly, cell cultures at confluence were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. After blocking overnight with 4% albumin, the cells were incubated overnight with anti-GFAP (1:400), anti-vimentin (1:1000) or anti-Sox10 (1:500) at 4°C, followed by PBS washes and incubation with a specific secondary antibody conjugated with Alexa Fluor® 488 or 594 for 1 h at room temperature. Cell nuclei were stained with 0.2 mg/mL of 4',6'-diamidino-2-phenylindole (DAPI). The cells were visualized with a Nikon inverted microscope and the images were transferred to a computer with a digital camera (Sound Vision Inc.).

Intracellular redox parameters

Mitochondrial membrane potential - ΔΨm (JC-1 assay)

To determine the ΔΨm, cells were incubated for 30 min with JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'tetraethylbenzimidazolylcarbocyanine iodide, 2 µg/mL) as previously described (Reers *et al.* 1995). Fluorescence was measured using an excitation wavelength of 485 nm and emission wavelengths of 540 and 590 nm. The ΔΨm was calculated using the ratio of 590 nm (red fluorescent J-aggregates) to 540 nm (green monomers). The results are expressed as percentages relative to the control conditions.

DCFH oxidation

Intracellular ROS production was detected using DCFH-DA, as previously described (Quincozes-Santos *et al.* 2014). The fluorescence was measured in a plate reader with excitation at 485 nm and emission at 520 nm. The results are expressed as percentages of the control.

MitoSOX

Mitochondrial superoxide generation was determined using MitoSOX red (Molecular Probes), a mitochondrial superoxide-specific indicator, according to the manufacturers' protocols. Fluorescence was measured using excitation wavelength of 510 nm and emission wavelength of 580 nm. The results are expressed as percentages relative to the control conditions.

NOX activity

NADPH oxidase (NOX) activity was measured in cell lysate suspended in a sodium phosphate buffer with 140 mM KCl using a modified assay (Abid *et al.* 2007). This luminescence assay used lucigenin as the electron acceptor generated by the NADPH oxidase complex. The data were converted to relative light units/min/mg of protein, using a standard curve generated with xanthine/xanthine oxidase. The results are expressed as percentages relative to the control conditions.

Creatine kinase (CK) Activity

Cells were homogenized with a 0.9% saline solution and pre-incubated for 15 min at 37°C in a mixture containing: 7 mM phosphocreatine, 9 mM $MgSO_4$, 60 mM Tris–HCl buffer (pH 7.5), as previously described (Hughes 1962). The color was developed by the addition of 20% *α*-naphthol and 20% diacetyl and read after 20 min at 540 nm. Results were obtained as µmol of creatine formed/min/mg protein. The results are expressed as percentages relative to the control conditions.

Superoxide dismutase (SOD) activity

SOD activity was determined using the RANSOD kit from Randox (Autrim, UK). The results are expressed as percentages relative to the control conditions.

Catalase (CAT) activity

CAT activity was assayed by the method described previously (Aebi 1984). The results are expressed as percentages relative to the control conditions.

Glutathione peroxidase (GPx) activity

GPx activity was measured using the RANSEL kit from Randox (Autrim, UK). The results are expressed as percentages relative to the control conditions.

Protein determination

Protein content was measured using Lowry's method with bovine serum albumin as a standard (Lowry *et al.* 1951).

Statistical analyses

All data are presented as the mean ± S.E.M. Comparisons were analyzed statistically using a one-way analysis of variance (ANOVA). Tukey's test was employed for pos-hoc analysis. P-values < 0.05 were considered significant.

Results

Cortical astrocyte primary cultures were derived from mature cells

Flow cytometry was used to measure the positive cells for GFAP and vimentin in *ex vivo* cells. Comprehensively, the percentage of GFAP+ cells was significantly lower in newborn than in mature cells (Fig. 1Aa). Vimentin+ cells were increased in newborn and aged compared with adult cells (Fig. 1Ab). When

analyzing *in vitro* cells, the levels of GFAP+ cells did not differ among groups (Fig. 1Ac). The levels of vimentin were significantly higher in newborn than in mature cells (Fig. 1Ad). After, we showed that the astrocyte cultures presented immunolabeling for these glial markers (Fig. 1B).

To ensure that our adult and aged culture model was derived from mature astrocytes, we determined the number of positive cells for Sox10 in *ex vivo* and *in vitro* preparations. We observed that adult and aged astrocytes as well as *ex vivo* tissues from adult and aged rats presented less Sox10+ cells compared to newborn cultures and animals (Fig. 2A). Astrocytes from different ages presented immunolabeling for Sox10 (Fig. 2B). In addition, mature astrocytes were able to divide until reach the confluence, such as neonatal astrocytes (data not shown).

Age-dependent changes in redox parameters

Mitochondria are the primary site of reactive oxygen species (ROS) production (Droge 2002). We observed an age-dependent decrease in the ΔΨm in *ex vivo*, 25% and 32% for adult and aged rats, respectively (Fig. 3A). In addition, the same profile was found in adult (23%) and aged (35%) astrocytes *in vitro* compared to newborn cultures (Fig. 3A). As possible consequence of changes in ΔΨm, we also observed an increase with age in intracellular ROS production in *ex vivo* tissue and *in vitro* astrocytes (cellular suspension: 35% and 45% vs. astrocytes: 30% and 45% (Fig. 3B); for adult and aged, respectively).

ROS production is also closely associated with superoxide anion radical produced via the superoxide-generating enzyme, NOX. In this sense, superoxide

levels increased in cellular suspension from adult (25%) and aged (45%) rats as well as in adult (30%) and aged (50%) astrocytes, (Fig. 3C). Furthermore, NOX activity showed an increase with age in cellular suspension (adult 30% and aged 55%) and in cultured astrocytes (adult 35% and aged 55%), (Fig. 3D). The enzyme CK is a target of ROS and may undergo changes in aging process. Thus, as expected, we observed a decreased CK activity in cellular suspension (adult 18% and aged 33%, Fig. 3E) and in astrocyte cultures (adult 15% and aged 25%, Fig. 3E).

We evaluated the activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in astrocyte cultures and *ex vivo* tissue. Thus, SOD (Fig. 3F) and CAT (Fig. 3G) activities decreased with age in cellular suspension derived from adult and aged rats as well as in astrocyte cultures. Opposite trend was observed for GPx activity (Fig. 3H) in *in vitro* astrocytes. Interestingly, GPx activity did not change in cellular suspension.

Discussion

Cell cultures have significantly contributed to the understanding of brain properties. In this sense, our group has characterized the main glial functions of cultured astrocytes from mature Wistar rats (Souza et al. 2016, Souza et al. 2013, Bellaver *et al.* 2016). We previously demonstrated significant alterations in astrocytes marker proteins, metabolic and amino acid profiles, redox homeostasis, inflammatory response and signaling pathways in adult rat astrocyte cultures (Souza et al. 2016, Souza et al. 2013, Bellaver *et al.* 2016).

Here we compared our *in vitro* astrocytes at confluence with the *ex vivo* tissue that was used to prepare the cultures, and found that mature astrocytes were derived from mature cells insofar the mitochondrial functionality *in vitro* reproduces the profile observed *in vivo*. Thus, with this approach we expected better understand the role of astrocytes in brain aging under physiological and pathological conditions.

Cultured astrocytes may co-express GFAP and vimentin (Bignami & Dahl 1989). GFAP expression in newborn *ex vivo* is significantly lower compared with mature astrocytes, because glia development is boosted after birth, however, after a few days in culture, there is no longer difference between GFAP expression between ages (*in vitro* data). Vimentin expression in *ex vivo* tissue, accordingly to previously demonstrated, is increased in newborn and aged tissue compared to adult, moment when a part of vimentin is transiently replaced by GFAP. Additionally, these proteins appear to be involved in cell shape maintenance, CNS cytoarchitecture and synaptic function, properties that are affected by aging and dependent on cerebral region.

Astrocyte cultures are more likely derived from mature cells, because Sox10, a glial progenitor marker (Kim *et al.* 2003, Kuhlbrodt *et al.* 1998), decreased in mature animals and in *in vitro* cultures. Sox10 is a transcriptional modulator with predominant expression in glial cells. This marker was originally confined to glial precursors and later detected in oligodendrocytes of the adult brain (He *et al.* 2016). In this sense, oligodendrocytes have a non-significant expression of GFAP, different than our cultured astrocytes. Comparing Sox10+

cells between *ex vivo* and *in vitro* preparations, we observed that the astrocyte cultures presented twice positive cells than the *ex vivo* tissue. Our data corroborated studies that showed that Sox10 protein was detected in primary cultures of Schwann cells and oligodendrocytes as well as in immature cell lines derived from glia (Kuhlbrodt et al. 1998). In addition, astrocyte cultures may have a small number of stem cells or progenitors cells that have the capacity to generate differentiated cells. Sox10 performs many essential functions during development and may drive gliogenesis in nervous system. The cellular suspension from mature rats showed less Sox10+ cells than newborn rats, which is possibly related to a decline in brain plasticity. Therefore, our cellular culture model reproduces the changes observed along the brain aging.

Classically, mitochondrial dysfunction and excessive ROS generation were implicated in brain aging, because both are a common feature associated with excitotoxicity and inflammation (Halliwell 2001, Halliwell 2006). The $\Delta\Psi_m$ is a strong indicative of mitochondrial impairment and we observed an age-dependent decrease in both *ex vivo* and *in vitro* preparations. Further, mitochondrial dysfunction is associated with ROS overproduction and failure in NOX activity that induces cellular superoxide production (Droge 2002). Moreover, superoxide generated by NOX activation can lead to severe oxidative damage and neural death. Excessive oxidative/nitrosative stress may impair cellular biomolecules, consequently, these data support that the changes observed in redox homeostasis of mature Wistar rats were also detected in astrocyte

cultures, showing the remarkable relevance of this tool for understanding glial functionality.

Regarding CK, our results showed a reduced activity both in *ex vivo* and *in vitro*. CK plays an important role in cellular energy homeostasis, being mainly responsible for ATP buffering. It should be emphasized that CK contains essential cysteine thiol groups for its full operation that are highly vulnerable to oxidation. Although astrocytes are the major source of glutathione (GSH), the main thiol-reducing antioxidant in CNS, GSH decreases with aging and age-related neurological diseases as well as CK activity, thus, both play a critical role in brain pathologies related to redox imbalance (Dringen 2000).

The oxidative damage previously observed during aging may be related to changes in repair system, such as SOD, CAT and GPx (Droge 2002, Dringen *et al.* 2005). We demonstrated an age-dependent decrease in SOD and CAT activities in *ex vivo* tissue reproduced in cultured astrocytes that may indicate an overproduction of superoxide and H_2O_2, which may cause severe cellular injury. Remarkably, GPx activity did not differ between groups in *ex vivo* tissue, probably by the presence of other cell populations than astrocytes. The increased GPx activity observed in mature cells *in vitro* may decrease levels of GSH, which can contribute to astrocyte dysfunction associated to aging (Dringen 2000).

In summary, the results obtained from mature astrocyte cultures showed that this tool markedly represents the changes observed in astrocytes with the development as well as aging process. After a careful characterization of the

main astrocytic properties of adult and aged astrocytes, here, we demonstrated that these cells are derived from mature cells; being a powerful tool to study aging, allowing a better comparison between *in vivo* and *in vitro* interventions. Moreover, adult and aged cultured astrocytes emerge as target for studies involving age-related neurological diseases.

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Conflict of interest

The authors declare there are no conflicts of interest.

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Figure legends

Fig 1. GFAP and vimentin in astrocytes. Cells were obtained from tissue dissociation (*ex vivo*) or from confluent astrocyte cultures (*in vitro*) and evaluated as described in Materials and Methods section. A) Percentage of GFAP+ and vimentin+ cells is relative to total number of events and represent mean + SEM of three independent experimental determinations, performed in duplicate, and analyzed statistically by one-way ANOVA followed by Tukey's test. *$p < 0.05$; #$p < 0.001$ refers to statistically significant differences from NB. B) At confluence, astrocytes show cytoskeleton immunolabeling to GFAP and vimentin, which colocalize. Scale bar = 50 μm.

Fig 2. Sox10 in astrocytes. Cells were obtained from tissue dissociation (*ex vivo*) or from confluent astrocyte cultures (*in vitro*) and evaluated as described in Materials and Methods section. A) Percentage of Sox10+ cells is relative to total number of events and represents mean + SEM of three independent experimental determinations, performed in duplicate, and analyzed statistically by one-way ANOVA followed by Tukey's test. #$p < 0.001$ refers to statistically significant differences from NB. B) At confluence, astrocytes show faint immunolabeling to SOX10. Scale bar = 50 μm.

Fig 3. Mitochondrial parameters *ex vivo* and *in vitro*. Cells were obtained from tissue dissociation (*ex vivo*) or from confluent astrocyte cultures (*in vitro*)

and evaluated as described in Materials and Methods section. A) Membrane potential ($\Delta\Psi_m$); B) ROS levels; C) Superoxide levels; D) NOX activity; E) CK activity; F) SOD activity; G) CAT activity; H) GPx activity. Data represents mean + SEM of three independent experimental determinations, performed in duplicate, and analyzed statistically by one-way ANOVA followed by Tukey's test. *$p < 0.05$ refers to statistically significant differences from NB.

Figure 1.

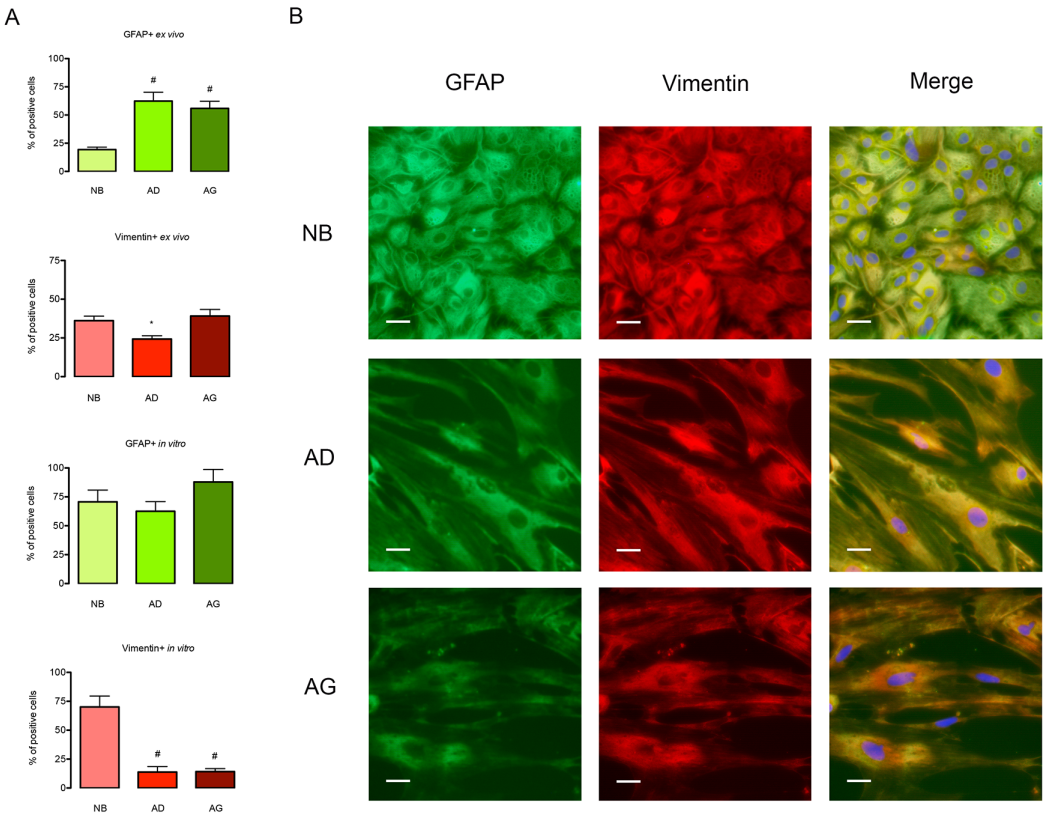
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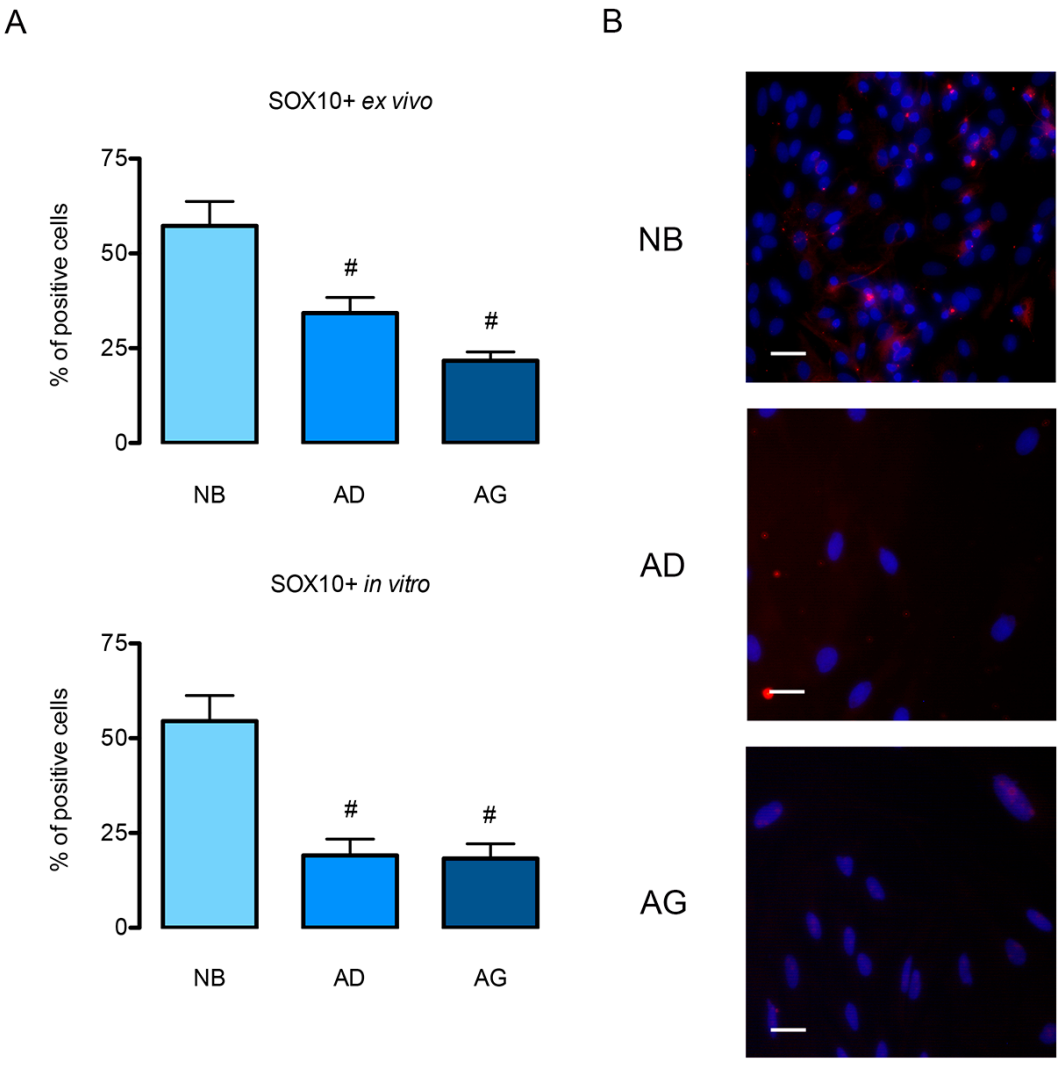
Figure 2.


