UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

EFEITOS DA EXPOSIÇÃO À AMÔNIA EM CÉLULAS ASTROGLIAIS E NEURONAIS: MECANISMOS PROTETORES DO RESVERATROL E DO ÁCIDO LIPOICO

Larissa Daniele Bobermin

Orientador: Prof. Carlos Alberto Saraiva Gonçalves

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do grau de Doutor em Bioquímica.

Porto Alegre

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Dedico esta tese aos meus pais, Nelci e Cláudio.

"Foi o tempo que dedicaste à tua rosa que a fez tão importante".

O Pequeno Príncipe – Antoine de Saint-Exupéry

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RESUMO

Os astrócitos são células conhecidas por sua capacidade dinâmica e versatilidade, participando não somente da manutenção da homeostase cerebral em condições fisiológicas, mas também respondendo em condições patológicas, como por exemplo, na encefalopatia hepática (EH). Esta patologia neurológica está associada principalmente à hiperamonemia, decorrente de uma falência hepática aguda ou crônica. A toxicidade da amônia no sistema nervoso central (SNC) é mediada por uma série de alterações celulares e metabólicas, principalmente nas células astrogliais. Nesse sentido, a busca por moléculas com potencial terapêutico no SNC é extremamente relevante. Trabalhos do nosso grupo mostraram que o resveratrol e o ácido lipoico, duas moléculas conhecidas pelos seus efeitos antioxidantes, modulam importantes funções gliais, relacionadas principalmente ao metabolismo glutamatérgico, à defesa antioxidante e à resposta inflamatória. Neste sentido, esta tese teve como objetivo avaliar os efeitos do resveratrol e do ácido lipoico em células astrogliais e neuronais expostas à amônia, assim como seus possíveis mecanismos protetores. Primeiramente, nós observamos que o resveratrol e o ácido lipoico foram capazes de prevenir as alterações induzidas pela amônia em células astrogliais C6, sobre parâmetros do metabolismo glutamatérgico, dentre os quais podemos destacar a captação de glutamato, a atividade da enzima glutamina sintetase (GS) e o conteúdo de glutationa (GSH). Além disso, o ácido lipoico também exerceu um efeito anti-inflamatório nestas células, prevenindo a liberação de citocinas pró-inflamatórias (TNFα, IL-1β, IL-6, IL-18) e da proteína S100B, através da diminuição da ativação do fator de transcrição NFκB. Nós também verificamos que a maioria dos efeitos protetores do resveratrol e do ácido lipoico foram dependentes da heme oxigenase 1 (HO1), uma enzima associada com a defesa celular em situações de estresse. Por fim, foram avaliados os efeitos do resveratrol e do ácido lipoico sobre células neuronais expostas à amônia. Novamente, ambos apresentaram um efeito benéfico, prevenindo tanto o aumento da produção de espécies reativas de oxigênio (ERO) quanto a diminuição do conteúdo de GSH induzidos pela amônia. Nas células neuronais, a proteína HO1 também participou dos efeitos protetores do resveratrol e do ácido lipoico. Em conjunto, esses resultados nos mostram que o resveratrol e o ácido lipoico são capazes de modular positivamente o funcionamento tanto de células astrogliais quanto de células neuronais em situações de hiperamonemia, compartilhando também alguns mecanismos de ação, como a indução da HO1. Este estudo *in vitro* sugere que o resveratrol e o ácido lipoico são potenciais agentes terapêuticos para doenças do SNC, as quais envolvam produção de espécies reativas e resposta inflamatória, como a EH.

ABSTRACT

Astrocytes are dynamic and versatile cells which participate in the maintenance of brain homeostasis in physiological conditions and also in response to pathological conditions, such as hepatic encephalopathy. This neurological disease is mainly associated with hyperamonnemia, resulting from acute or chronic liver failure. Ammonia toxicity in the central nervous system (CNS) is mediated by several cellular and metabolic alterations, primarily in astroglial cells. In this sense, the search for molecules with therapeutical potential for CNS becomes highly relevant. Our group has shown that resveratrol and lipoic acid, two molecules known for their antioxidant activities, are able to modulate important glial functions mainly related to glutamate metabolism, antioxidant defense and inflammatory response. In this sense, this study aimed to evaluate the effects of resveratrol and lipoic acid in astroglial and neuronal cells exposed to ammonia, as well as their possible protective mechanisms. Firstly, we observed that resveratrol and lipoic acid were able to prevent ammonia-induced alterations in C6 astroglial cells functioning, such as glutamate uptake, glutamine synthetase (GS) activity and intracellular GSH content. Moreover, lipoic acid also exerted an anti-inflammatory effect in C6 astroglial cells, preventing the ammonia-stimulated release of pro-inflammatory cytokines (TNF, IL-1β, IL-6, IL-18) and S100B protein, by decreasing the activation of the transcription factor NFκB. We also verified that the most protective effects of resveratrol and lipoic acid involved the heme oxygenase 1 (HO1), an enzyme associated with protection against stressful conditions. Finally, we evaluated the effects of resveratrol and lipoic acid on neuronal cells exposed to ammonia. Again, both showed beneficial roles, preventing the increase of reactive oxygen species (ROS) and decrease of GSH content induced by ammonia. In neuronal cells, HO1 also mediated the protective effects of resveratrol and lipoic acid. Taken together, these results show that resveratrol and lipoic acid are able to positively modulate the functioning of both astroglial and neuronal cells in hyperamonemmia conditions, sharing some mechanisms of action, such as HO1. This study *in vitro* suggests that resveratrol and lipoic acid may represent potential therapeutic agents for CNS, during oxidative and inflammatory damages, as the induced by ammonia.

LISTA DE ABREVIATURAS

ADP	Adenosina Disfofato
AMPA	Ácido α-amino-3-hidróxi-5-metil-isoxazolenopropionato
ATP	Adenosina trifosfato
DHLA	Ácido dihidrolipoico
DNA	Ácido desoxirribonucléico
EAAC	Carreador de aminoácidos excitatórios
EAAT	Transportador de aminoácidos excitatórios
EH	Encefalopatia hepática
ERK	Cinase regulada por sinal extracelular
ERN	Espécies reativas de oxigênio
ERO	Espécies reativas de oxigênio
GABA	Ácido gama-aminobutírico
GLAST	Transportator Glutamato-Aspartato
GLT1	Transportador de glutamato tipo 1
GS	Glutamina sintetase
GSH	Glutationa
HO	Heme oxigenase
IL	Interleucinas
LPS	Lipopolissacarídeo
MAPK	Proteínas cinases ativadas por mitógenos
mGluR	Receptores de glutamate metabotrópicos
NAC	N-acetilcisteína
NFκB	Fator nuclear de cadeia κ de linfócitos B

NKCC	Co-transportador de Na^{+}-K^{+}-Cl^{-}
NMDA	N-metil-D-aspartato
NOS	Óxido nítrico sintase
Nrf2	Fator nuclear eritroide 2
$O_2^{\bullet-}$	Ânion superóxido
$OH^{\bullet-}$	Radical hidroxila
$ONOO^{-}$	Peroxinitrito
PI3K	Fosfatidil-inositol-3-cinase
PKC	Proteína cinase C
RNA	Ácido ribonucleico
SNC	Sistema nervoso central
SOD	Superóxido dismutase
TNFα	Fator de necrose tumoral α
VRAC	Canais de ânios regulados por volume
Xc^{-}	Trocador cistina-glutamato

INTRODUÇÃO

1. Sistema Nervoso Central

O parênquima do Sistema Nervoso Central (SNC) é constituído de células nervosas, ou neurônios, e suas aferências e eferências, as quais estão envolvidas pelas células gliais, permitindo uma íntima interação celular entre elas. Os neurônios são células especializadas na condução de sinais elétricos a longa distância, a qual é possibilitada devido a sua morfologia, com extensas ramificações, e à organização específica dos seus componentes de membrana, que possibilitam a propagação dos sinais elétricos (Brady et al., 2012; Purves, 2008).

O médico e patologista alemão Rudolph Virchow foi quem descreveu pela primeira vez, em 1846, a neuroglia. O termo glia (do inglês *glue*, que significa cola) reflete a presunção, na época, de que estas células eram responsáveis pela conexão entre as células nervosas. Um pouco depois, nos anos 1870, a natureza celular da glia foi identificada por Camilo Golgi e, posteriormente, Santiago Ramón y Cajal estudou uma classe específica de células gliais, os astrócitos, e suas interações com os outros elementos neurais (Parpura et al., 2012). Atualmente, são conhecidas inúmeras funções das células gliais, que muito mais do que suporte estrutural, garantem proteção e nutrição aos neurônios, além de participarem da modulação da transmissão sináptica, apesar de não serem eletricamente excitáveis. Além disso, as células gliais são capazes de se dividir ao longo da vida, principalmente em resposta a danos (Brady et al., 2012; Purves, 2008; Somjen, 1988; Young, 1991).

Existem três categorias principais de células gliais no SNC: (1) macroglia, composta por astrócitos e oligodendrócitos; (2) microglia; (3) células ependimais. Os oligodendrócitos são responsáveis principalmente pela síntese de mielina, as células microgliais são as células fagocíticas envolvidas na resposta inflamatória e as células

ependimais revestem os ventrículos cerebrais (Jessen, 2004; Perea & Araque, 2005; Vallejo et al., 2010). As células astrogliais foram o alvo deste estudo e serão abordadas detalhadamente a seguir.

1.1. Astrócitos

Os astrócitos são células conhecidas por sua capacidade dinâmica e versatilidade, participando não somente da manutenção da homeostase em condições fisiológicas, mas também respondendo em condições patológicas ou de dano (Parpura et al., 2012; Wang and Bordey, 2008). As principais funções desempenhadas pelos astrócitos são: (1) formação e manutenção da barreira hematoencefálica (Abbott et al., 2006; Alvarez et al., 2013); (2) suporte metabólico aos neurônios, através do metabolismo da glicose e do glicogênio e do ciclo glutamato-glutamina (Bélanger et al., 2011; Pellerin, 2005); (3) metabolismo de neurotransmissores, particularmente o glutamato e o GABA (ácido gama-aminobutírico) (Anderson and Swanson, 2000; Brady et al., 2012; Coulter & Eid, 2012); (4) manutenção da homeostase iônica, principalmente do K^+, e regulação do volume extracelular (Nagelhus & Ottersen, 2013; Walz, 1989); (5) participação na resposta imune inata encefálica, produzindo e liberando diversos mediadores inflamatórios (Jensen et al., 2013); (6) defesa contra o estresse oxidativo e agentes tóxicos para os neurônios (Fernandez-Fernandez et al., 2012; Rao et al., 2005; Wilson, 1997); (7) síntese e liberação de fatores tróficos e substâncias neuroativas, as quais também podem atuar sobre outras células gliais (Allen & Barres, 2009; Pellerin, 2005); (8) atuação como guias na migração de neurônios durante o desenvolvimento (Paixão & Klein, 2010); entre outras.

Atualmente, os astrócitos são reconhecidos como o terceiro elemento da sinapse, e sabe-se que são capazes de responder de maneira seletiva à liberação de

neurotransmissores, os quais disparam cascatas de sinalização através de receptores astrocíticos (Allen & Barres, 2009; Perea et al., 2009; Pérez-Alvarez & Araque, 2013). Esta comunicação recíproca não somente com neurônios, mas também com outras células do SNC, possui uma importância fundamental para a manutenção da homeostase cerebral e plasticidade (Ben Achour & Pascual, 2012; Horner & Palmer, 2003). Dessa forma, o entendimento das alterações celulares e bioquímicas nos astrócitos durante condições patológicas e a busca por moléculas com potencial terapêutico em patologias do SNC, através da modulação da funcionalidade destas células, torna-se extremamente relevante.

1.1.1. Proteína S100B

A S100B é uma proteína de 21 kDa ligante de cálcio, zinco e cobre pertencente à família de proteínas S100, as quais recebem esta denominação devido a sua solubilidade em uma solução 100% saturada de sulfato de amônio. Estruturalmente, se apresenta como homodímeros constituídos por duas subunidades unidas por pontes dissulfeto (Donato et al., 2009; Moore, 1965; Van Eldik & Wainwright, 2003).

No SNC, a S100B é predominantemente expressa e secretada pelos astrócitos. Intracelularmente, a proteína S100B se localiza no citoplasma e participa da modulação do citoesqueleto (Frizzo et al., 2004; Sorci et al., 2000), da regulação da proliferação e diferenciação celular, da homeostase do cálcio e da degradação de proteínas (Donato, 2001; Donato et al., 2009). No meio extracelular, essa proteína pode exercer efeitos autócrinos e parácrinos sobre outras células gliais e neurônios.

Em baixas concentrações, a S100B possui efeitos neurotróficos, estimulando o crescimento e regeneração de neuritos e aumentando a sobrevivência de neurônios (Donato et al., 2009; Kleindienst et al., 2013; Kleindienst & Ross Bullock, 2006; Van Eldik & Wainwright, 2003).

Em altas concentrações, a S100B pode exercer efeitos tóxicos. Em astrócitos e microglia, ela estimula a produção e secreção de citocinas pró-inflamatórias, como por exemplo, o fator de necrose tumoral α (TNFα), a interleucina 1β (IL-1β) e a interleucina 6 (IL-6), além de estimular a produção de óxido nítrico (NO) através da indução da enzima óxido nítrico sintase induzível (iNOS) (Donato et al., 2009; Liu et al., 2005; Schmitt et al., 2007; Villarreal et al., 2014).

Trabalhos do nosso grupo de pesquisa também evidenciam que a S100B pode ser um componente importante na neuroinflamação. A IL-1β, através da sinalização pela via da ERK (cinase regulada por sinal extracelular), é capaz de estimular a secreção de S100B em cultura de astrócitos, células astrogliais C6 e fatias hipocampais (de Souza et al., 2009). Além disso, a secreção de S100B também é estimulada, tanto *in vitro* como *in vivo*, por LPS (lipopolissacarídeo), um componente da parede celular de bactérias gram-negativas que induz respostas inflamatória e imune (Guerra et al., 2011).

1.2. Glutamato

O glutamato é um neurotransmissor excitatório essencial para o funcionamento do SNC, mediando processos como cognição, memória e aprendizado; formação e eliminação das sinapses; migração, diferenciação e morte neuronal; e regulação do metabolismo energético cerebral (Anderson & Swanson, 2000; Bélanger et al., 2011; Hertz, 2006; Luján et al., 2005; Morris, 2013; Paoletti et al., 2013).

Existem duas classes distintas de receptores glutamatérgicos: os ionotrópicos e os metabotrópicos. Os receptores ionotrópicos recebem essa denominação por serem canais de cátions, e são subdivididos em NMDA (N-metil-D-aspartato), AMPA (ácido α-amino-3-hidróxi-5-metil-isoxazolenopropionato) e cainato. Já os receptores metabotrópicos

(mGluR) pertencem a uma família de receptores que são acoplados à proteína G (Brady et al., 2012; Lau & Tymianski, 2010; Luján et al., 2005).

Os astrócitos também expressam alguns tipos de receptores glutamatérgicos (Parpura & Verkhratsky, 2013). Os receptores glutamatérgicos melhor caracterizados em astrócitos são os mGluR, e sua ativação promove um aumento intracelular de Ca^{2+} (Aronica et al., 2000; Kirischuk et al., 1999; Parpura & Verkhratsky, 2012). Os receptores AMPA também são funcionalmente expressos na astroglia, e representam o tipo de receptor glutamatérgico ionotrópico dominante nesse tipo celular (Lalo et al., 2011; Parpura & Verkhratsky, 2013). Porém, em relação aos receptores NMDA, ainda existem controvérsias. Alguns trabalhos têm mostrado a expressão desse tipo de receptor em cultura de células astrogliais e existem indícios de que eles sejam funcionais (Lalo et al., 2006; López et al., 1997; Parpura & Verkhratsky, 2013). No entanto, o papel do receptor NMDA em astrócitos, em condições fisiológicas e/ou patológicas, não está totalmente esclarecido.

Um papel bem estabelecido dos astrócitos é a sua participação na regulação da transmissão sináptica, inclusive glutamatérgica, através da captação, do metabolismo e da liberação do glutamato. A manutenção de níveis extracelulares baixos de glutamato é essencial para o controle da homeostase cerebral, uma vez que em altas concentrações podem levar ao processo de excitotoxicidade (Anderson & Swanson, 2000; Coulter & Eid, 2012; Schousboe & Waagepetersen, 2005).

1.2.1. Captação de Glutamato pelas Células Gliais

Após ser liberado na fenda sináptica pelo neurônio, o glutamato interage com seus receptores ionotrópicos e/ou metabotrópicos, localizados nas membranas celulares pré- e pós-sinápticas dos neurônios e também nas células gliais. Para que a comunicação

sináptica seja encerrada, o glutamato deve ser removido da fenda sináptica por captação (Brady et al., 2012).

A captação de glutamato ocorre através de transportadores de alta afinidade presentes na membrana plasmática tanto de células gliais quanto de neurônios. Os transportadores GLAST (transportador glutamato-aspartato) e GLT1 (transportador de glutamato tipo 1) de roedores, altamente homólogos aos transportadores de aminoácidos excitatórios humanos (EAAT1 e EAAT2, respectivamente), estão localizados em astrócitos. Já os transportadores EAAC1 (carreador de aminoácidos excitatórios 1), correspondente ao EAAT3, são predominantemente neuronais. Existem também os transportadores EAAT4 e EAAT5 expressos pelas células de Purkinje e da retina, respectivamente (Danbolt, 2001; Zhou & Danbolt, 2014). Ainda, nas células gliais, existe o trocador cistina-glutamato (Xc^-), que realiza o transporte de uma molécula de cistina para dentro da célula com a saída de uma molécula de glutamato (Banerjee et al., 2008; Zhou & Danbolt, 2014). O papel fisiológico desse trocador é realizar a captação da cistina, que é necessária para a síntese de glutationa (GSH), a qual será abordada mais adiante.

É importante salientar que os astrócitos são as principais células responsáveis pela captação de glutamato (Danbolt, 2001). Esse processo é fortemente regulado pelo Na^+, uma vez que o gradiente transmembrana deste íon é o principal responsável por impulsionar esse processo nas células astrogliais. O transporte de uma molécula de glutamato é acompanhado pela entrada de três Na^+ e um H^+ e a saída de um K^+. Dessa maneira, a concentração de Na^+ intracelular nos astrócitos sofre flutuações regularmente. Como consequência, ocorre a ativação da enzima Na^+K^+-ATPase, uma proteína transmembrana responsável pela manutenção do gradiente eletroquímico de Na^+ e de K^+ nas células, às custas da hidrólise de ATP. Assim, o funcionamento adequado dessa

bomba nas células astrogliais possui uma importância fundamental para a manutenção da homeostase cerebral (Bélanger et al., 2011; Brady et al., 2012; Illarionava et al., 2014).

Uma vez no citosol das células astrogliais, o glutamato pode seguir diferentes destinos: conversão à glutamina; síntese de GSH; liberação para o meio extracelular; oxidação como substrato energético; síntese de proteínas. Os três primeiros destinos citados possuem uma grande relevância para esta tese, e serão detalhados nas próximas seções.

1.2.2. Glutamina Sintetase e o Ciclo Glutamato-Glutamina

O ciclo glutamato-glutamina é uma importante relação metabólica entre astrócitos e neurônios. Como mostrado na Figura 1, através desse ciclo o glutamato pode ser transportado de volta às células neuronais, na forma de glutamina, a qual é inerte para os receptores glutamatérgicos e, portanto, não provoca efeitos excitotóxicos (Bélanger et al., 2011; Parpura & Verkhratsky, 2012).

Após ser captado pelos astrócitos, o glutamato pode ser convertido em glutamina pela ação da enzima glutamina sintetase (GS) em um processo dependente de ATP. A glutamina, então, é transportada pelos astrócitos para o meio extracelular e é captada pelos terminais pré-sinápticos de neurônios, onde será convertida novamente em glutamato, através da enzima glutaminase (Bélanger et al., 2011; Hertz, 2013; Hertz et al., 1999). A atividade da GS nos astrócitos e a manutenção do ciclo glutamato-glutamina é essencial não só para o metabolismo glutamatérgico, mas também para a manutenção da neurotransmissão inibitória GABAérgica, uma vez que a glutamina é a fonte primária para a síntese de GABA (Hertz, 2013).

A GS é uma enzima predominantemente astrocitária (Martinez-Hernandez et al., 1977) e, além do seu papel no ciclo glutamato-glutamina, possui uma função crítica para

a detoxificação da amônia no SNC, uma vez que a síntese de glutamina envolve uma reação de amidação: glutamato + amônia + ATP → glutamina + ADP + P_i (Coulter & Eid, 2012; Hertz, 2006).

Evidências apontam que um declínio na expressão ou na atividade da GS pode estar relacionado a diversas doenças do SNC. Considerando o papel da GS no metabolismo glutamatérgico, alterações na sua funcionalidade podem afetar a concentração de glutamato nos astrócitos, prejudicando sua capacidade de captar o glutamato do meio extracelular (Coulter & Eid, 2012; Hertz, 2006; Matés et al., 2002).

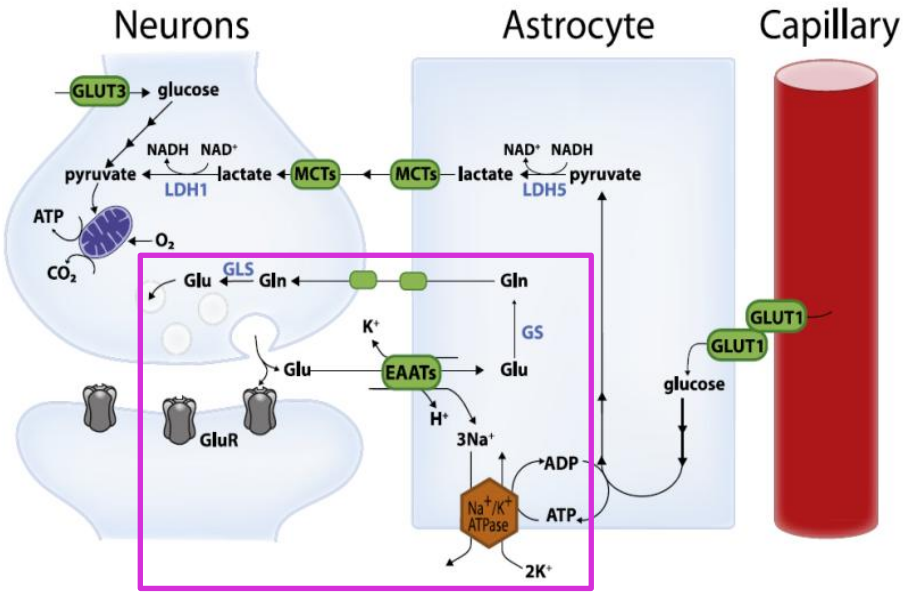

Figura 1. Representação esquemática de uma sinapse glutamatérgica. Em destaque (quadro rosa), alguns aspectos abordados no texto. Adaptado de Bélanger et al, 2011.

1.2.3. Síntese de Glutationa

Outro aspecto importante da funcionalidade astroglial é a proteção do SNC contra o estresse oxidativo e, nesse sentido, a GSH constitui uma importante defesa antioxidante cerebral. A GSH pode neutralizar radicais livres de forma não-enzimática ou servir de

substrato para a enzima antioxidante glutationa peroxidase (Dringen, 2000; Pope et al., 2008). Os astrócitos apresentam níveis mais elevados de GSH em relação aos neurônios. Logo, desempenham um papel na defesa antioxidante do SNC (Bélanger & Magistretti, 2009; Dringen, 2000).

A GSH é um tripeptídeo composto por três aminoácidos: glutamato, cisteína e glicina (γ-glutamil-cisteinil-glicina); e, por isso, sua síntese pode ser um dos destinos do glutamato captado pelos astrócitos (Robert et al., 2014). A primeira etapa da síntese de GSH é a formação do dipeptídeo γ-glutamil-cisteinil, a qual utiliza o glutamato e a cisteína como substratos. Após, este dipeptídeo se combina com a glicina, em uma reação catalisada pela enzima glutationa sintetase, originando a GSH (Banerjee et al., 2008; Dringen, 2000; Pope et al., 2008).

Tanto os astrócitos quanto os neurônios são capazes de sintetizar GSH. Porém, os neurônios são dependentes dos astrócitos para o suprimento dos aminoácidos precursores da GSH. Isso se deve, especialmente, ao fato de que os neurônios não são capazes de utilizar a cistina extracelular, forma oxidada da cisteína, a qual é precursora limitante da síntese de GSH. Então, parte da GSH sintetizada pelos astrócitos é secretada para o meio extracelular, onde sofre uma clivagem pela enzima γ-glutamil transpeptidase para produzir glutamato e cisteinil-glicina, a qual, por sua vez, sofre a ação de outra aminopeptidase produzindo glicina e cisteína, que são captadas pelos neurônios e utilizadas para a síntese de GSH neuronal (Bélanger et al., 2011; Dringen, 2000; Hertz, 2006).

Como mencionado anteriormente, o trocador cistina-glutamato (Xc^-), presente nas células astrogliais, desempenha um papel importante para a síntese de GSH. Através dele, os astrócitos internalizam a cistina, a qual dentro das células é reduzida a cisteína, substrato limitante para a síntese de GSH. Dessa forma, o trocador Xc^- e os demais

transportadores glutamatérgicos são críticos para a manutenção dos níveis de GSH no SNC (Lewerenz et al., 2006; Robert et al., 2014).

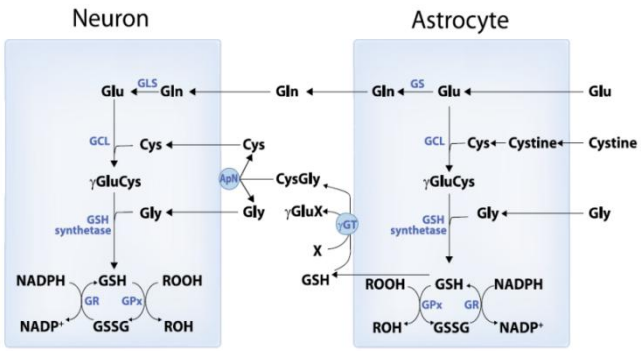

Figura 2. A integração metabólica entre astrócitos e neurônios para a síntese de GSH. Retirado de Bélanger et al, 2011.

1.2.4. Liberação de Glutamato pelas Células Gliais

Embora os terminais sinápticos neuronais sejam os principais responsáveis pela liberação de glutamato para o meio extracelular no SNC, mais recentemente mostrou-se que os astrócitos também são capazes de liberá-lo. Diferentes mecanismos pelos quais o glutamato pode ser liberado a partir de células astrogliais vêm sendo descritos, e podem ou não envolver um aumento de Ca^{2+} intracelular e transporte vesicular (Hamilton & Attwell, 2010; Malarkey & Parpura, 2008).