A
B
SOX10+ ex vivo
% of positive cells
75
50
25
0
#
#
NB
AD
AG
SOX10+ in vitro
% of positive cells
75
50
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NB
AD
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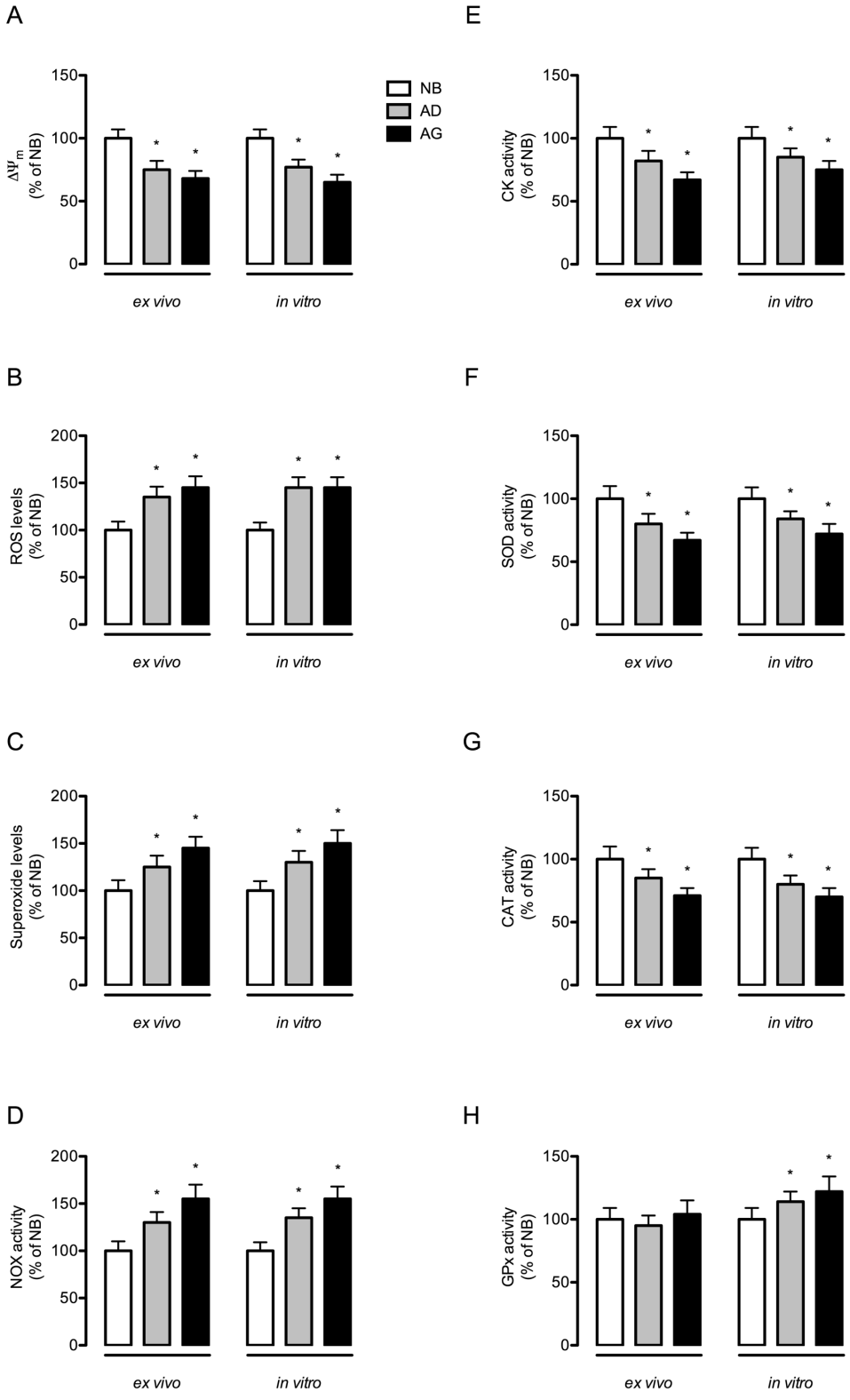
NB
AD
AG

Figure 3.


A
B
C
D
E
F
G
H
NB
AD
AG
$\Delta\Psi_m$ (% of NB)
ROS levels (% of NB)
Superoxide levels (% of NB)
NOX activity (% of NB)
CK activity (% of NB)
SOD activity (% of NB)
CAT activity (% of NB)
GPx activity (% of NB)
ex vivo
in vitro

CAPÍTULO 3

Artigo a ser submetido ao periódico Neuroscience

Differential age-dependent effects of a short glutamate exposure on primary cultures of rat cortical astrocytes

Débora Guerini Souza, Bruna Bellaver, Larissa Daniele Bobermin, Luc Pellerin, Diogo Onofre Souza, André Quincozes-Santos

Differential age-dependent effects of a short glutamate exposure on primary cultures of rat cortical astrocytes

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Abstract

The excitatory neurotransmitter glutamate (Glu) plays a major role in brain function and its recycling depends on neuron-glia interactions. Astrocytes express high-affinity Glu transporters, allowing them to remove the neurotransmitter from the synaptic cleft. Indeed, they are central players in Glu metabolism. Thus, astrocytes are the main site for glutathione (GSH) biosynthesis (with Glu as precursor) and they express the enzyme responsible for converting Glu into glutamine (glutamine synthetase – GS). Considering their central role in brain homeostasis, astrocytic function might undergo changes during aging, which might contribute to some neuropathologies. In this study, we evaluated the age-dependent effects of a short-term Glu exposure on specific metabolic and inflammation-related parameters in astrocyte cortical cultures from newborn (NB), adult (AD) and aged (AG) Wistar rats. NB and AD astrocytes exhibited increased glucose uptake and utilization following Glu exposure compared to their basal levels, although their Glu uptake capacity was reduced by the treatment. In contrast, glucose uptake and utilization was reduced in AG astrocytes following Glu exposure to undetectable levels, while their Glu uptake capacity was unaffected by the treatment. In parallel, AG astrocytes presented higher reactive oxygen species (ROS) generation than NB cells. Interestingly, AD and AG astrocytes showed higher GSH content than NB cells. Additionally, AG astrocytes showed a markedly increased inflammatory response after Glu exposure. Indeed, changes in NFκB activation and p38 levels were observed, suggesting that these pathways may explain at least in part the age-dependent responses to Glu exposure in astrocytes. Our results suggest that

various astrocytic responses to a Glu stimulus are dependent of cellular maturity. Therefore, primary cultures derived from adult and/or aged animals represent a useful and powerful tool to investigate the biochemical and physiological properties of brain development and aging.

Key words: Adult astrocytes, Glutamate, Age-dependent metabolism, Glial function

Abbreviations used: Glu, L-Glutamate; GS, glutamine synthetase; GSH, glutathione; NB, newborn; AD, adult; AG, aged; ROS, reactive oxygen species; NFκB, nuclear factor κB; MAPK, mitogen-activated protein kinase; CNS, central nervous system; 2DG, 2-deoxyglucose; GLAST, glutamate-aspartate transporter; GLT1, glutamate transporter 1; IL1β interleukin 1β; TNFα tumor necrosis factor α.

Introduction

Glutamate (Glu) is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) (Danbolt, 2001). Glu activates neuronal ionotropic and metabotropic receptors at excitatory synapses, and high affinity transporters predominantly located on astrocytes contribute to remove the neurotransmitter from the synaptic cleft, allowing the recycling of Glu and the maintenance of its physiological roles in the brain (Anderson & Swanson, 2000; Benarroch, 2010; Danbolt, 2001). Thus, astrocytes play a pivotal role in preserving glutamatergic signaling and protecting neurons, since overactivation of neuronal receptors can be detrimental to cells, leading to excitotoxicity with the generation of oxidative stress, an inflammatory response and, consequently, cell death (Coyle & Puttfarcken, 1993; Lewerenz & Maher, 2015; Mehta et al, 2013; Sattler & Rothstein, 2006).

As previously shown in astrocyte cultures, Glu increases 2-Deoxy-D-[1,2-^{3}H]glucose (2DG) uptake and phosphorylation, a metabolic effect triggered by Glu uptake, via high affinity transporters (Pellerin & Magistretti, 1994; Souza et al, 2013). The main Glu transporters present *in vitro* in mature astrocyte cultures are the glutamate-aspartate transporter (GLAST) and the glutamate transporter 1 (GLT1) (Benarroch, 2010; Danbolt, 2001; Souza et al, 2013). The nuclear factor κB (NFκB) is a transcription factor strongly associated to inflammatory, immune and stress-related responses (Farina et al, 2007; Kaltschmidt & Kaltschmidt, 2009; Mattson & Camandola, 2001), and is an essential effector of Glu actions in the CNS. Ghosh and colleagues have suggested a role for the NFκB signaling pathway in the modulation of GLT1 expression in astrocytes (Ghosh et al, 2011). In addition, this transcription

factor has previously been shown to be involved in excitatory signaling in hippocampal neurons, being required in processes of learning and memory (Boersma et al, 2011; Gutierrez et al, 2005). Moreover, oxidative/nitrosative stress and inflammatory responses increase with aging and NFκB may have an essential role in these events, together with p38, which may regulate NFκB activation (Gorina et al, 2011; Jiang & Cadenas, 2014; Miller & O'Callaghan, 2005).

Astrocyte cultures from adult (AD) and aged (AG) rats represent interesting *in* vitro models to study the features of mature brain. The mature cells show distinct properties compared to astrocytes derived from newborn (NB) rats, since they are less plastic to respond to different challenges, being therefore, more representative of the mature cerebral tissue. In this sense, our group has previously shown the usefulness of this routine protocol to study glial cell functions (Bellaver et al, 2014; Souza et al, 2016; Souza et al, 2013). Although NB astrocytes have been a very useful tool to study Glu metabolism, astrocyte cultures from mature animals may allow a better understanding of acute events, such as ischemia, or chronic events, such as neurodegenerative diseases, classically associated with the adult and aging brain.

In vivo, astrocytes are exposed to Glu concentrations in the micromolar to the milimolar range (Belanger & Magistretti, 2009; Lewerenz & Maher, 2015), since they are active players of the tripartite synapse. In order to investigate how astrocytes prepared from animals at different ages respond to a short-term exposure to an average Glu concentration, we challenged astrocytes from NB (1 day old), AD (90 days old) and AG (180 days old)

Wistar rats with 500 μM Glu for 20 min and investigated 2DG and Glu uptake, intracellular reactive oxygen species (ROS) generation, glutamine synthetase (GS) activity, glutathione (GSH) content, cytokines levels and the putative mechanisms associating Glu effects with NFκB transcriptional activity and p38 mitogen-activated protein kinase (MAPK) signaling pathway.

Experimental procedures

Chemicals

Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) and other materials for cell culture were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Papain was acquired from Merck (Darmstadt, Germany). DNase, cysteine, glutamate, albumin, polyclonal anti-β-tubulin III and monoclonal anti-GAPDH were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-[^{3}H]-glutamate, 2-Deoxy-D-[1,2^{3}H]glucose ([^{3}H]2DG), nitrocellulose membrane and ECL kit were purchased from Amersham (Buckinghamshire, UK). Monoclonal anti-NeuN was from Merck Millipore (Darmstadt, Germany). Monoclonal anti-CD11 was from Invitrogen (CA, USA). Polyclonal anti-GLT1 was from Alpha Diagnostic (San Antonio, TX, USA). Polyclonal anti-GFAP was from Dako (Glostrup, Denmark). Alexa Fluor® 488 (A_{max} = 493; E_{max} = 519)-conjugated AffiniPure antibody was from Jackson ImmunoResearch (West Grove, PA, USA). All other chemicals were from common commercial suppliers.

Animals

Male Wistar rats (1, 90 and 180 days old) were obtained from our breeding colony (Department of Biochemistry, UFRGS, Brazil), maintained under a controlled environment (12 h light/12 h dark cycle; 22 ± 1°C; *ad libitum* access to food and water). All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Federal University of Rio Grande do Sul Animal Care and Use Committee (process number 24419).

Cell culture preparation and maintenance

Male Wistar rats (1, 90 and 180 days old) were euthanized by decapitation, had their cerebral cortices aseptically dissected and meninges removed (Souza et al, 2015). The tissue was digested using trypsin only – for newborn tissue – or trypsin and papain – for adult tissue – at 37ºC as previously described (Souza et al, 2013). After mechanical dissociation and centrifugation, the cells were resuspended in DMEM/F12 [10% fetal bovine serum (FBS), 15 mM HEPES, 14.3 mM $NaHCO_3$, 1% Fungizone and 0.04% gentamicin], plated on 6- or 24-well plates pre-coated with poly-L-lysine and cultured at 37°C in a 5% CO_2 incubator. The cells were seeded at a density of 3–5 x 10^5 cells/cm^2. Twenty-four hours later, the culture medium was exchanged; during the 1st week, the medium was replaced once every two days and from the 2nd week on, once every four days. From the 3rd week on, the astrocytes received medium supplemented with 20% FBS until they reached confluence (at approximately the 4th week). No dibutyryl cAMP was added to the culture medium in order to observe the naive response of the cells. Specific proteins markers for neurons (NeuN and β-tubulin III) and

microglia (CD11) were tested in order to determine the purity of the astrocytes culture, which was around 95% (data not shown).

Immunofluorescence

This assay was performed as described previously (Souza et al, 2013). Briefly, cell cultures were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. After blocking overnight with 4% albumin, the cells were incubated overnight with anti-GFAP (1:400) at 4°C, followed by PBS washes and incubation with a specific secondary antibody conjugated with Alexa Fluor® 488 for 1 h at room temperature. Cell nuclei were stained with 0.2 mg/ml of 4',6'-diamidino-2-phenylindole (DAPI). The cells were visualized with a Nikon inverted microscope and the images were transferred to a computer with a digital camera (Sound Vision Inc.).

Glutamate treatment

In order to investigate how primary astrocytes respond to a brief treatment with glutamate, we exposed the cells to 500 μM L-Glutamate (Glu) in DMEM/F12 1% FBS for 20 min at 37°C before all measurements. Prior to Glu exposure, cells were rinsed once with PBS and incubated with DMEM/F12 1% FBS for 2 h at 37°C. Alternatively, whenever indicated, the same procedure was carried out in the presence of 500 μM D-Aspartate (D-Asp).

2-Deoxy-D-[1,2-^{3}H]glucose (2DG) uptake and phosphorylation assay

After cells reached confluence, basal Glu-stimulated and D-Asp-stimulated 2DG uptake and phosphorylation was assessed as previously described (Souza et al, 2013). Briefly, the cells were rinsed once with PBS and incubated with DMEM/F12 1% FBS for 2 h at 37°C. For basal uptake, the medium was replaced by DMEM/F12 1% FBS containing 1 mCi/ml [^{3}H]-2DG for 20 min at 37°C. For stimulated 2DG uptake and phosphorylation, the medium was then replaced by DMEM/F12 supplemented with 1% FBS containing 1 mCi/mL [^{3}H]-2DG, in the presence of 500 µM Glu or D-Asp for 20 min at 37°C. After incubation, the cells were rinsed with HBSS and lysed overnight with NaOH 0.5 M. Intracellularly accumulated radioactivity was measured in a scintillation counter. Cytochalasin B (10 µM) was employed as a specific glucose transporter inhibitor. 2DG uptake and phosphorylation was determined by subtracting uptake with cytochalasin B from total uptake. Results were calculated as fmol/mg protein and expressed as percentage of NB under basal condition.

Glutamate uptake assay

After Glu challenge, the Glu uptake was performed as previously described (Souza et al, 2013). Briefly, the cells were rinsed once with PBS and were incubated at 37°C in HBSS containing the following components (in mM): 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 Na_2HPO_4, 0.44 KH_2PO_4, 4.17 $NaHCO_3$ and 5.6 glucose, adjusted to pH 7.4. The assay was started by the addition of 0.1 mM Glu and 0.33 mCi/mL L-[2,3-^{3}H] glutamate. The incubation was stopped after 7 min by removal of the medium and rinsing the cells twice with ice-cold HBSS. The cells were then

lysed in a solution containing 0.5 M NaOH. Incorporated radioactivity was measured in a scintillation counter. Sodium-independent uptake was determined using ice-cold N-methyl-D-glucamine instead of sodium chloride. Sodium-dependent Glu uptake, considered ‘‘specific uptake’’, was obtained by subtracting the sodium-independent uptake from the total uptake. Results were calculated as nmol/mg protein/min and expressed as percentage of NB under basal conditions.

Glutamine Synthetase Activity

After Glu exposure, the enzymatic assay was performed as previously described (Souza et al, 2013). Briefly, 0.1 mL of cell homogenate was added to 0.1 mL of the reaction mixture containing (in mM): 10 $MgCl_2$, 50 Glu, 100 imidazole-HCl buffer (pH 7.4), 10 2-mercaptoethanol, 50 hydroxylamine-HCl and 10 ATP, and incubated for 15 min (37°C). The reaction was stopped by the addition of 0.4 mL of a solution containing (in mM): 370 ferric chloride, 670 HCl, and 200 trichloroacetic acid. After centrifugation, the absorbance of the supernatant was measured at 530 nm and compared to the absorbance generated using standard quantities of γ-glutamylhydroxamate treated with a ferric chloride reagent. Results were calculated as μmol/mg protein/h and expressed as percentage of NB under basal conditions.

Western blot

Cells were solubilized in lyses solution containing 4% SDS, 2 mM EDTA, 50 mM Tris-HCl (pH 6.8). Protein content was measured, the samples were standardized in sample buffer [62.5 mM Tris-HCl (pH 6.8), 4% (v/v)

glycerol, 0.002% (w/v) bromophenol blue] and boiled at 95°C for 5 min. Samples were separated by SDS/PAGE (30 µg protein per sample), and transferred to nitrocellulose membranes, as previously described (Souza et al, 2016). Adequate loading of each sample was confirmed using Ponceau S staining. Membranes were incubated overnight (4°C) with anti-GLT1 (1:1000) and GAPDH (1:1000). The membranes were then washed and incubated with a peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulin in a dilution of 1:3000 for 2 h. Chemiluminescence signals were detected in an Image Quant LAS4000 system (GE Healthcare) using ECL kit.

Glutathione content

After Glu exposure, GSH levels were assessed as previously described (Souza et al, 2015). Cell lisates were diluted in 100 mM sodium phosphate buffer (pH 8.0) containing 5 mM EDTA, and the protein was precipitated with 1.7% meta-phosphoric acid. The supernatant was assayed with o-phthaldialdehyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0 – 500 mM). GSH concentrations were calculated as nmol/mg protein.

Intracellular ROS levels

Intracellular ROS production was detected using the non-fluorescent cell permeating compound, 2'-7'-dichlorofluorescein diacetate (DCFH-DA) on cells under basal conditions or treated with 500 µM Glu for 20 min (Bellaver et

al, 2015). DCFH-DA is hydrolyzed by intracellular esterases to dichlorofluorescin (DCFH), which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by the action of cellular oxidants. Astrocytes were treated with DCFH-DA (10 μM) for 30 min at 37°C. Following DCFH-DA exposure, the cells were scraped into PBS containing 0.2% Triton X-100. Fluorescence was measured using a plate reader (Spectra Max M5, Molecular Devices) at excitation and emission wavelengths of 485 nm and 520 nm, respectively.

NFκB and p38 MAPK measurement

The levels of NFκB p65 in the nuclear fraction, which had been isolated from lysed cells with Igepal CA-630 and centrifugation (following manufacturer's instructions), were measured using an ELISA commercial kit from Invitrogen. p38 MAPK levels were measured in cell lysate using rat p38 MAPK ELISA commercial kit from Sigma Aldrich. The results are expressed as percentages relative to the basal conditions.

Inflammatory response

The tumor necrosis factor α (TNFα) levels were measured in extracellular medium using rat TNFα ELISA kit from Peprotech. The levels of interleukin 1β (IL1β) and interleukin 6 (IL6) were measured in extracellular medium using rat ELISA kits from eBioscience. The results are expressed in ng/mL.

Protein assay

Protein content was measured using Bicinchoninic Acid method with bovine serum albumin as a standard (Smith et al, 1985).

Statistical analyses

Data were statistically analyzed using one or two-way analysis of variance (ANOVA), followed by the Tukey's test. P-values < 0.05 were considered significant. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software version 15.0.