Entre os mecanismos independentes de Ca^{2+} estão: (1) abertura de canais iônicos induzida por inchaço celular (Kimelberg et al., 1990; Wang et al., 2013); (2) transporte reverso através dos transportadores de glutamato (Rossi et al., 2000; Szatkowski et al., 1990); (3) trocador Xc^-, o qual realiza o transporte de um glutamato para o meio extracelular em troca de uma molécula de cistina (Baker et al., 2002; Warr et al., 1999); (4) liberação através de receptores purinérgicos ionotrópicos (Duan et al., 2003); (5) hemicanais na superfície celular dos astrócitos (Ye et al., 2003).

No entanto, o glutamato também pode ser liberado a partir de astrócitos por um mecanismo dependente do aumento de Ca^{2+} intracelular. Essa dependência de Ca^{2+} intracelular sugere um mecanismo de exocitose regulado, o qual provavelmente envolve uma maquinaria secretória que utiliza proteínas complexas, responsáveis pelo controle da fusão das vesículas contendo glutamato com a membrana da célula astroglial (Araque et al., 2000; Görg et al., 2010; Parpura et al., 2011; Parpura & Zorec, 2010).

Em contextos patológicos, o aumento da liberação de glutamato por astrócitos pode ser induzido por alguns fatores como mediadores inflamatórios e perturbação na homeostase iônica. Como consequência, pode ocorrer uma ativação excessiva dos receptores glutamatérgicos neuronais e uma morte celular excitotóxica, agravando o processo patológico (Bezzi et al., 2001, 1998; Hamilton & Attwell, 2010).

2. Amônia

A amônia é um produto residual, formado por todos os tecidos, proveniente da degradação de proteínas e outros compostos nitrogenados. Apesar de ser estruturalmente simples, a amônia é uma molécula biologicamente potente, e seu acúmulo pode causar disfunções graves, principalmente no SNC. Por isso, a remoção de quantidades excessivas de amônia torna-se crítica e fundamental para a homeostasia celular (Bosoi & Rose, 2009; Cooper & Plum, 1987; Lehninger, 2005). Em solução aquosa, a amônia (NH_3) está em equilíbrio com o íon amônio (NH_4^+), e em condições fisiológicas (pH 7,4), 98% está na forma protonada (Bosoi & Rose, 2009; Bromberg et al., 1960; Felipo & Butterworth, 2002). O termo amônia será utilizado neste texto para se referir tanto a amônia em sua forma neutra quanto protonada.

Entre as principais vias para detoxificação de amônia no corpo está o ciclo da ureia, nas células hepáticas. Dessa forma, a amônia é convertida em ureia, que

posteriormente será eliminada pelos rins (Morris, 2002). Outra via pela qual a amônia pode ser metabolizada é através da sua incorporação ao glutamato formando glutamina, por ação da enzima GS hepática (Stadlbauer et al., 2009; Verhoeven et al., 1983). Assim, o fígado é responsável pela manutenção das concentrações séricas de amônia em níveis baixos (0,05 – 0,1 mM), a fim de evitar sua toxicidade (Felipo & Butterworth, 2002).

Doenças hepáticas que provoquem uma redução na capacidade de remoção da amônia, como desordens inatas do ciclo da ureia ou doenças hepáticas adquiridas (hepatite, cirrose e doenças autoimunes), resultam em um aumento da concentração sérica de amônia, o qual está frequentemente acompanhado por disfunções neurológicas (Albrecht & Jones, 1999; Butterworth, 2014; Häussinger & Schliess, 2008; Leber et al., 2009; Leonard & Morris, 2002).

2.1. Toxicidade da Amônia no SNC

A encefalopatia hepática (EH) é a principal patologia neurológica associada à hiperamonemia, e resulta da detoxificação incompleta da amônia no fígado, que pode ser aguda ou crônica. Nessas condições, a concentração de amônia no SNC pode atingir até 5 mM. A EH é uma desordem complexa, dinâmica e progressiva, manifestada por uma série de sintomas que podem variar entre o grau 0 (sem anormalidades detectadas) até o grau 4 (referido ao paciente em coma, irresponsivo à estímulos externos) (Ferenci et al., 2002; Weissenborn et al., 2001). Entre as anormalidades neurológicas apresentadas na EH estão, por exemplo, alterações comportamentais (como euforia, ansiedade, perda de consciência), letargia, desorientação, sonolência e confusão (Bajaj et al., 2011).

A amônia encontrada no SNC pode se originar tanto de rotas enzimáticas cerebrais, como glutamato desidrogenase e a glutaminase, quanto proveniente da circulação, principal fonte em situações de hiperamonemia (Felipo & Butterworth, 2002).

O transporte da amônia através da barreira hematoencefálica se deve à combinação da difusão passiva de NH_3 e transporte de NH_4^+ por canais de cátions e pela aquaporina (Ott et al., 2005; Saparov et al., 2007). No SNC, o metabolismo da amônia é realizado pela enzima GS, através da conversão do glutamato a glutamina com a incorporação de NH_4^+. Essa enzima é encontrada apenas nos astrócitos e, em situações de hiperamonemia, a GS pode não realizar a detoxificação completa da amônia, que pode se acumular nos astrócitos, fazendo com que essas células se tornem particularmente suscetíveis à sua toxicidade e sejam o seu principal alvo de ação (Martinez-Hernandez et al., 1977; Norenberg et al., 2009).

Inúmeros estudos tanto *in vitro* quanto *in vivo* demonstram uma série de alterações celulares fisiológicas e metabólicas que podem ser causadas direta ou indiretamente pelo aumento da concentração de amônia no SNC. A redução do pH intracelular e alterações na homeostase iônica, com consequente acúmulo intracelular de água, levam ao inchaço astroglial característico em situações de hiperamonemia (Bosoi & Rose, 2009; Braissant et al., 2013; Jayakumar et al., 2008). Outros efeitos tóxicos importantes são o estresse oxidativo/nitrosativo, ocasionado por um aumento na geração de espécies reativas e/ou por uma redução na capacidade antioxidante celular (Bobermin et al., 2012; Görg et al., 2008; Hilgier et al., 1991; Kosenko et al., 1997a, 1997b; Skowrońska et al., 2010), e uma deficiência na produção de ATP para as células (Qureshi et al., 1998; Rama Rao & Norenberg, 2012). Além disso, estudos também demonstram que um processo inflamatório está associado à EH, e que a amônia pode induzir a liberação de citocinas pró-inflamatórias como TNFα, IL-1β e IL-6 por células astrogliais (Bobermin et al., 2012; Butterworth, 2013, 2011a; Odeh et al., 2005). Uma vez que o estresse oxidativo e a resposta inflamatória estão intimamente relacionados, eles podem agir sinergicamente agravando os danos provocados pela amônia.

A amônia também provoca importantes alterações no sistema glutamatérgico, e evidências apontam para um papel dos receptores de glutamato do tipo NMDA no desenvolvimento da neurotoxicidade (Monfort et al., 2002). A ativação desses receptores pela amônia pode resultar em um influxo de Ca^{2+} e a consequente ativação de diversas enzimas, como a óxido nítrico sintase (NOS). O consequente aumento dos níveis de NO pode levar à formação de moléculas potencialmente nocivas às células, como o peroxinitrito, e à nitração de proteínas (Bemeur et al., 2010; Calabrese et al., 2007; Skowrońska & Albrecht, 2013). Além disso, já foi demonstrado que a amônia provoca um aumento da concentração extracelular de glutamato, principalmente em decorrência de uma diminuição na sua captação pelas células astrogliais (Bender & Norenberg, 1996; Kelly et al., 2009; Ohara et al., 2009; Rose, 2006).

3. Estresse oxidativo

Radicais livres são moléculas que possuem um elétron desemparelhado no orbital mais externo. Essa característica os torna instáveis e extremamente reativos, com uma grande capacidade de combinar-se inespecificamente com moléculas integrantes da estrutura celular, a fim de completar seus orbitais (Augusto, 2006; Halliwell, 2007, 2006a).

Em condições fisiológicas, o metabolismo celular aeróbio reduz o oxigênio molecular (O_2) à água (H_2O). No entanto, uma pequena parcela do oxigênio consumido pela cadeia de transporte de elétrons na mitocôndria não é totalmente reduzido, dando origem, então, a radicais livres de oxigênio, ou espécies reativas de oxigênio (ERO). O termo ERO é frequentemente utilizado para incluir não apenas radicais livres que contenham o oxigênio em sua estrutura, como o ânion superóxido ($O_2^{\cdot -}$) e o radical hidroxila ($OH^{\cdot}$), mas também compostos que facilmente possam gerar radicais livres,

como é o caso do peróxido de hidrogênio (H_2O_2). Além das ERO, existem também as espécies reativas que contêm nitrogênio na sua estrutura e, portanto, são denominadas de espécies reativas de nitrogênio (ERN), sendo os seus principais representantes o NO e o peroxinitrito ($ONOO^-$) (Augusto, 2006; Halliwell, 2007; Lieberman, 2009).

Em diversas condições patológicas, a produção de ERO/ERN pelo metabolismo celular pode aumentar significativamente, ocasionando uma condição denominada de estresse oxidativo. Por definição, o estresse oxidativo é um desequilíbrio entre a produção de ERO/ERN e a capacidade antioxidante celular, favorecendo a formação de moléculas pró-oxidantes (Halliwell, 2006a; Schieber & Chandel, 2014; Sies, 1991).

O estresse oxidativo pode promover adaptação, dano ou morte celular. No processo de adaptação, as células podem compensar a produção de ERO/ERN com um aumento da síntese de antioxidantes. No entanto, quando isso não é possível, pode ocorrer dano oxidativo a biomoléculas como lipídeos, proteínas e DNA com comprometimento de processos biológicos importantes. Caso não haja um sistema de reparo adequado, o dano pode ser irreversível e levar à morte celular (Halliwell, 2007; Kaludercic et al., 2014; Lieberman, 2009).

Evidências de danos por ERO/ERN estão descritas em inúmeras patologias, inclusive do SNC, como por exemplo, nas doenças de Parkinson, Alzheimer, Huntington e também na EH, como anteriormente comentado (Guo et al., 2013; Halliwell, 2006b, 2001; Schapira et al., 2014). No entanto, é difícil estabelecer se o dano oxidativo é uma causa ou uma consequência do curso dessas doenças. Como o SNC é particularmente suscetível ao dano oxidativo, estratégias que possam atuar para sua prevenção ou controle podem ser bastante úteis nessas patologias.

4. Antioxidantes

Para evitar os efeitos danosos da produção contínua de ERO/RNS durante os processos metabólicos, as células dispõem de vários mecanismos de defesa antioxidante, para a manutenção do funcionamento celular normal (Gutteridge & Halliwell, 2010; Halliwell, 2006a; Lieberman, 2009). Halliwell e Gutteridge definiram antioxidante como "alguma substância que, presente em baixas concentrações quando comparada ao substrato oxidável, atrase ou iniba a oxidação deste substrato de maneira eficaz" (Halliwell & Gutteridge, 1999). As defesas antioxidantes celulares podem ser classificadas em enzimáticas e não-enzimáticas.

As enzimas antioxidantes reagem com as ERO para convertê-las em produtos não tóxicos. Entre elas estão a superóxido dismutase (SOD), a catalase, a glutationa peroxidase e a glutationa redutase. Em relação ao SNC, uma característica importante é que a SOD localiza-se predominantemente nos astrócitos, os quais são capazes de secretá-la para o meio extracelular (Barbeito et al., 2004; Pope et al., 2008). Entre as defesas antioxidantes não-enzimáticas, a mais importante é a GSH. Juntamente com ela, outras moléculas antioxidantes como, por exemplo, o ácido ascórbico, o α-tocoferol e a bilirrubina são capazes de sequestrar ERO/ERN evitando o estresse oxidativo (Halliwell et al., 2005; Harrison & May, 2009; Niki & Traber, 2012).

No entanto, em adição aos efeitos protetores dos antioxidantes endógenos, o uso de antioxidantes exógenos tem sido alvo de inúmeros estudos em diversas situações, tanto fisiológicas quanto patológicas. O antioxidante N-acetilcisteína (NAC) é um dos mais clássicos e amplamente utilizados para o tratamento de diversas doenças periféricas e cerebrais. O principal efeito antioxidante do NAC se dá através da síntese de GSH, já que é um precursor acetilado da cisteína (Samuni et al., 2013). Nesse contexto, a busca por

moléculas com potencial antioxidante pode representar avanços terapêuticos em doenças que de alguma maneira envolvam o estresse oxidativo.

4.1. Resveratrol

O resveratrol (3,4',5-triidroxi-*trans*-estilbeno) é um composto polifenólico não-flavonoide da classe dos estilbenos, encontrado naturalmente em diversas espécies de plantas, e que apresenta importantes propriedades benéficas à saúde (Pervaiz, 2003). O resveratrol é encontrado nas isoformas *cis* e *trans*, sendo que o isômero *trans* é o principal responsável pelos efeitos biológicos do resveratrol em mamíferos (Soleas et al., 1997a).

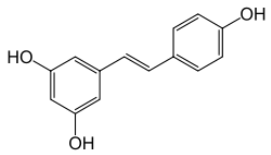

Figura 3. Estrutura química do *trans*-resveratrol.

O resveratrol foi primeiramente isolado das raízes de *Veratrum grandiflorum* O. Loes em 1940 e, posteriormente, em 1963, das raízes de *Polygonum cuspidatum*, uma planta utilizada na tradicional medicina oriental (Baur & Sinclair, 2006; Nonomura et al., 1963). Atualmente, sabe-se que o resveratrol está presente em mais de 70 espécies vegetais, dentre as quais componentes da nossa dieta como amendoins, ameixas, frutas vermelhas (como morango, amora e framboesa) e, principalmente, em cascas e sementes de uvas (Baur & Sinclair, 2006; de la Lastra & Villegas, 2005; Markus & Morris, 2008). Consequentemente, está presente também em vinhos, especialmente em vinhos tintos (de la Lastra & Villegas, 2005).

Diversos estudos, desde cultura de células a modelos animais, mostram que o resveratrol exerce inúmeras atividades biológicas, entre as quais está o seu efeito antioxidante. O resveratrol pode agir diretamente como *scavenger* de ERO/ERN e

também como quelante de metais. Além disso, é capaz de aumentar a atividade de enzimas antioxidantes, como a glutationa peroxidase, e o conteúdo de GSH (Bastianetto et al., 2000; Belguendouz et al., 1997; Mokni et al., 2007; Quincozes-Santos et al., 2013). Além disso, o resveratrol apresenta um importante efeito anti-inflamatório através da inibição da enzima ciclooxigenase e da redução da ativação do fator nuclear da cadeia κ de linfócitos B (NFκB) (Bellaver et al., 2014; Bobermin et al., 2012; Chen et al., 2005; Sarkar et al., 2009).

Embora o resveratrol não tenha um receptor específico, ele pode exercer seus efeitos benéficos sobre a resposta celular através da modulação de enzimas e vias de sinalização celular envolvidas na resposta ao estresse. Entre elas está a quinona redutase 2, uma enzima que pode aumentar a produção de ERO e encontrar-se superexpressa em situações patológicas como a Doença de Alzheimer, e que pode ser inibida pelo resveratrol (Bastianetto et al., 2014). Outra via de sinalização que pode ser modulada pelo resveratrol são as proteínas sirtuínas, que constituem uma família de histona desacetilases, cujos efeitos podem estar relacionados com o aumento da expectativa de vida em diversas espécies (Baur, 2010; Della-Morte et al., 2009). O resveratrol também pode exercer seus efeitos através da ativação da heme oxigenasse 1 (HO1), uma enzima endógena que pode regular a resistência celular em situações de estresse oxidativo (Quincozes-Santos et al., 2013; Sakata et al., 2010). Além disso, vias relacionadas à sobrevivência celular, como a fosfatidil-inositol-3-cinase (PI3K), podem ser ativadas e mediar os efeitos biológicos do resveratrol (Simão et al., 2012a).

Quando administrado de forma oral, o resveratrol é metabolizado no intestino e no fígado (Walle, 2011), sendo seus principais metabólitos o resveratrol glicuronídeo e o resveratrol sulfato (Wenzel & Somoza, 2005). Além disso, ocorrem dois picos plasmáticos de resveratrol, indicando que há um processo de recirculação entero-hepática

(Boocock et al., 2007). Pequenas quantidades de resveratrol livre (aproximadamente 30 nM) podem ser detectadas após uma dose oral de 25 mg, enquanto a quantidade total de resveratrol (livre ou conjugado) pode ser bem maior, aproximadamente 2 μM (Goldberg et al, 2003). Estudos em animais mostram que o resveratrol de uma maneira geral é bem tolerado. Além disso, estudos com administração aguda de resveratrol em humanos mostram que ele pode induzir efeitos colaterais, como diarreia, em uma dose diária de 4 g (la Porte et a, 2010). Porém, doses menores, como 270 mg, não causam desconfortos (Wong et al., 2010).

O SNC também é alvo terapêutico do resveratrol, pois ele atravessa a barreira hematoencefálica. Muitos estudos em modelos animais de isquemia e doenças neurodegenerativas mostram que o resveratrol exerce importantes efeitos neuroprotetores, mediados tanto por seu efeito direto quanto pela modulação de vias de sinalização celular (Sakata et al., 2010; Sharma & Gupta, 2002; Simão et al., 2012b).

Nosso grupo tem mostrado que o resveratrol modula importantes parâmetros relacionados a funções gliais, como a captação de glutamato, atividade da GS, níveis de GSH e liberação de citocinas pró-inflamatórias (Bobermin et al., 2012; de Almeida et al., 2007; dos Santos et al., 2006; Quincozes-Santos et al., 2013). Assim, o resveratrol surge como um potencial agente farmacológico, mediando importantes efeitos sobre células gliais, representando uma nova estratégia terapêutica em patologias do SNC.

4.2. Ácido Lipoico

O ácido lipoico (1,2-ditiolano-3-ácido pentanoico) é um composto dissulfeto que ocorre naturalmente, o qual é sintetizado enzimaticamente na mitocôndria a partir do ácido octanoico. Ele atua como cofator para enzimas α-cetoácido desidrogenases mitocondriais, exercendo um importante papel no metabolismo energético mitocondrial (Liu et al., 1995; Packer et al., 1995; Schmidt et al., 1969).

Estruturalmente, apresenta um centro quiral e, portanto, pode existir como isômeros R e S. O isômero R é sintetizado endogenamente e permanece ligado a proteínas. Porém, para fins terapêuticos, normalmente é administrado como uma mistura racêmica de R e S, sendo que ambos exercem atividade biológica. O ácido lipoico contém dois grupos tióis, que podem estar oxidados ou reduzidos. A forma oxidada é usualmente definida como ácido lipoico (LA) e a forma reduzida é denominada ácido diidrolipoico (DHLA), e ambas apresentam efeitos biológicos (Haramaki et al., 1997; Packer & Cadenas, 2011; Singh & Jialal, 2008).

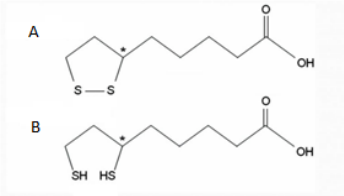

Figura 4. Estrutura química do ácido lipoico nas formas oxidada (A) e reduzida (B).

O ácido lipoico foi primeiramente isolado e identificado quimicamente em 1951 por Lester Reed et al (Reed et al., 1951). Os humanos podem sintetizá-lo *de novo* a partir de ácidos graxos e cisteína, porém, apenas em pequenas quantidades. No entanto, o ácido lipoico pode ser absorvido de fontes exógenas, dentre as quais se destacam a carne vermelha e o fígado, e também vegetais como espinafre, brócolis, tomate, couve e batata (Gorąca et al., 2011; Kataoka, 1998). Quando administrado exogenamente, é totalmente absorvido dentro de 30 minutos a 1 hora (Breithaupt-Grögler et al., 1999). O metabolismo primário do ácido lipoico acontece no fígado, através da oxidação mitocondrial, e os principais metabólitos identificados foram o ácido bisnorlipoico, ácido tetranorlipoico e ácido hidroxi-bisnorlipoico (Biewenga et al., 1997; Harrison & McCormick, 1974).

O efeito biológico do ácido lipoico melhor descrito é o antioxidante, o qual é mediado principalmente por sua habilidade direta de sequestrar ERO, interagir com outros antioxidantes (como ácido ascórbico, α-tocoferol e GSH), regenerando-os, e de quelar metais. O ácido lipoico também é capaz de induzir a síntese de GSH e a expressão de enzimas antioxidantes celulares (Bast & Haenen, 2003; Bilska & Włodek, 2005; Packer et al., 1995; Packer & Cadenas, 2011; Shay et al., 2009). É importante salientar que o DHLA pode ser liberado pelas células, atuando como antioxidante no meio extracelular (Packer & Cadenas, 2011). O ácido lipoico também desempenha um importante papel anti-inflamatório, diminuindo a sinalização através do NFκB e, consequentemente, a produção de mediadores inflamatórios (Demarco et al., 2004; Kim et al., 2014; Maczurek et al., 2008; Scumpia et al., 2014).

Devido ao fato de ser uma molécula de pequeno tamanho e solúvel em lipídeos, o ácido lipoico pode atravessar facilmente a barreira hematoencefálica, e exercer seus efeitos também no SNC (Roy & Packer, 1998; Schreibelt et al., 2006). Diversos estudos têm mostrado efeitos neuroprotetores do ácido lipoico em situações patológicas como doenças neurodegenerativas e isquemia (Gorąca et al., 2011; Maczurek et al., 2008; Shay et al., 2009). Além disso, o ácido lipoico também é capaz de agir em células gliais (Bramanti et al., 2010; Rocamonde et al., 2013), e nosso grupo recentemente demonstrou sua ação sobre o metabolismo do glutamato (Kleinkauf-Rocha et al., 2013). Dessa forma, representa uma molécula com potencial terapêutico em condições patológicas que envolvam estresse oxidativo e alterações gliais.

OBJETIVOS

Objetivo geral

Avaliar os potenciais efeitos protetores do resveratrol e do ácido lipoico em células astrogliais e neuronais expostas à amônia.

Objetivos específicos

1. Investigar o potencial efeito protetor do resveratrol sobre parâmetros relacionados à comunicação glutamatérgica em células astrogliais C6 expostas à amônia;
2. Avaliar os potenciais efeitos protetores do ácido lipoico sobre a secreção de S100B, captação de glutamato, atividade da GS e conteúdo intracelular de GSH em células C6 expostas à amônia, explorando alguns possíveis mecanismos envolvidos;
3. Investigar os efeitos do ácido lipoico, bem como da N-acetilcisteína, sobre a liberação de citocinas pró-inflamatórias por células C6 expostas à amônia;
4. Estudar as consequências da exposição de células da linhagem de neuroblastoma humano SH-SY5Y e de culturas primárias de neurônios granulares cerebelares à amônia, assim como efeitos neuroprotetores do resveratrol e do ácido lipoico.

PARTE II

CAPÍTULO I

Artigo submetido no periódico Molecular Neurobiology

Ammonia triggers impairment in glutamatergic communication in astroglial cells: the protective role of resveratrol

Larissa Daniele Bobermin, Gisele Hansel, Emilene Barros da Silva Sherer, Angela T.S. Wyse, Diogo Onofre Souza, André Quincozes-Santos, Carlos Alberto Gonçalves

Molecular Neurobiology

AMMONIA TRIGGERS IMPAIRMENT IN GLUTAMATERGIC COMMUNICATION IN ASTROGLIAL CELLS: THE PROTECTIVE ROLE OF RESVERATROL

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Abstract:	Ammonia is a key toxin in the precipitation of hepatic encephalopathy (HE), a neuropsychiatric disorder associated with liver failure. In response to ammonia, various toxic events are triggered in astroglial cells, and alterations in brain glutamate communication are common. Resveratrol is a polyphenolic compound that has been extensively studied in pathological events because it presents several beneficial effects, including some in the central nervous system (CNS). We previously described that resveratrol is able to significantly modulate glial functioning and has a protective effect during ammonia challenge in vitro. In this study, we addressed the mechanisms by which resveratrol can protect C6 astroglial cells from glutamatergic alterations induced by ammonia. We observed that ammonia decreased glutamate uptake and increased glutamate release in a manner dependent on the activation of the Na+-K+-Cl- co-transporter NKCC1. Ammonia also decreased the levels of EAAC1, the main glutamate transporter present in C6 cells. Reductions in GS activity, the intracellular GSH content and Na+K+-ATPase functioning were also consequences of ammonia cytotoxicity. On the other hand, resveratrol was able to prevent all of these effects triggered by ammonia. Moreover, resveratrol, per se, positively modulated the evaluated astroglial functions. We demonstrated that heme oxygenase 1 (HO1), an enzyme that is part of the cellular defense system, mediated some of the effects of resveratrol. In conclusion, the mechanisms of the putative protective role of resveratrol against ammonia toxicity involve the modulation of pathways and molecules related to glutamate communication in astroglial cells.

AMMONIA TRIGGERS IMPAIRMENT IN GLUTAMATERGIC COMMUNICATION IN ASTROGLIAL CELLS: THE PROTECTIVE ROLE OF RESVERATROL

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Abstract

Ammonia is a key toxin in the precipitation of hepatic encephalopathy (HE), a neuropsychiatric disorder associated with liver failure. In response to ammonia, various toxic events are triggered in astroglial cells, and alterations in brain glutamate communication are common. Resveratrol is a polyphenolic compound that has been extensively studied in pathological events because it presents several beneficial effects, including some in the central nervous system (CNS). We previously described that resveratrol is able to significantly modulate glial functioning and has a protective effect during ammonia challenge *in vitro*. In this study, we addressed the mechanisms by which resveratrol can protect C6 astroglial cells from glutamatergic alterations induced by ammonia. We observed that ammonia decreased glutamate uptake and increased glutamate release in a manner dependent on the activation of the Na^+-K^+-Cl^- co-transporter NKCC1. Ammonia also decreased the levels of EAAC1, the main glutamate transporter present in C6 cells. Reductions in GS activity, the intracellular GSH content and Na^+K^+-ATPase functioning were also consequences of ammonia cytotoxicity. On the other hand, resveratrol was able to prevent all of these effects triggered by ammonia. Moreover, resveratrol, *per se*, positively modulated the evaluated astroglial functions. We demonstrated that heme oxygenase 1 (HO1), an enzyme that is part of the cellular defense system, mediated some of the effects of resveratrol. In conclusion, the mechanisms of the putative protective role of resveratrol against ammonia toxicity involve the modulation of pathways and molecules related to glutamate communication in astroglial cells.