Results

Effects of Glutamate exposure on 2DG uptake and phosphorylation in primary cultures of astrocytes from newborn, adult and aged animals

Primary cultures of rat cortical astrocytes prepared from newborn, adult and aged animals exhibited the typical morphology of astrocytes *in vitro* and comparable GFAP expression as revealed by immunofluorescence (Fig. 1). Glucose uptake (and its eventual conversion to lactate to sustain neuronal energy needs) is a well-established role of astrocytes and it is facilitated by the strategic position of astrocytic end-feet, which cover almost the totality of blood vessels surface in the brain parenchyma (Benarroch, 2014). Glu, a classical activator of glucose metabolism in astrocytes, was able to increase 2DG uptake and phosphorylation in NB and AD astrocytes, by approximately 260% and 360% compared to basal uptake and phosphorylation, respectively (Fig. 2). In contrast, AG astrocytes did not exhibit any 2DG uptake and phosphorylation in presence of Glu, although they had a basal 2DG uptake and phosphorylation, but smaller than NB and AD astrocytes. Alternatively,

cells were exposed to D-Asp (a transported but non-metabolizable analog of Glu). A comparable effect to Glu was observed in NB astrocytes while in AD astrocytes, D-Asp induced a significant higher 2DG uptake and phosphorylation compared to Glu, increasing it by approximately 1250% compared to basal 2DG uptake and phosphorylation. Upon D-Asp exposure, AG cells did not display detectable 2DG uptake and phosphorylation. It is important to note that there were no changes in cellular membrane integrity in NB, AD or AG cultures (data not shown).

Glutamate exposure reduced subsequent glutamate uptake in NB and AD astrocytes but not in AG astrocytes while it did not alter the GS activity profile at any age

In order to evaluate Glu metabolism, we first determined the age-dependent expression of GLT1 in astrocytes (Fig. 3A). AG presented significantly higher expression of GLT1 ($P<0.0001$) compared to newborn and adult astrocytes. In addition, GLT1 protein levels did not change after a short-term (20 min) Glu treatment (data not shown). Next, we investigated Glu uptake and GS activity following Glu exposure. First, AD and AG astrocytes exhibited an age-dependent decrease of Glu uptake, compared to NB (Fig. 3B). Interestingly, Glu exposure decreased Glu uptake in NB and AD astrocytes. In contrast, AG astrocytes did not respond to Glu exposure as their Glu uptake remained the same as without Glu exposure. Additionally, GS activity also decreased in an age-dependent manner, but it did not change following Glu exposure (Fig. 3C).

Glutamate exposure triggered oxidative stress in AG astrocytes

In order to evaluate the involvement of oxidative stress in Glu effects, and, consequently, a putative Glu fate, we measured GSH content, which is one of the major reducing defenses in the CNS. Higher GSH levels were found in AD and AG astrocytes compared to NB cells (Fig. 4). Surprisingly, Glu treatment had no effect in GSH levels although Glu is a precursor for GSH synthesis. When the intracellular ROS production was evaluated, AG cells presented higher intracellular ROS levels compared to the other groups, and they were further enhanced after Glu exposure (Fig. 4B).

Glutamate exposure activated NFkB and p38 MAPK pathways in aged astrocytes

In order to identify the putative signaling pathways activated by Glu exposure that could explain the previous effects, we measured the expression levels of both NFκB and p38 MAPK. Glu induced an age-dependent increase in NFκB levels (approximately 10% in NB, 25% in AD and 60% in AG, Fig. 5A). AG astrocytes exposed to Glu also presented higher NFκB levels compared to NB/AD cells (Fig. 5A). Concomitantly, higher p38 MAPK levels were observed in AG astrocytes compared to NB/AD cells, which were further enhanced after Glu treatment (Fig. 5B).

Glutamate exposure induced an inflammatory response in AG astrocytes

Since there was a significant activation of the NFκB signaling pathway, which typically stimulates pro-inflammatory cytokine production and release,

we measured the levels of the main pro-inflammatory cytokines, TNFα, IL1β and IL6 (Fig. 6). TNFα and IL1β levels significantly increased after Glu exposure of AG astrocytes, showing a strong inflammatory response. IL6 levels did not change following Glu exposure, and the levels of cytokines did not differ between groups under basal conditions.

Discussion

Glutamate (Glu) plays an important role in the CNS both as a neuro and a gliotransmitter (Harada et al, 2016; Willard & Koochekpour, 2013; Zorec et al, 2015). As the major excitatory neurotransmitter in the brain, Glu is involved in several functions such as development, learning, memory and behavior. It also fulfills other roles such as a metabolic substrate and precursor of GSH and GABA (Bak et al, 2006; Dringen, 2000). It was previously shown that astrocyte functions might undergo changes with aging (Jiang & Cadenas, 2014; Stanimirovic et al, 1999). Consequently, astrocytes might differentially respond to stimuli in an age-dependent manner. The purpose of this study was to show the age-dependent effects of Glu on primary cultures of rat cortical astrocyte prepared from NB, AD and AG Wistar rats.

Astrocytes, the most versatile glial cells, play several roles in brain homeostasis, such as neurotransmitter and energetic substrate metabolism, antioxidant defenses and inflammatory response (De Pitta et al, 2015; Halassa & Haydon, 2010; Parpura et al, 2012; Pellerin, 2005; Wang & Bordey, 2008). Moreover, these cells are intimately related to the Glu production and clearance. They possess the pyruvate carboxylase enzyme,

which allows the replenishment of Glu from glucose, and they express high affinity Glu transporters (GLAST and GLT1), which remove the neurotransmitter from the synaptic cleft, thus avoiding excitotoxicity (Anderson & Swanson, 2000; Danbolt, 2001).

Astrocytes are involved in the regulation of energy metabolism and Glu has been shown to modulate some energetic properties of astrocytes *in vitro* (Porras et al, 2004; Tescarollo et al, 2014). Glu has been proven to stimulate 2DG uptake and phosphorylation by astrocytes, since Na^+ ions are co-transported with Glu through GLAST and GLT1, in an energy-coupled mechanism (Benarroch 2010, Pellerin 1994). The intracellular concentration of Na^+ increases following Glu uptake and activation of the Na^+/K^+ ATPase is responsible for reestablishing the Na^+ gradient at the expense of ATP consumption (Chatton et al, 2000; Pellerin & Magistretti, 1997). As previously shown, AD astrocytes take up less 2DG than NB astrocytes under basal conditions (Souza et al, 2013). Our data showing that AD but also AG astrocytes take up less 2DG than NB cells confirm and further extend this observation. As expected, there was an increase in 2DG uptake and phosphorylation following Glu exposure in both NB and AD cells, but interestingly, AG cells exhibited reduced 2DG uptake and phosphorylation to undetectable levels upon Glu exposure.

Alternatively, we employed D-Asp as a stimulus for 2DG uptake measurements. NB astrocytes exhibited an increase in 2DG uptake and phosphorylation of similar magnitude than with Glu. However, AD cells had a significantly more important increase in 2DG uptake and phosphorylation following D-Asp exposure compared to the response obtained with Glu. A

possible explanation for this effect could be that since Glu is an energetic substrate and D-Asp is not, astrocytes would need to derive more energy from glucose in the case of D-Asp uptake. This also indicates that AD cells significantly rely more than NB cells on energy generation from Glu oxidation to meet energetic requirements during Glu exposure (Swanson et al, 1990). AG cells responded to D-Asp exposure with a similar reduction in 2DG uptake and phosphorylation as with Glu compared to the basal levels, showing that some other cellular aspects, common to both substances, are altered, although it is difficult to identify which ones at this stage.

Concerning Glu transport, only AG astrocytes preserved their uptake capacity after Glu exposure. Such an intact Glu transport capacity could be an attempt of AG cells to maintain this important function, although it appears dissociated from the metabolic coupling role observed in NB and AD cells. However, we cannot exclude that AG astrocytes might take longer to respond to a Glu stimulus with enhanced glucose utilization. Such a putative delayed metabolic response would need to be evaluated with longer exposure times. Of note, the activation of NFκB and p38 pathways could have an impact on the maintenance of Glu uptake in AG cells, since these signaling pathways have been implicated in GLT1 activation, the Glu transporter present in mature astrocytes (Souza et al, 2016; Souza et al, 2013).

NB and AD cells respond to Glu exposure by increasing 2DG uptake, although a decrease in Glu uptake is observed. For NB astrocytes, the Glu uptake decrease may be explained as a compensatory mechanism for maintenance of glucose levels for the cells. However, Glu uptake was reported to decrease with aging, most likely due to stress conditions

correlated with impairment in Glu transporter activity (Pertusa et al, 2007; Souza et al, 2013; Trotti et al, 1998).

The aging process is accompanied by a hypometabolic state (Jiang & Cadenas, 2014), and here we showed that in AG cells Glu exposure does not lead to a increased 2DG uptake and phosphorylation but rather to a decrease, which attests that these cells are hypometabolic, such as are aged brains. This can also be evident in the activities of some enzymes. GS, an intracellular enzyme of extreme importance for the glutamate-glutamine cycle, has a reduced activity in mature cells compared with NB astrocytes (Souza et al, 2013). Although GS is essential for recycling Glu and is exclusively found in astrocytes in the CNS, it did not show alteration after Glu exposure. Since Glu uptake was not increased after Glu exposure, it is not surprising that the activity of GS did not increase in any group, considering also that GSH synthesis is a possible fate of Glu in astrocytes, and GSH levels were indeed higher in AD and AG cells compared to NB astrocytes. This fact points to a change in mature astrocytes, since they show a preference for keeping high levels of antioxidant defenses than keeping high GS activity.

Oxidative stress, a common feature frequently present in neurodegenerative diseases, occurs when an increase in ROS takes place that cannot be neutralized by cellular defenses. Astrocytes have a critical role in the control of this process (Halliwell, 2001; Halliwell, 2006). When the GSH content and intracellular ROS production was assessed in astrocytes exposed to Glu, higher GSH levels in AD and AG astrocytes was found compared to NB. We have previously shown that mature astrocytes aged *in vitro* have a higher GSH content than NB astrocytes. Glu exposure was able to further

increase GSH levels in AD and AG cells, which might represent an effort of cells to protect themselves against ROS. Accordingly, our group and a previous study have shown that AD and AG astrocytes possess higher content of xCT, a subunit of system x_c^-, which contributes to intracellular availability of substrates for GSH synthesis (Lewerenz et al, 2006; Souza et al, 2015). Considering that aging causes an augmentation of ROS generation, the increased GSH content in mature cells might prevent oxidative stress-mediated damage to cellular biomolecules and inflammation (Halliwell, 2001; Lee et al, 2010).

AG cells exhibited elevated ROS production under basal conditions compared to NB and AD cells while it is further enhanced after Glu exposure. Considering changes brain undergoes throughout the aging process, it is expected that the levels of ROS increase in mature cells compared with neonatal cells (Bellaver et al, 2014; Klamt et al, 2002). ROS have a key role in the onset and progression of several neurodegenerative diseases. Our data demonstrated that AG astrocytes exhibited oxidative stress, exacerbated by a short-term exposure to metabolic concentrations of Glu, which is not the case for NB and AD astrocytes. These results highlight the usefulness of astrocyte cultures from rats of different ages to decipher the specific processes of aging.

To identify which mechanisms could be implicated in the effect of aging on Glu metabolism, we investigated the activation of NFκB, which was altered in AG astrocytes and in all groups after Glu exposure, showing that this pathway is pivotal for astrocytic handling of Glu (Ghosh et al, 2011). NFκB, in addition of being the major inflammatory mediator of the CNS, is also related

to the astrocytic response to Glu (Caccamo et al, 2005) and, importantly, to the increased expression and activity of GLT1. It is noteworthy that GLT1 presented an age-dependent level of expression in astrocytes under basal conditions. Here we provide the first evidence that Glu treatment at a non-toxic concentration activated NFκB pathway in an age-dependent manner.

The MAPK pathway also displays a critical role in the crosstalk between glutamate metabolism, oxidative stress and inflammation, because p38 MAPK regulates NFκB translocation (Gorina et al, 2011). p38 MAPK levels were higher in AG astrocytes than in NB and AD astrocytes, both under basal conditions and after Glu exposure. Since this signaling pathway is directly related to Glu uptake and NFκB (Ghosh et al, 2011), this high level of expression might explain why AG cells did not decrease Glu uptake after Glu exposure, similarly to the other groups. In line with the previous results, Glu also selectively elevated p38 MAPK levels in AG astrocytes, an effect which might be related to the distinct responses already observed in AG astrocytes, regarding Glu metabolism, oxidative stress and inflammation.

Key mediators of the inflammatory response were measured after Glu exposure and we observed a significant increase of TNFα and IL1β in AG astrocytes only. In this case, the glutamatergic stimulus elicited an increase in pro-inflammatory cytokine levels along with the increase in oxidative stress, two key factors related to the onset of brain pathologies (Coyle & Puttfarcken, 1993; Lewerenz & Maher, 2015). This result corroborates with our data about NFκB and p38 MAPK levels that are increased in these cells. It is important to note that glucose homeostasis might be affected under inflammatory

conditions and this might explain why AG cells respond to Glu exposure with a reduction in 2DG uptake and phosphorylation rather than an increase.

During the course of acute brain pathologies and long-term neurodegenerative diseases, *e.g.* stroke, epilepsy, Alzheimer's, Parkinson's and Huntington's diseases, it is common to find impairments of the glutamatergic system with major detrimental effects for brain function as a whole (Benarroch, 2010; Mehta et al, 2013; Segovia et al, 2001). In this study we showed that a short Glu exposure induces an age-dependent metabolic, oxidative and inflammatory responses, most likely through activation of the NFκB pathway in primary astrocytes. Moreover, it supports our previous findings which suggested that astrocyte cultures from mature animals are a powerful tool to understand the biochemical and physiological properties of brain development and aging.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1 **Astrocytic morphology.** Cells were prepared as described in the Materials and Methods section. **(a)** NB; **(b)** AD and **(c)** AG astrocytes showed GFAP expression and typical morphology under basal conditions. NB = newborn; AD = adult and AG = aged. Scale bar: 50 μM.

Fig. 2 **The effect of Glu and D-Asp challenge in 2-deoxyglucose (2DG) uptake.** Cells were pre-treated with 500 μM Glu or D-Asp for 20 min. 2DG uptake was measured as described in the Materials and Methods section. The data represent the mean ± SEM of three independent experimental determinations, performed in triplicate. The data were analyzed statistically using two-way ANOVA followed by Tukey's test. *a* indicates difference from NB under basal conditions; *b* indicates difference between unchallenged and challenged with Glu and *c* indicates age-dependent treatment difference.

Fig. 3 **Glu metabolism. (a)** under basal conditions, NB, AD and AG astrocytes express GLT1; the top panel shows representative images of the blots. In **b** and **c** cells were pre-treated with 500 μM Glu for 20 min. **(b)** Glu uptake and **(c)** GS activity were measured as described in the Materials and Methods section. The data represent the mean ± SEM of three independent experimental determinations, performed in triplicate. The data were analyzed statistically using one or two-way ANOVA followed by Tukey's test. NB = newborn; AD = adult and AG = aged. *a* indicates difference from NB under basal conditions; *b* indicates difference between unchallenged and challenged

with Glu and *c* indicates age-dependent treatment difference. *** indicates P<0.0001 compared with the other groups.

Fig. 4 **The effect of Glu challenge in GSH content and ROS generation.** Cells were pre-treated with 500 μM Glu for 20 min. (a) GSH content and (b) Intracellular ROS levels were measured as described in the Materials and Methods section. The data represent the mean ± SEM of three independent experimental determinations, performed in triplicate. The data were analyzed statistically using two-way ANOVA followed by Tukey's test. *a* indicates difference from NB under basal conditions and *c* indicates age-dependent treatment difference.

Fig. 5 **Glu challenge activated NFκB and p38 MAPK signaling pathways.** Cells were pre-treated with 500 μM Glu for 20 min. (a) NFκB and (b) p38 MAPK were measured as described in the Materials and Methods section. The data represent the mean ± SEM of three independent experimental determinations, performed in triplicate. The data were analyzed statistically using two-way ANOVA followed by Tukey's test. *a* indicates difference from NB under basal conditions; *b* indicates difference between unchallenged and challenged with Glu and *c* indicates age-dependent treatment difference.

Fig. 6 **The effect of Glu challenge in inflammatory response.** Cells were pre-treated with 500 μM Glu for 20 min. (a) TNFα; (b) IL1β and (c) IL6 levels were measured as described in the Materials and Methods section. The data represent the mean ± SEM of three independent experimental determinations,

performed in triplicate. The data were analyzed statistically using two-way ANOVA followed by Tukey's test. *a* indicates difference from NB under basal conditions, *b* indicates difference between unchallenged and challenged with Glu and *c* indicates age-dependent treatment difference.

Fig. 7 **Glu challenge induces age-dependent responses in astrocyte cultures.** Under Glu exposure, NB astrocytes increased 2DG uptake, decreased Glu uptake and activated NFκB transcription factor. AD astrocytes also increased 2DG uptake and decreased Glu uptake, however they rely on Glu as metabolic substrate. AG astrocytes did not take up 2DG under Glu challenge, however they sustained Glu uptake and showed higher levels of ROS and GSH. Additionally, NFκB transcription factor and p38 MAPK were activated and there was increased IL1β and TNFα, the most important pro-inflammatory mediators. *EAAT* excitatory amino acid transporter; *GLUT* glucose transporter; Na^+/K^+ sodium potassium ATPase.

Figure 1.

a) NB b) AD c) AG

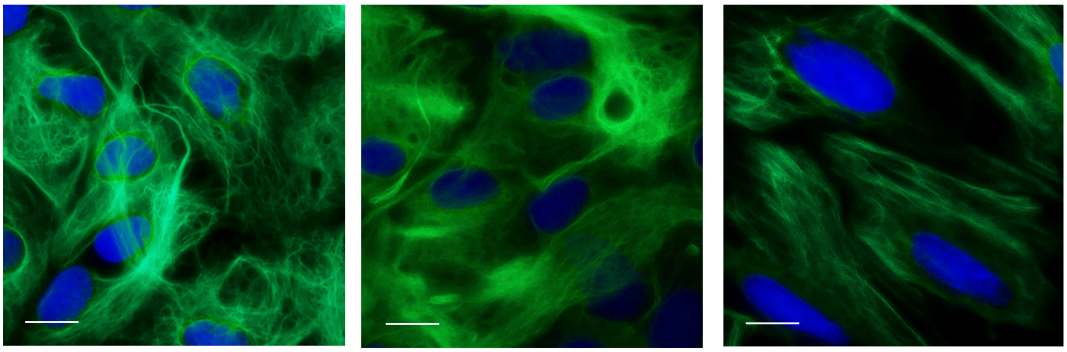


Figure 2.

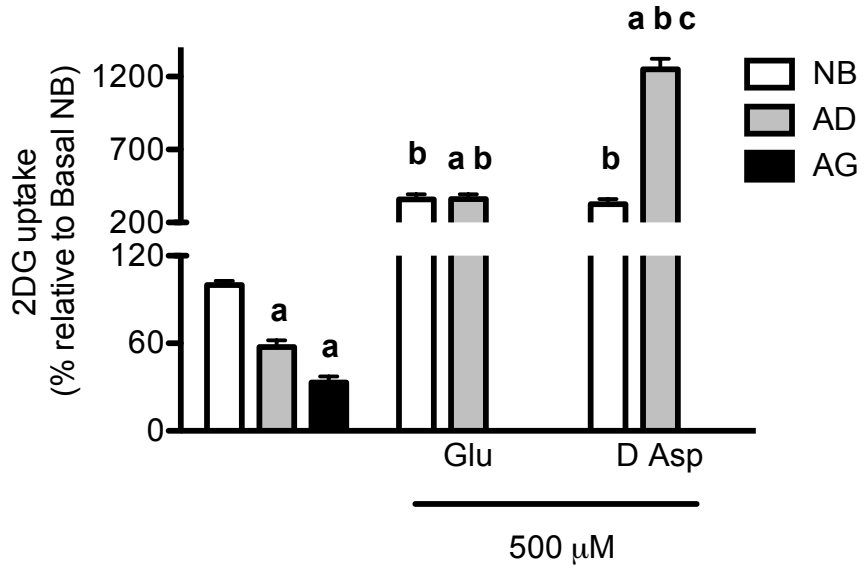

a b c
NB
AD
AG
1200
700
200
120
60
0
2DG uptake
(% relative to Basal NB)
b
a b
b
a
a
Glu
D Asp
500 μM

Figure 3.

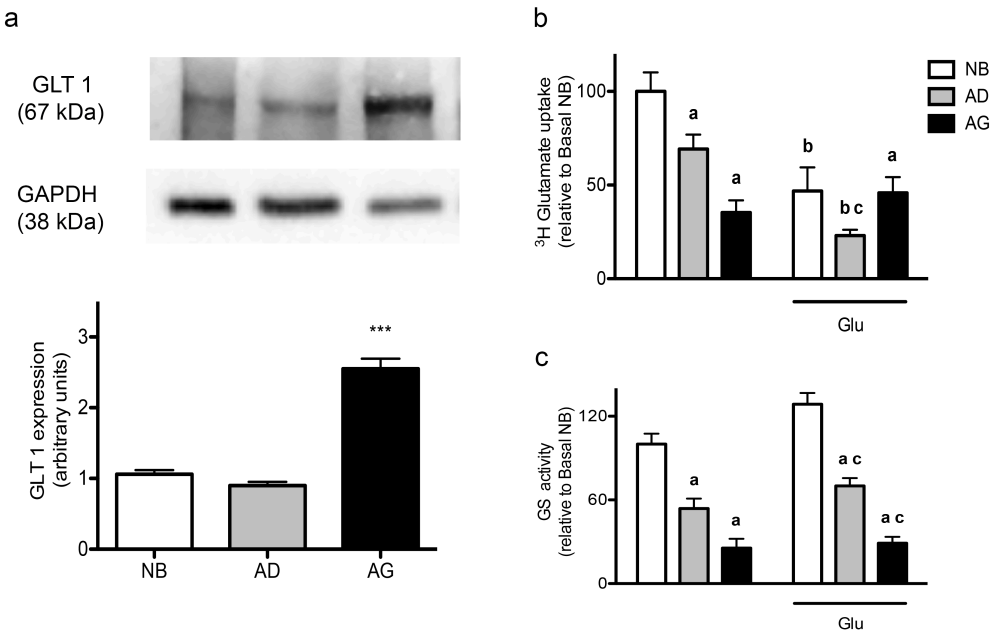
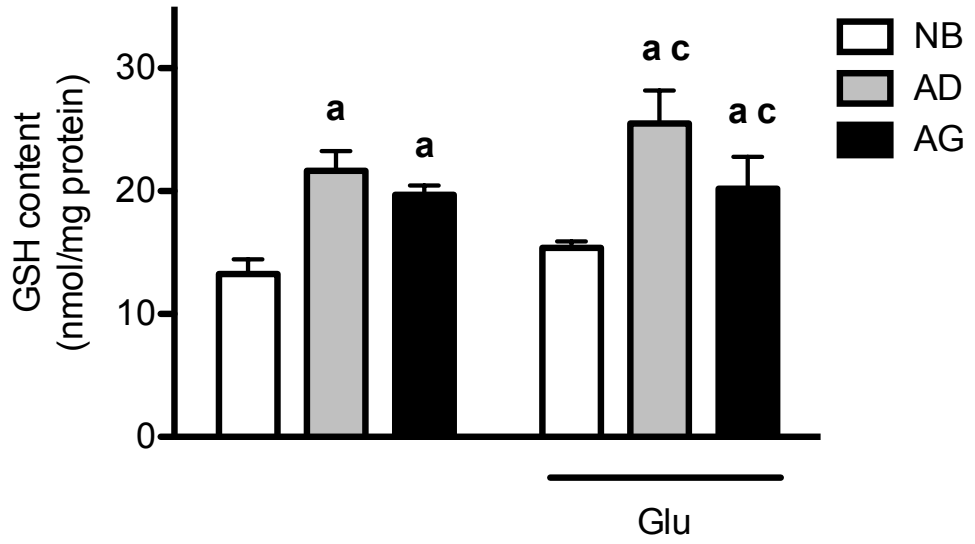

a
GLT 1
(67 kDa)
GAPDH
(38 kDa)
GLT 1 expression
(arbitrary units)
***
NB
AD
AG
b
^{3}H Glutamate uptake
(relative to Basal NB)
NB
AD
AG
a
a
b
b c
a
Glu
c
GS activity
(relative to Basal NB)
a
a
a c
a c
Glu

Figure 4.

a


GSH content
(nmol/mg protein)
30
20
10
0
a
a
a c
a c
NB
AD
AG
Glu

b

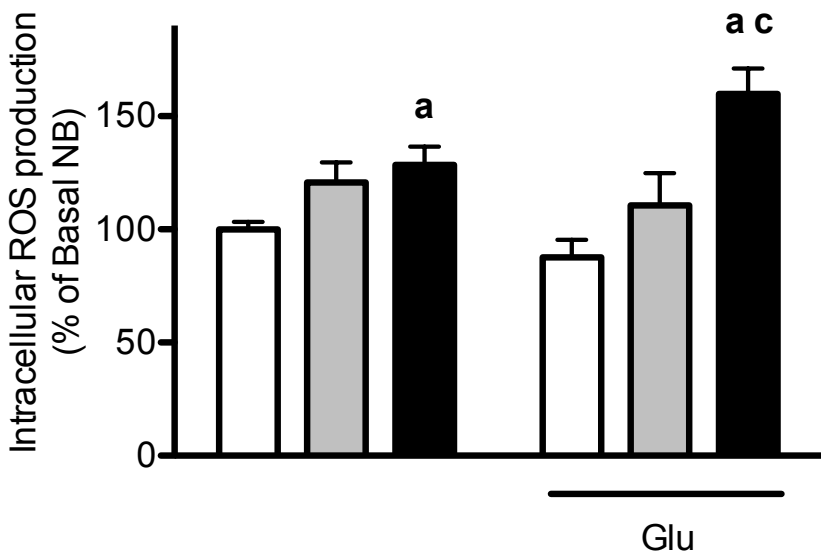
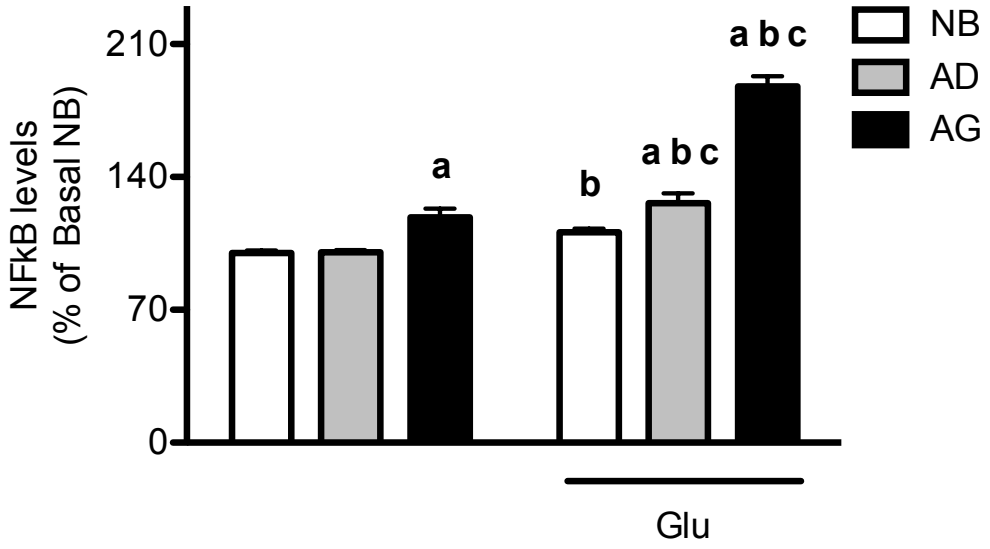

Intracellular ROS production
(% of Basal NB)
150
100
50
0
a
a c
Glu

Figure 5.

a


NB
AD
AG
NFkB levels
(% of Basal NB)
210
140
70
0
a
b
a b c
a b c
Glu

b

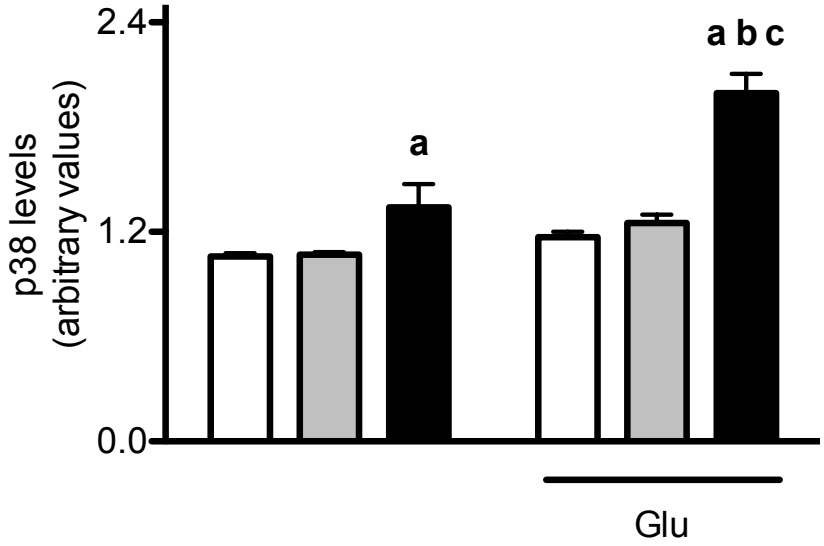

p38 levels
(arbitrary values)
2.4
1.2
0.0
a
a b c
Glu

Figure 6.

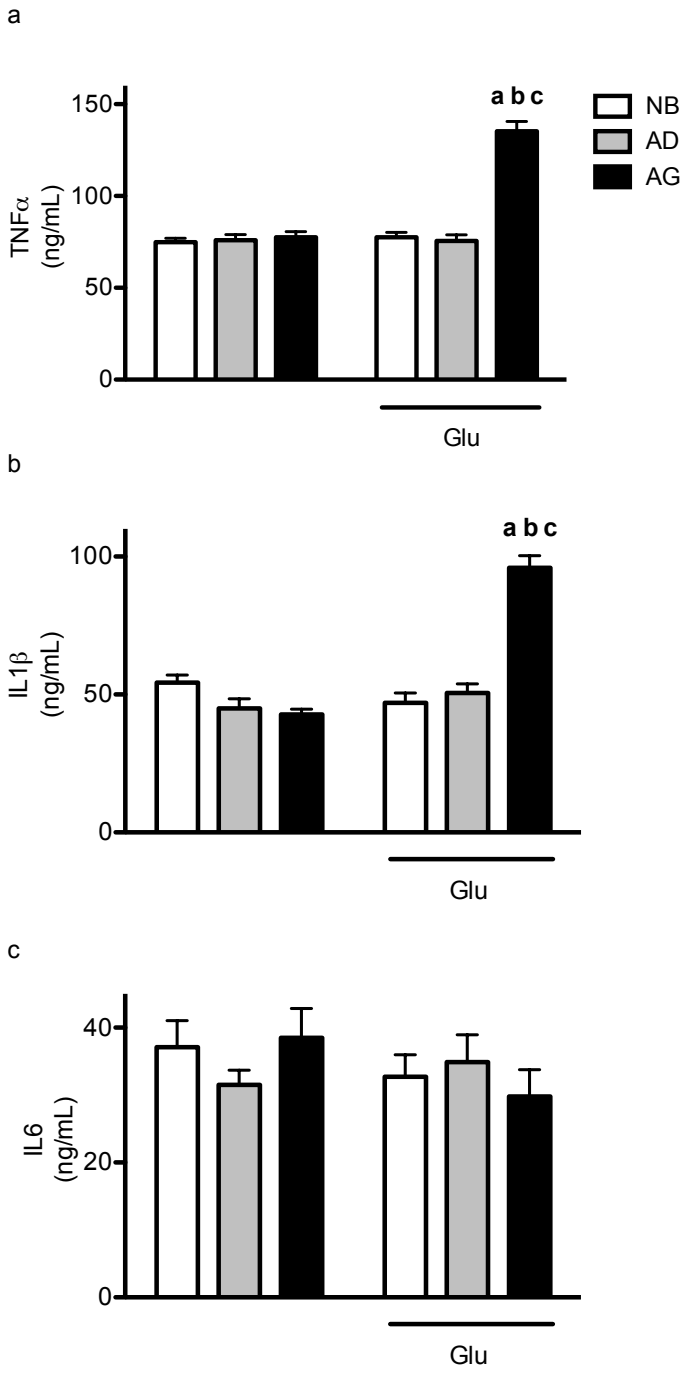
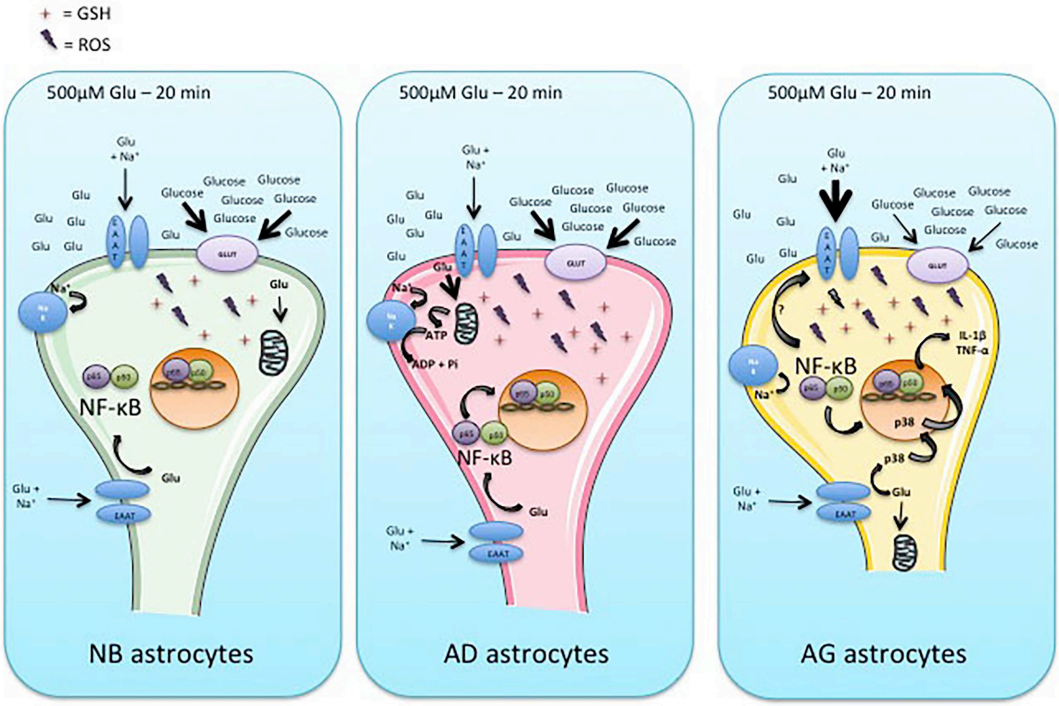

a
150
100
50
0
TNFα (ng/mL)
a b c
NB
AD
AG
Glu
b
100
50
0
IL1β (ng/mL)
a b c
Glu
c
40
20
0
IL6 (ng/mL)
Glu

Figure 7.


= GSH
= ROS
500µM Glu – 20 min
NF-κB
NB astrocytes
500µM Glu – 20 min
ATP
ADP + Pi
NF-κB
AD astrocytes
500µM Glu – 20 min
IL-1β
TNF-α
NF-κB
p38
p38
AG astrocytes

CAPÍTULO 4

Artigo submetido ao periódico Purinergic Signalling

Anti-aging effects of guanosine in glial cells

Débora Guerini Souza, Bruna Bellaver, Larissa Daniele Bobermin, Diogo Onofre Souza, André Quincozes-Santos

Anti-aging effects of guanosine in glial cells

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Abstract

Guanosine, a guanine-based purine, has been shown to exert several beneficial roles in *in vitro* and *in vivo* injury models of neural cells. Guanosine is released from astrocytes and modulates important astroglial functions, including glutamatergic metabolism, antioxidant and anti-inflammatory activities. Astrocytes are crucial for regulating neurotransmitter system and synaptic information process, ionic homeostasis, energy metabolism, antioxidant defenses and inflammatory response. Aging is a natural process able to induce numerous changes in the astrocyte functionality. Thus, the search for molecules able to reduce glial dysfunction associated to aging is an approach to avoid the onset of age-related neurological diseases. Thus, the aim of this study was to evaluate the anti-aging effects of guanosine, by using primary astrocyte cultures from newborn (NB), adult (AD) and aged (AG) Wistar rats. Concomitantly, we evaluated the role of heme oxygenase 1 (HO-1) in guanosine-mediated glioprotection. We observed an age-dependent changes in glutamate uptake, glutamine synthetase (GS) activity, glutathione (GSH) content, pro-inflammatory cytokines (TNF-α and IL-1β) release and nuclear factor κB (NFκB) p65 levels, which were prevented by guanosine in a HO-1 dependent way. Our results support that guanosine is a promisor therapeutic agent able to afford glioprotection in aging process. Thus, this study contributes to understand the cellular and molecular mechanisms of guanosine on aging.

Key-words: Aging, Adult/aged astrocytes, Guanosine, Heme oxygenase 1

Introduction

Guanine-based purines are known to act as extracellular signaling molecules, exerting trophic and neuroprotective roles in *in vitro* and *in vivo* experimental models [1-5]. Guanosine, more specifically, has previously shown to induce numerous beneficial cellular responses in several brain injuries such as seizures, hypoxia, anxiety-like behavior, ischemia and glucose deprivation [1,6-9]. Guanosine has demonstrated ability to modulate glutamatergic metabolism, avoiding the overactivation of glutamate receptors, as well as exerting antioxidant and anti-inflammatory activities [10-12]. Guanosine has also shown to modulate several signaling pathways to afford neuroprotection [1,13,14]. Although there is increasing evidence for the protective effects of guanosine in neural cells, its mechanism of action is not fully understood.

Our group has previously demonstrated the interplay between guanosine and the enzyme heme oxigenase-1 (HO-1) [12,14], which is the major responsible for the conversion of heme into CO and the antioxidant products biliverdin and bilirubin [15,16]. It has been reported that HO-1 may be a therapeutic target in aging process and/or neurodegenerative diseases. Increased HO-1 activity is correlated with protection against stressful conditions, such as hypoxia/ischemia, oxidative stress and neuroinflammation [12,15,16]. Furthermore, HO-1 counteracts the transcriptional activity of nuclear factor kappa B (NFκB), which is the master regulator of oxidative/nitrosative stress and inflammatory response [17-19].

Aging is a natural process able to induce numerous changes in the brain functionality, *e.g.* alterations in synaptic efficacy; changes in neuron-glia

communication with consequent impairment in cerebral activities; increases in reactive oxygen species (ROS) and inflammatory mediators [20-22]. Understand and manage these alterations may be an important strategy to extend healthy lifespan. In this sense, the association among oxidative stress, inflammation and aging is based on complex molecular and cellular changes that have only just begun to be understood. Since HO-1 signaling pathway appears to play a role in some of these changes, this enzyme emerges an important therapeutic target to protect against the aging process [15].

Astrocytes, the main class of glial cells, participate in a diverse range of central nervous system (CNS) parameters, including the regulation of neurotransmitter systems, synaptic information processing (as part of the tripartite synapse), ionic homeostasis, energy metabolism, antioxidant defenses and inflammatory response [23-27]. In line with this, to study features of adult and aged brain, our group has established a routine methodology of primary culture of astrocytes obtained from non-neonatal rats, since the metabolic, oxidative and inflammatory properties of cells derived from newborn and adult/aged animals are distinct [21,22,28,29]. We have previously shown that this tool can be employed to study different astrocytic roles in CNS, since the cells present classical astroglial markers, such as glial fibrillary acidic protein (GFAP), S100B and glutamine synthetase (GS) expression and activity. Moreover, they express glutamate transporters such as glutamate-aspartate transporter (GLAST) and glutamate transporter 1 (GLT-1), being able to respond to neuroprotective and neurotoxic stimuli [27,30,31].

Considering the essential role of astrocytes in brain functionality and the differences between immature and aged brain as well as the protective effects of guanosine on glial cells, the aim of this study was to evaluate the anti-aging effects of guanosine in primary astrocyte cultures. We evaluated glutamate uptake, GS activity, glutathione (GSH) content, levels of tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β), and the transcriptional activity of NFκB p65 in cortical primary astrocyte cultures from newborn (NB), adult (AD) and aged (AG) obtained from 1, 90, 180 days old, respectively, Wistar rats. Additionally, we explored whether HO-1 was involved in the glioprotective effects of guanosine.

Materials and methods

Chemicals

Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) and other materials for cell culture were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Papain was acquired from Merck (Darmstadt, Germany). L-[^{3}H]-glutamate was from Amersham/GE Healthcare (Little Chalfont, UK). DNase, cysteine, albumin, γ-glutamylhydroxamate, reduced glutathione, zinc protoporphyrin IX (ZnPP IX) and guanosine were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were from common commercial suppliers.