Key Words: resveratrol, ammonia, C6 astroglial cells, glutamate

Introduction

Ammonia is considered to be the major etiological factor in the development of hepatic encephalopathy (HE), a neuropsychiatric syndrome associated with acute or chronic liver failure, and astroglial cells are believed to be the primary target of ammonia toxicity in the brain [1–3]. Metabolic effects of ammonia in the CNS include reactive oxygen and nitrogen species (ROS/RNS) production alterations, energy deficits due to ATP reduction, glutamine synthetase (GS) activity changes, pro-inflammatory cytokine release and astrocyte swelling [4–8]. Moreover, acute ammonia toxicity can be mediated by an excitotoxic mechanism involving the glutamatergic system [9–12].

Glutamate is the main excitatory neurotransmitter in the CNS, and its extracellular accumulation has deleterious effects on the functioning and survival of neurons. Thus, maintaining low concentrations of this amino acid in the extracellular environment is a critical function, which is exerted by astrocytes [13–15]. Astrocytes take up extracellular glutamate through specialized transporters on their cell surfaces [16]. These transporters use the electrochemical Na^+ and K^+ gradients across the plasma membrane as the driving force of glutamate uptake [17]. Glutamate uptake, along with the associated cotransport of Na^+ that raises $[Na^+]_i$, substantially activates the Na^+K^+-ATPase pump, a membrane protein responsible for maintaining the Na^+ and K^+ electrochemical gradients [18, 19]. Ion homeostasis is also controlled by ion channels and transporters such as the Na^+-K^+-Cl^- co-transporter NKCC, which is a membrane protein that transports Na^+, K^+ and Cl^- ions into and out of the cell under physiological and pathological conditions [20, 21]. NKCC1, the isoform present in astroglial cells, is also involved in the influx of ammonium (NH_4^+) into these cells [9, 22, 23].

Once taken up by astrocytes, glutamate can be directed to different routes, for example: (1) enzymatic amidation to glutamine by GS [24–26]; (2) biosynthesis of glutathione (GSH), the major antioxidant molecule of the brain [27–29]; (3) release to the extracellular space as a gliotransmitter [15, 30–32]. As mentioned above, ammonia toxicity is closely associated with the glutamatergic system, and the disruption of glutamate homeostasis may occur through changes in the uptake and/or the release of glutamate by astroglial cells [11, 12, 33, 34].

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a natural polyphenol found in a variety of dietary sources such as grapes and red wine [35–39]. Many healthy properties have been attributed to this compound, including antioxidant and anti-inflammatory activities, which modulate the cell phenotype [40–42]. Several beneficial effects of resveratrol in the central nervous system (CNS) have also been described [43–45]. Our group has shown that, in addition to its neuroprotective action, resveratrol affects the functioning of glial cells. Resveratrol improves important astroglial functions, and the modulation of glutamatergic metabolism may

play an important role in their cytoprotective action [46–49]. Although there is increasing evidence of the protective effects of resveratrol in neural cells, the mechanisms of these effects are not fully understood. It is believed that resveratrol exerts its antioxidant activity due either to its direct scavenger effect or to its activation of pathways that upregulate cellular antioxidant defenses such as heme oxygenase 1 (HO1), an endogenous enzyme that provides resistance against oxidative stress-related damage [47, 50, 51].

C6 astroglial cells are widely used as an astrocyte-like cell line to study astrocytic parameters such as glutamate uptake, GS activity, GSH content, oxidative response and signaling pathways [4, 47, 48, 52–54]. In this sense, this study was undertaken to investigate the outcomes of ammonia challenge on parameters related to glutamatergic communication in C6 astroglial cells and the potential protective role of resveratrol. For this, the following parameters were assessed: (i) glutamate uptake; (ii) EAAC1 immune content; (iii) glutamate release; (iv) GS activity; (v) GSH content; and (vi) Na^+K^+-ATPase activity. Some possible mechanisms are also addressed, pointing to the participation of the NKCC1 co-transporter in ammonia toxicity and the activation of HO-1 for cellular protection by resveratrol.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and other cell culture materials were purchased from Gibco (Carlsbad, CA, USA). Resveratrol, ZnPP IX, furosemide, GSH standard, *o*-phthaldialdehyde, ouabain and peroxidase-conjugated anti-rabbit immunoglobulin (IgG) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-EAAC1 was obtained from Novus Biologicals (Littleton, CO, USA). L-[2,3-^{3}H]-glutamate, nitrocellulose membranes and enhanced chemiluminescence kits were purchased from Amersham (Buckinghamshire, UK). All other chemicals were purchased from common commercial suppliers.

C6 astroglial cells

The C6 astroglial cell line was obtained from the American Type Culture Collection (Rockville, MA, USA) and was cultured according to a previously described procedure [48]. The cells were seeded in flasks and cultured in DMEM (pH 7.4) containing 5% FBS, 1% amphotericin B and 0.032% gentamicin. The cells were maintained at a temperature of 37°C in an atmosphere of 5% CO_2/95% air. Exponentially growing cells were detached from the culture flasks using 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA), seeded at a density of $5x10^3$ cells/cm^2 and maintained until they reached confluence (on the 3rd day under *in vitro* conditions).

Experimental treatments

After the cells reached confluence, the culture medium was exchanged with serum-free DMEM, and the cells were pre-incubated in the absence or presence of 100 μM resveratrol for 1 h. After pre-incubation, resveratrol was maintained, and 5 mM ammonia was added for 24 h. During all treatments, the cells were maintained at 37°C in an atmosphere of 5% CO_2/95% air. For all parameters analyzed, the results obtained with vehicle (ethanol) were not different from those obtained under basal conditions. Where indicated, C6 astroglial cells were also incubated with ZnPP IX (10 μM) or furosemide (20 μM), inhibitors of HO1 and NKCC1, respectively, to assess the involvement of these proteins in the effects of ammonia and/or resveratrol in some parameters analyzed.

Glutamate uptake

Glutamate uptake was performed as previously described [48]. Briefly, C6 cells were incubated at 37°C in Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 $Na_2HPO_4 \cdot 7H_2O$, 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 μCi/ml L-[2,3-^{3}H] glutamate. The incubation was stopped after 10 min by removing 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 *N*-methyl-D-glucamine instead of sodium chloride. Sodium-dependent glutamate uptake was obtained by subtracting the non-specific uptake from the total uptake. The results are expressed as percentages of the control value.

Measurement of extracellular glutamate

High-performance liquid chromatography (HPLC) was performed with culture medium aliquots to quantify the extracellular glutamate levels [55]. Briefly, 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 Class VP Shimadzu Instruments liquid chromatograph with auto-injection (SIL-10AF). The mobile phase flowed at a rate of 1.4 mL/ min, and the column temperature was 24°C (column oven, CTO-20AC). The buffer compositions were as follows: A: 0.04 mol/L sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 20% methanol; B: 0.01 mol/L sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 80% methanol. The gradient profile was modified according to the content of buffer B in the mobile phase: 0% at 0.01 min, 29.1% at 16.00 min, 100% at 17.00– 21.00 min, 0% at 21.01–25.00 min. Absorbance was read at an excitation of 360 nm and an emission of 455 nm

in a Shimadzu fluorescence detector (RF-10AxL). Samples of 5 μL were used, and concentrations were calculated in μM and expressed as percentages of the control value.

Glutamine synthetase (GS) activity

The enzymatic assay was performed as previously described [48]. Briefly, the homogenate was added to a reaction mixture containing 10 mM $MgCl_2$, 50 mM L-glutamate, 100 mM imidazole-HCl buffer (pH 7.4), 10 mM 2-mercaptoethanol, 50 mM hydroxylamine–HCl and 10 mM ATP and incubated for 15 min at 37°C. The reaction was stopped by adding a solution containing 370 mM ferric chloride, 670 mM HCl and 200 mM trichloroacetic acid. After centrifugation, the absorbance of the supernatant was measured at 530 nm and compared to a calibration curve of γ-glutamyl hydroxamate treated with ferric chloride reagent. The results are expressed as percentages of the control value.

Glutathione (GSH) intracellular levels

GSH levels were assessed as previously described [56]. Cell lysate, suspended in a sodium phosphate buffer with 140 mM KCl, was diluted with a 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 (at a concentration of 1 mg/ml methanol) at 22ºC for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. A calibration curve was performed with standard GSH solutions at concentrations ranging from 0 μM to 500 μM. GSH concentrations were calculated as nmol/mg protein.

Na^+K^+-ATPase activity assay

The reaction mixture for the Na^+K^+-ATPase assay contained (in mM) 5 $MgCl_2$, 80 NaCl, 20 KCl and 40 Tris–HCl, pH 7.4 in a final volume of 200 μl. The reaction was initiated by addition of ATP to a final concentration of 3 mM. Control reactions were carried out under the same conditions with the addition of 1 mM ouabain. Na^+K^+-ATPase activity was calculated as the difference between the two assays according to the method of Wyse et al. (2000) [57]. Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986) [58]. The specific activity of the enzyme was calculated as nmol Pi released per min per mg of protein and expressed as a percentage of the control value.

Western blot analysis

Cells were homogenized using lysis solution with 4% SDS, 2 mM EDTA, 50 mM Tris–HCl, pH 6.8. Equal amounts of proteins from each sample were boiled in sample buffer [62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 5% β-mercaptoethanol, 10% (v/v) glycerol, 0.002%

(w/v) bromophenol blue] and submitted to electrophoresis in a 10% (w/v) SDS–polyacrylamide gel [52]. The separated proteins were then blotted onto a nitrocellulose membrane. Equal loading of each sample was confirmed with Ponceau S staining (Sigma). The membranes were incubated with anti-EAAC1 (1:1000) or anti-Na^+K^+-ATPase α-1 (1:1000). After incubating overnight with the primary antibody at room temperature, the membrane was washed and incubated with peroxidase-conjugated anti-rabbit immunoglobulin (IgG) at a dilution of 1:1000 for 1 h. The chemiluminescence signal was detected using ECL (Amersham); the films were then scanned, and the bands were quantified using the Scion Image software.

Protein assay

Protein content was measured using Lowry's method, with bovine serum albumin as a standard [59].

Statistical analyses

Differences among groups were statistically analyzed using two-way analysis of variance (ANOVA), followed by Tukey's test. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software, version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Ammonia-induced changes in glutamate uptake and glutamate release: protection by resveratrol

Ammonia exposure for 24 h decreased the glutamate uptake activity in C6 astroglial cells by approximately 22% compared with the control (Fig. 1A). On the other hand, resveratrol prevented the reduction caused by ammonia, changing the levels from 78 ± 4% to 103 ± 10%. To test the role of HO1 in the protective effect of resveratrol on glutamate uptake during ammonia exposure, the cells were pre-incubated with the HO1 inhibitor ZnPP IX. The positive effects of resveratrol were blocked by the HO1 inhibitor, suggesting that this enzyme can mediate the action of resveratrol (Fig. 1A). Additionally, resveratrol *per se* increased glutamate uptake (23% compared with the control), and these effect were also mediated by HO1.

The immune content of EAAC1, which is the primary glutamate transporter present in C6 astroglial cells [60], was also modulated by ammonia and resveratrol. Ammonia decreased EAAC1 expression, whereas resveratrol inhibited the downregulation by ammonia and increased the EAAC1 immune content, *per se* (Fig. 1B). Thus, resveratrol can effectively modulate the glutamate uptake in the presence of ammonia, modulating both the activity and the expression of glutamate transporters.

To investigate further effects of ammonia and resveratrol on the glutamatergic system, we also evaluated glutamate release by C6 astroglial cells (Fig. 1C and D). Fig. 1C depicts the HPLC chromatogram of the culture medium, showing that ammonia markedly increases extracellular glutamate levels compared with basal conditions. Resveratrol was able to prevent this effect of ammonia, maintaining the levels of glutamate release similar to the basal levels (from 171 ± 19% to 95 ± 10%, Fig. 1D). Resveratrol *per se* had no effect on the extracellular glutamate concentration.

Modulation of glutamate uptake and release involves NKCC1

Glutamate uptake and release by astroglial cells can be altered by ion gradients and cellular swelling, respectively. Both of these mechanisms are under the regulation of NKCC1. In this sense, we evaluated the effect of furosemide, an NKCC1 activity inhibitor, on glutamate uptake and release by C6 astroglial cells in the presence of ammonia and/or resveratrol (Table 1). Furosemide inhibited the effect of ammonia on the reduction in glutamate uptake, elevating the value to above the basal level (from 78 ± 4% to 120 ± 10%). However, furosemide did not modulate the effect of resveratrol, alone or in the presence of ammonia, on glutamate uptake.

Furthermore, furosemide reduced the ammonia-induced glutamate release (from 171 ± 19% to 122 ± 3%). Interestingly, when furosemide was co-incubated with resveratrol in the presence of ammonia, the extracellular glutamate concentration was even lower, returning to basal values (106 ± 8%).

Ammonia decreases GS activity and intracellular GSH content in C6 astroglial cells, and these effects are reversed by resveratrol through HO1

In agreement with our previous studies [4, 5], ammonia leads to a 20% decrease in the GS activity after 24 h (Fig. 2A). Ammonia also caused a decrease in the intracellular GSH content (30%) (Fig. 2B). Resveratrol was able to prevent the ammonia-induced effects on both GS activity and GSH levels. We verified that the action of resveratrol was abolished by the HO1 inhibitor (Fig. 2A and B), indicating that the outcome of resveratrol treatment was dependent on HO1. In addition, resveratrol increased the GS activity (22%) and GSH content (15%) *per se,* also through HO1.

Ammonia-induced impairment of Na^+K^+-ATPase activity is prevented by resveratrol

As shown in Fig. 3, ammonia decreased the Na^+K^+-ATPase activity (30%), and resveratrol prevented this effect, keeping the activity equal to that at basal conditions. Moreover, resveratrol alone significantly increased the Na^+K^+-ATPase activity (23%). Despite the alterations in enzyme activity, no difference was found in Na^+K^+-ATPase catalytic subunit

protein expression following ammonia or resveratrol treatment (data not shown). Additionally, the modulation of the Na^+K^+-ATPase activity by resveratrol was not dependent upon HO1 (data not shown).

Discussion

At high concentrations, ammonia has toxic effects in the CNS and plays a critical role in the manifestation of HE, which is a disorder associated with neurological and psychiatric alterations [2, 3]. However, the impaired neurotransmission that occurs in HE seems to be a consequence of astroglial dysfunction [61, 62]. As show in this study, ammonia triggers several cellular alterations related to the glutamatergic system in C6 astroglial cells such as: (i) decreased glutamate uptake activity and EAAC1 glutamate transporter expression; (ii) increased glutamate release; (iii) reduced GS activity and intracellular GSH levels; and (iv) impaired Na^+K^+-ATPase activity. The results suggest that ammonia affects glutamate uptake and release in an NKCC1-dependent manner. On the other hand, resveratrol was able to prevent all of these effects triggered by ammonia, thereby protecting C6 astroglial cells from damage. We also demonstrated the role of HO1 in the mechanism of action of resveratrol.

Resveratrol is a polyphenol that presents a wide range of beneficial effects in the CNS, including the improvement of glial functioning *in vitro* [43, 46–48]. We previously described that resveratrol is able to prevent ammonia toxicity in C6 astroglial cells by modulating oxidative stress, glial and inflammatory responses [4]. However, the cellular mechanisms underlying resveratrol-induced protection need to be better elucidated; here, we evaluated astroglial parameters related to the glutamatergic system during ammonia exposure.

In agreement with previous reports [5, 63, 64], we observed that ammonia induced a decrease in glutamate uptake by C6 astroglial cells. Glutamate transporters are vulnerable to oxidation [65, 66] and may be, at least in part, responsible for the reduced glutamate uptake function, as consequence of the oxidative/nitrosative stress generated by ammonia [4, 7]. This functional loss was accompanied by a reduction in the immune content of EAAC1, which is the main glutamate transporter present in C6 cells [60]. Resveratrol, in contrast, caused an increase of glutamate uptake activity and EAAC1 content. Additionally, in the presence of ammonia, resveratrol prevented the detrimental effects of ammonia, restoring the expression and activity of glutamate transporters, likely due to its antioxidant action. Because the homeostatic control over the extracellular glutamate concentration in the brain is an aspect of astroglial function [13, 30], the effects of resveratrol on glutamate uptake may be an important point for preventing the ammonia-mediated glutamatergic excitotoxicity.

In addition, the actions of resveratrol may also depend on their interplay with a cellular target, which would be a downstream controller of cellular events generated in the presence of resveratrol. One candidate target is HO1, an enzyme that is part of a defensive mechanism for cells exposed to oxidant challenge [67, 68]. The products of heme degradation by HO1 − carbon monoxide (CO) and biliverdin/bilirubin – are biologically active antioxidant molecules that exert cytoprotective effects [69, 70]. Thus, to verify whether the effects of resveratrol could be mediated by this protein, cells were treated with ZnPP IX, a potent and selective inhibitor of HO1 activity [52, 71, 72]. The effects of resveratrol on glutamate uptake were abolished in the presence of the HO1 inhibitor, indicating the participation of this enzyme and/or its metabolites in the protective mechanism of resveratrol. Similarly, other polyphenols (e.g. epicatechin and curcumin) have been shown to exert their beneficial effects via HO1 production and/or activity [73].

NKCC co-transporters are a class of membrane proteins that transport Na^+, K^+ and Cl^- ions across the cell membrane with a stoichiometry of $1Na^+$:1 K^+:$2Cl^-$. The NKCC1 isoform is present in many cell types, including astrocytes, and is involved in the maintenance and regulation of cell volume and ion gradients [20]. Ammonia can activate NKCC1, decreasing the intracellular pH and increasing the intracellular Na^+ concentration. The resulting reductions in the transmembrane electrochemical gradients of Na^+ and H^+ reduce the driving force for astroglial glutamate transporters and may inhibit glutamate uptake [9, 74]. According to our previous finding [5] and to Kelly et al. (2009) [9], we observed that the decrease of glutamate uptake induced by ammonia in C6 astroglial cells involved NKCC1 because the effect was abolished by furosemide treatment. Moreover, the activation of NKCC1 contributes to astroglial osmotic swelling, which is a characteristic of hyperammonemic conditions [21, 75, 76]. One possible consequence of the swelling is glutamate release [77, 78]. In agreement with previous studies [11, 33, 77], we observed that ammonia induced a substantial increase of glutamate release by C6 astroglial cells, which may represent a key element of a self-amplifying cycle of ammonia toxicity. We also verified that furosemide, an NKCC1 inhibitor, partially prevented the ammonia-induced glutamate release, showing that NKCC1 activation is related to this effect. Moreover, during ammonia exposure, resveratrol was able to prevent the increase of extracellular glutamate, indicating that oxidative stress may be involved in the observed ammonia-induced glutamate release [11]. In fact, NKCC1 activity is increased by oxidation, and antioxidants prevent this effect [21, 26, 78, 79]. Thus, in addition to increasing glutamate uptake, resveratrol can modulate glutamate release by astroglial cells exposed to ammonia, thereby preventing NKCC1 activation.

Astrocytes play an important role in glutamate metabolism and the removal of brain ammonia via glutamine synthetase (GS) activity, which catalyzes the ATP-dependent amidation

of up-taken glutamate to form glutamine [26, 79]. Glutamine is then exported to neurons, allowing for the synthesis of glutamate; this characterizes the metabolic glutamate-glutamine cycle [18, 26]. Additionally, in our previous works, we found that ammonia decreased GS activity in C6 astroglial cells, thereby compromising their own detoxification ability [4, 5]. One explanation to the limitation of GS activity in the presence of ammonia is that GS is very sensitive to oxidative and nitrosative stress, which is a well-known effect of ammonia [4, 79]. Furthermore, the decrease in GS activity could occur due to alterations in cerebral energy metabolism and ATP depletion during ammonia exposure [6, 80].

Resveratrol prevented GS failure in the presence of ammonia and caused an increase of GS activity *per se*, also via HO1. This enzyme is able to inhibit inducible nitric oxide synthase (iNOS) activity and, thus, nitric oxide (NO) synthesis, which can inactivate GS [4, 47, 69]. Importantly, a decline in GS activity has been reported in many neurological disorders [81, 82]. Due the role of GS in glutamate conversion, changes in GS activity may affect the glutamate concentration in astroglial cells, therefore affecting the uptake/release of glutamate [81, 82]. In this context, GS exerts a protective function against excitotoxicity in astroglial that which is enhanced by resveratrol.

Another possible destination of glutamate in astroglial cells is the synthesis of tripeptide GSH (glutamate, cysteine and glycine), which is the most abundant antioxidant in the brain [83]. GSH is a critical molecule that protects the CNS against oxidative stress, and its loss is associated with neurotoxicity and neuroinflammation [84–86]. Resveratrol increased the intracellular GSH levels in C6 astroglial cells and prevented the ammonia-induced decrease of GSH content. The decrease of intracellular GSH content could be related to the inflammatory response induced by ammonia because the resulting pro-oxidant environment activates a number of cellular pathways that are involved in the synthesis and release of inflammatory cytokines, which are also prevented by resveratrol [4]. We observed that the increase of intracellular GSH by resveratrol was also mediated by HO1 in astroglial cells, in accordance with previous works demonstrating that HO1 can increase GSH levels in other cell types [87, 88]. Resveratrol could decrease the oxidation and modulate the synthesis of GSH, an important target to reduce the impact of ammonia toxicity, via the participation of HO1.

Many physiological processes in the cells are dependent on the Na^+ and K^+ concentrations, which are maintained through the proper operation of Na^+K^+-ATPase. This is particularly important in astroglial cells because, as mentioned above, glutamate uptake is associated with the cotransport of Na^+, and the activation of the Na^+K^+-ATPase pump is necessary to maintain the ion gradients [18, 19]. However, Na^+K^+-ATPase is also a target for ammonia toxicity and, similarly to what happens with NKCC1, ammonium ions (NH_4^+) can be

transported into astrocytes by Na^+K^+-ATPase [22, 89]. In contrast with previous reports [90, 91], we verified that ammonia reduced the Na^+K^+-ATPase activity in C6 astroglial cells. On the other hand, resveratrol *per se* increased the Na^+K^+-ATPase activity and prevented the effects of ammonia. The impairment of the enzyme activity may result from ammonia-induced increased ROS production [4] because Na^+K^+-ATPase is highly susceptible to free radical attack [90, 91]. Similarly to resveratrol, other antioxidants also modulate Na^+K^+-ATPase activity and prevent cellular damage [90, 92–94]. Another probable cause of the decreased Na^+K^+-ATPase activity is the energy deficit caused by ammonia; this enzyme has a high ATP requirement, and, in the presence of resveratrol, the detoxification of ammonia may occur faster.

The main conclusions of this study are depicted in Fig. 4. Taken together, the results showed that ammonia might induce the accumulation of extracellular glutamate in the CNS because it provokes a reduction of glutamate uptake and increase of glutamate release from astroglial cells. Furthermore, other parameters that can influence glutamatergic homeostasis are altered by ammonia, including GS activity, GSH content and Na^+K^+-ATPase functioning. In this context, we found that resveratrol modulates several steps and molecules involved in glial glutamate metabolism, thereby contributing to excitotoxicity prevention and ammonia detoxification. Moreover, the data show that HO1 participates in the mechanism of action of resveratrol. Collectively, our findings support that the effects of resveratrol in targeting glutamate communication in astroglial cells can help in the recovery of brain homeostasis and the improvement of synaptic plasticity during ammonia challenge.

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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. The effects of ammonia on glutamate uptake and glutamate release: protection by resveratrol. C6 astroglial cells were pre-treated for 1 h with 100 μM resveratrol, followed by the addition of 5 mM ammonia for 24 h. Cells also were pre-incubated with ZnPP IX (10 μM) to assess the involvement of HO1 in the parameters analyzed. A – Glutamate uptake; B – EAAC1 content; C and D – Glutamate release. The data are expressed as percentages relative to the control conditions (indicated by the line) and represent the mean ± S.E.M of at least three independent experimental determinations, performed in triplicate and statistically analyzed by two-way ANOVA followed by Tukey's test. (a) indicates significant differences from the control ($P < 0.05$). (b) indicates significant differences from ammonia ($P < 0.05$). *B* basal, *A* ammonia, *R* resveratrol, *A*+*R* ammonia plus resveratrol.

Fig. 2. Ammonia decreases the GS activity and GSH intracellular content in C6 astroglial cells, and these effects are reversed by resveratrol through HO1. C6 astroglial cells were pre-treated for 1 h with 100 μM resveratrol, followed by the addition of 5 mM ammonia for 24 h. Cells also were pre-incubated with ZnPP IX (10 μM) to assess the involvement of HO1 in the parameters analyzed. A – GS activity; B – GSH levels. The data are expressed as percentages relative to the control conditions (indicated by the line) and represent the mean ± S.E.M of at least three independent experimental determinations, performed in triplicate and statistically analyzed by two-way ANOVA followed by Tukey's test. (a) indicates significant differences from the control ($P < 0.05$). (b) indicates significant differences from ammonia ($P < 0.05$).

Fig. 3. Ammonia-induced impairment of Na^+K^+-ATPase activity is prevented by resveratrol. C6 astroglial cells were pre-treated for 1 h with 100 μM resveratrol, followed by the addition of 5 mM ammonia for 24 h. The data are expressed as percentages relative to the control conditions (indicated by the line) and represent the mean ± S.E.M of at least three independent experimental determinations, performed in triplicate and statistically analyzed by two-way ANOVA followed by Tukey's test. (a) indicates significant differences from the control ($P < 0.05$). (b) indicates significant differences from ammonia ($P < 0.05$).

Fig. 4. Schematic illustration of the outcomes of ammonia challenge in C6 astroglial cells − the protective role of resveratrol. Ammonia may activate NKCC1 entering the cell through this transporter, triggering several changes, such as decrease of glutamate uptake and glutamate transporter expression, as well as GS activity, GSH content and Na^+K^+-ATPase activity, and increase glutamate release. The alterations in glutamate uptake/release by ammonia involve NKCC1 activation (gray dashed arrows). Resveratrol prevents all of these effects. The HO1 signaling pathway is involved in the protective mechanism of resveratrol (red dashed arrow).

Table 1. The modulation of glutamate uptake and release involves NKCC1.

Treatments	**Glutamate uptake** (% of control)	**Glutamate release** (% of control)
Ammonia	78 ± 4 [(a)]	171 ± 19 [(a)]
Ammonia + Furosemide	120 ± 10 [(b)]	122 ± 3 [(b)]
Resveratrol	123 ± 7 [(a)]	96 ± 13
Resveratrol + Furosemide	116 ± 11	104 ± 7
Ammonia + Resveratrol + Furosemide	117 ± 13 [(b)]	106 ± 8 [(b)]

C6 astroglial cells were pre-treated for 1 h with 100 μM resveratrol, followed by the addition of 5 mM ammonia for 24 h. The cells also were pre-incubated with furosemide (20 μM) to assess the involvement of NKCC1 in the parameters analyzed. The data are expressed as percentages relative to the control conditions and represent the mean ± S.E.M of at least three independent experimental determinations, performed in triplicate and statistically analyzed by two-way ANOVA followed by Tukey's test. (a) indicates significant differences from the control ($P < 0.05$). (b) indicates significant differences from ammonia ($P < 0.05$).

Figure 1
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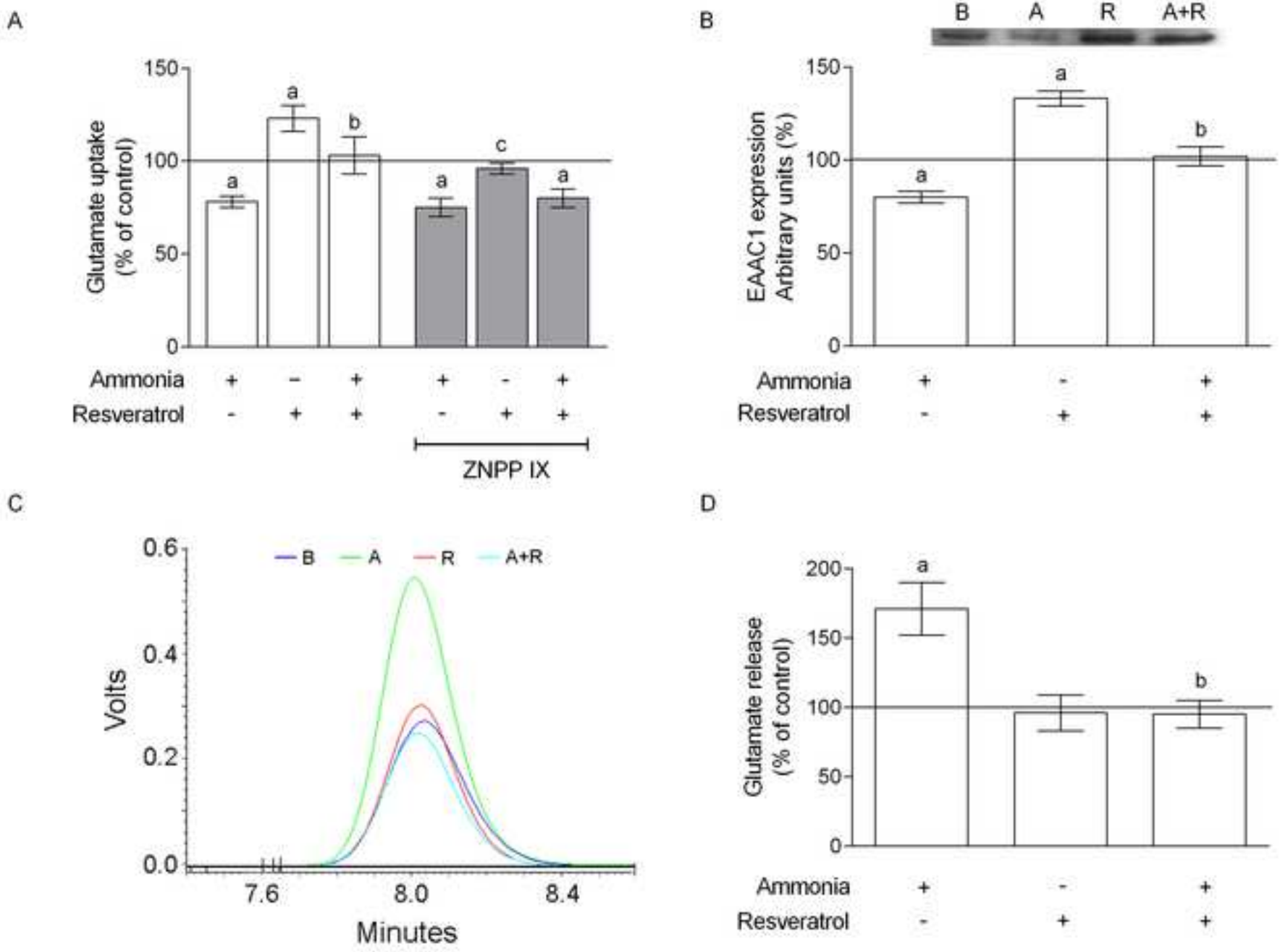

Figure 2
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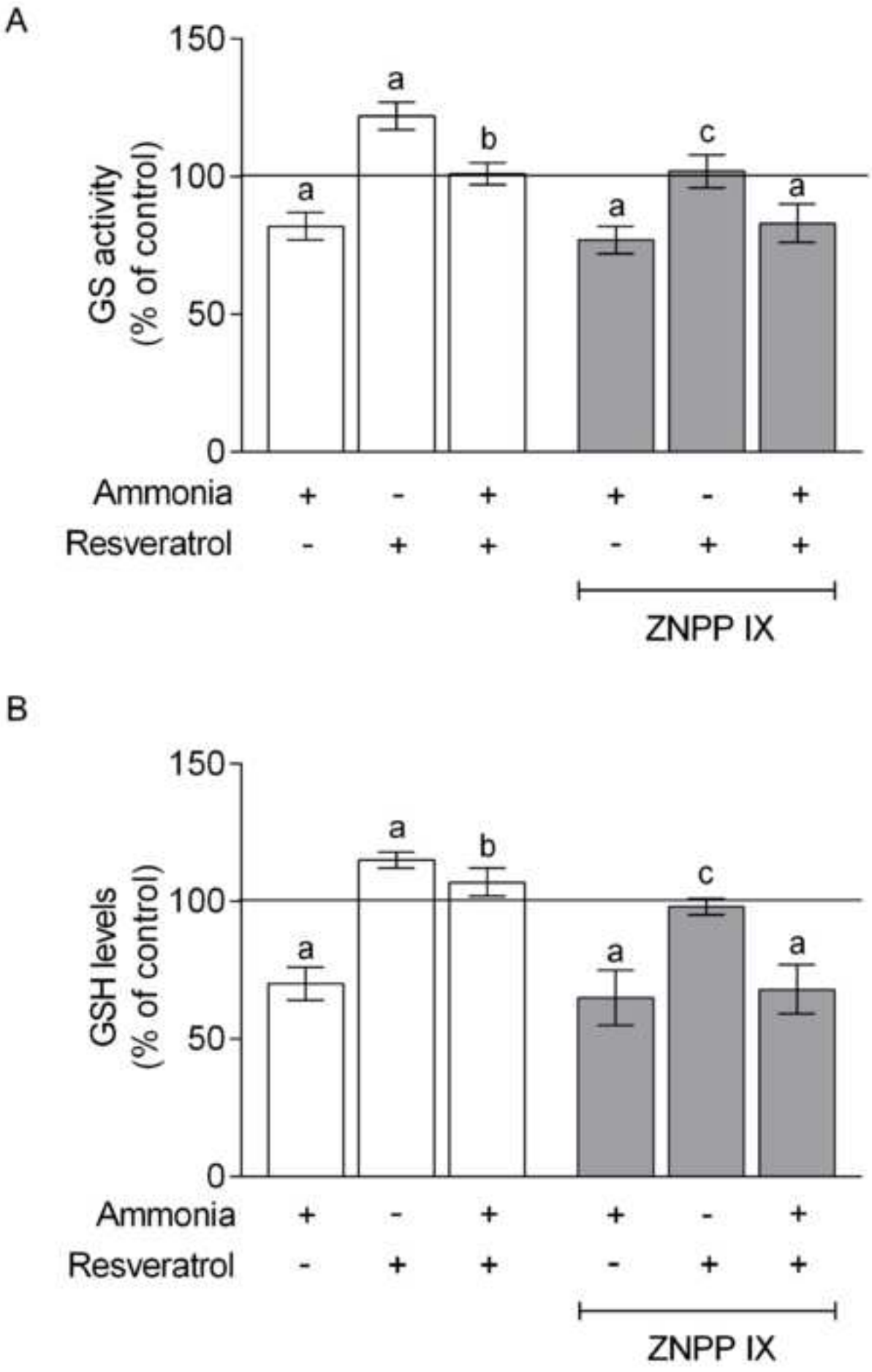

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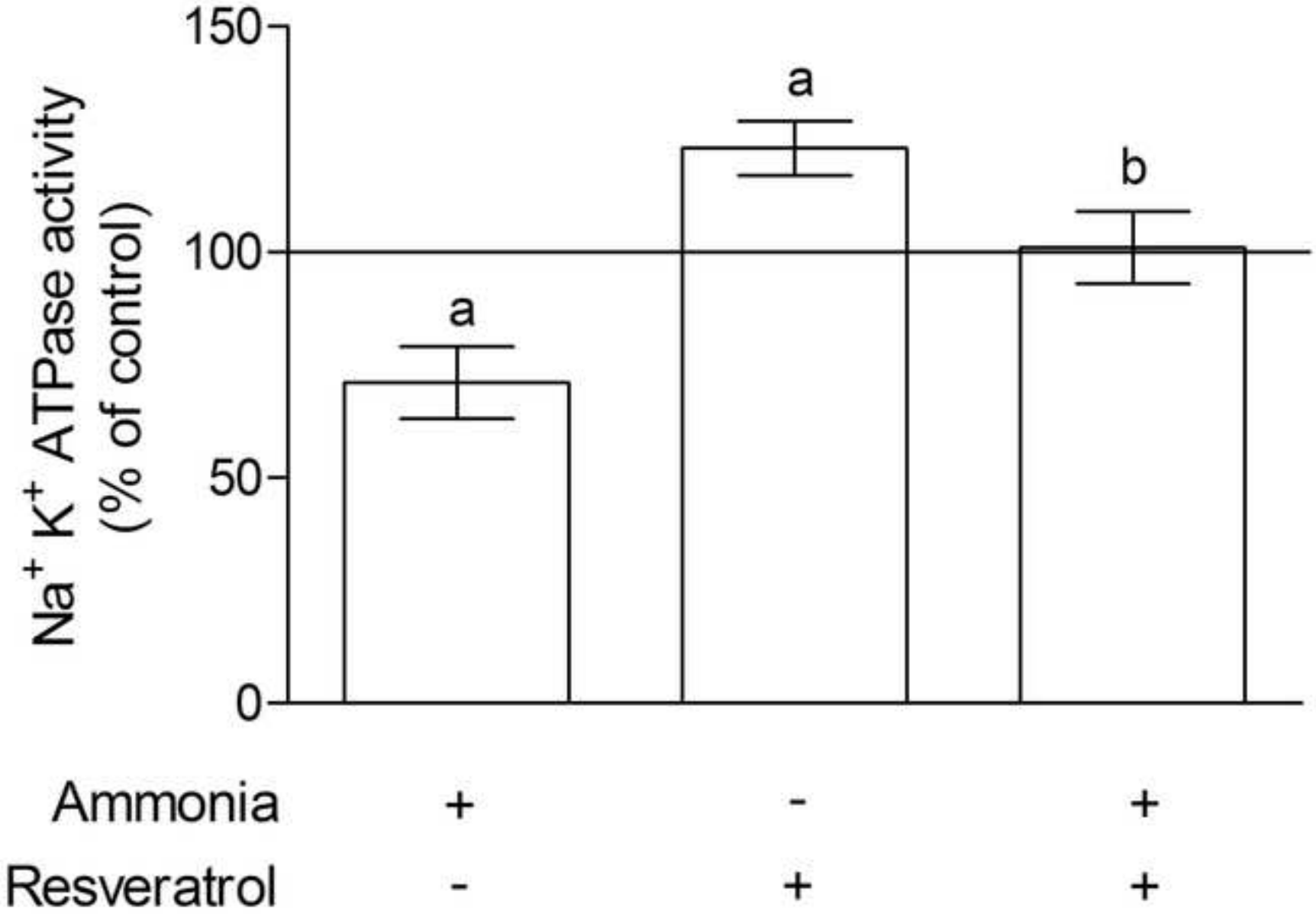

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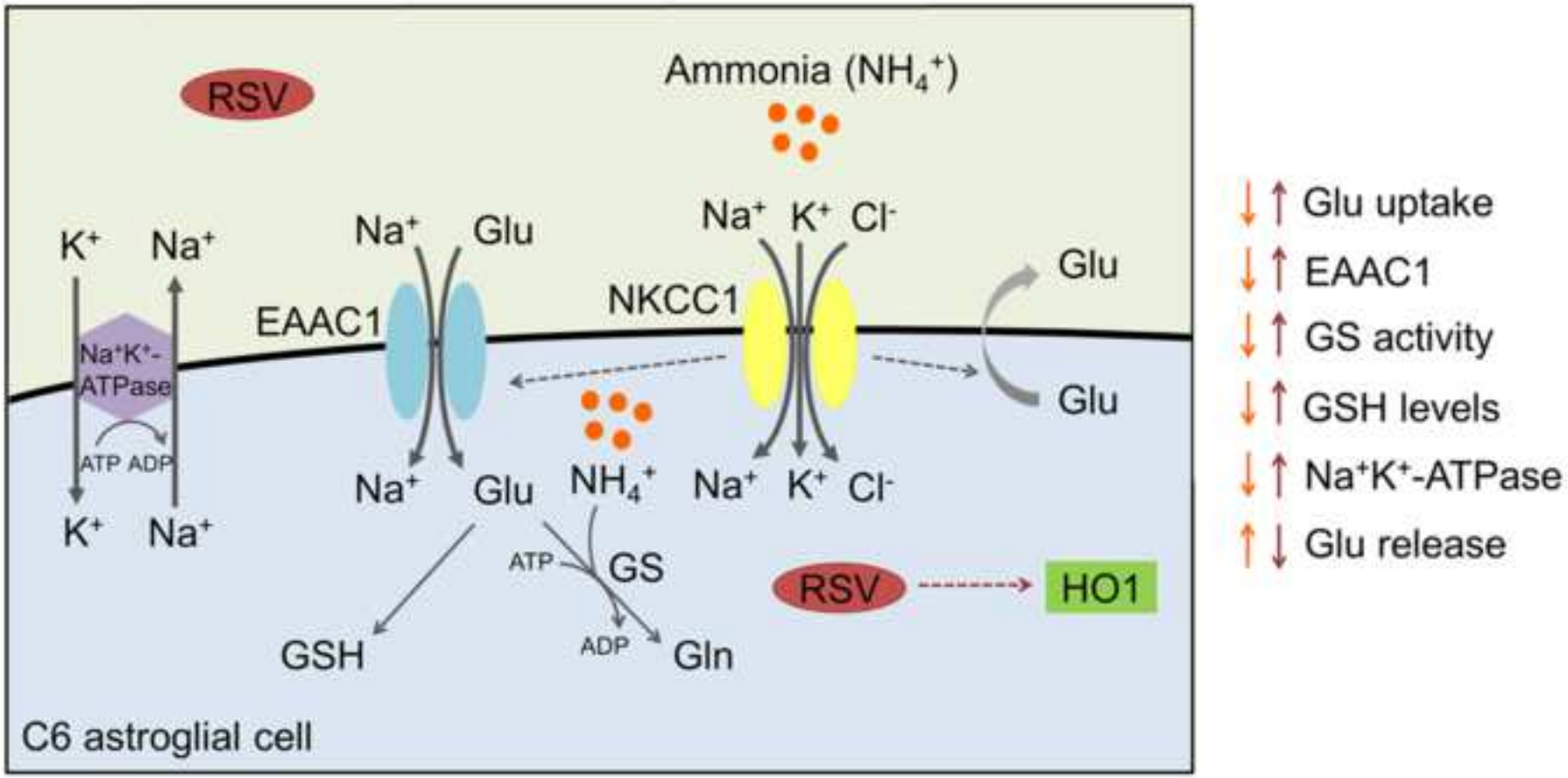

CAPÍTULO II

Artigo publicado no periódico Toxicology in Vitro

Lipoic acid protects C6 cells against ammonia exposure through Na^+-K^+-Cl^- co-transporter and PKC pathway

Larissa Daniele Bobermin, Diogo Onofre Souza, Carlos Alberto Gonçalves, André-Quincozes Santos

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Lipoic acid protects C6 cells against ammonia exposure through Na^+-K^+-Cl^- co-transporter and PKC pathway

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ABSTRACT

Astrocytes play an essential role in the central nervous system (CNS) homeostasis. They providing metabolic support and protecting against oxidative stress and glutamatergic excitotoxicity. Glutamate uptake, an electrogenic function, is driven by cation gradients and the Na^+-K^+-Cl^- co-transporter (NKCC1) carries these ions into and out of the cell. Elevated concentrations of ammonia in the brain lead to cerebral dysfunction. Ammonia toxicity can be mediated by an excitotoxic mechanism, oxidative stress and ion discharged. Astrocytes also convert excess ammonia and glutamate into glutamine, via glutamine synthetase (GS). Lipoic acid (LA) is a modulator of the cellular redox status potentially beneficial in neurodegenerative diseases. In this study, we investigated the effect of LA on glial parameters, in C6 cells exposed to ammonia. Ammonia increased S100B secretion and decreased glutamate uptake, GS activity and glutathione (GSH) content. LA was able to prevent these effects. LA exerts its protective effect on glutamate uptake and S100B secretion via mechanisms dependent of NKCC1 and PKC. These findings show that LA is able to modulate glial function impairments by ammonia *in vitro*, indicating a potential therapeutic agent to improve glutamatergic metabolism and oxidative stress against hyperammonemia.

1. Introduction

Astrocytes play a critical role in regulation of central nervous system (CNS) homeostasis. These cells interact with neurons, providing structural, metabolic, trophic support and antioxidant defenses (Barbeito et al., 2004; Belanger et al., 2011; Ransom and Ransom, 2012; Wang and Bordey, 2008). In pathological circumstances, however, they have the potential to induce neuronal dysfunction (Markiewicz and Lukomska, 2006; Parpura et al., 2012). Astrocytes also participate of the amino acids uptake, particularly glutamate, maintaining glutamate extracellular concentration below toxic levels (Anderson and Swanson, 2000; Coulter and Eid, 2012; Danbolt, 2001). Furthermore, glutamate uptake is driven by cation gradients (Kelly et al., 2009). The glutamate transporter couples the uptake of one glutamate and two Na^+ with the export of one K^+, and when the cellular ion gradients are discharged, the driving force for glutamate uptake is lost. Since the transporter is electrogenic, i.e. normally transferring a positive charge inward, membrane depolarization astrocytes can lead to reversal of the transporter, producing glutamate efflux (Siegel et al., 2006). Moreover, astrocytes have a specific enzyme, glutamine synthetase (GS – EC 6.3.1.2), which catalyzes the amidation reaction of glutamate to form glutamine, thereby protecting neurons against excitotoxicity (Mates et al., 2002; McKenna, 2007). The glutamate taken up by astrocytes may also be used to the biosynthesis of glutathione (GSH), the major antioxidant molecule in the brain (Banerjee et al., 2008; Hertz, 2006).

Ammonia is produced by amino acids and other nitrogenous compounds metabolism. Conditions as acute and chronic liver failure or deficiencies in enzymes of the urea cycle may increase the levels of ammonia in blood and, consequently, in CNS, leading to deleterious effects and development of hepatic encephalopathy (HE), a neuropsychiatric syndrome (Bjerring et al., 2009; Braissant et al., 2012; Felipo and Butterworth, 2002). Ammonia neurotoxicity can be mediated by an excitotoxic mechanism involving the glutamatergic system, including elevation of extracellular glutamate content, decreased glutamate transporters expression, NMDA receptor activation and subsequent increases in intracellular calcium concentration (Felipo et al., 1998; Hillmann et al., 2008; Monfort et al., 2002; Vaquero and Butterworth, 2006). Furthermore, under hyperammonemia, GS activity is decreased (Bobermin et al., 2012; Felipo and Butterworth, 2002; Leite et al., 2006). Besides its importance for glutamate metabolism, GS is responsible for detoxify ammonia in the CNS (Cooper and Plum, 1987). Ammonia toxicity in the brain also involves increase in reactive oxygen and nitrogen species (ROS/RNS) levels (Bobermin et al., 2012; Norenberg, 2003; Norenberg et al., 2009), change in nitric

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oxide (NO) metabolism (Buzanska et al., 2000), disturbance in energy metabolism (Rama Rao and Norenberg, 2012) and activation of mitogen-activated protein kinase (MAPK) pathway (Jayakumar et al., 2006; Norenberg et al., 2009). Increased levels of S100B secretion, an important glial marker in CNS, often used as an indicator of glial activation (Rothermundt et al., 2003; Steiner et al., 2011), and inflammatory cytokines (TNF-α, IL-1β, IL-6) were also observed in astroglial cells exposed to ammonia (Bobermin et al., 2012; Leite et al., 2006).

The Na^+-K^+-Cl^- co-transporter (NKCC) is a membrane protein that transport Na^+, K^+ and Cl^- ions into and out of the cell (Haas and Forbush, 1998) and also is involved in the ammonium (NH_4^+) influx in astrocytes (Kelly et al., 2009; Kelly and Rose, 2010; Worrell et al., 2008). NKCC1, the isoform present in astroglial cells, is related to regulation of cell volume in many conditions and contributes to astrocyte swelling induced by ammonia (Chassande et al., 1988; Jayakumar and Norenberg, 2010).

Lipoic acid (LA) is a naturally occurring compound that is synthesized in small amounts by plants and animals, including humans, and acts as cofactor in mitochondrial energy metabolism (Smith et al., 2004). LA exogenously administrated as dietary supplement is a potent modulator of the cell's redox status and presents anti-inflammatory and neuroprotective effects (Aguirre et al., 1999; Bramanti et al., 2010; Kleinkauf-Rocha et al., 2013; Perera et al., 2011; Salinthone et al., 2011; Shay et al., 2009; Tomassoni et al., 2013). LA contains two thiol (sulfur) groups, which may be reduced to dihydrolipoic acid (DHLA), and both the oxidized and reduced forms possess biological activity. The oxidized form is easily absorbed and taken by cells, being finally reduced to DHLA with participation of the system NAD(P)H (Haramaki et al., 1997; Packer and Cadenas, 2010). As DHLA can be released, it acts as antioxidant within the cell, mainly in mitochondria, and also in extracellular space, protecting cells against oxidative damage (Busse et al., 1992; Packer and Cadenas, 2010). In addition to their direct antioxidant activity, DHLA has the ability to regenerate other antioxidants, such as GSH, vitamin E and vitamin C (Biewenga et al., 1997; Kolgazi et al., 2007), and metal chelating activity (Ou et al., 1995), resulting in reduced ROS production. In this sense, LA has emerged as potential therapeutic agent in pathologies involving oxidative stress, including neurodegenerative diseases.

Recently, our group demonstrated that LA increases glutamate uptake, GS activity and GSH content in C6 astroglial cells (Kleinkauf-Rocha et al., 2013). This cell line is strongly stained for GFAP (95% GFAP positive) and has been widely used as an astroglial model to study glial functions and cellular signaling (Benda et al., 1968; dos Santos et al., 2006; Han et al., 1997; Mangoura et al., 1989; Quincozes-Santos and Gottfried, 2011; Steiner et al., 2010). Because ammonia exposure induces impairment in astroglial functions, in the present study, we investigated whether LA could protect C6 astroglial cells from ammonia toxicity. Thus, the S100B secretion, glutamate uptake, GS activity and GSH intracellular content was assessed, as well as, the possible mechanisms involved in the modulation of glutamate uptake and S100B secretion.

2. Materials and methods

2.1. Materials

Racemic (±) lipoic acid, standard GSH, *o*-phthaldialdehyde, bisindolylmaleimide II (Bis II), PD98059, furosemide and monoclonal anti-S100B (SH-B1) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM) and other materials for cell culture were purchased from Gibco BRL (Carlbad, CA, USA). Polyclonal anti-S100B from Dako (Glostrup, Denmark); anti-rabbit peroxidase and L-[^{3}H] glutamate were purchased from Amersham (Buckinghamshire, UK). All other chemicals were purchased from common commercial suppliers.

2.2. C6 astroglial cell culture and treatments

The C6 astroglial cell line was obtained from the American Type Culture Collection (Rockville, MA, USA) and was cultured according to a previously described procedure (dos Santos et al, 2006). The cells were seeded in flasks and cultured in DMEM (pH 7.4) containing 5% FBS, 0.1% amphotericin B and 0.032% gentamicin. Cells were maintained at a temperature of 37 °C in an atmosphere of 5% CO_2/95% air. At log phase, cells were detached from the culture flasks using 0.05% trypsin/ethylene-diaminetetracetic acid (EDTA) and seeded (5×10^3 cells/cm^2) in 24- or 6-well plates. At confluence, the culture medium was removed by suction, and the cells were pre-treated for 1 h with lipoic acid (10 μM) at 37 °C in an atmosphere of 5% CO_2/95% air in DMEM without serum. Subsequently, 5 mM ammonia (NH_4Cl) was added in the presence or absence of lipoic acid (10 μM) for 24 h.

Cells also were treated for 1 h or 24 h with furosemide (20 μM), which inhibits NKCC activity, to verify the involvement of this co-transporter in the modulation of S100B secretion and glutamate uptake by ammonia and LA. Moreover, to study the role of PKC (protein kinase C) and ERK (extracellular-signal regulated kinase) pathways in the glutamate uptake, cells were treated for 1 h, before ammonia and LA treatments for 24 h, with Bis II (1 μM) or PD98059 (5 μM), the specific PKC and MEK/ERK inhibitors, respectively.

2.3. Membrane integrity and metabolic activity assays

2.3.1. Propidium iodide (PI) incorporation assay

Cells were treated simultaneously with 7.5 μM PI and incubated for up 24 h in an atmosphere of 5% CO_2/95% air, at 37 °C. The optical density of fluorescent nuclei (labeled with PI), indicative of loss membrane integrity, was determined with Optiquant software (Packard Instrument Company). Density values obtained are expressed as a percentage of the control value.

2.3.2. Lactate dehydrogenase (LDH) assay

Was conducted in 50 μL of extracellular medium using a commercial colorimetric assay from Doles (Brazil). Results are expressed as percentages of the control value.

2.3.3. MTT reduction assay (metabolic activity)

MTT (50 μg/mL) was added after the treatments and cells were incubated for 30 min in an atmosphere of 5% CO_2/95% air, at 37 °C. Subsequently, the medium was removed and the MTT crystals were dissolved in DMSO. Absorbance values were measured at 560 and 650 nm. Results are expressed as percentages of the control value.

2.4. S100B measurement

S100B secretion and intracellular content were measured by an enzyme-linked immune sorbent assay (ELISA). Briefly, 50 μL of sample and 50 μL of Tris buffer were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B (SH-B1). Next, the samples were incubated with polyclonal anti-S100B for 30 min, and then, peroxidase-conjugated anti-rabbit antibody was added for a further 30 min incubation period. A colorimetric reaction with *o*-phenylenediamine was observed at 492 nm. Results are expressed as percentages of the control value.

2.5. Glutamate uptake assay

Glutamate uptake was performed as previously described (Gottfried et al., 2002) with some modifications. Briefly, C6 cells were incubated at 37 °C in a Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 $Na_2HPO_4{\cdot}7H_2O$, 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 μCi/ml L-[2,3-^{3}H] glutamate. The incubation was stopped after 10 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. Radioactivity was measured in a scintillation counter. Sodium-independent uptake was determined by using N-methyl-d-glucamine instead of sodium chloride. Sodium-dependent glutamate uptake was obtained by subtracting the non-specific uptake of the total uptake. Results are expressed as percentages of the control value.