Animals

Male Wistar rats (1, 90 and 180 days old) were obtained from our breeding colony (Department of Biochemistry, UFRGS, Brazil), maintained

under a controlled environment (12 h light/12 h dark cycle; 22 ± 1°C; *ad libitum* access to food and water). All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Federal University of Rio Grande do Sul Animal Care and Use Committee (process number 24419).

Cell culture preparation and maintenance

Male Wistar rats (1, 90 and 180 days old) were sacrificed by decapitation, had their cerebral cortices aseptically dissected and meninges removed. The tissue was digested using trypsin only – for newborn tissue – or trypsin and papain – for adult tissue – at 37ºC as previously described [21]. After mechanical dissociation and centrifugation, the cells were resuspended in DMEM/F12 [10% fetal bovine serum (FBS), 15 mM HEPES, 14.3 mM $NaHCO_3$, 1% Fungizone® and 0.04% gentamicin], plated on 6- or 24-well plates pre-coated with poly-L-lysine and cultured at 37°C in a 5% CO_2 incubator. The cells were seeded at a density of 3–5 x 10^5 cells/cm^2. Twenty four hours later, the culture medium was exchanged; during the 1st week, the medium was replaced once every two days and from the 2nd week on, once every four days. From the 3rd week on, the astrocytes received medium supplemented with 20% FBS until they reached confluence (at approximately the 4th week). No dibutyryl cAMP was added to the culture medium in order to observe the naive response of the cells. Specific proteins of neurons and microglia were examined in order to determine the purity of the astrocytes culture, which was around 95% (data not shown).

Cellular treatments

In order to investigate how astrocytes react to guanosine stimulus, we treated astrocyte cultures with 100 μM of guanosine for 24 h in DMEM/F12 with 1% FBS at 37°C in a 5% CO_2 incubator [14]. To explore the involvement of HO-1 signaling pathway in the glioprotective effects of guanosine, we previously incubated astrocytes with 10 μM of ZnPP IX (a HO-1 inhibitor) for 1 h, in the presence or absence of guanosine. After cellular treatments, the evaluations described below were performed. To measure membrane integrity, cells were incubated with propidium iodide. For evaluate glutamate uptake after L-[2,3-^{3}H] glutamate incorporation, cells were lysed with NaOH. For GS activity and GSH levels, cells were lysed in a sodium phosphate buffer with KCl (140 mM). The extracellular medium was used to measure cytokines release. The nuclear fraction from cell cultures was isolated and used to measure NFκB p65 levels.

Membrane Integrity

Membrane integrity was assessed by fluorescent image analysis (Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory) of propidium iodide (PI) uptake (at 7.5 μM) at 37°C in an atmosphere of 5% CO_2 in DMEM/F12 supplemented with 1% FBS.

Glutamate uptake

The glutamate uptake was performed as previously described with some modifications [27]. Briefly, the cells were rinsed once with PBS and were incubated at 37°C in HBSS containing the following components (in

mM): 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 Na_2HPO_4, 0.44 KH_2PO_4, 4.17 $NaHCO_3$ and 5.6 glucose, adjusted to pH 7.4. The assay was started by the addition of 0.1 mM L-glutamate and 0.33 mCi/mL L-[2,3-^{3}H] glutamate. The incubation was stopped after 7 min by removal of the medium and rinsing twice the cells with ice-cold HBSS. The cells were then lysed in a solution containing 0.5 M NaOH. Incorporated radioactivity was measured in a scintillation counter. Sodium-independent uptake was determined using ice-cold N-methyl-D-glucamine instead of sodium chloride. Sodium-dependent glutamate uptake was obtained by subtracting the sodium-independent uptake from the total uptake.

Glutamine Synthetase Activity

The enzymatic assay was performed as previously described [27]. Briefly, cell homogenate (0.1 mL) was added to 0.1 ml of the reaction mixture containing (in mM): 10 $MgCl_2$, 50 L-glutamate, 100 imidazole-HCl buffer (pH 7.4), 10 2-mercaptoethanol, 50 hydroxylamine-HCl and 10 ATP, and incubated for 15 min (37°C). The reaction was stopped by the addition of 0.4 mL of a solution containing (in mM): 370 ferric chloride, 670 HCl, and 200 trichloroacetic acid. After centrifugation, the absorbance of the supernatant was measured at 530 nm and compared to the absorbance generated using standard quantities of γ-glutamylhydroxamate treated with a ferric chloride reagent. The activity was expressed as μmol/mg protein/h.

Glutathione content

GSH levels were assessed as previously described [27]. Astrocyte homogenates were diluted in 100 mM sodium phosphate buffer (pH 8.0) containing 5 mM EDTA, and the protein was precipitated with 1.7% meta-phosphoric acid. The supernatant was assayed with o-phthaldialdehyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0–500 mM). GSH concentrations were calculated as nmol/mg protein.

Inflammatory response

The TNF-α levels were measured in extracellular medium using rat TNF-α ELISA kit from Peprotech. The levels of IL-1β were measured in extracellular medium using rat ELISA kit from eBioscience. The results are expressed in ng/mL. The average minimum sensitivity of the ELISA kits detection is 0.4 ng/mL of cytokines.

NFκB measurement

The levels of NFκB p65 in the nuclear fraction ultimately determines the activity of NFκB. Astrocytes nuclear fraction were isolated from lysed cells with Igepal CA-630 and centrifugation (following manufacturer's instructions), and assayed using an ELISA commercial kit from Invitrogen (USA). The results were expressed as percentages relative to the basal conditions. The ELISA kit detects a minimum of 50.0 pg/mL.

Protein assay

Protein content was measured using Bicinchoninic Acid method with bovine serum albumin as a standard [32].

Statistical analyses

Data were statistically analyzed using two-way analysis of variance (ANOVA), followed by the Tukey's test. P-values < 0.05 were considered significant. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software version 17.0.

Results

Glutamate management in astrocyte cultures was assessed through glutamate uptake (Fig. 1a) and GS activity (Fig. 1b). Firstly, we demonstrated an age-dependent decrease in the control glutamate uptake in cortical astrocytes (Fig. 1a). In line with this, guanosine treatment partially prevented this age effect, inducing an increase (around 130%, $p < 0.01$) of glutamate uptake in AD and AG cultures, compared to control conditions. Since guanosine modulates HO-1 [12,14], we investigated whether its effect on glutamate uptake was dependent on HO-1 activity. In this sense, HO-1 inhibitor (ZnPP IX) totally abolished the guanosine effect. It is noteworthy that NB astrocytes did not show significant changes after guanosine exposure.

Regarding the GS activity, we observed an age-dependent decrease in its control activity (Fig.1b), compared to NB cultured astrocytes. In addition, guanosine induced an age-related increase in GS activity in AD and AG astrocytes (17% and 42%, respectively), compared to control conditions. The presence of HO-1 inhibitor was also able to block this effect, restoring the

activity to the control values. Guanosine presented no effect in NB cells. The HO-1 inhibitor *per se* had no effect on glutamate uptake and GS activity (data not shown).

The levels of GSH, the main non-enzymatic antioxidant defense of CNS and closely associated to glutamate metabolism, significantly decreased with age (Fig. 2). Guanosine prevented this effect, increasing the levels of GSH in AD and AG cultured astrocytes (approximately 16% and 30%, respectively). The HO-1 inhibitor once again abolished the effect of guanosine.

As shown in Fig. 3a and b, in contrast to GSH levels, there was an age-dependent increase in pro-inflammatory cytokines (TNF-α and IL-1β) release. Guanosine was able to induce a decrease in both TNF-α and IL-1β levels in AD and AG cultured astrocytes. This anti-inflammatory effect of guanosine was mediated by HO-1. Because changes in cell integrity were not observed through PI incorporation (data not shown), the increased levels of cytokines most likely resulted from secretion.

The levels of NFκB, a transcription factor involved in many biological activities, including inflammatory response, showed a significant increase with aging (Fig. 4). Guanosine decreased NFκB p65 activation in AD and AG astrocytes; the levels of NFκB p65 dropped to values similar to NB astrocytes. In the presence of the HO-1 inhibitor, guanosine did not reduce the transcriptional activity of NFκB. Although HO-1 pathway is an upstream signaling of NFκB, the HO-1 inhibitor *per se* did not present any effect on NFκB levels (data not shown).

Discussion

Aging process is correlated with brain biochemical, cellular and molecular changes, including alterations in glutamate metabolism, oxidative stress, inflammatory response, and these events involve the modulation of several signaling pathways [33-35]. It is important to develop preventive treatments that could protect from brain aging, since the cells may become dysfunctional and lead to the onset of neurodegenerative diseases.

Guanosine has been studied in a variety of experimental models, including seizures, hypoxia, glucose deprivation, oxidative injury and inflammatory conditions [4,5,36-39]. Since guanosine may be released from glial cells, modulating important glial functions, astrocytes emerge as central players in the protective actions of guanosine [12,14]. However, the precise mechanism of guanosine neural/glioprotection is not completely understood, being relevant elucidate its cellular and molecular targets. Some preliminary data support the existence of specific receptor-like binding sites for guanosine and data also indicated that the extracellular effects of guanosine might involve the activation of intracellular signaling pathways, such as G proteins, MAPK and HO-1 [12-14].

HO-1 pathway has been reported to be active and to operate as a fundamental defensive mechanism for cells exposed to stressful conditions [15,40,41]. In line with this, HO-1 is closely associated to nuclear factor erythroid-derived 2-like 2 (Nrf-2), which controls the master regulator of redox state and inflammatory response, the transcription factor NFκB [17]. In our previous reports, we demonstrated that HO-1 expression is increased by guanosine; this way, HO-1 is the putative mediator of glutamatergic,

antioxidant and anti-inflammatory effects of guanosine [12,14]. Additionally, HO-1 may induce GSH biosynthesis that protects glutamate transporters from oxidative damage.

Moreover, it was recently shown the protective role of HO-1 in Alzheimer's disease by the modulation of MAPK signaling pathway [42]; another study suggested an improvement in learning and a decrease in brain oxidative stress by increasing HO-1 in a preclinical model of Alzheimer's disease [43]. There is a wide range of beneficial effects that HO-1 may exert and it is important to highlight that HO-1 is predominantly expressed in the mature brain, therefore, it is likely that the cultured astrocyte from AD/AG rats provide answers about guanosine role in aging and neurodegenerative diseases.

Recently, we proposed the glioprotective effects of guanosine and, here, we showed for the first time the anti-aging effect of guanosine on classical astroglial markers, analyzing the effect of a guanosine treatment on primary astrocytes prepared from animals with different ages. Cellular responses under the presence of ZnPP IX, a HO-1 inhibitor, were also evaluated.

Effects of aging in astrocytes – the role of guanosine on glutamatergic transmission

We showed that glutamate uptake activity decreased with aging. The removal of glutamate from synaptic cleft by high-affinity transporters is considered one of the most important functions of astrocytes [44,45], since it may avoid the excitotoxicity, a common condition correlated with

neurodegenerative diseases, which incidence increases with aging [22,33,46]. Thus, we demonstrated that guanosine modulated glutamate uptake in an age-dependent manner, emerging as a promisor pharmacological tool for prevention of age-related/neurodegenerative diseases. Reinforcing our data, the effects of guanosine were exclusively from mature astrocyte cultures, which presented an increase in oxidative/nitrosative stress that may impair the glutamate transport. For over 25 years, our group has studied the effects of guanosine on glutamatergic system and, recently, we proposed its glioprotective activity that may improve the neuronal functionality, supporting the neuroprotective action of guanosine [1,6,9,10,47]. Interestingly, as previously demonstrated under oxidative and inflammatory conditions, the anti-aging effect of guanosine in glial cells was dependent on HO-1 pathway.

Glutamate may be a precursor of GSH as well as of glutamine via the enzyme GS, thus entering in the glutamate-glutamine cycle [48,49]. GS activity presented an age-dependent decrease in astrocyte cultures, which may impair neuronal activity. In addition, aging process is correlated to increased oxidative/nitrosative stress and GS activity is very sensitive to this condition [50,51]. Our data revealed that guanosine was able to prevent the decreased GS activity, reinforcing its antioxidant activity as well as the role in glutamate metabolism. Corroborating with our previous data, the effect of guanosine on GS was mediated by HO-1.

GSH is the main non-enzymatic antioxidant defense of CNS and, as expected, the levels of GSH decreased with aging [27,52]. Guanosine prevented this effect, which was also regulated by HO-1. The increase in GSH content in glial cells confers protection against age-related neurological

diseases, such as Alzheimer's and Parkinson's diseases [53]. In this context, the antioxidant effect of guanosine upon aged cells could improve the scavenger activity of GSH, supporting its *de novo* biosynthesis as well as protecting glutamate transporters from oxidation promoted by increased oxidative/nitrosative stress related to aging. Regarding to neural cells, the upregulation of EAAC1 glutamate transporter through Nrf2/HO-1 facilitates GSH synthesis [54]. Thus, GSH availability is strongly correlated to putative protective effect of HO-1.

Effects of aging in astrocytes – the role of guanosine on inflammatory mediators

Together with the increase in oxidative species levels, aging is commonly associated with increased inflammatory response in CNS. Accordingly, astrocytes are key players in the brain immunity, because they sense and amplify inflammatory signals [26,55]. Moreover, the depletion of GSH in glial cells also induces neuroinflammation [52]. Thus, we showed that classical pro-inflammatory cytokines, such as TNF-α and IL-1β, were increased with aging in cortical astrocytes. These cytokines act in acute inflammatory response and also have a critical role in chronic inflammation, commonly correlated with age-related neurological diseases. Guanosine was able to prevent the increase in TNF-α and IL-1β levels through HO-1 pathway, and, for the first time, we demonstrated the anti-inflammatory effect of guanosine associated to the aging process. Moreover, recently, we showed that guanosine inhibits TNF-α and IL-1β release induced by lipopolysaccharide (LPS) in newborn hippocampal astrocytes, independently

on adenosine receptors, although adenosine also presents a well-characterized anti-inflammatory effect [12].

TNF-α and IL-1β are clear activators of the NFκB signaling pathway, which is closely related to inflammation [18,56]. Thus, in accordance with increased inflammatory response in aging, we also showed increased transcriptional activity of NFκB p65. Guanosine controlled NFκB activation in mature astrocytes in a mechanism dependent on HO-1. In this way, HO-1 is upstream of NFκB, inhibiting its translocation from the cytoplasm to the nucleus. NFκB exerts a key role in inflammatory response and oxidative stress and the ability of guanosine to control its activation, as well as its regulator protein (HO-1), support the relevance of our data that indicates the glioprotective actions of guanosine. Additionally, numerous studies have shown that suppressing pro-inflammatory astroglial NFκB signaling could improve clinical outcomes in cases of neuroinflammatory-related diseases [57,58].

Conclusion

Here we demonstrated that the astrocyte culture model developed by our group is a powerful tool to investigate age-related changes in astrocytic functionality, such as glutamate metabolism, oxidative stress and inflammatory response. Additionally, we showed for the first time the anti-aging effect of guanosine on these functions. Our results reinforce that guanosine is a promisor glioprotective molecule in aging process, modulating HO-1, a fundamental pathway related to the cellular defenses. Although aging is a biological condition, this study suggests the exciting possibility to manage

this process, and changes in glial activity may be a way to achieve this purpose. Considering the main effects of guanosine were from mature cells, this endogenous nucleoside emerges as an important anti-aging molecule. Thus, this study contributes to understand the cellular and molecular mechanism of guanosine on brain aging.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Legends of figures

Fig. 1 Guanosine alters glutamatergic metabolism in mature astrocytes

Cells were incubated in DMEM/F12 1% FBS in the presence or absence of 100 μM guanosine (Guo) for 24 h. Alternatively, cells were co-incubated with a HO-1 inhibitor. **(a)** Glutamate uptake and **(b)** GS activity were measured as described in the "Materials and methods" section. Data represent the mean + S.E.M. of four independent experiments performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey's test. Values of $P<0.05$ were considered significant. *a* indicates differences from NB control; *b* indicates difference within the same group

Fig. 2 Guanosine increases GSH content in mature astrocytes

Cells were incubated in DMEM/F12 1% FBS in the presence or absence of 100 μM guanosine (Guo) for 24 h. Alternatively, cells were co-incubated with a HO-1 inhibitor. GSH content was measured as described in the "Materials and methods" section. Data represent the mean + S.E.M. of four independent experiments performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey's test. Values of $P<0.05$ were considered significant. *a* indicates differences from NB control; *b* indicates difference within the same group

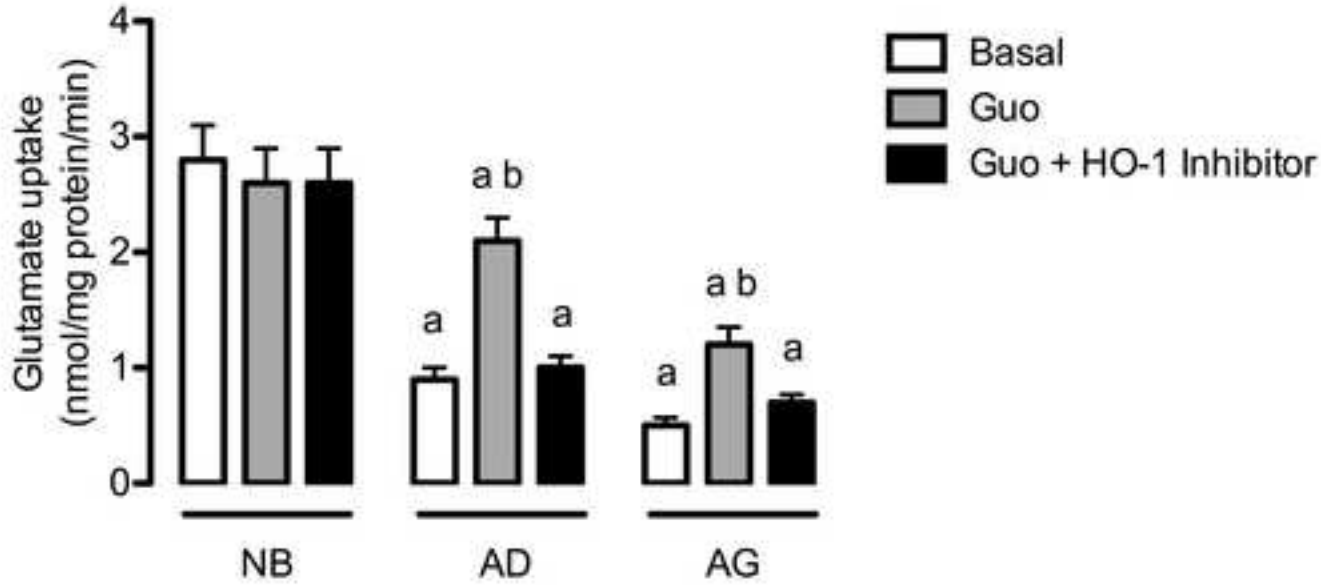
Fig. 3 Guanosine decreases pro-inflammatory cytokines levels in mature astrocytes

Cells were incubated in DMEM/F12 1% FBS in the presence or absence of 100 μM guanosine (Guo) for 24 h. Alternatively, cells were co-incubated with a HO-1 inhibitor. The levels of **(a)** TNF-α and **(b)** IL-1β were measured as described in the "Materials and methods" section. Data represent the mean + S.E.M. of four independent experiments performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey's test. Values of $P<0.05$ were considered significant. *a* indicates differences from NB control; *b* indicates difference within the same group