2.6. Glutamine synthetase (GS) activity

The enzymatic assay was performed as previously described (dos Santos et al, 2006). Briefly, homogenate was added to a reaction mixture containing 10 mM $MgCl_2$, 50 mM L-glutamate, 100 mM imidazole-HCl buffer (pH 7.4), 10 mM 2-mercaptoethanol, 50 mM hydroxylamine-HCl and 10 mM ATP and incubated for 15 min at 37 °C. The reaction was stopped by adding a solution containing 370 mM ferric chloride, 670 mM HCl and 200 mM trichloroacetic acid. After centrifugation, the absorbance of the supernatant was measured at 530 nm and compared to a calibration curve of γ-glutamylhydroxamate treated with ferric chloride reagent. Results are expressed as percentages of the control value.

2.7. Glutathione (GSH) content assay

GSH levels were performed as previously described with some modifications (Browne and Armstrong, 1998). C6 astroglial cells homogenates were diluted in 10 volumes of 100 mM sodium phosphate buffer pH 8.0 containing 5 mM EDTA and protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with *o*-phthaldialdeyde (1 mg/ml methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively, Spectra Max GEMINI XPS, Molecular Devices, USA. A calibration curve was performed with standard GSH solutions (0–500 μM). GSH concentrations were calculated as nmol/mg protein and results are expressed as percentages of the control value.

2.8. Protein determination

Protein content was measured by Lowry's method using bovine serum albumin as the standard.

2.9. Statistical analysis

Data are presented as mean ± S.E.M. Each experiment was performed in triplicate from at least three independent cultures. The data were subjected to two-way analysis of variance (ANOVA) followed by the Tukey's test. Values of $P < 0.05$ were considered significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSSs) software.

3. Results

The exposure to ammonia (5 mM) for 24 h increased by about 46% ($P < 0.01$) the S100B secretion in C6 astroglial cells, Fig. 1.

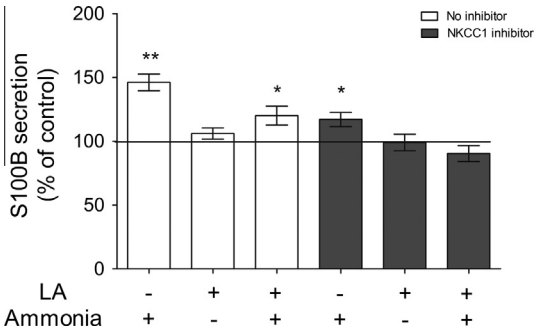

Fig. 1. Effects of ammonia and LA on S100B secretion. Cells were pre-treated for 1 h with LA (10 μM). After, ammonia (5 mM) was added in the presence or absence of LA for 24 h. Cells were also incubated with furosemide (20 μM), a NKCC1 inhibitor, followed by treatment with LA and ammonia for 24 h, in the presence of inhibitor. The basal secretion level, assumed to be 100%, is indicated by the line. Data represent mean ± S.E.M of three independent experimental determinations performed in triplicate, analyzed statistically by ANOVA followed by the Tukey's test. * Indicates significant differences from control, * for $P < 0.05$, ** for $P < 0.01$.

The intracellular S100B content was not affected by ammonia (data not shown). LA prevented this effect, dropped the levels from 146 ± 13% to 120 ± 13% ($P < 0.05$), Fig. 1. Because changes in cell integrity (as evaluated by measuring PI incorporation, extracellular LDH content and MTT reduction, data not shown) were not observed, the increased levels of extracellular S100B most likely resulted from secretion. We also investigated whether NKCC1 activity is related to S100B secretion in the presence of ammonia for 24 h. Furosemide, an inhibitor of NKCC1, *per se* did not modify the basal S100B secretion, but prevented the increase induced by ammonia exposure, Fig. 1. Moreover, co-incubation of furosemide and LA strongly reduced S100B secretion (from 146 ± 13% to 90 ± 8%, $P < 0.01$), Fig. 1. Additionally, morphological studies showed that ammonia exposure for 1 h induced astroglial swelling and cell body retraction, which were totally prevented by LA and furosemide, but at the same time S100B release was not affect (data not shown).

Ammonia decreased glutamate uptake (83 ± 8%, $P < 0.05$) in C6 astroglial cells after 1 h incubation (table inset in Fig. 2). LA *per se* did not alter the glutamate uptake, but avoided the decrease caused by ammonia (97 ± 8%). Furosemide alone protected cells against ammonia damage and potentiated the effect of LA on glutamate uptake (109 ± 8%, $P < 0.05$). Following 24 h of exposure to ammonia, glutamate uptake decreased 21% compared to control conditions ($P < 0.01$), Fig. 2. LA *per se* induced an increase in glutamate uptake (123 ± 11%, $P < 0.01$) and also was able to prevent the reduction induced by ammonia (113 ± 9%, $P < 0.05$). We also observed that decrease in glutamate uptake by ammonia is dependent of NKCC1 (from 79 ± 6% to 91 ± 7%). Furosemide also potentiated the LA-induced increase in glutamate uptake, indicating that LA may modulate NKCC1 co-transporter.

Moreover, in a previous work, we demonstrated that the modulation of glutamate uptake by LA is dependent of PKC (Kleinkauf-Rocha et al., 2013). Then, we evaluated whether PKC is able to modulate this function in hyperammonemia conditions. As expected, the PKC inhibitor (Bis II, 1 μM) blocked the increase in glutamate uptake induced by LA and also avoided the protective effect of LA against ammonia-induced glutamate uptake decrease, Fig. 2. As MAPK signaling pathway has been also described as modulator of glutamate uptake (Lee et al., 2012; Zhou et al., 2011), we tested the possible involvement of ERK, a member of MAPK family, in this parameter. The MEK/ERK inhibitor (PD98059, 5 μM) prevented the decrease in glutamate uptake induced by ammonia. However, did not alter the effect of LA, Fig. 2. These results indicate that another mechanism by which LA exerts its protective effect

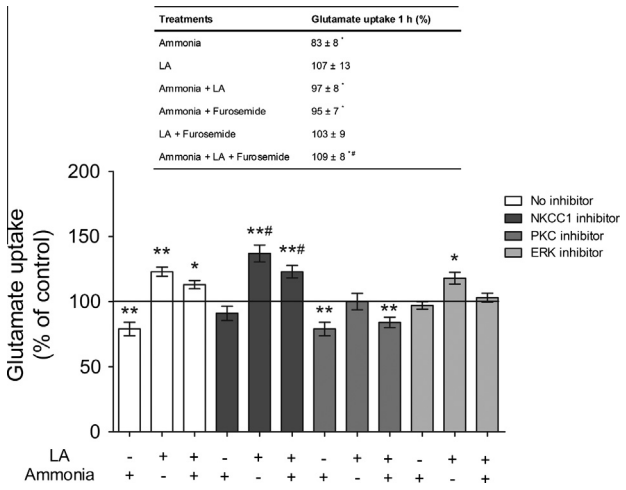

Fig. 2. Effects of ammonia and LA on glutamate uptake – involvement of NKCC1 co-transporter, PKC and ERK signaling pathways. Cells were pre-treated for 1 h with LA (10 μM). After, ammonia (5 mM) was added in the presence or absence of LA for 24 h. Cells were also pre-incubated with 20 μM furosemide, 1 μM Bis II (a PKC inhibitor) or 5 μM PD98059 (a MEK/ERK inhibitor), followed by treatment with LA and ammonia for 24 h in the presence of inhibitors. The line indicates the control value. The table inset shows the effect of ammonia, LA and furosemide on glutamate uptake after 1 h of treatment. Data represent mean ± S.E.M of three independent experimental determinations performed in triplicate, analyzed statistically by ANOVA followed by the Tukey's test. * Indicates significant differences from control, * for $P < 0.05$, ** for $P < 0.01$ and # for $P < 0.05$ compared to furosemide.

per se and against ammonia toxicity is PKC, while ammonia alone reduces glutamate uptake through NKCC1 and ERK pathway. For glutamate uptake, all inhibitors tested did not change the basal levels of uptake.

Because GS is primarily responsible for clearing ammonia and the main destination of glutamate after taken up by astroglial cells is conversion to glutamine by GS, we measure the activity of this enzyme. Following exposure to ammonia for 24 h, GS activity was decreased by about 17% (100 ± 9–83 ± 6%, $P < 0.01$), Fig. 3. LA *per se* increased the activity of GS (20%, $P < 0.05$), and completely prevented the ammonia-induced decrease in GS activity (99 ± 7%), Fig. 3.

Finally, we evaluated the GSH intracellular content, Fig. 4. Ammonia decreased GSH content by about 22% ($P < 0.01$). LA increased GSH (33%, $P < 0.01$) and, under ammonia exposure, restored the GSH intracellular levels (109 ± 9%).

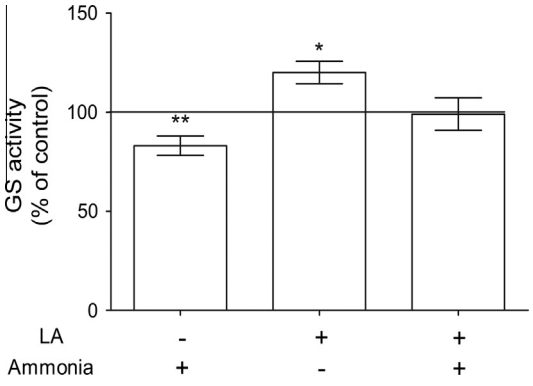

Fig. 3. Effects of ammonia and LA on GS activity. Cells were pre-treated for 1 h with LA (10 μM). After, ammonia (5 mM) was added in the presence or absence of LA for 24 h. The line indicates the control value. Data represent mean ± S.E.M of three independent experimental determinations performed in triplicate, analyzed statistically by ANOVA followed by the Tukey's test. * Indicates significant differences from control, * for $P < 0.05$ and ** for $P < 0.01$.

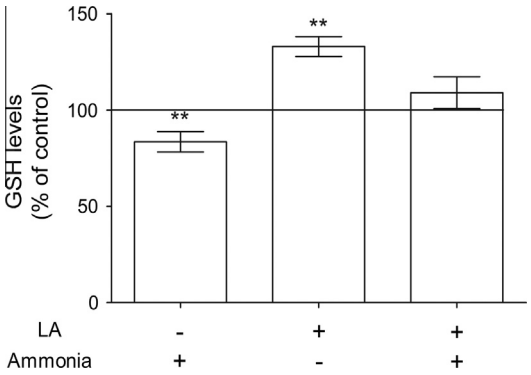

Fig. 4. Effects of ammonia and LA on GSH intracellular content. Cells were pre-treated for 1 h with LA (10 μM). After, ammonia (5 mM) was added in the presence or absence of LA for 24 h. The line indicates the control value. Data represent mean ± S.E.M of three independent experimental determinations performed in triplicate, analyzed statistically by ANOVA followed by the Tukey's test. * Indicates significant differences from control, ** for $P < 0.01$.

4. Discussion

LA has gained considerable attention due their role as antioxidant. There is increasing interest in potential therapeutic uses of LA in diverse conditions, such as neurodegenerative diseases, neuropathy, ischemia-reperfusion injury, metabolic syndrome and diabetes (De Araujo et al., 2011; Evans and Goldfine, 2000; Goraca et al., 2011; Packer et al., 1997; Shay et al., 2009; Tomassoni et al., 2013; Ziegler, 2009). Our group demonstrate, in a previous work, that besides exerting a well described antioxidant effect, LA is also able to modulate important glial functions and improve glutamatergic metabolism *in vitro* (Kleinkauf-Rocha et al., 2013). As hyperammonemia conditions result in glial alterations (Bobermin et al., 2012), we investigated the protective effect of LA in C6 astroglial cells exposed to ammonia.

Astrocytes display a wide range of adaptative functions in CNS (Markiewicz and Lukomska, 2006). During brain pathological conditions, injury or insults, one the biochemical responses of astroglial cells is an increase in S100B secretion (Van Eldik and Wainwright, 2003). Elevated extracellular concentration of this protein may be associated with brain damage, and persistent high levels could be involved in neurodegenerative disorders (Rothermundt et al., 2003). Previous works showed elevated S100B serum levels in patients with HE (Wiltfang et al., 1999) and increased levels of S100B secretion in astrocytes and C6 astroglial cells exposed to ammonia *in vitro* (Bobermin et al., 2012; Leite et al., 2006).

Here, we also observed that ammonia stimulated an increase of S100B release in C6 astroglial cells. However, when cells were incubated with ammonia and LA, the S100B secretion was lower, indicating a protective effect of LA. It is important to mention that the mechanism of S100B secretion still remains unclear, but is known that secretion is affected by redox conditions and metabolic stress (Donato et al., 2009; Nardin et al., 2007; Quincozes-Santos et al., 2009; Quincozes-Santos et al., 2010). Resveratrol, another molecule with antioxidant and neuroprotective properties, also is able to prevent the increase of S100B secretion provoked by ammonia, through a mechanism that may involve the decrease in NO production (Bobermin et al., 2012). Since LA modulates the cellular redox status and decreases NO levels (Kleinkauf-Rocha et al., 2013), which can be a possible mechanism whereby LA exerted their protective effect on S100B secretion in C6 astroglial cells exposed to ammonia.

In addition, furosemide prevented the increase on S100B secretion under ammonia exposure, and potentiates the effect of LA. NKCC1 is involved with the transport of NH_4^+ into the astroglial

cells (Kelly et al., 2009; Kelly and Rose, 2010) and stimulation of NKCC1 activation may indirectly modulate the intracellular Ca^{2+} concentration (Chen et al., 2008; Lenart et al., 2004; Liu et al., 2010). In this regard, NKCC1 inhibitor could decrease S100B secretion either by NH_4^+ influx and/or intracellular Ca^{2+} levels, because Ca^{2+} has been proposed to mediate S100B release (Davey et al., 2001; Nardin et al., 2009). This data indicates that the mechanism of S100B secretion probably involves Na^+, K^+ and Cl^- ion transport. Moreover, Jayakumar et al. (2008) reported that NKCC1 activation is involved in astroglial swelling under hyperammonemia and we observed that LA and furosemide totally prevented this effect (data not shown).

Glutamate is the main neurotransmitter in mammalian CNS and abnormalities of glutamatergic neurotransmission play a substantial role in the mechanisms of neurotoxicity of different toxic agents, including ammonia (Monfort et al., 2002). Astrocytes are intimately related to glutamatergic neurotransmission, because are the major cells responsible for glutamate clearance from the synaptic cleft by specific glutamate transporters (or excitatory amino acids transporter – EAAT) (Danbolt, 2001; Hertz, 2006; Veenman et al., 2012). There is evidence from *in vitro* and *in vivo* studies to suggest that ammonia exposure results in alterations in expression and activity of these transporters, leading to limitations in the capacity of glutamate uptake in astrocytes (Braissant et al., 2012; Felipo and Butterworth, 2002; Felipo et al., 1998; Rose, 2006; Vaquero and Butterworth, 2006). We showed that ammonia decreased glutamate uptake in C6 astroglial cells and LA prevented this effect. Furthermore, LA *per se* caused enhance in glutamate uptake. The glutamate uptake may be affected by oxidative stress (Volterra et al., 1994a,b). Ammonia increase ROS/RNS production (Bobermin et al., 2012; Jayakumar et al., 2006; Norenberg, 2003), which can oxidize the glutamate transporters, resulting in reduced uptake function (Anderson and Swanson, 2000; Trotti et al., 1998). Thus, one way by which LA can modulates glutamate uptake is decreasing oxidizing conditions.

Ammonia also activates NKCC1, leading to decrease in intracellular pH and increase in intracellular Na^+ concentration. As glutamate transporters activity is pH- and Na^+-dependent (Danbolt, 2001; Kanai and Hediger, 2004), ammonia can reduce glutamate uptake via alteration of ion gradients, reducing inward transmembrane gradients of Na^+ and H^+, which provide the driving force for glutamate uptake (Kelly et al., 2009; Kelly and Rose, 2010). In addition, reductions in the driving force for glutamate uptake, an electrogenic function associated to depolarizing astrocytes, may accompany an inhibition of GS, the enzyme responsible for detoxify ammonia in the CNS. According to Kelly et al. (2009), we found that the decrease in glutamate uptake induced by ammonia was dependent of NKCC1, because furosemide attenuates this effect *per se* and potentiates in the presence of LA. Additionally, ROS/RNS have an important role to increase NKCC1 activity and antioxidants prevent this effect (Jayakumar et al., 2008). LA can restore the glutamate uptake levels under ammonia exposure through NKCC1, preventing its oxidation/nitration and then, decreasing its activation (Jayakumar and Norenberg, 2010).

In addition to vulnerability to the action of biological oxidants and ionic gradient, some signaling pathways may also affect glutamate transport in astroglial cells. The activation of PKC regulates the expression of EAAT3, the main glutamate transporter present in C6 cells (Davis et al., 1998), and also increases the activity and the membrane expression of this transporter (Bianchi et al., 2006; Gonzalez et al., 2002). MAPK signaling pathway has been also described as modulator of glutamate uptake (Lee et al., 2012; Zhou et al., 2011). During this study, we investigated the involvement of PKC and ERK pathways on the modulation of glutamate uptake by ammonia and LA. In agreement with Kleinkauf-Rocha et al. (2013), we demonstrated that PKC inhibitor prevented the effects of LA, under basal and ammonia exposure conditions, indicating that the modulation of LA on glutamate uptake is dependent of PKC. Jayakumar et al. (2006) reported that inhibition of ERK did not reverse glutamate uptake decrease induced by ammonia in primary astrocyte cultures. However, here, the ERK inhibitor abolished the reduction of glutamate uptake caused by ammonia, showing that ammonia can impair glutamate uptake through ERK pathway. Interestingly, ERK is also responsible, at least in part, by other toxic effects of ammonia as the increased proinflammatory cytokines (TNF-α, IL-1β and IL-6) and S100B release by astroglial cells (Bobermin et al., 2012).

It is important to note that after taken up by astroglial cells, glutamate has many fates, for example glutamine and GSH biosynthesis (McKenna, 2007). The glutamate can be converted by the enzyme GS into glutamine, which is exported to neurons allowing the synthesis of glutamate, characterizing the glutamate-glutamine cycle (Mates et al., 2002). GS also is the major CNS pathway of ammonia removal (Braissant et al., 2012; Felipo and Butterworth, 2002), because synthesizes glutamine from glutamate and ammonium (NH_4^+), and is very sensitive to oxidative and nitrosative stress (Hertz, 2003). We reported that ammonia decreased GS activity, while LA prevented this effect and increased GS activity *per se*, contributing to the ammonia detoxification. Augment in NO production seems to be related to GS activity failure induced by ammonia (Bobermin et al., 2012; Rose and Felipo, 2005). Thus, a possible mechanism by which LA could modulate the activity of this enzyme is scavenging ROS/RNS. Additionally, the increase in the GS activity could be related to increased production of GSH. Glutamine formation is essential for glutamate metabolism and ammonia detoxification, and also helps to maintain GSH levels in CNS (Allaman et al., 2011; Banerjee et al., 2008; Dringen and Hirrlinger, 2003; Mates et al., 2002).

Glutamate is a precursor for synthesis of tripeptide GSH (glutamate, cysteine, and glycine), the main non-enzimatic antioxidant in the CNS (Wang and Bordey, 2008). Therefore, glial cells have an important role for antioxidant defenses in the CNS because it produce and secrete GSH, which serves as a substrate to neuronal GSH synthesis (Dringen, 2000; Pope et al., 2008). We observed that ammonia decreased GSH intracellular content. The depletion of GSH in glial cells induces neurotoxicity and impairment in glutamate transporters, and has been described as the basis of neurodegenerative disorders, such as Alzheimer's and Parkinson's disease (Lee et al., 2010; Schulz et al., 2000). As above mentioned, ammonia also decreases the glutamate uptake. Increase in extracellular glutamate levels affect cystine transport, essential for GSH synthesis, because glutamate and cystine share the same transport system – the cystine/glutamate (xc) antiporter (Lewerenz et al., 2006).

On the other hand, we found that LA increased the GSH intracellular content and completely recovered the GSH levels in the presence of ammonia. In addition its ability to regenerate glutathione oxidized (GSSG) to GSH, LA enhance GSH intracellular levels in several cell types and tissues (Busse et al., 1992; Suh et al., 2004b). DHLA can stimulate GSH synthesis by reduction of cystine to cysteine. LA may also increase cellular cysteine levels by enhancing cystine uptake, followed by its reduction to cysteine (Han et al., 1997, 1995; Packer and Cadenas, 2010). Additionally, LA can mediate the induction of GSH through transcription factor Nrf2 (nuclear factor erythroid-2 related factor-2) (Moini et al., 2002; Suh et al., 2004a), which facilitates the GSH synthesis (Escartin et al., 2011). The activation of Nrf2 signaling by LA also induces the antioxidative protein heme oxygenase 1 (HO1) expression in neural cells (Koriyama et al., 2013). This protein acts as a scavenger of NO and plays a crucial role against oxidative stress in a wide range of toxic insults (Bastianetto and Quirion, 2010; Li et al., 2007; Quincozes-Santos et al., 2013). Furthermore, the mechanism of

the Nrf2 activation and consequent HO1 induction involves PI3K pathway (Kim et al., 2010; Koriyama et al., 2013), which is modulated by LA (Wang et al., 2010; Yamada et al., 2011; Zhang et al., 2007). Thus, the modulation of GSH content and other antioxidative systems by LA reinforce their antioxidant and neuroprotective roles.

LA is a versatile bioactive compound, and their neuroprotective effects may be independent of its antioxidant activity. In summary, our results suggest that LA could protect important glial functions that are impaired under hyperammonemia conditions. Ammonia induced an increase in S100B secretion and a decrease in glutamate uptake, GS activity and GSH intracellular content in C6 astroglial cells and LA was able to prevent all these effects, suggesting that LA may be able to improve the clearance of glutamate and ammonia in the brain. The data presented in this study suggest that ammonia and LA may modulate S100B secretion and glutamate uptake through NKCC1. Moreover, the effects of LA and ammonia on glutamate uptake also occur via mechanisms probably dependent of PKC and ERK activation, respectively. Electrophysiological studies in progress, in our lab, will evaluate LA protective effects on ammonia exposure in hippocampal slices. Although further studies in animal models, to better clarify the mechanisms of action, and clinical trials are necessary, LA may represent a potential therapeutic agent to improve glutamatergic metabolism and oxidative stress, associated to neurological disorders, such as HE.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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CAPÍTULO III

Artigo submetido no periódico Toxicology in Vitro

Lipoic acid and N-acetilcysteine prevents ammonia-induced inflammatory response in C6 astroglial cells through ERK and HO1 signaling pathways

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Abstract: Hyperammonemia induces significant changes in central nervous system (CNS) directly associated to astroglial functions, such as oxidative damage, glutamatergic excitotoxicity, impairment in glutamine synthetase (GS) activity and pro-inflammatory cytokines release. Classically, lipoic acid (LA) and N-acetylcysteine (NAC) exhibit antioxidant and anti-inflammatory activities, by increasing glutathione (GSH) biosynthesis and decreasing pro-inflammatory mediators in glial cells. In this sense, here we evaluated the protective effect of LA and NAC against ammonia cytotoxicity in C6 astroglial cells. Ammonia decreased GSH content and increased cytokines release and NFκB levels. LA and NAC prevented these effects via ERK and HO1 signaling pathways. Taken together, these observations show that LA and NAC are able to prevent ammonia-induced inflammatory response.

Lipoic acid and N-acetylcysteine prevent ammonia-induced inflammatory response in C6 astroglial cells through ERK and HO1 signaling pathways

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Abstract

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Keywords: ammonia cytotoxicity, LA, NAC, inflammatory response, ERK, HO1

1. Introduction

Ammonia is a key factor in the pathogenesis of hepatic encephalopathy (HE), which is a major complication in acute and chronic liver failure (Felipo and Butterworth, 2002; Gorg et al., 2010). In this neuropsychiatric syndrome, the concentrations of ammonia in the brain can rise as high as 5 mM (Felipo and Butterworth, 2002). Hyperammonemia induces strong metabolic effects in central nervous system (CNS), including glutamatergic excitotoxicity, mitochondrial dysfunction with consequent overproduction of reactive oxygen and nitrogen species (ROS/RNS) and failure in ATP production, impairment in glutamine synthetase (GS) activity and release of pro-inflammatory cytokines (Bobermin et al., 2012; Bobermin et al., 2013; Gorg et al., 2010; Hillmann et al., 2008; Jayakumar et al., 2006; Leite et al., 2006; Norenberg, 2003; Norenberg et al., 1997; Norenberg et al., 2009).

Astrocytes are the primary target of ammonia toxicity in the brain, because GS is responsible for detoxify ammonia (Felipo and Butterworth, 2002). Furthermore, astrocytes contribute to maintenance of synaptic information processing and ionic homeostasis; regulate energy metabolism and release of neurotrophic factors; modulate the biosynthesis and release of antioxidant defenses, like glutathione (GSH), and the main anti- and pro-inflammatory cytokines (Belanger et al., 2011; Dringen, 2000; Farina et al., 2007; Maragakis and Rothstein, 2006; Parpura et al., 2012; Pope et al., 2008; Ransom and Ransom, 2012).

Our group have reported the beneficial effects of antioxidants, such as resveratrol and lipoic acid (LA) on glial cells as well as proposed some mechanisms for their activities (Bobermin et al., 2012; Bobermin et al., 2013;

Kleinkauf-Rocha et al., 2013; Quincozes-Santos et al., 2013; Quincozes-Santos et al., 2014b; Quincozes-Santos and Gottfried, 2011). LA is an essential cofactor for mitochondrial enzymes and has been widely studied as a neuroprotective compound when exogenously administrated (Packer and Cadenas, 2011; Perera et al., 2011; Salinthone et al., 2011). Numerous reports ranging from cell cultures to animal models have demonstrated that LA exhibits antioxidant and anti-inflammatory activities, by increasing GSH biosynthesis and decreasing pro-inflammatory mediators (Rocamonde et al., 2013; Rocamonde et al., 2012; Xia et al., 2014). Recently, we demonstrated that LA protects astroglial cells against ammonia toxicity improving glutamatergic metabolism (Bobermin et al., 2013).

N-acetylcysteine (NAC), a classical antioxidant, has been clinically used for the treatment of several peripheral and brain disorders (Berk et al., 2012; Samuni et al., 2013). Moreover, NAC can sustain the synthesis of GSH, because it is the acetylated precursor of the amino acid cysteine, a constituent of tripetide GSH, the major antioxidant defense of the CNS (Beloosesky et al., 2012; Reliene et al., 2009; Samuni et al., 2013). GSH is synthesized and secreted by astrocytes and serves as a substrate to neuronal GSH synthesis (Dringen, 2000; Hertz and Zielke, 2004). In this sense, Lee et al, 2010 demonstrated that a depletion of GSH in glial cells induced neuroinflammation. Although the increase of GSH intracellular levels is commonly associated to the beneficial roles of NAC, the GSH-independent mechanisms underlying its activity are only partially understood.

LA and NAC have been used in several clinical trials associated to neuropsychiatric disorders, mainly because these diseases have a multi-

factorial etiology that involves inflammatory pathways, oxidative stress, glutamatergic transmission and GSH metabolism, which are closely associated to glial cells (Beloosesky et al., 2012; Berk et al., 2008; Berk et al., 2012; Han et al., 2012; Packer and Cadenas, 2011; Samuni et al., 2013). However, the cellular and molecular mechanisms by which LA and NAC exerts their diverse effects are complex and still unclear. In this sense, the aim of this study was to evaluate the effect of ammonia on GSH content and cytokines release in C6 astroglial cells as well as the putative mechanisms involved in the protective effect of LA and NAC on hyperammonemia. Thus, we assessed GSH content, TNF-α, IL-1β, IL-6, IL-18, S100B and nuclear factor κB (NFκB) levels as well as the role of extracellular signal-regulated kinase (ERK) and heme oxygenase 1 (HO1) signaling pathways.

2. Materials and Methods

2.1. Materials

Lipoic acid (LA), N-acetylcysteine (NAC), PD98059, ZnPP IX, standard GSH, o-phthaldialdeyde, monoclonal anti-S100B antibody (SH-B1) and o-phenylenediamine (OPD) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and other materials for cell culture were purchased from Gibco (Carlsbad, CA, USA). All other chemicals were purchased from common commercial suppliers.

2.2. C6 astroglial cell cultures and treatments

C6 astroglial cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and was cultured according to a previously

described procedure (dos Santos et al., 2006). The cells were seeded in flasks and cultured in DMEM (pH 7.4) containing 5% fetal bovine serum (FBS), 0.1% amphotericin B and 0.032% gentamicin. Cells were kept at a temperature of 37ºC in an atmosphere of 5% CO_2/95% air. At confluence, the cells were pre-treated for 1 h with LA (10 μM) or NAC (100 μM) in DMEM without serum. Subsequently, 5 mM ammonia (NH_4Cl) was added in the presence or absence of LA (10 μM) or NAC (100 μM) for 24 h. To study the role of ERK and HO1 on the effects of LA and/or NAC, cells were treated for 1 h (before ammonia and LA/NAC treatments) with PD98059 (5 μM) – a specific MEK/ERK inhibitor and ZnPP IX (10 μM) – a specific HO1 inhibitor.

2.3. Glutathione (GSH) content assay

C6 astroglial cells homogenates were diluted in 100 mM sodium phosphate buffer with 140 mM KCl (pH 8.0) containing 5 mM EDTA and the protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with *o*-phthaldialdeyde (at a concentration of 1mg/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 μM to 500 μM) (Browne and Armstrong, 1998). The results are expressed as the percentage of the control levels.

2.4. Cytokines and S100B measurement

The levels of cytokines were carried out in the extracellular medium, using ELISA kits for TNF-α (PeproTech, USA), IL-1β, IL-6 and IL-18 (eBioscience, USA). The S100B secretion was also measured by ELISA

(Leite et al., 2008). The results are expressed as the percentage of the control levels.

2.5. Nuclear factor-kB levels

The levels of NF-kB p65 in the nuclear fraction, which had been isolated from lysed cells by centrifugation, were measured using an ELISA commercial kit from Invitrogen (USA). The results are expressed as percentages relative to the control levels.

2.6. Protein determination

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

2.7. Statistical analysis

Differences among groups were statistically analyzed using two-way analysis of variance (ANOVA), followed by Tukey's test. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software, version 16.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. LA and NAC prevented the ammonia-induced GSH decrease

Ammonia decreased GSH levels, by approximately 25% ($P < 0.01$) compared to control conditions (Fig. 1). LA and NAC restored the GSH intracellular content near to basal values. Moreover, LA and NAC *per se* increased GSH levels, 30% and 70% ($P < 0.01$), respectively.

3.2. LA and NAC prevented the ammonia-induced TNF-α, IL-1β, IL-6, IL-18 and S100B release

Ammonia induced a significant release of TNF-α (50%), IL-1β (35%), IL-6 (35%), IL-18 (30%) and S100B (55%) and LA and NAC prevented all of these effects (Table 1). LA and NAC *per se* did not affect cytokines release. Because changes in cellular integrity (evaluated by PI incorporation and MTT reduction, data not shown) were not observed, the increased levels of cytokines most likely resulted from secretion.

3.3. LA and NAC inhibited NFκB activation through ERK and HO1 signaling pathways

To elucidate the possible mechanisms of LA and NAC on the inhibition of inflammatory response, we determined the NFκB activation. Ammonia increased NFκB p65 levels by 45% (Fig. 2) and LA and NAC decreased the NFκB levels from 145% to 110% and 112%, respectively. The antioxidants *per se* not changed NFκB activation levels. When cells were incubated with ERK inhibitor (PD98059) LA and NAC potentiated the effect of the inhibitor, decreasing NFκB levels lower than ammonia plus PD98059. The HO1 inhibitor (ZnPP IX) totally abolished the effects of LA and NAC.

4. Discussion

The results of this study showed that LA and NAC could protect astroglial cells against ammonia-induced cytotoxicity. Ammonia decreased GSH levels and induced NFκB activation with consequent increase in pro-inflammatory cytokines and S100B secretion. LA and NAC protected glial cells through ERK and HO1 signaling pathways.

The antioxidative effect of LA and NAC were supported by modulating the homeostasis of GSH (Packer and Cadenas, 2011; Rocamonde et al., 2012; Samuni et al., 2013). The increase of GSH levels in glial cells confers protection against neuropsychiatric disorders, such as epilepsy, Alzheimer's and Parkinson's diseases (Halliwell, 2006; Lee et al., 2010b). In this sense, ammonia decreases GSH levels and, in agreement with other findings from our group, also induces oxidative and nitrosative stress and impairment in glutamate transporter activity (Bobermin et al., 2012; Bobermin et al., 2013). Accordingly, the depletion of GSH in glial cells induces neuroinflammation with significant augment of pro-inflammatory cytokines (Lee et al., 2010b). Furthermore, oxidative stress (associate to GSH depletion) plays a critical role in inflammatory response and, the pathophysiology of brain disorders like HE, involves oxidative and inflammatory pathways (Butterworth, 2011a, b; Norenberg, 2003).

Thus, herein, we demonstrated that ammonia-stimulated TNF-α, IL-1β, IL-6 and IL-18 levels. TNF-α is synthesized mainly by glial cells, and has several important functions in the CNS, including astrocytes activation and glutamatergic gliotransmission (Santello et al., 2011; Tanabe et al., 2010). Additionally, TNF-α and IL-1β, classical pro-inflammatory cytokines, have a major role in initiating a cascade of activation of other cytokines, such as IL-6 and IL-18 (Tanabe et al., 2011; Tanabe et al., 2010). IL-6 is also produced by glial cells and has a pivotal role in pathological brain inflammation as well as IL-18, a member of IL-1 superfamily, which induces inflammatory process and is directly associated to neurodegenerative diseases (Bossu et al., 2010; de Rivero Vaccari et al., 2014). Recent studies showed that pro-inflammatory

cytokines are increased in patients with HE and may be used as a marker for encephalopathy grade (Butterworth, 2011a). Assuming the role of S100B in neuroinflammation, it presented the same profile of pro-inflammatory cytokines (Donato, 2001; Mrak and Griffin, 2005). Moreover, elevated serum levels of S100B were observed in HE and findings from our group in primary astrocytes and C6 cells showed increase levels of S100B secretion (Bobermin et al., 2012; Bobermin et al., 2013; Leite et al., 2006; Wiltfang et al., 1999). LA and NAC strongly down-regulated ammonia-stimulated cytokines release. Thus, the results observed for LA and NAC may be promisor as therapeutic agents against ammonia toxicity.

NFκB is a transcriptional factor considered the major inflammatory mediator in the CNS (Gloire et al., 2006). As expected, ammonia challenge increased NFκB activation and LA and NAC were able to inhibit NFκB p65 levels. Regarding to antioxidants, both have been shown to suppress NFκB cascade signaling and subsequently production of pro-inflammatory cytokines (Packer and Cadenas, 2011; Samuni et al., 2013; Shay et al., 2009). In this study, we focused on testing a mechanistic hypothesis whether the attenuation of pro-inflammatory cytokines by antioxidants was through ERK and HO1 signaling pathways.

ERK pathway has been implicated in the regulation of glial inflammatory response following an insult and is an upstream signal transduction of NFκB (Bobermin et al., 2012; Lee et al., 2010a). LA and NAC potentiated the effect of ERK inhibitor, indicating that this pathway may be involved in the anti-inflammatory action of LA and NAC.

Concerning to HO1, it is the rate-limiting enzyme in the pathway through which pro-oxidant heme is degraded into the antioxidants biliverdin and bilirubin (Bastianetto and Quirion, 2010; Dore, 2005; Wakabayashi et al., 2010). Increases in HO1 activity are associated with protection against stressful conditions, like oxidative stress and inflammation, commonly found in HE (Bastianetto and Quirion, 2010; Sakata et al., 2010). HO1 through its transcription factor, Nrf2, mediates neuroprotection by modulation of several genes that encode antioxidant proteins, such as GSH system (Calkins et al., 2009; Vargas and Johnson, 2009; Wakabayashi et al., 2010). HO1 also facilitates the GSH synthesis by regulation of EAAC1, the main glutamate transporter present in C6 cells (Escartin et al., 2011). Additionally, HO1 signaling is upstream of NFκB, impeding its translocation to the nucleus (Wakabayashi et al., 2010), and the copresence of HO1 inhibitor blocked the positive effects of LA and NAC on NFκB activation in astroglial cells. NFκB activation also stimulates NO production via iNOS expression with consequent stimuli for pro-inflammatory cytokines release (Wakabayashi et al., 2010). HO1 inhibits iNOS activity, which in turn, is able to scavenge NO (Quincozes-Santos et al., 2013; Quincozes-Santos et al., 2014a; Wakabayashi et al., 2010). Thus, we demonstrated that LA and NAC decreased NFκB levels via HO1 signaling pathway and, consequently, this inhibition may be associated to attenuation in inflammatory response and oxidative stress.

In summary, our data reinforce the antioxidant effects of LA and NAC in glial cells against ammonia toxicity. Moreover, the findings show that ERK and HO1 signaling pathways participate in the mechanism of action of LA and

NAC. In addition, it is worthy of note that LA and NAC classically increase GSH, offering protection against neuroinflammation, including in clinical trials about HE (Butterworth, 2011b; Samuni et al., 2013; Shay et al., 2009). Thus, our results improve the understanding about glioprotection of LA and NAC from hyperammonemia. Studies in progress in our lab will investigate the outcomes of LA and NAC in the *in vivo* hyperammonemia animal model, focusing on glial properties.

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Figure legends

Fig. 1. Effects of LA and NAC on ammonia-induced GSH decrease. Cells were incubated for 1 h with LA (10 μM) or NAC (100 μM), followed by the addition of 5 mM ammonia for 24 h in serum-free DMEM. Data represent the mean ± S.E.M of four independent experimental determinations performed in triplicate and differences among 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 control conditions and *b* differences from ammonia exposure.

Fig. 2. LA and NAC prevented the ammonia-stimulated NFκB activation levels. Cells were incubated for 1 h with LA (10 μM) or NAC (100 μM), followed by the addition of 5 mM ammonia for 24 h in serum-free DMEM. 5 μM PD98059 or 10 μM ZnPP IX were coincubated with LA and NAC. Data represent the mean ± S.E.M of four independent experimental determinations performed in triplicate and differences among 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 control conditions and *b* differences from ammonia exposure.

Table 1. Effects of LA and NAC on cytokines and S100B release.

	TNF-α	IL-1β	IL-6	IL-18	S100B
Ammonia	150 ± 13 [a]	135 ± 10 [a]	135 ± 11 [a]	130 ± 15 [a]	155 ± 12 [a]
LA	104 ± 9	98 ± 8	102 ± 9	95 ± 10	105 ± 8
NAC	102 ± 10	96 ± 10	107 ± 10	101 ± 9	108 ± 10
Ammonia + LA	110 ± 10 [b]	111 ± 9 [b]	103 ± 10 [b]	108 ± 13 [b]	118 ± 8 [b]
Ammonia + NAC	106 ± 9 [b]	105 ± 8 [b]	110 ± 10 [b]	98 ± 8 [b]	120 ± 10 [b]

Cells were incubated for 1 h with LA (10 μM) or NAC (100 μM), followed by the addition of 5 mM ammonia for 24 h in serum-free DMEM. Data represent the mean ± S.E.M of four independent experimental determinations performed in triplicate and differences among 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 control conditions and *b* differences from ammonia exposure.

Figure 1
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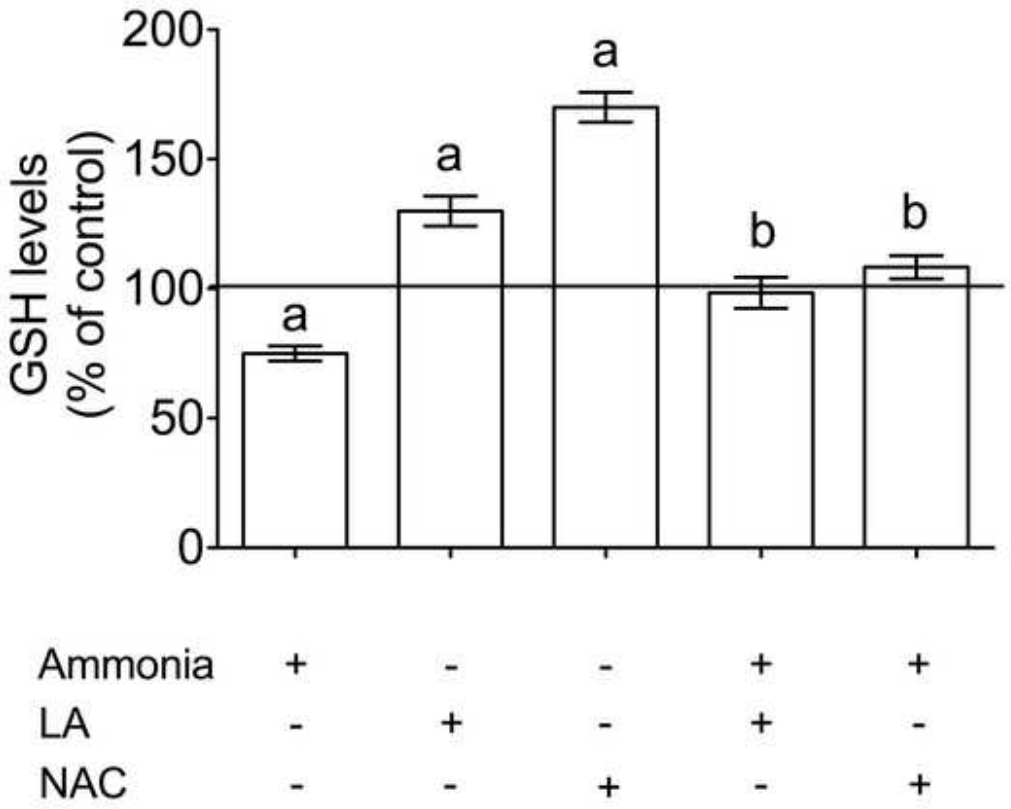

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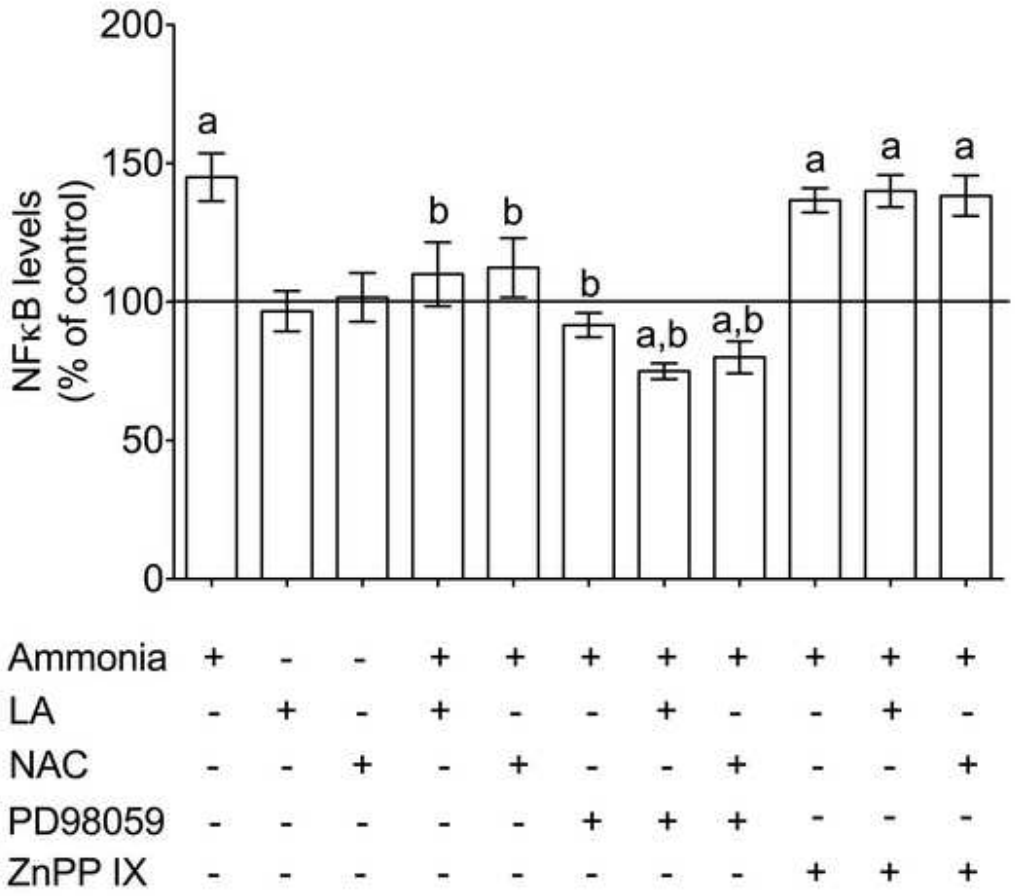

CAPÍTULO IV

Artigo submetido no periódico NeuroToxicology

Ammonia-induced oxidative damage in neurons is prevented by resveratrol and lipoic acid with participation of heme oxygenase 1

Larissa Daniele Bobermin, Krista Minéia Wartchow, Marianne Pires Flores, Marina Concli Leite, André Quincozes-Santos, Carlos Alberto Gonçalves

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Keywords: ammonia; neurotoxicity; oxidative stress; resveratrol; lipoic acid; heme oxygenase 1

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Abstract: Ammonia is a metabolite that at high concentrations is implicated with neurological disorders, such as hepatic encephalopathy (HE), which is associated with acute or chronic liver failure. Astrocytes are considered the primary target of ammonia toxicity in the central nervous system (CNS) because glutamine synthetase (GS), responsible for detoxify ammonia in CNS, is an astrocytic enzyme. Thus, neuronal dysfunction has been associated as secondary to astrocytic impairment. However, we demonstrated that ammonia can induce direct effects on neuronal cells. The cell viability was decreased by ammonia in SH-SY5Y cells and cerebellar granule neurons. In addition, ammonia induced increase of ROS production and decrease of GSH intracellular content, the main antioxidant in CNS. As ammonia neurotoxicity is strongly associated with oxidative stress, we also investigated the potential neuroprotective roles of antioxidants resveratrol (RSV) and lipoic acid (LA) against ammonia toxicity in cerebellar granule neurons. RSV and LA were able to prevent the oxidative damage induced by ammonia, maintaining the levels of ROS production and GSH near to the basal values. Both antioxidants also decreased the ROS production and increased GSH content in basal conditions (in the absence of ammonia). Moreover, we showed that heme oxygenase 1 (HO1), a protein associated with protection against stressful conditions, is involved in the beneficial effects of RSV and LA in cerebellar granule neurons. Thus, this study reinforces the neuroprotective effects of RSV and LA. Although more studies in vivo are required, RSV and LA could represent interesting therapeutic strategies in the management of HE.

AMMONIA-INDUCED OXIDATIVE DAMAGE IN NEURONS IS PREVENTED BY RESVERATROL AND LIPOIC ACID WITH PARTICIPATION OF HEME OXYGENASE 1

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Abstract

Ammonia is a metabolite that at high concentrations is implicated with neurological disorders, such as hepatic encephalopathy (HE), which is associated with acute or chronic liver failure. Astrocytes are considered the primary target of ammonia toxicity in the central nervous system (CNS) because glutamine synthetase (GS), responsible for detoxify ammonia in CNS, is an astrocytic enzyme. Thus, neuronal dysfunction has been associated as secondary to astrocytic impairment. However, we demonstrated that ammonia can induce direct effects on neuronal cells. The cell viability was decreased by ammonia in SH-SY5Y cells and cerebellar granule neurons. In addition, ammonia induced increase of ROS production and decrease of GSH intracellular content, the main antioxidant in CNS. As ammonia neurotoxicity is strongly associated with oxidative stress, we also investigated the potential neuroprotective roles of antioxidants resveratrol (RSV) and lipoic acid (LA) against ammonia toxicity in cerebellar granule neurons. RSV and LA were able to prevent the oxidative damage induced by ammonia, maintaining the levels of ROS production and GSH near to the basal values. Both antioxidants also decreased the ROS production and increased GSH content in basal conditions (in the absence of ammonia). Moreover, we showed that heme oxygenase 1 (HO1), a protein associated with protection against stressful conditions, is involved in the beneficial effects of RSV and LA in cerebellar granule neurons. Thus, this study reinforces the neuroprotective effects of RSV and LA. Although more studies *in vivo* are required, RSV and LA could represent interesting therapeutic strategies in the management of HE.

Key words: ammonia; neurotoxicity; oxidative stress; resveratrol; lipoic acid; heme oxygenase 1

1. Introduction

Ammonia is a metabolite that at high concentrations is implicated with neurological disorders, such as hepatic encephalopathy (HE), which is associated with acute or chronic liver failure (Albrecht and Jones 1999; Haussinger and Schliess 2008; Felipo 2013; Butterworth 2014). Ammonia crosses the blood-brain barrier readily (Ott and Larsen 2004) and, in the central nervous system (CNS), the predominant route for its metabolism is the amidation of glutamate to form glutamine through the glutamine synthetase (GS), an enzyme located in the astrocytes (Norenberg and Martinez-Hernandez 1979; Cooper 2011). This feature makes the astrocytes the primary target of ammonia-induced damage, and neuronal dysfunction has been associated as secondary to astrocytic impairment.

However, several *in vitro* and *in vivo* evidences show that ammonia induces direct effects on neuronal cells (Yang et al. 2004; Klejman et al. 2005; Rangroo Thrane et al. 2013; Chen et al. 2014). Moreover, NMDA receptors activation and their downstream effects, e.g. reactive oxygen and nitrogen (ROS/RNS) production, seem to be related with ammonia neurotoxicity (Kosenko et al. 2000; Hilgier et al. 2003; Zielińska et al. 2003; Giordano et al. 2005). In this sense, was observed that glutathione (GSH), a major antioxidant in the CNS (Dringen 2000), prevents ammonia toxicity in neurons (Klejman et al. 2005).

Antioxidants are substances that delay, prevent or reverse oxidative damage to a target molecule (Gutteridge and Halliwell 2010). A great number of molecules with antioxidant activity have demonstrated neuroprotective roles, such as resveratrol (RSV) and lipoic acid (LA) (Shay et al. 2009; Albarracin et al. 2012; Pallàs et al. 2013; Virmani et al. 2013). RSV (3,5,4'-trihydroxy-trans-stilbene) is a naturally occurring polyphenol present in grapes, berries and also red wines (Baur and Sinclair 2006). The protective effects of RSV in the brain has been studied in a variety of pathological events, including tumors (Leone et al. 2008; Gagliano et al. 2010; Filippi-Chiela et al. 2011), ischemic injury (Raval et al. 2008; Shin et al. 2012; Wang et al. 2014) and neurodegenerative disorders (Vingtdeux et al. 2008; Huang et al. 2011; Richard et al. 2011). RSV

presents important antioxidant properties, possibly by its direct scavenging effect and/ or activation of cellular antioxidant defenses (Bastianetto et al. 2014).

LA is another compound with beneficial effects, which is synthesized in small amounts by plants and animals, including humans, and is an essential cofactor for mitochondrial enzymes (Haramaki et al. 1997; Smith et al. 2004). When exogenously administrated, is a potent modulator of the cell redox status and presents anti-inflammatory effect (Perera et al. 2011; Kleinkauf-Rocha et al. 2013; Rocamonde et al. 2013; Tomassoni et al. 2013). Thus, LA has emerged as a potential therapeutic agent in pathologies involving oxidative stress.

Heme oxygenase 1 (HO1) is the rate-limiting enzyme in the pathway through pro-oxidant heme is degraded into antioxidants biliverdin and bilirubin. HO1, the inducible isoform, is activated not only by its physiological substrate heme, but also by various stress and noxious conditions, such as oxidative stress, hypoxia and inflammation (Doré 2005; Ryter et al. 2006; Pae et al. 2008; Jazwa and Cuadrado 2010). In the CNS, HO1 has been reported to operate an important cytoprotective/defense mechanism for cells exposed to oxidant challenges (Le et al. 1999; Scapagnini et al. 2004). Interestingly, this enzyme can participate in the positive effects of antioxidants RSV and LA (Sakata et al. 2010; Yamada et al. 2011; Kim et al. 2013; Koriyama et al. 2013; Lin et al. 2013; Quincozes-Santos et al. 2013).

Previously, we demonstrated that both RSV and LA exert beneficial effects in astroglial cells exposed to ammonia, avoiding oxidative stress, proinflammatory cytokines release and impairment of important astroglial functions, e.g. glutamate uptake, GS activity and GSH levels (Bobermin et al. 2012, 2013). Here, we investigated the potential neuroprotective roles of antioxidants RSV and LA, as well as the participation of HO1, against oxidative damage-mediated ammonia toxicity on SH-SY5Y cells and cerebellar granule neurons.

2. Materials and methods

2.1. Chemicals

Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12), Minimum Essential Media (MEM), fetal bovine serum (FBS) and other materials for cell cultures were purchased from Gibco (Carlsbad, CA, USA). DNase, poly-D-lysine, resveratrol, lipoic acid, ZnPP IX, methylthiazolyldiphenyltetrazolium bromide (MTT), 2'-7'-dichlorofluorescein diacetate (DCFH-DA), GSH standard and *o*-phthaldialdehyde were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were purchased from common commercial suppliers.