Fig. 4 Guanosine decreases NFκB transcriptional activity in mature astrocytes

Cells were incubated in DMEM/F12 1% FBS in the presence or absence of 100 μM guanosine (Guo) for 24 h. Alternatively, cells were co-incubated with a HO-1 inhibitor. The levels of NFκB were measured as described in the "Materials and methods" section. Data represent the mean + S.E.M. of four independent experiments performed in triplicate. Differences between groups were statistically analyzed using two-way ANOVA, followed by Tukey's test. Values of $P<0.05$ were considered significant. *a* indicates differences from NB control; *b* indicates difference within the same group

a



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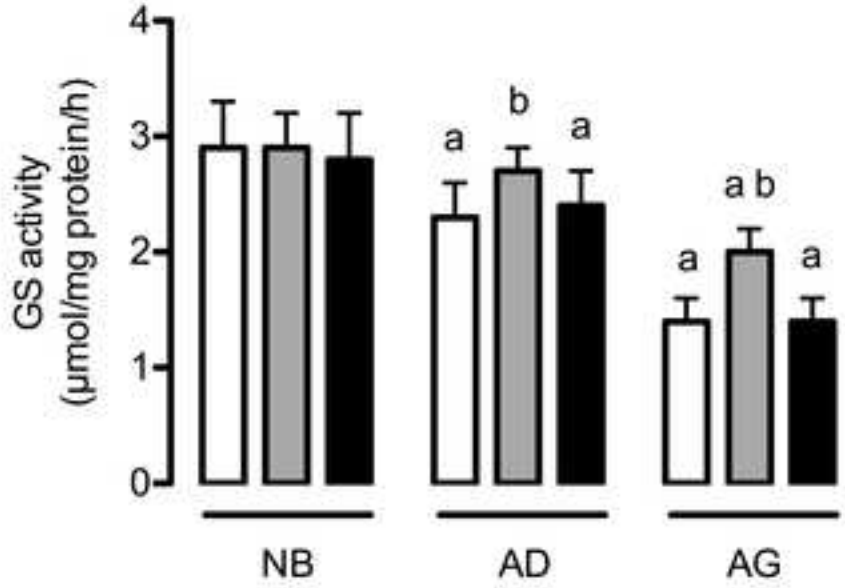

Figure 2

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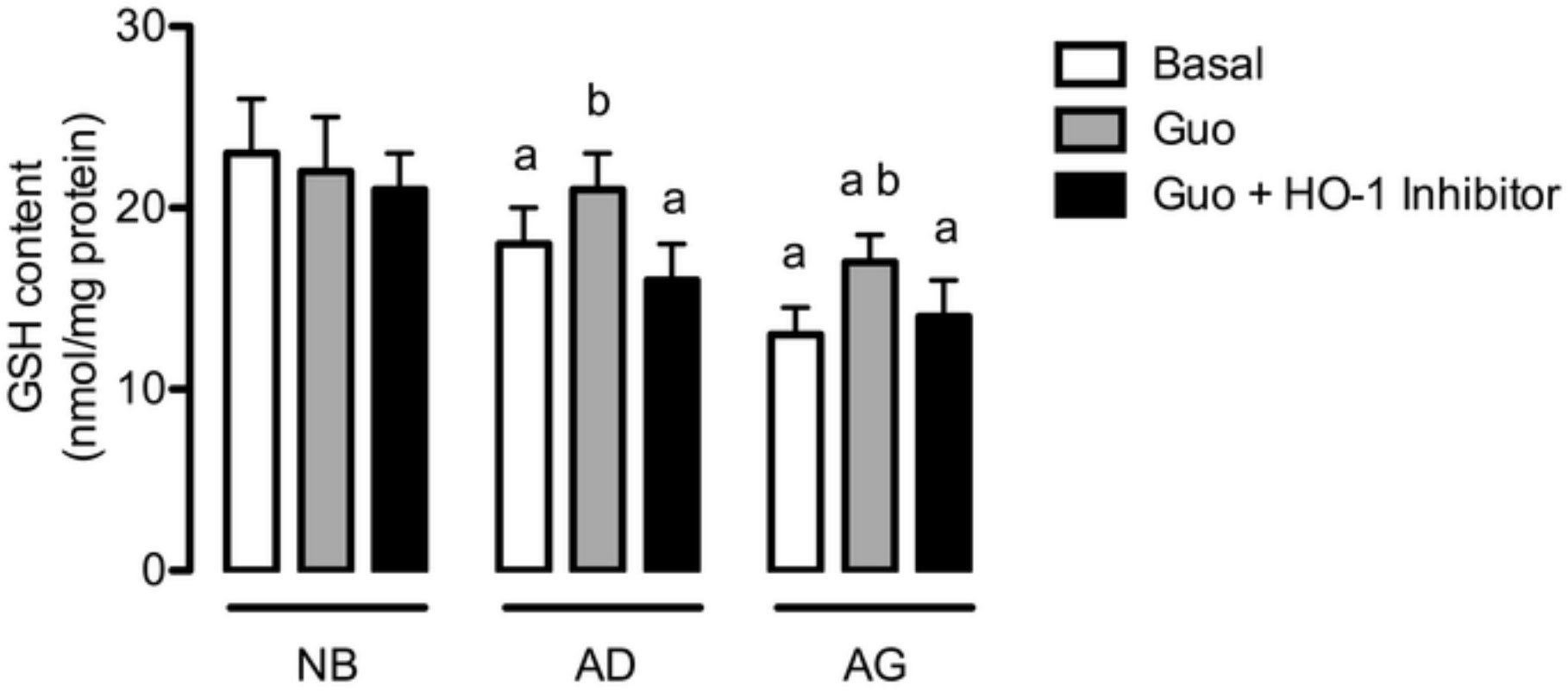

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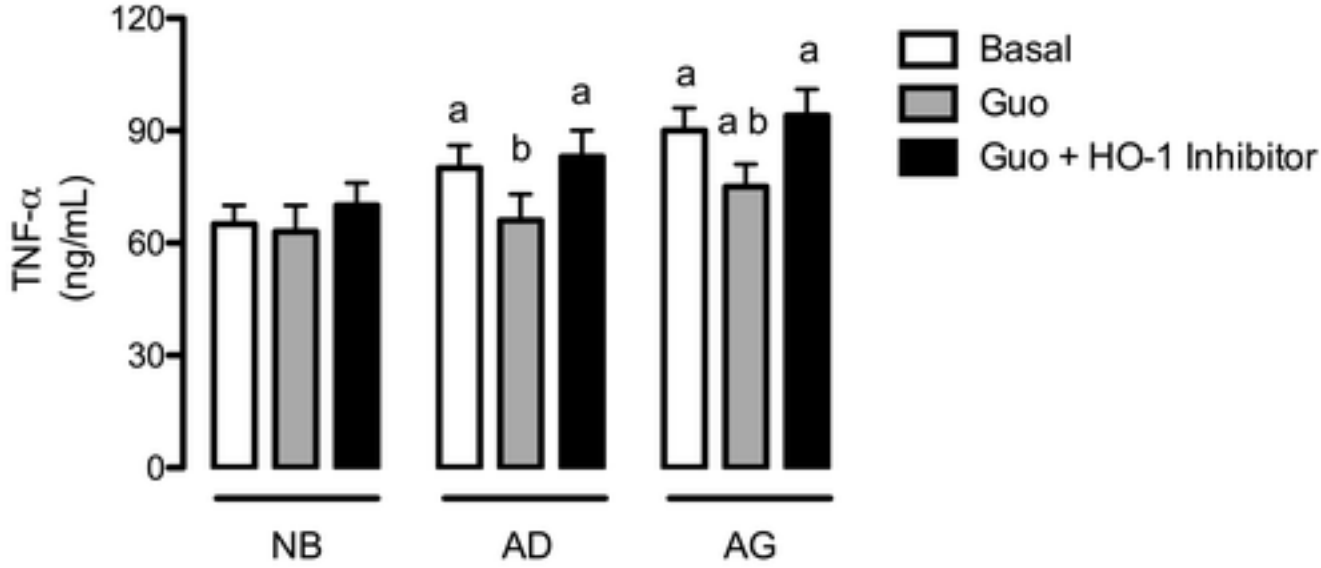

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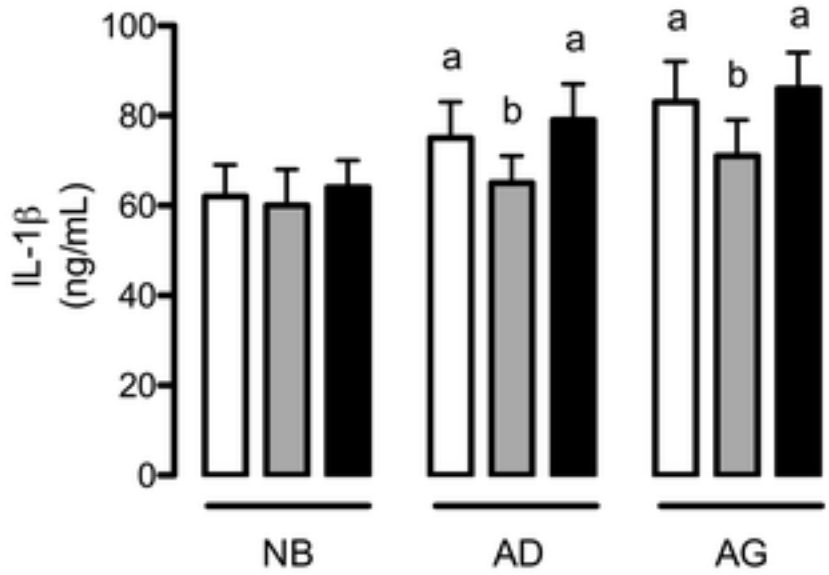

Figure 4

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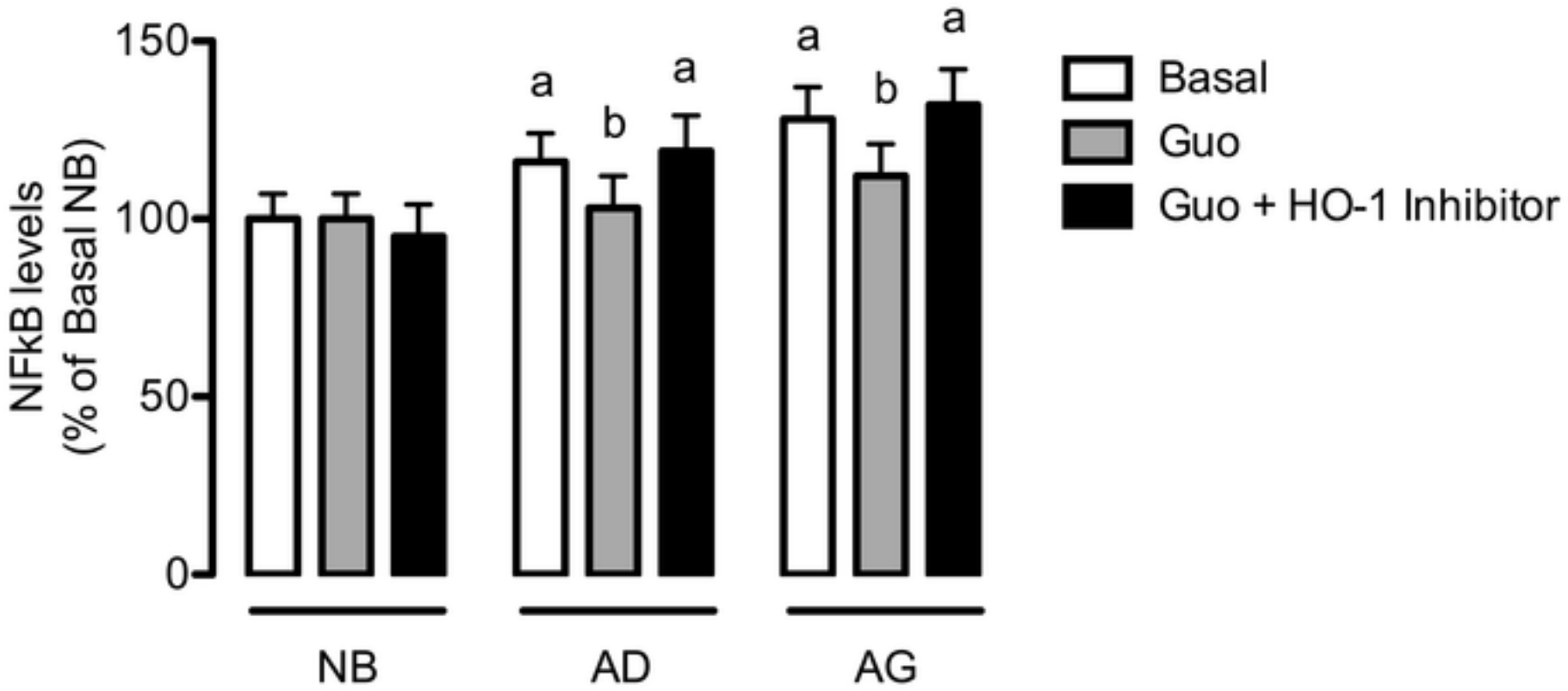

CAPÍTULO 5

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Astrocytes from adult Wistar rats aged in vitro show changes in glial functions

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Astrocytes from adult Wistar rats aged *in vitro* show changes in glial functions

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ABSTRACT

Astrocytes, the most versatile cells of the central nervous system, play an important role in the regulation of neurotransmitter homeostasis, energy metabolism, antioxidant defenses and the anti-inflammatory response. Recently, our group characterized cortical astrocyte cultures from adult Wistar rats. In line with that work, we studied glial function using an experimental *in vitro* model of aging astrocytes (30 days *in vitro* after reaching confluence) from newborn (NB), adult (AD) and aged (AG) Wistar rats. We evaluated metabolic parameters, such as the glucose uptake, glutamine synthetase (GS) activity, and glutathione (GSH) content, as well as the GFAP, GLUT-1 and xCT expression. AD and AG astrocytes take up less glucose than NB astrocytes and had decreased GLUT1 expression levels. Furthermore, AD and AG astrocytes exhibited decreased GS activity compared to NB cells. Simultaneously, AD and AG astrocytes showed an increase in GSH levels, along with an increase in xCT expression. NB, AD and AG astrocytes presented similar morphology; however, differences in GFAP levels were observed. Taken together, these results improve the knowledge of cerebral senescence and represent an innovative tool for brain studies of aging.

1. Introduction

Astrocytes are robust glial cells that play several roles in central nervous system (CNS) homeostasis. They are responsible for neurotransmitter management, synaptic processing, ionic homeostasis, antioxidant defenses and the anti-inflammatory response as well as energy metabolism (Jiang and Cadenas, 2014; Maragakis and Rothstein, 2006; Parpura et al., 2012; Perea et al., 2014; Wang and Bordey, 2008). Their strategically positioned processes are able to reach both blood capillaries and other cells, allowing them to carry energy substrates that provide metabolic fuel for brain activity (Belanger et al., 2011; Schousboe et al., 2011). These functions can likely be attributed to an organized cytoskeleton, which implicates the presence of characteristic intermediate filaments, such as glial fibrillary acidic protein (GFAP), a classical cytoskeletal marker of astrocytes. GFAP is thought to contribute to a broad number of functions, such as mechanical strength and astrocytic shape (Menet et al., 2001; Middeldorp and Hol, 2011).

Astrocytes are also known to be both highly oxidative and glycolytic, and through glucose transporter 1 (GLUT 1), they are able to transport glucose from the blood to the inside of the cell and thereby provide metabolic fuel for the CNS (Benarroch, 2014; Nedergaard et al., 2003). Moreover, astrocytes are critical cells in glutamatergic transmission homeostasis. They take up glutamate through their high-affinity glutamate transporters GLT-1 and GLAST (Anderson and Swanson, 2000; Benarroch, 2010; Danbolt, 2001; Ye and Sontheimer, 2002), which may then be used as a substrate for oxidation in the tricarboxylic acids cycle, to synthesize either the tripeptide glutathione (GSH), the major antioxidant of the brain, or glutamine through glutamine synthetase (EC 6.3.1.2) (Dringen, 2000; Uwechue et al., 2012; Mates et al., 2002; Lee et al., 2010). Astrocytes are not only able to take up glutamate but also able to release it in a non-vesicular manner through a cystine-glutamate antiporter (system x_c^-), allowing it to activate extrasynaptic receptors and shape synaptic activity. System x_c^- is an important source of cystine, which is intracellularly converted to cysteine, the rate-limiting substrate in GSH biosynthesis (Lewerenz et al., 2006; Sato et al., 1999).

Previous studies from our group have shown that adult and aged

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rat astrocyte cultures are a novel tool to study the age-related roles of astrocytes under physiological and pathological conditions (Bellaver et al., 2014; Souza et al., 2013). Our culture model demonstrates the correct functioning of various astrocytic features, such as the expression of GFAP, and other proteins related to glial function (GLT-1, GLAST, GS, S100B and vimentin), glutamate and glucose uptake, antioxidant defenses and the release of inflammatory mediators. Furthermore, these cells have also demonstrated age-related cellular alterations and responses to neurotoxic and neuroprotective stimuli.

Considering the importance of the changes that brain undergo during aging and the lack of models to better comprehend these changes, the aim of this study was to evaluate glial parameters in primary cortical cultures from newborn (NB), adult (AD) and aged (AG) Wistar rats, 30 days after the cultures reached confluence, which is referred to in the text as aged *in vitro* (AIV). Thus, we evaluated glucose uptake, GS activity and GSH content, as well as cellular morphology, GFAP, GLUT-1 and xCT (a functional subunit of system x_c^-).

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) and other materials for cell culture were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Papain was acquired from Merck (Darmstadt, Germany). DNase, cysteine, albumin and monoclonal anti-GAPDH were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2-Deoxy-D-[1,2–^{3}H]glucose ([^{3}H]2DG), nitrocellulose membrane and an ECL kit were purchased from Amersham (Buckinghamshire, UK). Polyclonal anti-GLUT1 and polyclonal anti-xCT were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were from common commercial suppliers.

2.2. Animals

Male Wistar rats (1, 90 and 180 days old) were obtained from our breeding colony (Department of Biochemistry, UFRGS, Brazil), maintained under a controlled environment (12 h light/12 h dark cycle; 22 ± 1 °C; *ad libitum* access to food and water). All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Federal University of Rio Grande do Sul Animal Care and Use Committee (process number 24419).

2.3. Cell culture obtainment and maintenance

Male Wistar rats (1, 90 and 180 days old) had their cerebral cortices aseptically dissected and meninges removed. The tissue was digested using trypsin only at 37 °C, for newborns or using trypsin and papain at 37 °C for adult and aged rats as previously described (Souza et al., 2013). After mechanical dissociation and centrifugation, the cells were resuspended in DMEM/F12 [10% fetal bovine serum (FBS), 15 mM HEPES, 14.3 mM $NaHCO_3$, 1% Fungizone and 0.04% gentamicin], plated on 6- or 24-well plates pre-coated with poly-L-lysine and cultured at 37 °C in a 5% CO_2 incubator. The cells were seeded at a density of 3–5 × 10^5 cells/cm^2. Twenty four hours later, we performed a medium exchange; during the first week, we replaced the medium once every two days and from the 2ndweek on, we replace it once every four days; at each medium exchange we submit the cells to mechanical shaking, therefore, depleting the cultures from microglial presence (data not shown). At that time, the newborn cells reached confluence. From the 3rd week on, the adult and aged astrocytes received medium supplemented with 20% FBS until they reached confluence (at approximately the 4th to 5th week). Experiments were performed thirty days after the cultures reached confluence. In order to maintain cells in a naive state, no dibutyryl cAMP was added to the culture medium.

2.4. Cell morphology

Morphological studies were performed using phase contrast optics (Nikon inverted microscope).

2.5. Western blot analyses

Cells were solubilized in lyses solution containing 4% SDS, 2 mM EDTA, 50 mM Tris–HCl (pH 6.8). Protein content was measured, the samples were standardized in sample buffer [62.5 mM Tris–HCl (pH 6.8), 4% (v/v) glycerol, 0.002% (w/v) bromophenol blue] and boiled at 95 °C for 5 min. Samples were separated by SDS/PAGE (45 mg protein per sample), and transferred to nitrocellulose membranes. Adequate loading of each sample was confirmed using Ponceau S staining. Membranes were incubated overnight (4 °C) with one of the following antibodies: anti-GFAP (1:1000), anti-GLUT-1 (1:100), anti-xCT (1:100) or anti-GAPDH (1:3000). Then, the membranes were washed and incubated with a peroxidase-conjugated anti-rabbit immunoglobulin (IgG) (for anti-GFAP, anti-GLUT-1 and anti-xCT) or with peroxidase-conjugated anti-mouse IgG (for anti-GAPDH) at a dilution of 1:3000 for 2 h at room temperature. Chemiluminescence signals were detected in an Image Quant LAS4000 system (GE Healthcare) using an ECL kit.