2.2. SH-SY5Y cell culture

Human neuroblastoma cell line SH-SY5Y, obtained from the American Type Culture Collection (ATCC; USA), was cultured in DMEM/F12 (pH 7.4) containing 10% FBS, 15 mM HEPES, 14.3 mM $NaHCO_3$,1% amphotericin B and 0,032% gentamicin, at 37ºC in a humidified atmosphere of 5% CO_2/95% air. When cells reached approximately 90% confluence, they were sub-cultured using 0.05% trypsin/ethylene-diaminetetracetic acid (EDTA) and seeded in 24-well plates ($6x10^4$ cells/well) (Lopes et al. 2010).

2.3. Cerebellar granule neurons culture

Cerebellar granule neurons cultures were prepared from 7-day-old Wistar rats as previously described with some modifications (Boeck et al. 2005). Animals 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). The 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 21215). Briefly, cerebella were aseptically dissected and dissociated enzymatically (with trypsin 0.05%) and mechanically, in Krebs-Ringer buffer containing DNAse (0.003%). After decantation for 20 min, the supernatant was collected and centrifuged for 5 min (1000 rpm). The cells from pellet were resuspended in MEM supplemented with 10% FBS, 14.3 mM $NaHCO_3$, 25 mM KCl and 0,032% gentamicin, plated in 6- or 24-well plates pre-coated with poly-D-lysine (10 µg/mL) at a density of $3\text{-}10x10^5$ cells/well and maintained at 37ºC in a humidified atmosphere of 5% CO_2/95% air. The non-neuronal cells were

inhibited by the addition of 20 μM cytosine arabinofuranoside 20–24 h after seeding. These cultures contain >90% granule cells and a small number of glial (2–3%) and endothelial cells (<1%) (Kingsbury et al. 1985).

2.4. Experimental treatments

SH-SY5Y cells were treated when reached approximately 75% confluence. The culture medium was replaced by DMEM/F12 1% FBS and cells were incubated with ammonia at indicated concentrations (1–10 mM) for 24 h. Cerebellar granule neurons were treated at 7 days in vitro (DIV) with ammonia (1–10 mM) for 24 h. To study the effects of antioxidants against ammonia neurotoxicity, cells were pre-treated for 1 h with resveratrol (RSV – 10 μM) or lipoic acid (LA – 10 μM) and then, 2 mM ammonia was added for 24 h. During all treatments, the cells were maintained at 37°C in an atmosphere of 5% CO_2/95% air. To study the role of HO1 in the effects of antioxidants, cells were co-incubated with ZnPP IX (10 μM), a specific inhibitor for HO1.

2.5. MTT assay

SH-SY5Y cells and cerebellar granule neurons were incubated with 50 μg/mL MTT for 3 h or 0.5 h, respectively, at 37°C in 5% CO_2/95% air. Subsequently, the medium was removed and the MTT crystals were dissolved in dimethylsulfoxide (DMSO). Absorbance values were measured at 560 nm and 650 nm. The results are expressed as percentages relative to the control conditions.

2.6. DCFH oxidation

Intracellular ROS levels were detected using DCFH-DA. DCFH-DA was added to the medium at a concentration of 10 μM and cells were incubated for 30 min at 37°C. Following DCFH-DA exposure, the cells were scraped into phosphate-buffered saline with 0.2% Triton X-100. The fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm (Quincozes-Santos et al. 2009). The results are expressed as percentages relative to the control conditions.

2.7. Glutathione (GSH) intracellular levels

GSH levels were assessed as previously described (Browne and Armstrong 1998). Cell lysate suspended in 100 mM sodium phosphate buffer

with 140 mM KCl (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 (at a concentration of 1 mg/ml methanol) at 22ºC for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. A calibration curve was performed with standard GSH solutions at concentrations ranging from 0 to 500 µM. GSH concentrations were calculated as nmol/mg protein. The results are expressed as percentages relative to the control conditions.

2.7. Protein assay

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

2.8. Statistical analyses

Differences among groups were statistically analyzed using one- or two-way analysis of variance (ANOVA), followed by Tukey's test. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software, version 16.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of ammonia on SH-SY5Y cells and cerebellar granule neurons viability

Cellular viability of the SH-SY5Y cells and cerebellar granule neurons challenged with ammonia (1–10 mM) for 24 h was evaluated by measuring MTT reduction. Ammonia decreased SH-SY5Y viability only at higher concentration (10 mM), Figure 1A. However, in cerebellar granule neurons, decreased cell viability was already found at 5 mM (19%), with an important loss at 10 mM (40%), Figure 1B. Cellular morphology of cerebellar granule neurons was evaluated by phase contrast microscopy. Consistent with the result of cell viability, was observed a reduced number of neurites and increased number of cells with bodies and processes disintegrated in the cultures exposed to ammonia, as illustrated in Figure 1C.

3.2. Effects of ammonia on DCFH oxidation and intracellular GSH levels in SH-SY5Y cells and cerebellar granule neurons

The production of ROS was measured using DCFH oxidation. The DCFH oxidation in SH-SY5Y cells were not altered by any ammonia concentrations tested (Figure 2A). However, in cerebellar granule neurons, DCFH oxidation increased by approximately 23% and 54% following the treatment with ammonia 1 and 2 mM, respectively, indicating an increase in ROS production (Figure 2B).

As well as in the DCFH oxidation, ammonia not changed the intracellular GSH content in SH-SY5Y cells (Figure 3A). In cerebellar granule neurons, ammonia decreased the GSH levels at 1 mM (20%) and 2 mM (28%), Figure 3B. Higher concentrations were not tested because caused a significant loss of cell viability.

3.3. Ammonia-induced oxidative damage in cerebellar granule neurons was prevented by RSV and LA

We further investigated the role of antioxidants RSV and LA against ammonia-induced oxidative damage in cerebellar granule neurons. RSV was able to prevent the ammonia-induced ROS production, changing the levels from 154% to basal values (Figure 4A). RSV also avoided the decrease of GSH content, maintaining the levels near to the basal values (Figure 4B). In addition, resveratrol *per se* exerted positive effects in neurons, decreasing ROS production and increasing GSH levels.

In the presence of 2 mM ammonia, LA also reduced the ROS production from 154% to control values (Figure 5A). The intracellular GSH content also was modulated by LA, with prevention of decrease (from 80% to 97%), Figure 5B. As RSV, LA alone decreased the DCFH oxidation by approximately 20% and increased the GSH content (22%) in neurons.

3.4. Role of HO1 in the effects of RSV and LA on cerebellar granule neurons

In order to verify the involvement of HO1 in the neuroprotection by RSV and LA against oxidative damage in cerebellar granule cells, we used a specific

inhibitor of HO1, ZnPP IX (10 μM). Interestingly, the inhibitor blocked the effects of both antioxidants in DCFH oxidation and intracellular GSH content during ammonia exposure (Figure 6A and 6B). Moreover, when HO1 was inhibited, the positive modulation of RSV and LA on DCFH oxidation and GSH levels also was abolished (Figure 6C and 6D), reinforcing the participation of this protein in their mechanisms of action.

4. Discussion

HE is the most common disorder associated with hyperammonemmia, as result of impaired ammonia detoxification in the liver (Felipo, 2013). Here, we observed that ammonia may induce neurotoxicity, which seems to be related to oxidative stress, and that the antioxidants RSV and LA prevented these effects via HO1.

We firstly tested the effects of ammonia in SH-SY5Y cells, a human neuroblastoma cell line which has been widely used in neuroscience research as a neuron-like model (Molina-Holgado et al. 2008; Cheung et al. 2009; Xie et al. 2010). At higher concentration (10 mM), ammonia caused a loss of SH-SY5Y viability. However, ammonia did not change ROS production and intracellular GSH content, the main antioxidant in CNS, in these cells. These results may be due to an decreased susceptibility of the SH-SY5Y cells to toxic stimuli, owing their biochemical properties (Gilany et al. 2008), in relation to neurons primary cultures.

Hence, we investigated the effect of ammonia in primary cultures of cerebellar granule neurons, a good model system for molecular and biological studies of neuronal development and function (Bilimoria and Bonni 2008). We observed an increased cell death from the concentration of 5 mM ammonia and, at 10 mM, morphological alterations was evidenced, such as disintegrated processes and reduced number of neurites (Figure 1C). Even though, further evaluations was performed with ammonia concentrations that not caused loss of cell viability (1 and 2 mM), but that can trigger cellular alterations and dysfunctions, which ultimately can lead to this outcome.

Ammonia induced an increase of ROS production in cerebellar granule neurons, evaluated by DCFH oxidation. Oxidative stress is strongly associated with ammonia toxicity (Norenberg et al. 2004; Lemberg and Fernández 2009; Bemeur et al. 2010; Skowrońska and Albrecht 2013). Oxidative stress is characterized by an excess of ROS/RNS production or impairment in their detoxification by endogenous mechanisms. During this condition can occurs oxidation of lipid, protein and DNA, causing cell damage to some cellular constituents and impairment of the cellular functioning (Halliwell 2006, 2007).

Moreover, ammonia also resulted in a decrease of intracellular GSH content, which is the most abundant antioxidant in the brain (Dringen 2000; Lewerenz and Maher 2011). GSH is a critical molecule that provide the major line of defense for the protection of CNS from oxidative stress and its loss is associated with neurotoxicity and neuroinflammation (Li et al. 1997; Lee et al. 2010; Currais and Maher 2013). Our data are in agreement with Klejman et al. (2005), who showed that ammonia treatment in rat cortical neurons caused a decrease in the GSH/GSSG ratio indicating enhanced GSH consumption. Moreover, they found that GSH supply counteracted ammonia-induced cell death (Klejman et al. 2005).

In this sense, antioxidant molecules can promote a protective effect during ammonia challenge. We found that RSV and LA, two important and well-established antioxidants prevented ammonia-induced oxidative damage. RSV is a polyphenolic compound that displays antioxidant activity by its direct scavenger of free radicals effect and also by activation of pathways and enzymes that up regulate cellular antioxidants defenses, such as HO1 (Quincozes-Santos et al. 2009, 2013; Sakata et al. 2010). We showed that RSV was able to attenuate ROS production and prevent GSH decrease in cerebellar granule neurons exposed to ammonia. Additionally, these effects were mediated, at last in part, by HO1 because incubation of neurons with ZnPP IX, a potent and selective inhibitor of HO1 activity (Vargas et al. 2008; Chao et al. 2014; Quincozes-Santos et al. 2014), blocked the protective effects of resveratrol. The positive effects of resveratrol alone, reducing ROS production and increasing GSH levels, also were avoided by ZnPP IX. HO1 is an enzyme that provides resistance against oxidative stress and its activity results in

profound changes in the abilities of the cells to protect themselves against oxidative injury, and is strongly associated to neuroprotective effects of resveratrol (Bastianetto et al. 2014). Our data are consistent with work of Sakata et al. (2010), which showed that resveratrol increased HO1 protein levels in primary neuronal cells exposed to glutamate (Sakata et al. 2010).

LA, another molecule that acts as free radical scavenger, also protected the neurons against ammonia in relation to ROS production and recovered the GSH levels. In addition, as RSV, LA *per se* decreased DCFH oxidation and increased GSH content. The antioxidant effects of LA are due to its free radical scavenger action and interaction with other antioxidant molecules, e.g. GSH, preventing their oxidation and participating in their regeneration (Packer and Cadenas 2011). Previous works have demonstrated that LA enhances intracellular GSH levels in several cell types and tissues. These effects on GSH induction can be mediated through transcription factor Nrf2 (nuclear factor erythroid-2 related factor-2), which facilitates the GSH synthesis (Suh et al. 2004a, 2004b; Kleinkauf-Rocha et al. 2013; Moraes et al. 2014; Yang et al. 2014). Nrf2 is upstream to HO1 (Wakabayashi et al. 2010) and induces its expression in neural cells (Fujita et al. 2008; Koriyama et al. 2013). In fact, our data support this evidence, since ZnPP IX inhibited the role of LA, indicating that HO1 mediated the neuroprotection by LA.

During hyperammonemia, astroglial cells, responsible for ammonia detoxification in the CNS, can failure to support the proper functioning of neurons (Pérez-Alvarez and Araque 2013). Our previous works (Bobermin et al. 2012, 2013) showed that both RSV and LA are able to enhance important astroglial functions in the presence of ammonia, such as glutamate uptake, GS activity and GSH content, which could improve glia-neuron interaction and synaptic plasticity (Quincozes-Santos and Gottfried 2011). Here, we showed that the same antioxidants present positive effects on cerebellar granule neurons exposed to ammonia, which reinforce their neuroprotective effects. Moreover, RSV and LA have been used in several clinical trials in progress associated to neuropsychiatric disorders and another antioxidant, N-acetylcysteine (NAC) also has been tested in HE (Vaquero and Butterworth 2007; Butterworth 2011). In this sense, RSV and LA need to be better studied in

models of HE *in vivo*, but could represent interesting therapeutic strategies in the management of HE.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. Effect of ammonia on SH-SY5Y cells and cerebellar granule neurons viability. SH-SY5Y cells (A) and cerebellar granule neurons (B) were treated with ammonia at indicated concentrations for 24 h, and cell viability was assessed by MTT reduction. The morphology of cerebellar granule neurons was also evaluated by phase contrast microscopy (C). The data are expressed as percentages relative to the control conditions (indicated by the line) and represent the mean ± S.E.M of at least three independent experimental determinations, performed in triplicate and statistically analyzed by one-way ANOVA followed by Tukey's test. * indicates significant differences from the control ($P < 0.05$).

Figure 2. Effects of ammonia on DCFH oxidation and intracellular GSH levels in SH-SY5Y cells. Cells were treated with ammonia at indicated concentrations for 24 h. DCFH oxidation (A) and intracellular GSH content (B) were evaluated. The data are expressed as percentages relative to the control conditions (indicated by the line) and represent the mean ± S.E.M of at least three independent experimental determinations, performed in triplicate and statistically analyzed by one-way ANOVA followed by Tukey's test. * indicates significant differences from the control ($P < 0.05$).

Figure 3. Effects of ammonia on DCFH oxidation and intracellular GSH levels in cerebellar granule neurons. Cells were treated with ammonia at indicated concentrations for 24 h. DCFH oxidation (A) and intracellular GSH content (B) were evaluated. The data are expressed as percentages relative to the control conditions (indicated by the line) and represent the mean ± S.E.M of at least three independent experimental determinations, performed in triplicate and statistically analyzed by one-way ANOVA followed by Tukey's test. * indicates significant differences from the control ($P < 0.05$).

Figure 4. Effects of RSV against ammonia-induced oxidative damage in cerebellar granule neurons. Cells were pre-treated with RSV (10 μM) for 1 h, followed by the addition of 2 mM ammonia for 24 h. DCFH oxidation (A) and intracellular GSH content (B) were evaluated. The data are expressed as percentages relative to the control conditions (indicated by the line) and

represent the mean ± S.E.M of at least three independent experimental determinations, performed in triplicate and statistically analyzed by two-way ANOVA followed by Tukey's test. * indicates significant differences from the control ($P < 0.05$).

Figure 5. Effects of LA against ammonia-induced oxidative damage in cerebellar granule neurons. Cells were pre-treated with LA (10 μM) for 1 h, followed by the addition of 2 mM ammonia for 24 h. DCFH oxidation (A) and intracellular GSH content (B) were evaluated. The data are expressed as percentages relative to the control conditions (indicated by the line) and represent the mean ± S.E.M of at least three independent experimental determinations, performed in triplicate and statistically analyzed by two-way ANOVA followed by Tukey's test. * indicates significant differences from the control ($P < 0.05$).

Figure 6. Role of HO1 in the effects of RSV and LA on cerebellar granule neurons. Cells were pre-incubated with ZnPP IX (10 μM) prior the treatments with antioxidants and ammonia to assess the involvement of HO1 in the parameters analyzed. DCFH oxidation (A and C) and intracellular GSH content (B and D) were evaluated. The data are expressed as percentages relative to the control conditions (indicated by the line) and represent the mean ± S.E.M of at least three independent experimental determinations, performed in triplicate and statistically analyzed by two-way ANOVA followed by Tukey's test. * indicates significant differences from the control ($P < 0.05$).

Figure 1

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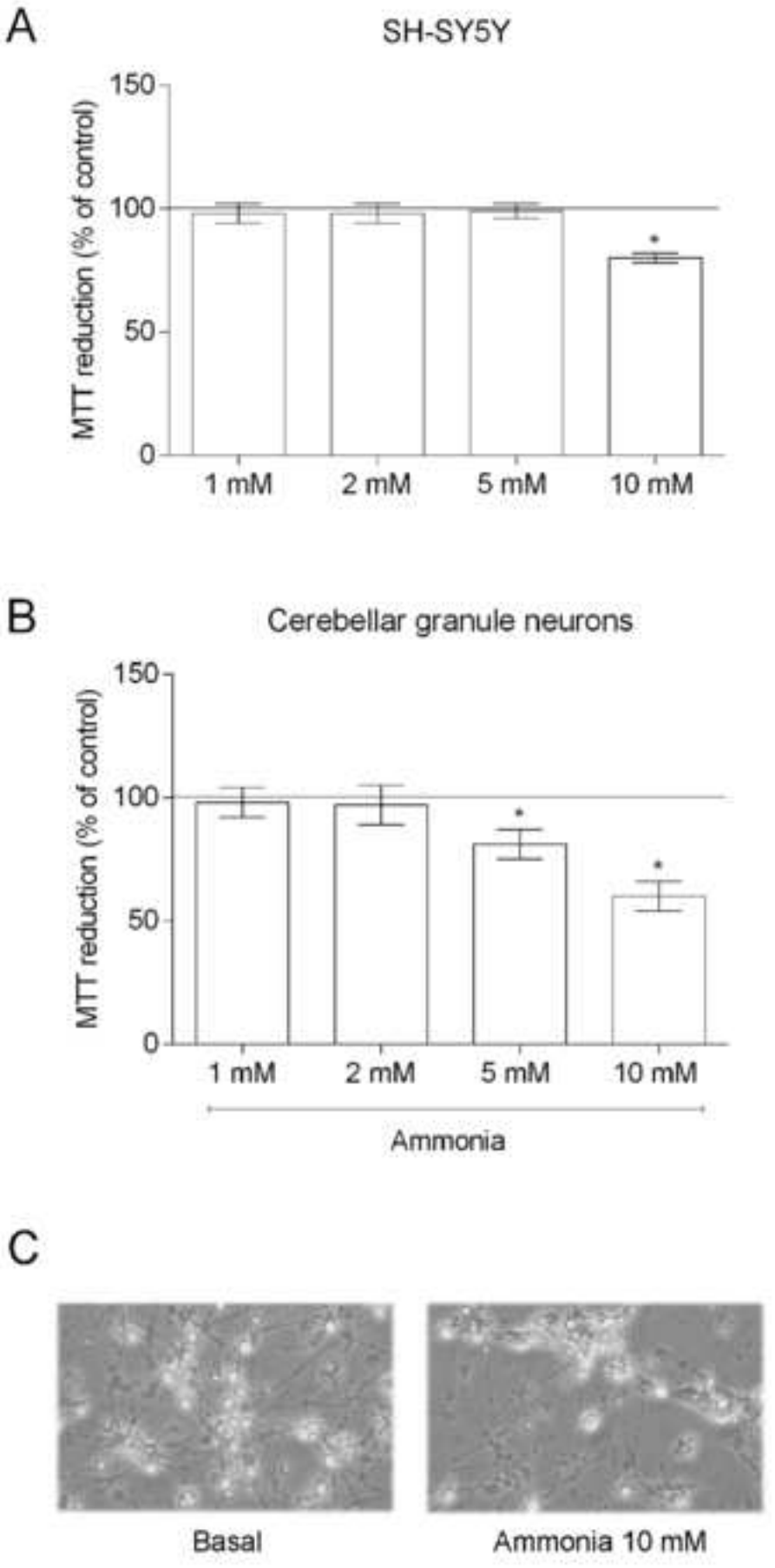

Figure 2
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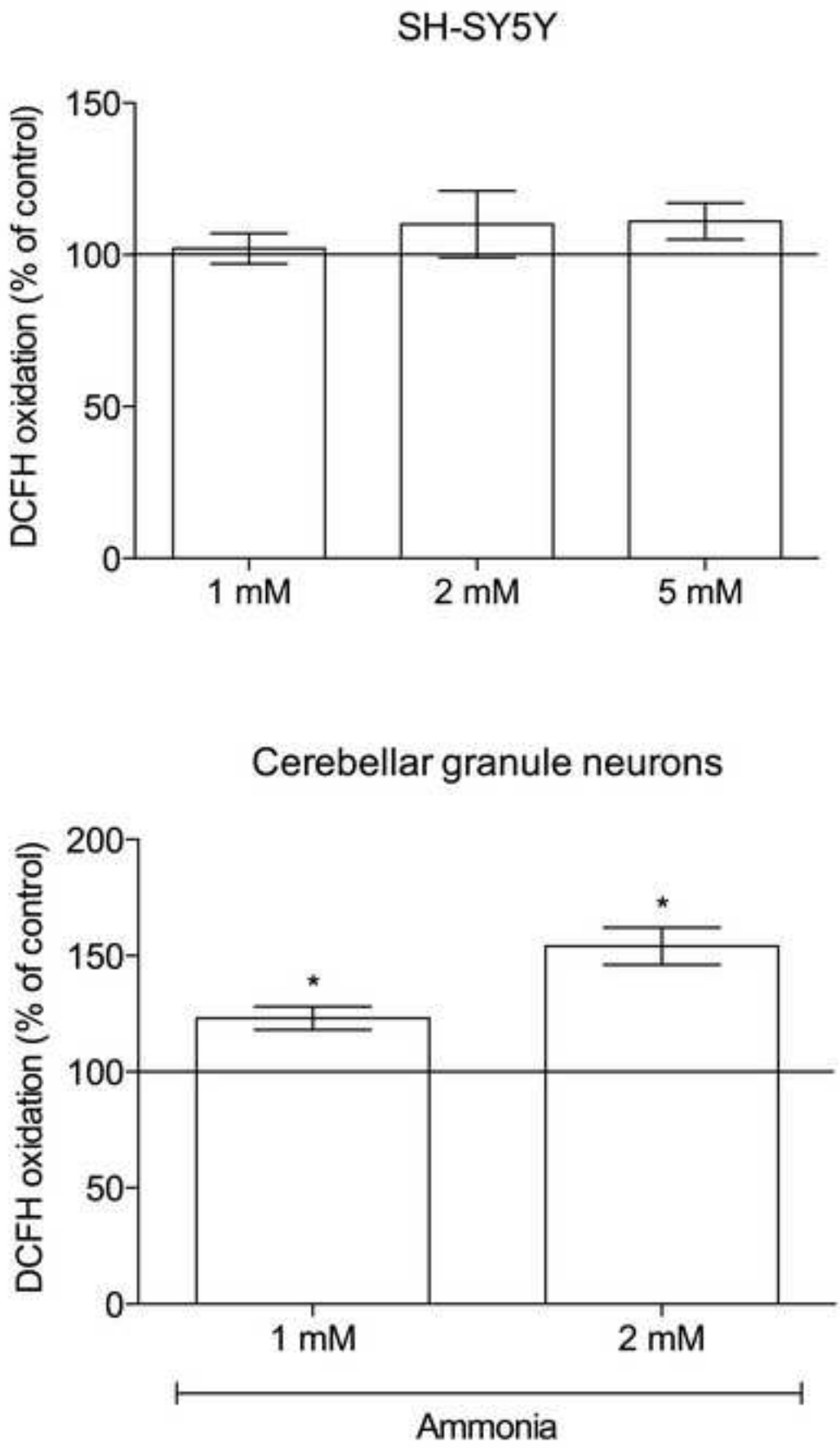

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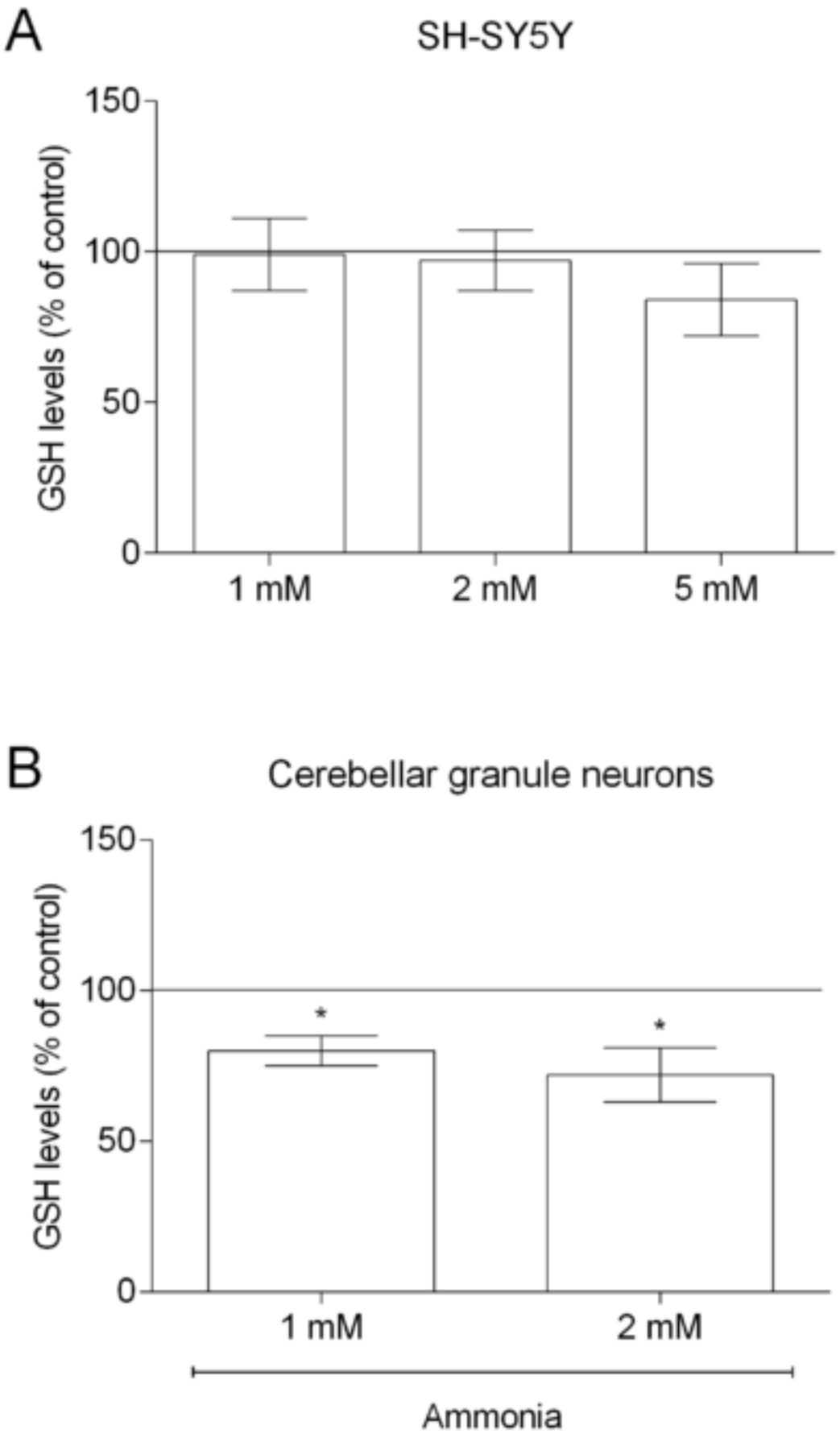