2.6. 2-Deoxy-D-[1,2-^{3}H]glucose ([^{3}H]2DG) uptake

Basal glucose uptake was assessed as previously described (Souza et al., 2013). Briefly, the cells were rinsed once with HBSS and then the medium was replaced with fresh DMEM/F12 1% FBS for 2 h at 37 °C. Astrocytes were incubated with DMEM/F12 1%FBS containing 1 mCi/ml [^{3}H]2DG for 20 min at 37 °C. After incubation, the cells were rinsed with HBSS and lysed overnight with NaOH 0.3 M. The incorporated radioactivity was measured in a scintillation counter. Cytochalasin B (10 mM) was used as a specific glucose transporter inhibitor. Glucose uptake was determined by subtracting the uptake in the presence of cytochalasin B from the total uptake.

2.7. Glutamine synthetase activity

The enzymatic assay was performed as previously described (Souza et al., 2013). Thirty days after the cultures reached confluence, the cell homogenate (0.1 mL – about 50 mg) was added to 0.1 mL of the reaction mixture containing (in mM): 10 $MgCl_2$, 50 L-glutamate, 100 imidazole-HCl buffer (pH 7.4), 10 2-mercaptoethanol, 50 hydroxylamine-HCl and 10 ATP, and incubated for 15 min (37 °C). The reaction was stopped by the addition of 0.4 mL of a solution containing (in mM): 370 ferric chloride, 670 HCl, and 200 trichloroacetic acid. After centrifugation, the absorbance of the supernatant was measured at 530 nm and compared to the absorbance generated using standard quantities of γ-glutamylhydroxamate treated with the ferric chloride reagent. The results were calculated as μmol/mg protein/h and expressed as the percentage of NB expression.

2.8. Glutathione content

GSH levels were assessed as previously described (Souza et al.,

2013). Thirty days after the cells reached confluence, astrocytes were homogenized (about 50 mg) and diluted in 100 mM sodium phosphate buffer (pH 8.0) containing 5 mM EDTA, and the protein was precipitated with 1.7% meta-phosphoric acid. The supernatant was assayed with ophthaldialdehyde (1 mg/ml methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0–500 mM). The results were calculated as nmol GSH/mg protein and expressed as the percentage of NB expression.

2.9. Protein assay

Protein content was measured using Lowry's method with bovine serum albumin as a standard (Lowry et al., 1951).

2.10. Statistical analyses

Data were statistically analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test. P values < 0.05 were considered significant. For normality analyses, the Kolmogorov–Smirnov test was applied. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software version 15.0 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Adult and aged astrocytes exhibited changes in GFAP expression levels

Despite being derived from animals of different ages, the cultured astrocytes presented a specific morphology (Fig. 1A). As shown by phase contrast microscopy, their shape varied from polygonal to fusiform. Fig. 1B reveals that GFAP expression decreased in an age-dependent manner in AD and AG astrocytes compared to NB cultures (NB = 100 ± 4.3%; AD = 83.6 ± 2.8%; AG = 62.4 ± 5.3%; $P < 0.01$).

3.2. Adult and aged astrocytes take up less glucose and present decreased expression of GLUT1

As shown in Fig. 2A, astrocytes of all ages take up glucose under basal conditions; however, AD and AG astrocytes take up less glucose than NB astrocytes (NB = 100 ± 3.2%; AD = 59.2 ± 7.4%; AG = 47.6 ± 5.9%; $P < 0.05$). The absolute value obtained for glucose uptake in the NB cells was 184.99 ± 5.91 fmol/mg protein and it was considered 100%. As glucose uptake is primarily a function of

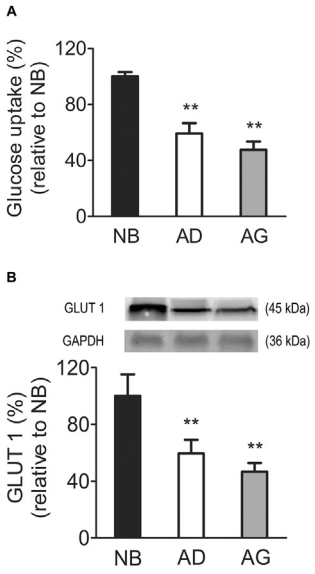

Fig. 2. Changes in glucose uptake and GLUT1 expression in AIV cultures. Astrocytes take up glucose. A) AD and AG astrocytes take up less glucose than NB astrocytes ($P < 0.05$). B) NB astrocytes present higher GLUT1 expression ($P < 0.01$). The top of the panel shows representative images of the blots. Data are presented as the mean + S.E. from 4 experimental trials performed in triplicate. NB = newborn, AD = adult and AG = aged.

GLUT1 in astrocytes; Fig. 2B shows that AD and AG astrocytes exhibited decreased GLUT1 expression levels compared to that of NB cultures (NB = 100 ± 15%; AD = 59.5 ± 9.6%; AG = 46.8 ± 6.0%; $P < 0.01$).

3.3. Adult and aged astrocytes exhibited decreased GS activity and increased GSH levels

Fig. 3A reveals that AD and AG astrocytes presented decreased GS activity compared to that of NB (NB = 100 ± 7.0%; AD = 61.5 ± 5.5%; AG = 59.7 ± 2.7%; $P < 0.01$). AD and AG astrocytes presented increased GSH content compared to that of NB (NB = 100 ± 2.6%; AD = 117.4 ± 6.9%; AG = 118.2 ± 3.6%; Fig. 3B, $P < 0.05$). The absolute values obtained for GS activity and GSH content assays in the NB cells were, respectively, 4.61 ± 0.32 μmol/mg protein/h and 91.83 ± 3.8 nmol/mg protein and it were considered 100%. Accordingly, we found increased expression of the subunit xCT of system x_c^-, (NB = 100 ± 3.6%; AD = 402.5 ± 25.2%; 415.3 ± 31.3%; Fig. 3C, $P < 0.01$).

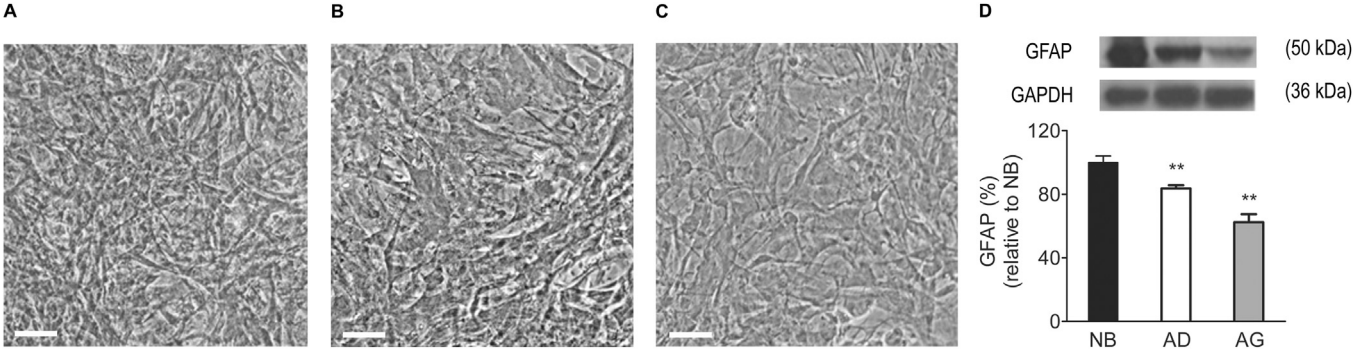

Fig. 1. Cellular morphology and GFAP expression levels in AIV cultures. Astrocyte cultures present a characteristic morphology by phase contrast. A) NB; B) AD; C) AG. D) AD and AG astrocytes presented decreased expression of GFAP ($P < 0.01$). The top of panel shows representative images of the blots. Data are presented as the mean + S.E. from 4 experimental trials performed in triplicate. NB = newborn, AD = adult and AG = aged. Scale bar = 50 μm, magnification = 200×.

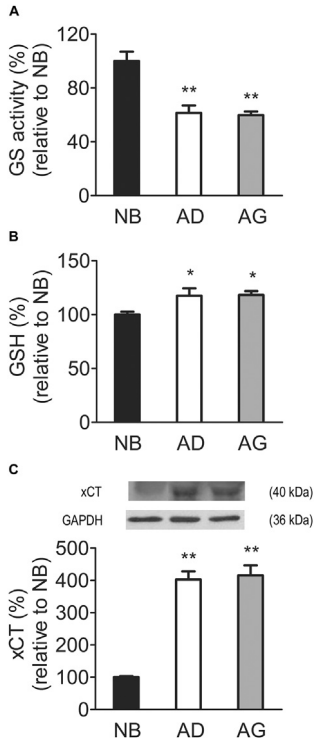

Fig. 3. Changes in glial functionality. A) GS activity was decreased in AD and AG astrocytes compared to that in NB astrocytes ($P < 0.01$). B) GSH content was increased in AD and AG astrocytes compared with that in NB astrocytes ($P < 0.05$). C) xCT expression was increased in AD and AG cells compared with NB cells ($P < 0.01$). The top of the panel shows representative images of the blots. Data are presented as the mean + S.E. from 4 experimental trials performed in triplicate. NB = newborn, AD = adult and AG = aged.

4. Discussion

Aging causes a number of alterations in the properties of cells. Although cerebral homeostasis is a tightly controlled process, during aging, certain properties of this system may become dysfunctional (Pertusa et al., 2007; Shen et al., 2014). Therefore, the possibility to reliably study the features of the brain during development and senescence has become a necessity, considering the continuous increase in human lifespan and the concomitant high incidence of neurodegenerative disorders. In this paper, we proposed to simulate the aging process by extending the culture time of astrocytes to 30 days after confluence (AIV), and we have shown that astrocyte primary cultures derived from newborn, adult and aged Wistar rats show distinct metabolic properties.

Astrocytes are the most versatile cells in the CNS and are fundamentally related to the maintenance of brain physiology. They are involved in neurotransmitter management, ionic homeostasis, and neuronal plasticity; in addition, they are located near the blood vessels and participate in the blood–brain barrier, enabling them to manage the input of glucose and other substances into the brain (Bellaver et al., 2014; Jiang and Cadenas, 2014; Perea et al., 2014; Souza et al., 2013). In this sense, our group has previously shown that astrocyte cultures from AD and AG Wistar rats are a reliable tool to study brain development under physiological and pathological conditions. Other studies using this methodology were carried out at confluence, but this is the first study to analyze cellular responses 30 days after confluence, a condition that some authors have suggested more closely mimics the process of aging (Gottfried et al., 2002; Klamt et al., 2002). Authors have hypothesized that primary dysfunction in astrocytes leads to compromised functions in other cells, culminating in a loss of brain homeostasis (Pertusa et al., 2007).

Morphological analysis of confluent astrocyte cultures shows that NB, AD and AG cells present a similar morphology. However, NB cultures proliferate faster than AD and AG cultures. NB cells tend to be much more plastic than AD and AG cells are, due to the fact that they are placed in a developing tissue, hence, susceptible to changes until reach maturity. Therefore, these adult astrocyte cultures may truly represent developed and mature cells, allowing them to respond more reliably to stimuli during the study of aging models. Accordingly, the AIV model may represent an important tool to study senescence. GFAP, an important astrocytic marker, is related to migration and proliferation, glutamate transport and glutamine synthesis (Middeldorp and Hol, 2011). In this sense, our astrocyte cultures present intense GFAP expression (Fig. 1B). It is noteworthy that herein, we evaluated GFAP under the same conditions (AIV) but from rats of different ages.

One important property of these cells is that they take up glucose, as they are in close contact with capillaries at their end-feet processes (Benarroch, 2014). This is an essential activity because it allows the astrocytes to synthesize glycogen and to metabolize glucose into lactate for export to other cells – either to use for their energy requirements or as a signaling molecule (Dienel and Cruz, 2014; Pellerin, 2005). We observed that AD and AG cells take up less glucose than NB cells. This finding is in accordance with the fact that NB astrocytes present more stimuli to grow and proliferate, requiring more energy generation and a consequent greater need to take up energetic substrates until confluence (Kuzawa et al., 2014). In accordance with their higher glucose uptake, the expression of glucose transporter 1 (GLUT 1) was also higher in NB astrocytes compared to AD and AG astrocytes.

GS is an enzyme that is expressed only in astrocytes in the CNS, and its activity tends to decrease with the aging process, as shown by Bellaver et al., 2014. Herein, we found decreased GS activity in AD and AG cells compared to that of NB cells. Astrocytes are key cells in glutamate homeostasis, and a decrease in GS activity has been reported in neuropsychiatric disorders, in which glutamatergic excitotoxicity plays an important role (Sheldon and Robinson, 2007). Moreover, GS is markedly sensitive to oxidative stress, which is increased under these conditions (Klamt et al., 2002).

Notwithstanding, AD and AG astrocytes showed increased GSH content compared to that of NB astrocytes. This finding is in accordance with the findings of previous studies of cultured astrocytes aged *in vitro*, which showed that enzymatic antioxidant defenses increased as aging occurred, probably stimulated by the increase in reactive oxygen/nitrogen species generation (Klamt et al., 2002). GSH synthesis is closely related to system x_c^- (Lewerenz et al., 2006). Therefore, we showed in Fig. 3C that AD and AG astrocytes present augmented expression of the xCT subunit of system x_c^-, a cystine-glutamate antiporter that contributes both to extracellular glutamatergic signaling and to the intracellular availability of substrates to GSH synthesis; consequently, alterations in this transporter may contribute to aging.

The complexity of the aging process depends on several variables, and *in vitro* conditions are very different from those *in vivo*. Still, cell cultures remain a source of answers for the numerous questions posed by researchers about brain function. This work represents an innovative tool in the study of brain aging, because astrocytic features are a key determinant of brain homeostasis. In conclusion, we showed here that AD and AG astrocytes respond differently compared with NB astrocytes in several respects, which helps to elucidate the effect of astrocytes physiology on aging. Taken together, our data suggest a role of astrocytes in senescence.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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PARTE III

DISCUSSÃO

O cultivo de células do SNC tem sido amplamente utilizado nas últimas décadas como ferramenta imprescindível na elucidação de funções bioquímicas, celulares e moleculares, especialmente relacionadas à atividade astrocitária (Hertz et al, 1998; Lange et al, 2012). Classicamente, culturas primárias de astrócitos são obtidas do cérebro de roedores em estágio neonatal e são facilmente cultiváveis, devido ao seu grande potencial de proliferação e desenvolvimento. Isto se deve ao fato de o ambiente tecidual do neonato ser altamente enriquecido em estímulos para que ocorra aumento do número e tamanho das células, que levarão à aquisição de funções especializadas de cada tipo celular e à realização de atividades mais complexas (Herculano-Houzel, 2014).

De maneira inversa, o cérebro de animais adultos apresenta pouca variação na quantidade de células e inúmeras conexões celulares já estabelecidas. Por isso, as células cerebrais adultas em cultivo apresentam menor capacidade de adaptação a novas condições do que as células cerebrais neonatais, sendo, portanto, mais adequadas para o estudo de respostas fisiológicas e patológicas que acometem o cérebro adulto (Souza et al, 2013; Souza et al, 2016).

Mudanças sutis na plasticidade funcional no SNC e alterações neurológicas dependentes da idade podem ser observadas durante o envelhecimento cerebral normal, sem necessariamente serem relacionadas a uma doença específica, entretanto, podem levar a um declínio cognitivo que frequentemente acomete idosos (Burke & Barnes, 2006).

Cabe ressaltar que nosso grupo de pesquisa já possui experiência com a cultura de astrócitos derivados do cérebro de animais adultos (Bellaver et al, 2014; Bellaver et al, 2016; Souza et al, 2015; Souza et al, 2013). Demonstramos que culturas derivadas do córtex e do hipocampo de ratos Wistar adultos apresentam os principais marcadores astrocitários – GFAP, GS, GLT1, GLAST, ALDH1L1, S100B, vimentina – e funções astrocitárias clássicas como captação de glutamato, produção de GSH, atividade da GS. Estas células respondem à intervenções com compostos protetores, tóxicos e a estímulos em vias de sinalização. Para reforçar a importância da cultura de astrócitos adultos como uma ferramenta de estudos fidedigna e versátil, nos propusemos a estudar mais aspectos e respostas astrocitárias sob diferentes condições e estímulos.

Inicialmente, analisamos características basais de culturas preparadas a partir de animais de 90 dias, para revelar outros aspectos da funcionalidade destas células e possivelmente dar abertura a novos campos de estudos com esta ferramenta. Demonstramos através da técnica de cromatografia líquida de alta eficiência (CLAE) que o cultivo astrocitário adulto apresenta variado conteúdo de aminoácidos e purinas, alguns dos quais podem atuar como gliotransmissores, importantes moduladores da atividade neural (Harada et al, 2016). Várias moléculas são conhecidas por serem gliotransmissores e eles podem ser trocados através de junções GAP para comunicação entre os próprios astrócitos ou podem ser liberados no meio extracelular para comunicação entre astrócitos e outros tipos celulares (Parpura et al, 2012). Estudos demonstram que astrócitos secretam gliotransmissores para responder ou modular a atividade neuronal, os quais ativam receptores

localizados nas células circundantes, levando a alterações na transmissão e plasticidade sinápticas, portanto transformando astrócitos em elementos ativos da funcionalidade cerebral (Perea et al, 2014).

Os principais aminoácidos presentes intra e extracelularmente nas culturas foram a glutamina e a glicina. Isto é compreensível devido a grande importância destes aminoácidos em atividades específicas astrocitárias. A glutamina é o principal carreador de grupamentos amino no cérebro, sendo um produto do ciclo glutamato-glutamina, que tem como enzima principal a GS, uma enzima exclusivamente astrocitária no SNC (Norenberg & Martinez-Hernandez, 1979). A glutamina é fundamental na homeostase do SNC, sendo um importante substrato energético e repositor de glutamato e GABA para neurônios. A glicina, aminoácido com a segunda maior concentração nos astrócitos adultos, é fundamental na gliotransmissão, sendo um substrato direto para a síntese de glutationa, o principal antioxidante astrocitário e um dos mais importantes no cérebro como um todo (Dringen, 2000).

O ATP, uma molécula essencial ao funcionamento celular, pode atuar também como gliotransmissor, regulando a excitabilidade neuronal dependendo da concentração (Zorec et al, 2015). Demonstramos que os níveis de ATP extracelular são bastante elevados, especialmente comparados ao ADP e adenosina, sendo que, *in vivo*, o mais comum é o inverso, pois geralmente o ATP é rapidamente hidrolisado no meio extracelular, o que indica que, na cultura adulta, o funcionamento ou a expressão das nucleotidases podem estar diminuídos, por isso, sugerimos que a baixa atividade das nucleotidases pode ser um indicativo de

envelhecimento. Dessa forma, demonstramos que astrócitos maduros são uma importante ferramenta para estudar gliotransmissão no envelhecimento.

A principal consumidora de ATP no astrócito e em todo o SNC é a enzima Na^+/K^+-ATPase, a qual mantém níveis intracelulares de Na^+ extremamente baixos em comparação ao meio extracelular, permitindo uma relação sinal/ruído adequada à uma rápida e eficiente comunicação sináptica, momento em que ocorre o influxo de Na^+ através de canais presentes na membrana sináptica, gerando uma complexa sinalização celular. Para os astrócitos, a importância da baixa concentração de Na^+ extracelular se dá pelo fato de que os transportadores de glutamato utilizam a energia do gradiente iônico para transportar o glutamato para dentro da célula, evitando o excesso de glutamato na fenda sináptica que pode induzir a superativação dos receptores glutamatérgicos, o que pode desencadear excitotoxicidade e morte neuronal (Anderson & Swanson, 2000; Danbolt, 2001). Pela extrema importância da Na^+/K^+-ATPase, nós dosamos sua atividade sob condições basais em astrócitos adultos e o valor obtido demonstrou o correto funcionamento desta enzima, validando com mais este parâmetro o uso da cultura como ferramenta de estudos do cérebro adulto.

Pela importância dos astrócitos no manuseio dos neurotransmissores, nós estudamos na cultura adulta se os astrócitos têm papel relevante na manutenção de níveis de outros neurotransmissores além do glutamato. Astrócitos adultos apresentaram GAT1, o principal transportador astrocitário de GABA, o qual tem como destino a geração de energia (Schousboe et al, 2013). Demonstramos também que astrócitos adultos apresentam atividade da AchE, capaz de metabolizar a Ach e modular o sistema colinérgico. Desta

maneira, fornecemos evidências que astrócitos adultos mantêm algumas características do cérebro adulto e apresentam funcionalidade na manutenção de níveis de neurotransmissores.

O receptor canabinóide astrocitário CB1 não foi detectado nas células, o que indica que, compreensivelmente, nem todas as características são mantidas *in vitro*. A sinalização canabinóide está relacionada à plasticidade sináptica em muitas regiões do SNC e sua desregulação pode contribuir para desordens psiquiátricas, como ansiedade e depressão (Rasooli-Nejad et al, 2014). A ausência da sinalização canabinóide pode promover dano neuronal, mais frequentemente observado no cérebro envelhecido, sendo assim mais uma razão para o uso da cultura adulta como modelo de estudos do cérebro que está mais suscetível a sofrer alguma injúria.

A seguir, apresentamos evidências de que as células do nosso modelo de cultura provêem, em sua maioria, de astrócitos do cérebro maduro que se organizam na forma de uma cultura monotípica, e não de precursores presentes no cérebro que, de forma quiescente, aguardam estímulos para se diferenciar e desenvolver. As células NB *ex vivo* apresentaram expressão significativamente maior de Sox10 em relação às células AD e AG, sendo a Sox10 um fator de transcrição associado a células gliais indiferenciadas (Kim et al, 2003; Kuhlbrodt et al, 1998). Observamos também a expressão significativamente maior de GFAP em AD e AG, que está de acordo com informações da literatura que indicam que a partir do nascimento a glia tem mais estímulos para proliferar e se desenvolver em roedores, sendo seu conteúdo proporcionalmente menor no momento do nascimento do que o conteúdo neuronal (Herculano-Houzel, 2014). Em relação ao conteúdo de

vimentina *ex vivo*, observamos que ela é significativamente menor em AD do que nos outros grupos, o que também corrobora estudos prévios que indicam que a vimentina é bastante expressa nos astrócitos jovens, após um decréscimo no cérebro adulto, há posterior aumento no cérebro envelhecido (Bignami et al, 1982; Eliasson et al, 1999). Cabe ressaltar que apesar do baixo conteúdo de Sox10 na suspensão celular, ou seja, baixa quantidade de progenitores, a cultura se desenvolve até atingir a confluência.

Em relação ao conteúdo *in vitro*, o qual foi analisado no momento da confluência das culturas, observamos que não há diferenças no conteúdo de GFAP entre os grupos, sendo compreensivelmente mais alto que no conteúdo *ex vivo*, demonstrando que de fato as condições de cultivo levam a um fenótipo astrocitário. O fator de transcrição Sox10, interessantemente, está presente numa proporção similar nos conteúdos *in vitro* e *ex vivo*, demonstrando que as células neonatais permanecem com características de indiferenciação, possivelmente devido à falta de estímulo neuronal, pois, à medida que neurônios e astrócitos coexistem e interagem no tecido cerebral, um tipo cerebral influencia/sinaliza o outro através da liberação de moléculas de comunicação celular, como neuro- e gliotransmissores, fatores de crescimento e outros (Swanson et al, 1997). O conteúdo de vimentina não sofre muitas alterações nos grupos AD e AG, permanecendo em valores baixos, entretanto o grupo NB apresenta presença de vimentina similar à de GFAP, demonstrando que, nesta cultura as duas proteínas são proporcionalmente expressas.

Classicamente, disfunção mitocondrial e geração excessiva de ERO/ERN estão implicadas no envelhecimento cerebral (Halliwell, 2001;

Halliwell, 2006). Através de algumas medidas de funcionalidade mitocondrial, demonstramos que as mitocôndrias tanto do animal (*ex vivo*) quanto da cultura celular (*in vitro*) apresentam prejuízo funcional de maneira idade-dependente. Aqui fornecemos a primeira evidência de que a cultura madura é representativa do cérebro maduro e pode fornecer respostas mais aproximadas da funcionalidade real dos astrócitos *in vivo*.

O aumento do status oxidativo observado durante o envelhecimento pode estar relacionado a mudanças no sistema enzimático de proteção antioxidante, representado principalmente pelas enzimas SOD, CAT e GPx. Nós demonstramos reduzida atividade de SOD e CAT de maneira idade-dependente, tanto *ex vivo* quanto *in vitro*. Em relação à GPx, observamos apenas alteração idade-dependente *in vitro*, possivelmente por alta atividade desta enzima em outros tipos celulares nos experimentos conduzidos *ex vivo*.

A seguir, realizamos um estudo comparativo entre culturas provenientes de animais de três idades diferentes (neonatos, adultos e envelhecidos) frente ao efeito de incubação com glutamato (500 μM), concentração que já demonstrou exercer efeitos metabólicos, por vinte minutos, um curto período de tempo. É importante ressaltar que já foi demonstrado que a funcionalidade astrocitária sofre alterações ao longo do desenvolvimento e envelhecimento, por isso é esperado que células de diferentes idades apresentem respostas diferentes a certos estímulos.

Conforme já demonstrado pelo nosso grupo (Souza et al, 2013), a presença de glutamato é capaz de aumentar a captação de glicose em astrócitos AD, e isto foi observado também em astrócitos NB. Entretanto, astrócitos AG não apresentaram captação específica de glicose frente ao

estímulo com glutamato. Considerando que este evento poderia estar relacionado a uma mudança no metabolismo energético e que as células poderiam estar usando glutamato como substrato energético no lugar da glicose, fornecemos D-Aspartato (D-Asp) em substituição ao glutamato para testar esta hipótese. Os valores de captação no grupo NB não se alteraram significativamente, enquanto que no grupo AD a captação se elevou consideravelmente, sugerindo que, nestas células, o glutamato é utilizado como substrato energético no lugar da glicose, uma vez que esteja presente no meio de incubação. O grupo AG não demonstrou captação de glicose mesmo na presença de D-Asp, sugerindo, além das diferenças metabólicas, outras alterações na sinalização celular.

O pré-tratamento com glutamato foi capaz de reduzir a captação de glutamato nos grupos NB e AD, contudo, interessantemente, o grupo AG não alterou a captação de glutamato comparado às condições controle, apesar de ter reduzido a captação de glicose. Sugerimos que estes fatos podem ter sido um esforço das células para evitar o possível efeito deletério do glutamato extracelular, mantendo a captação de glutamato e reduzindo a de glicose, considerando que astrócitos são capazes de utilizar outras fontes energéticas além de glicose recém captada, por exemplo, mobilizar o glicogênio estocado para suprir suas demandas energéticas por um curto período de tempo. Como o envelhecimento cerebral é acompanhado por um estado progressivo de hipometabolismo (Jiang & Cadenas, 2014), é compreensível que níveis de atividade enzimática e de transportadores sejam reduzidos em AD/AG comparados à NB.

Neste sentido, demonstramos que a atividade da GS é reduzida de maneira idade-dependente, entretanto o conteúdo de GSH é aumentado em astrócitos maduros comparados a astrócitos NB. Isto pode indicar uma mudança nas prioridades metabólicas/oxidativas das células maduras, que preferencialmente usam o glutamato como substrato para a síntese de GSH, e não de glutamina. O fato de a GSH estar aumentada em AG pode ter relação com a quantidade aumentada de ERO observada tanto em níveis basais quanto sob exposição ao glutamato, sendo que já foi demonstrado que as defesas antioxidantes aumentam proporcionalmente ao aumento de ERO.

Para compreender os mecanismos envolvidos nos efeitos do glutamato, nós investigamos as vias de sinalização NFκB e p38 MAPK, devido ao fato destas vias já haverem sido previamente relacionadas ao funcionamento do principal transportador de glutamato, GLT1, e à resposta ao estresse oxidativo celular (Ghosh et al, 2011; Shih et al, 2015). Em relação ao NFκB, em condições basais há uma maior atividade transcricional em AG comparado aos outros grupos. Sob exposição ao glutamato, todos os grupos apresentam ativação de NFκB, sendo que a ativação é proporcional à idade da cultura. Em relação a p38 MAPK, apenas AG apresenta níveis aumentados tanto de maneira basal quanto sob exposição ao glutamato. Estas vias de sinalização podem estar atuando para promover os efeitos observados, como captação de glutamato, aumento de GSH e aumento de ERO. A via da p38 MAPK é a responsável pela translocação do NFκB para o núcleo (Kaminska et al, 2009), e encontra-se alterada apenas em AG, o que

indica uma maior atividade deste fator de transcrição neste grupo, mesmo em condições basais.

Considerando as alterações supracitadas, investigamos por fim a resposta inflamatória nestes grupos. Sabe-se que as células microgliais são as principais responsáveis pelas respostas inflamatória e imune no SNC, entretanto, os astrócitos também estão altamente implicados neste processo, monitorando-o e amplificando-o (Farina et al, 2007). Observamos um aumento significativo nas principais citocinas pró-inflamatórias no grupo AG exposto ao glutamato, corroborando o fato de NFκB e p38 MAPK estarem ativados neste grupo. Isto indica uma resposta inflamatória idade-dependente a uma concentração atóxica de glutamato, o que pode ter contribuído para exacerbar o hipometabolismo associado à idade, o qual está presente em nossos resultados na forma de redução no metabolismo da glicose, glutamato e atividade da GS.

Com a intenção de não demonstrar apenas o uso da cultura adulta em condições basais ou neurotóxicas, a seguir demonstramos o potencial glioprotetor da guanosina sob condições basais, em que utilizamos astrócitos obtidos de animais de três idades diferentes (novamente NB, AD e AG), objetivando demonstrar que muitos dos efeitos benéficos da guanosina no cérebro adulto potencialmente têm como alvo os astrócitos.

Diversos estudos do nosso grupo e de outros grupos de pesquisa já evidenciaram que a guanosina exerce um efeito benéfico em modelos experimentais *in vivo* e *in vitro*, como por exemplo, convulsões induzidas por ácido caínico, insulto isquêmico, hipoglicemia e insultos oxidativos e inflamatórios (Bellaver et al, 2015; Dal-Cim et al, 2013; Quincozes-Santos et

al, 2013; Quincozes-Santos et al, 2014; Rathbone et al, 2011; Schmidt et al, 2009; Schmidt et al, 2000). Em relação ao mecanismo e alvos moleculares, não está bem definido se a guanosina de fato possui um receptor através do qual realiza suas ações, entretanto já foi demonstrado que ela modula vias de sinalização como proteínas G, MAPK e HO1.

Portanto, para este estudo, as células NB, AD e AG foram incubadas por 24 h com 100 μM guanosina na presença ou ausência do inibidor da via HO1, ZnPP IX (10 μM). Conforme já mencionado, o envelhecimento pode alterar propriedades celulares e, sendo os astrócitos essenciais para a homeostase cerebral, alterações nas funções astrocitárias podem comprometer todo o SNC (Burke & Barnes, 2006; Jiang & Cadenas, 2014; Stanimirovic et al, 1999). Da mesma forma, melhorias nas funções astrocitárias podem contribuir para que o SNC se mantenha equilibrado, o que seria especialmente desejável no envelhecimento, momento de surgimento das principais doenças neurodegenerativas.

Neste estudo demonstramos que o tratamento com guanosina foi capaz de melhorar funções astrocitárias de maneira HO1-dependente apenas em células maduras. O tratamento com guanosina promoveu o aumento na captação de glutamato, fator considerado glioprotetor por evitar excitotoxicidade, também já demonstrado anteriormente (Frizzo et al, 2001; Thomazi et al, 2008). Transportadores de glutamato são redox-sensíveis por possuírem muitos resíduos cisteína, e o potencial antioxidante da guanosina possivelmente exerce uma proteção da estrutura destas proteínas. Diretamente relacionado ao aumento da captação de glutamato, observamos

o aumento da atividade da enzima GS e do conteúdo do antioxidante GSH, sendo o glutamato precursor de ambas funções.

O aumento na GSH é um feito notável da guanosina, visto que, conforme já demonstrado, o cérebro maduro é mais suscetível ao estresse oxidativo e gera maior quantidade de ERO, o que pode culminar com perda de funcionalidade de proteínas e, consequentemente, de funções celulares. Desta forma, o aumento de GSH – que, nos astrócitos, é usada na defesa própria contra ERO, entretanto também pode ser exportada para uso por outros tipos celulares – sendo um fator que ajuda e compreender parcialmente os efeitos glioprotetores da guanosina.

Sob efeito do tratamento com guanosina, avaliamos também parâmetros inflamatórios como a liberação das principais citocinas pró-inflamatórias – TNFα e IL1β – e níveis de NFκB, o principal regulador da resposta oxidativa e inflamatória. De maneira similar ao metabolismo glutamatérgico, a resposta inflamatória foi modulada pela guanosina, que exerceu um efeito dependente da via da HO1, reduzindo a secreção de citocinas e a ativação do NFκB. Sendo o cérebro maduro mais inclinado a apresentar uma condição pró-infamatória e a resposta inflamatória uma condição prevalente na fisiopatologia de doenças neurodegenerativas (Farina et al, 2007; Lee et al, 2010), é de grande interesse para o envelhecimento saudável que esta situação seja controlada ou até mesmo revertida, conforme observamos neste estudo.

Efeitos de substâncias protetoras podem ser evidenciados devido à ação coordenada entre a ativação de vias de sinalização presentes no cérebro maduro e o aparato enzimático astrocitário, o que demonstra que, em

estudos *in vivo*, muitas vezes observam-se efeitos glioprotetores que levam a um benefício geral no SNC devido ao grande número de funções que estas células desempenham. Evidenciamos aqui que a guanosina, através de sua modulação da via HO1, é um potencial agente terapêutico, podendo atuar também como um agente antienvelhecimento. A guanosina exerce um efeito geral neuroprotetor em modelos *in vivo*, e nossos resultados sugerem que os astrócitos são o alvo primário da sua ação.

Como maneira de estudar a senescência, utilizamos culturas de astrócitos derivadas de NB, AD e AG e realizamos um modelo de envelhecimento *in vitro* (mencionadas no artigo e neste texto como AIV, do inglês “aged *in vitro*”) para verificar respostas celulares relacionadas à senescência. Os experimentos foram realizados trinta dias após a confluência. Sendo os astrócitos células determinantes da funcionalidade do SNC, é importante compreender as mudanças que estas células sofrem no processo de senescência, visto que mudanças nas funções astrocitárias afetam o SNC de maneira geral (Jiang & Cadenas, 2014).

A análise dos níveis proteicos de GFAP e GLUT1 demonstrou que há um decréscimo dependente da idade na expressão destas proteínas nas culturas AIV, em relação aos níveis de NB. A captação de glicose e a atividade da GS apresentaram-se diminuídas de maneira idade-dependente. Estes dados corroboram estudos que indicam que o cérebro maduro/envelhecido encontra-se num estado hipometabólico ou hipofuncional em comparação ao cérebro jovem, conforme já discutido anteriormente (Jiang & Cadenas, 2014).

Os níveis diminuídos de GLUT1 podem estar relacionados ao fato de o tecido neonato estar em fase de formação, proliferação e aquisição de funções no momento em que é utilizado para o preparo da cultura, necessitando, assim, de mais substrato energético para seu desenvolvimento (Benarroch, 2014). O fato de que, comparativamente, astrócitos NB possuem mais GLUT1 do que AD/AG pode indicar uma ˜memória de funcionalidade" celular, que faz com que as células continuem expressando níveis altos da proteína, mesmo estando em uma situação diferente. Com nossa pesquisa pretendemos mostrar que as células neonatais permanecem em um estágio de sub maturidade celular, e a diferença nos níveis de GLUT1 corrobora nossa hipótese e justifica o fato de astrócitos NB captarem mais glicose que AD/AG.

Demonstramos também que o conteúdo de GSH foi significativamente maior em AD/AG do que em NB, possivelmente porque durante o amadurecimento/envelhecimento ocorre maior geração de radicais livres e, consequentemente, estresse oxidativo, portanto as células podem estar sintetizando mais defesas antioxidantes para garantir o funcionamento celular mesmo com maior nível de ERO. A síntese de GSH está relacionada ao funcionamento do sistema x^-_c (Lewerenz et al, 2006; Sato et al, 1999). Consequentemente, evidenciamos que os níveis de xCT, uma subunidade do trocador cistina/glutamato (sistema x^-_c), que contribui para a importação de cisteína, um dos substratos da GSH, e exportação de glutamato, estão fortemente aumentados em AD/AG comparado a NB.

O sistema x^-_c já demonstrou estar envolvido na progressão e agressividade de tumores e em eventos epilépticos no SNC (Buckingham &

Robel, 2013), e o expressivo aumento da subunidade xCT nos astrócitos maduros AIV comparados aos neonatais pode demonstrar uma maior suscetibilidade do cérebro maduro ao surgimento destas condições patológicas, especialmente no envelhecimento.

Alguns estudos já sugeriram esta prática da extensão do tempo de cultivo como maneira de gerar células mais similares a células adultas ou senescentes (Gottfried et al, 2002), entretanto, conforme já demonstrado anteriormente nesta tese e em trabalhos do nosso grupo de pesquisa, a cultura de células proveniente do cérebro maduro é diferente da proveniente do cérebro neonato em diversos aspectos, portanto mais adequada para gerar células com características de envelhecimento.

Ao longo deste estudo demonstramos diferentes abordagens para estudar alterações de resposta astrocitária dependentes da idade e, desta forma, é possível evidenciar dois perfis de respostas antioxidantes astrocitárias. Temos que considerar que, em cada modelo estudado, o momento de coleta das células para dosagem de GSH varia, sendo nos experimentos com glutamato (onde demonstramos mais GSH em células maduras do que em neonatos) um tempo de incubação de vinte minutos e nos experimentos com guanosina (onde demonstramos mais GSH em neonatos do que em células maduras) um tempo de incubação de 24 horas. Da mesma forma, no modelo de senescência, em que observamos maior conteúdo de GSH nas células maduras do que em neonatos, estudamos respostas astrocitárias trinta dias após a confluência celular.

Assim, demonstramos com este estudo a importância da disponibilidade de uma ferramenta como a cultura adulta, pois com

abordagens neurotóxicas e glioprotetoras, analisando respostas celulares em condições basais na confluência do cultivo ou num modelo de envelhecimento, objetivamos desvendar características bioquímicas, celulares e moleculares da cultura de astrócitos adultos, demonstrando algumas de suas diferenças em comparação à cultura astrocitária preparada a partir de cérebro de ratos neonatos. Desta forma, a cada estudo, pretendemos ampliar a compreensão das propriedades e funções celulares desta ferramenta, para que ela possa ser amplamente empregada e fornecer respostas mais aproximadas às respostas fornecidas por astrócitos do cérebro maduro *in vivo*, especialmente no estudo do envelhecimento e das doenças neurodegenerativas.

CONCLUSÕES

- A cultura de astrócitos adultos é proveniente de células maduras, não de progenitores imaturos. Além disso, possui grande potencial de representatividade do cérebro maduro, conforme observamos nos parâmetros mitocondriais analisados;
- Algumas propriedades basais da cultura adulta foram determinadas, como, por exemplo, o perfil intra e extracelular de aminoácidos, a presença de neuro- e gliotransmissores, assim como, atividade enzimática;
- A resposta celular frente à exposição a uma concentração não-tóxica de glutamato é dependente do grau de maturidade da cultura e das vias de sinalização NFκB e p38 MAPK;
- A guanosina exerceu um efeito antienvelhecimento dependente da via da HO1 em astrócitos maduros, sugerindo possuir um papel glioprotetor;
- Propriedades astrocitárias analisadas em células de diferentes idades submetidas a um modelo de senescência demonstraram que, sendo a cultura proveniente de animais neonatos, o modelo de senescência não é a melhor abordagem para estudar astrócitos maduros, e sim a cultura preparada do animal adulto/envelhecido.

PERSPECTIVAS

- Investigar alterações metabólicas no modelo de insulto inflamatório por LPS;
- Analisar respostas astrocitárias em culturas preparadas a partir de animais alimentados com diferentes dietas;
- Analisar respostas astrocitárias relacionadas à inflamação em animais senescentes (2 anos) tratados com resveratrol;
- Padronizar o cultivo astrocitário a partir de ratas adultas e investigar a sinalização do estrogênio, bem como possíveis estratégias glioprotetoras;
- Analisar os papéis das vias de sinalização associadas ao metabolismo e envelhecimento como: GSK3β, AMPK, SIRT1 e da enzima PKM2 em parâmetros astrocitários.

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