Figure 4

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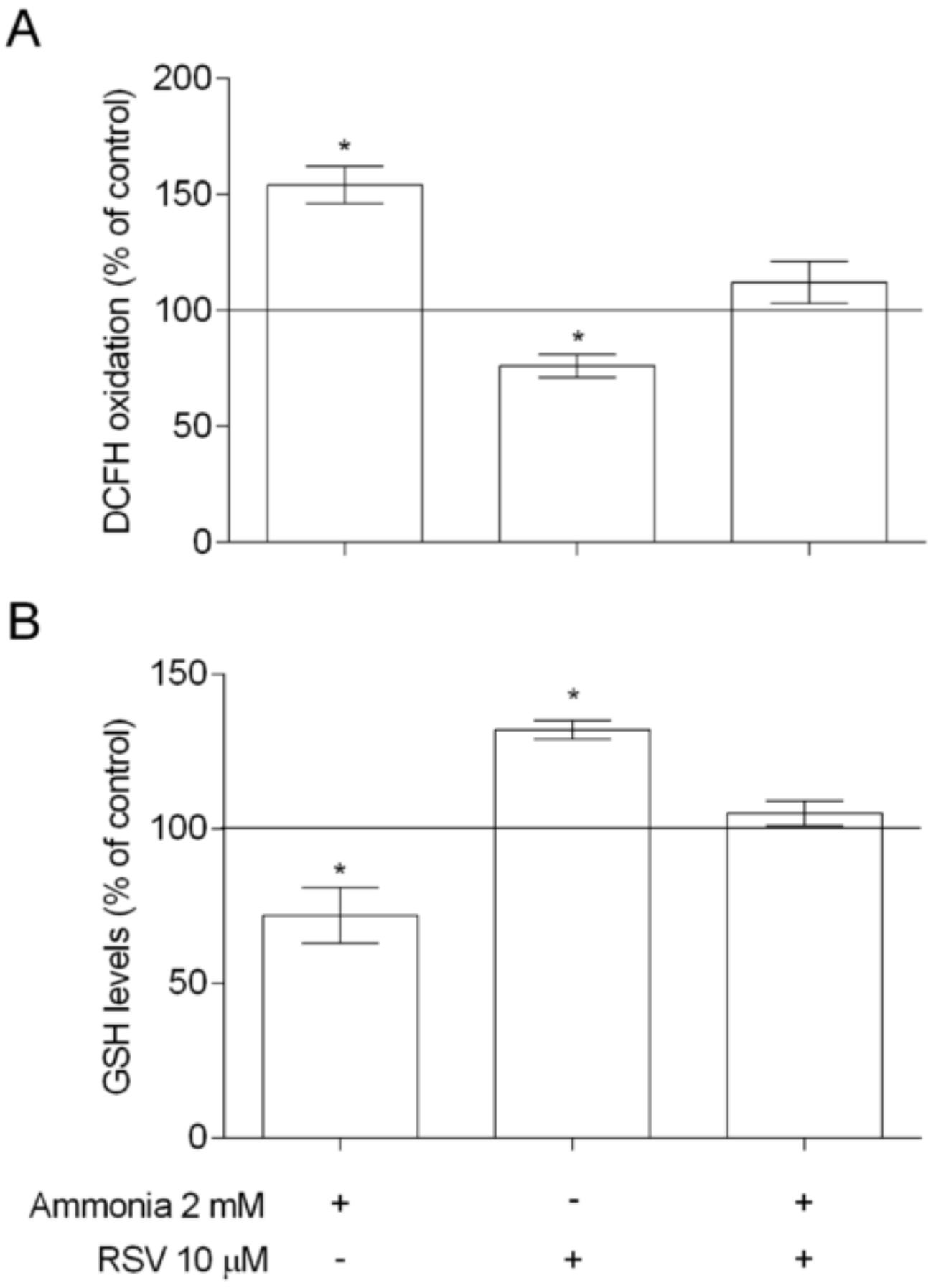

Figure 5
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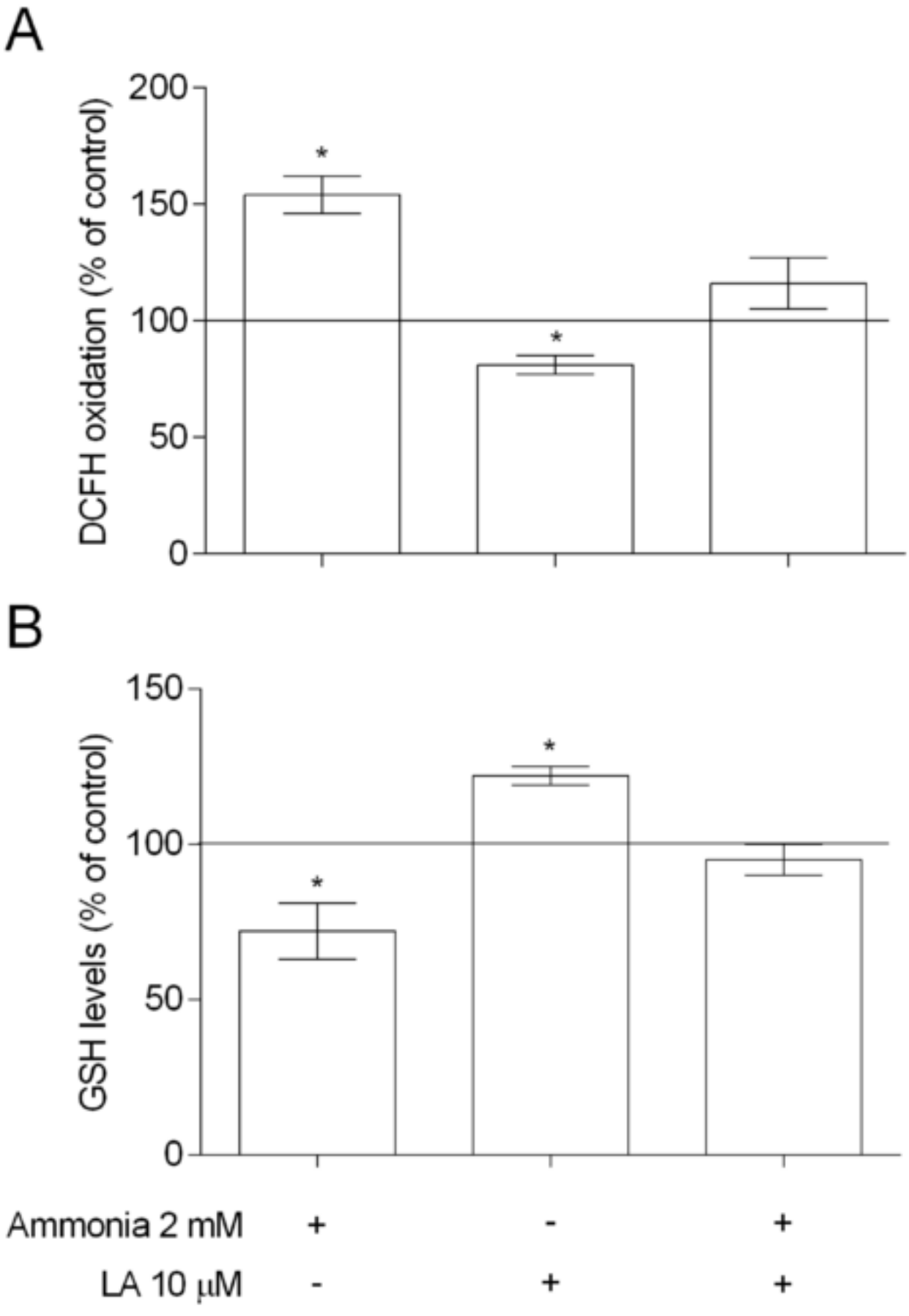

Figure 6

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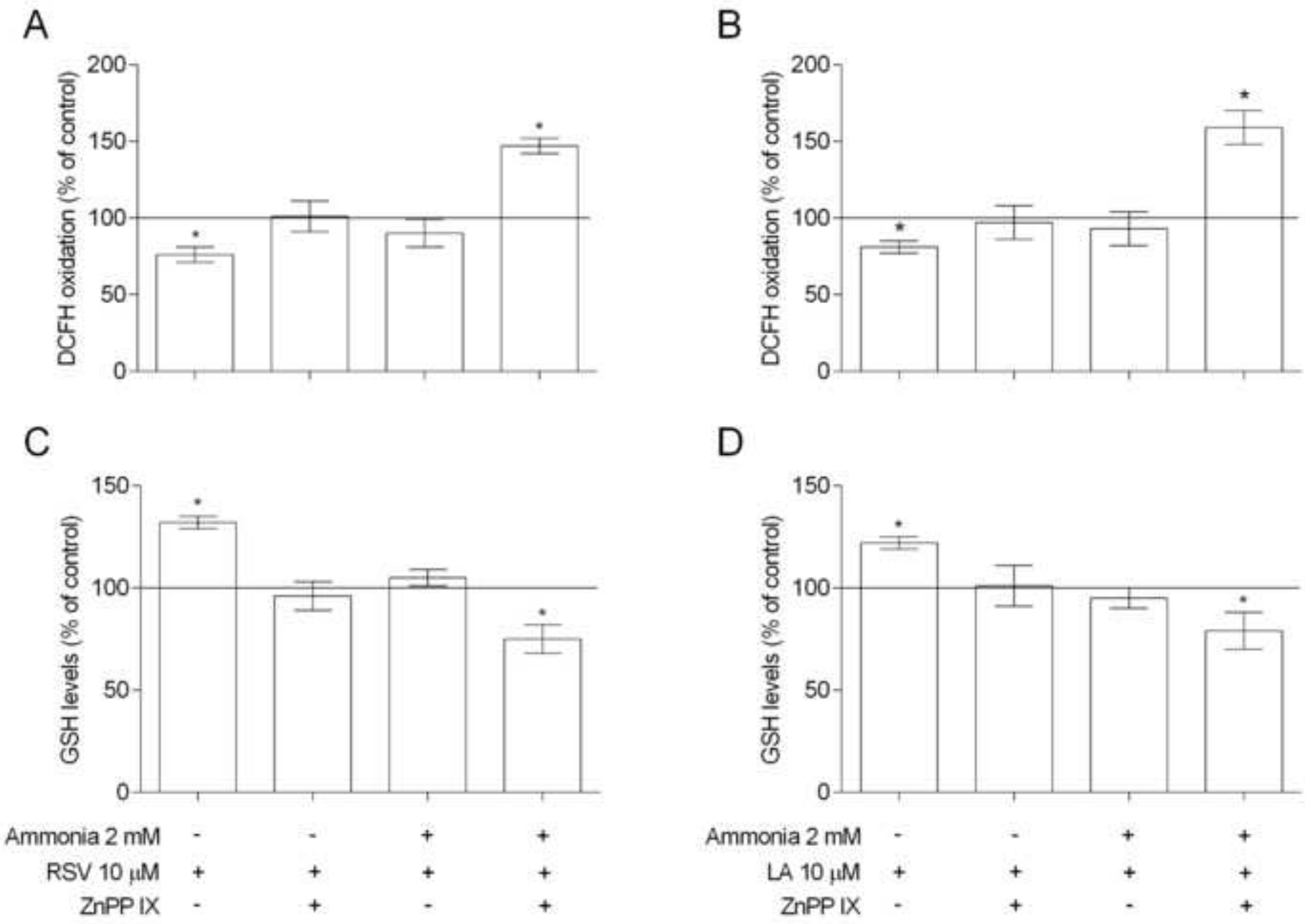

PARTE III

DISCUSSÃO

A hiperamonemia é o principal elemento no desenvolvimento da EH, uma desordem neurológica complexa, manifestada por diversos sintomas patofisiológicos e alterações bioquímicas. O acúmulo de amônia pode resultar de deficiências inatas em enzimas do ciclo da ureia, mas está normalmente associado a doenças hepáticas agudas ou crônicas (Häussinger and Schliess, 2008; Weissenborn et al., 2001).

O cérebro, na verdade, é o órgão mais suscetível à toxicidade da amônia e o primeiro a ser afetado em episódios de hiperamonemia. Em condições fisiológicas, a concentração de amônia no SNC é de aproximadamente 0,05 mM, podendo aumentar para 5 mM durante a EH (Felipo and Butterworth, 2002). No SNC, as células astrogliais são o principal alvo da toxicicidade da amônia. Tal sensibilidade é principalmente em função da enzima GS, responsável pela detoxificação da amônia no cérebro, estar localizada nos astrócitos. Somado a isso, o fato dos astrócitos fazerem parte da barreira hematoencefálica e circundarem os capilares do SNC faz com que eles sejam as células que primeiramente entram em contato com a amônia e, assim, metabolizem-na, a fim de poupar as outras células do SNC dos seus efeitos nocivos (Norenberg et al., 2009). Apesar da disfunção neuronal durante a hiperamonemia ser considerada secundária ao dano astroglial, um comprometimento direto das células nervosas pela ação da amônia não deve ser descartado.

Nesse sentido, esta tese se propôs a avaliar diversas alterações neuroquímicas induzidas pela amônia em células astrogliais e neuronais, visando contribuir para a elucidação dos seus mecanismos de ação no SNC, e também a buscar moléculas que possam ter um potencial terapêutico em condições de hipermonemia, como o resveratrol e o ácido lipoico.

Os modelos celulares de estudo

O modelo de estudo mais utilizado neste trabalho foi a linhagem celular astroglial C6. A linhagem C6 tem sua origem datada da década de 60, sendo uma das linhagens mais amplamente utilizadas em estudos neuroquímicos (Benda et al., 1968). Ela foi obtida após injeções em ratos do agente alquilante N-nitrosometilureia, e o termo C6 se refere ao sexto clone pós-injeção do agente alquilante. A expressão das proteínas GFAP (proteína glial fibrilar ácida) e S100B atesta o caráter astrocítico das células C6 (Benda et al., 1971).

Embora com algumas limitações, as células astrogliais C6 são utilizadas para a investigação de características bioquímicas e metabólicas que envolvam funções desempenhadas por astrócitos (Cechin et al., 2005; Feng and Zhang, 2004; Mangoura et al., 1989). Estas células também constituem um modelo de estudo para a liberação de fatores responsáveis pela comunicação celular e vias de transdução de sinal (Kim et al., 2006; Steiner et al., 2010; Tanabe et al., 2011).

As células C6 utilizadas neste estudo apresentam forte marcação para a proteína GFAP e vários trabalhos do nosso grupo mostram que elas exercem importantes funções astrocíticas, como secreção da proteína S100B, captação de glutamato, atividade da GS, síntese de GSH e resposta inflamatória (Bobermin et al., 2012; de Souza et al., 2009; dos Santos et al., 2006; Quincozes-Santos et al., 2014a, 2013).

No quarto capítulo, utilizamos tanto células SH-SY5Y quanto culturas primárias de neurônios granulares cerebelares como ferramenta de estudo. A linhagem de neuroblastoma humano SH-SY5Y é um subclone derivado das células SK-N-SH, as quais foram estabelecidas originalmente a partir da biópsia de um sítio metastático de medula óssea em um paciente com neuroblastoma (Biedler et al., 1973). Essa linhagem celular tem sido amplamente utilizada desde a década de 80 como um modelo de células

neuronais, devido a suas propriedades bioquímicas e funcionais (Xie et al., 2010). Assim como as células astrogliais C6, a linhagem SH-SY5Y é capaz de proliferar por longos períodos de tempo em cultura, um pré-requisito básico para uma boa ferramenta de estudo *in vitro*.

As células granulares do cerebelo são neurônios glutamatérgicos e constituem a maior população neuronal homogênea do cérebro. Além disso, o fato destas culturas primárias serem preparadas a partir de animais no período pós-natal e já estarem bem caracterizadas *in vitro* as tornam valiosas ferramentas para o estudo de vias de sinalização que controlam diversos processos neuronais como sobrevivência, migração e diferenciação. Estas culturas primárias também são muito exploradas para o estudo de respostas celulares a fatores de crescimento, mecanismos relacionados à neurodegeneração e à neuroproteção, bem como o estresse oxidativo (Bilimoria and Bonni, 2008; Canu and Calissano, 2003; Contestabile, 2002). As culturas de neurônios granulares cerebelares contêm mais de 95% de células granulares e um pequeno número de células gliais (2–3%) e endoteliais (<1%) (Kingsbury et al., 1985).

Os efeitos da amônia sobre células astrogliais C6

Nos primeiros três capítulos, nós avaliamos os efeitos da exposição à amônia (5 mM) por um período de 24 h em células astrogliais C6, sobre diversos parâmetros relacionados à funcionalidade glial. Cabe ressaltar que essas condições não provocam perda de viabilidade e integridade celular, como havia sido constatado anteriormente (Bobermin et al., 2012).

Os astrócitos estão intimamente relacionados com a comunicação glutamatérgica, pois são as principais células responsáveis pela remoção do glutamato da fenda sináptica e sua metabolização (Parpura and Verkhratsky, 2012; Zhou and Danbolt, 2014). Nós

observamos que a amônia desencadeou diversas alterações celulares relacionadas à comunicação glutamatérgica: (i) diminuição da captação de glutamato e do conteúdo do transportador EAAC1; (ii) aumento da liberação de glutamato para o meio extracelular; (iii) redução da atividade da enzima GS e do conteúdo intracelular de GSH; (iv) prejuízo no funcionamento da bomba Na^+K^+-ATPase.

Em concordância com a literatura (Bender and Norenberg, 1996; Jayakumar et al., 2006), nós verificamos que a amônia induziu um prejuízo na captação de glutamato. Sabe-se que os transportadores glutamatérgicos são bastante vulneráveis à oxidação, a qual provoca uma redução na sua funcionalidade (Anderson and Swanson, 2000; Trotti et al., 1998). Um dos efeitos bem relatados da amônia é a indução de estresse oxidativo, por aumento na geração de ERO/ERN e diminuição das defesas antioxidantes celulares (Bobermin et al., 2012; Skowrońska and Albrecht, 2013). Nesse sentido, nós acreditamos que o efeito da amônia sobre a captação de glutamato esteja relacionado, pelo menos em parte, ao estresse oxidativo/nitrosativo.

Nós também observamos que a diminuição da atividade dos transportadores de glutamato foi acompanhada por uma diminuição do conteúdo de EAAC1, o principal transportador das células C6 (Davis et al., 1998). A redução da expressão proteica desse transportador também pode estar diretamente relacionada ao estresse oxidativo. Uma das consequências da produção aumentada de ERO é a oxidação de ácidos nucleicos, tanto o DNA quanto o RNA (Nunomura et al., 2006), sendo que o RNA é mais suscetível à oxidação, pois ele apresenta uma estrutura de fita simples e não é protegido por proteínas empacotadoras. Assim, a oxidação do RNA pode diminuir a eficiência e a acurácia da sua tradução, resultando em um prejuízo tanto da síntese quanto da função das proteínas (Nunomura et al., 2006; Shan et al., 2007). De fato, Görg et al demonstraram que a amônia induz a oxidação de RNA tanto em culturas de astrócitos quanto no cérebro *in*

vivo (Görg et al., 2008). No entanto, esse aspecto do nosso trabalho permanece em aberto para futuras investigações.

A atividade dos transportadores glutamatérgicos também pode ser modulada por algumas vias de sinalização celular como, por exemplo, a via das proteínas cinases ativadas por mitógenos (MAPK) (Lee et al., 2012; Zhou et al., 2011). Além disso, a amônia leva a um aumento da fosforilação da ERK (cinase regulada por sinal extracelular), um membro da família das MAPK, em astrócitos (Jayakumar et al., 2006). Nós mostramos que a redução da captação de glutamato induzida pela amônia foi dependente da ativação da ERK, uma vez que inibindo a atividade dessa proteína com PD98059, a captação de glutamato não foi alterada pela amônia. No entanto, um trabalho anterior demonstrou que a inibição da ERK não foi capaz de reverter a redução da captação de glutamato induzida pela amônia em cultura primária de astrócitos (Jayakumar et al., 2006). Essa controvérsia pode ser explicada pelo uso de diferentes inibidores farmacológicos e modelos de estudo, já que este outro trabalho utilizou culturas primárias de astrócitos.

A manutenção do gradiente iônico transmembrana é um aspecto essencial para o processo de captação de glutamato nos astrócitos (Brady et al., 2012). Os co-transportadores de Na^+, K^+ and Cl^- (NKCC) são uma classe de proteínas de membrana que realizam o transporte desses íons através da membrana celular com uma estequeometria de $1Na^+:1K^+:2Cl^-$. A isoforma presente nos astrócitos, NKCC1, desempenha um papel importante na manutenção e regulação do gradiente iônico e do volume celular (Haas and Forbush, 1998). A amônia pode ativar esse tipo de transportador e também entrar nas células através dele, mimetizando o K^+, o que resulta em um aumento da concentração de Na^+ e uma redução no pH intracelular (Jayakumar et al., 2008; Kelly and Rose, 2010). O consequente prejuízo no gradiente transmembrana de

Na^+ e H^+ afeta a captação de glutamato, pois o gradiente desses íons é a força que impulsiona o transporte do glutamato contra o seu gradiente de concentração nos astrócitos (Kelly and Rose, 2010). Corroborando com dados anteriores da literatura (Kelly et al., 2009), nós observamos que a pré-incubação das células com um inibidor de NKCC1, a furosemida, preveniu a redução da captação de glutamato induzida por amônia, indicando que o NKCC1 está diretamente envolvido nesse efeito.

A ativação do NKCC1 pela amônia também contribui para o inchaço celular característico dessa situação (Jayakumar and Norenberg, 2010). Uma importante consequência desse inchaço pode ser a liberação de glutamato para o meio extracelular, decorrente da abertura de canais de ânions regulados por volume (VRAC) (Kimelberg et al., 1990). Nós observamos que a amônia induziu um grande aumento (aproximadamente 70%) na concentração de glutamato no meio extracelular. Esse aumento, porém, foi parcialmente prevenido pelo inibidor de NKCC1, apontando sua participação no efeito da amônia. Provavelmente, o glutamato esteja sendo liberado também de outras maneiras, que não envolvam necessariamente uma alteração do volume celular. Görg et al já haviam mostrado previamente que a amônia é capaz de induzir a liberação de glutamato em culturas primárias de astrócitos, através de um mecanismo de exocitose (Görg et al., 2010).

Como mencionado acima, a captação de glutamato é um processo associado ao cotransporte de Na^+ e, por isso, está diretamente relacionado ao correto funcionamento da bomba de Na^+K^+-ATPase, enzima responsável pela manutenção dos gradientes de Na^+ e K^+ nas células (de Lores Arnaiz and Ordieres, 2014; Lingrel, 1992). Existem evidências de que a Na^+K^+-ATPase também pode ser alvo da amônia, e de maneira similar ao NKCC1, estar envolvida com seu transporte para o interior da célula (Kelly and Rose, 2010). Em contraste com dados já existentes na literatura (Dai et al., 2013; Kala et al.,

2000), nós verificamos que a amônia reduziu a atividade da Na^+K^+-ATPase, sem alterar, no entanto, sua expressão. O prejuízo no funcionamento dessa enzima pode ser resultado do aumento do estresse oxidativo, uma vez que a Na^+K^+-ATPase é bastante suscetível ao ataque por radicais livres (Chakraborty et al., 2003; Quincozes-Santos et al., 2014b). Outra possibilidade é a deficiência energética celular provocada pela amônia, pois esta enzima possui uma grande demanda de ATP para sua atividade.

Aqui, cabe ressaltar que a redução da captação de glutamato juntamente com o aumento da liberação de glutamato induzidos pela amônia nas células astrogliais podem formar um ciclo de amplificação do dano no SNC. Concentrações excessivas de glutamato no meio extracelular resultam em uma superestimulação de seus receptores e uma entrada massiva de Ca^{2+} na célula. A concentração de Ca^{2+} intracelular elevada pode desencadear uma série de eventos, como o aumento da produção de ERO, que podem culminar com a morte celular. Esse processo caracterizado por uma excessiva estimulação excitatória é denominado de excitotoxicidade glutamatérgica, e está relacionado a uma série de patologias do SNC (Esposito et al., 2013; Plitman et al., 2014).

A seguir, estudamos um dos principais destinos do glutamato após ser captado pelas células astrogliais: sua conversão à glutamina pela ação da enzima GS. Essa enzima astrocítica possui uma importância particular em situações de hipermonemia, pois é responsável também pela detoxificação da amônia no SNC (Braissant et al., 2013). Estudos apontam um papel da glutamina na mediação dos danos da amônia em células astrogliais, sugerindo que seu acúmulo poderia produzir um efeito osmótico e induzir a produção de ERO. No entanto, outros estudos em pacientes com EH não conseguiram encontrar uma correlação entre a síntese aumentada de glutamina e o grau de severidade da doença ou do edema cerebral (Butterworth, 2014).

Nesse sentido, confirmando dados anteriores do nosso grupo, tanto em células astrogliais C6 quanto em culturas primárias de astrócitos (Bobermin et al., 2012; Leite et al., 2006), nós observamos uma diminuição da atividade da GS nas células expostas à amônia. A GS é uma enzima sensível à nitração e à oxidação (Matés et al., 2002), e uma das explicações para sua atividade limitada na presença da amônia é o aumento da produção de NO, um efeito bem estabelecido da amônia, e que também está envolvido na sua toxicidade (Bobermin et al., 2012). Ademais, a atividade da GS também é dependente de ATP e pode sofrer interferências pela deficiência no metabolismo energético celular na presença de amônia (Braissant et al., 2013).

Os astrócitos desempenham um importante papel para a defesa contra o estresse oxidativo no SNC, pois produzem e secretam o tripeptídeo GSH (γ-glutamil-cisteinil-glicina) para o meio extracelular, onde é clivado e utilizado como substrato para a síntese de GSH nos neurônios (Bélanger et al., 2011). Nós verificamos que a amônia provocou uma diminuição dos níveis intracelulares de GSH nas células astrogliais, provavelmente em razão de um aumento do seu consumo decorrente de uma maior produção de ROS/RNS. Também, não podemos descartar a possibilidade da diminuição dos níveis intracelulares de GSH ser consequência de um aumento de sua secreção pelas células.

Um dos possíveis destinos para o glutamato captado pelas células astrogliais é a síntese de GSH e, apesar de não ser a etapa limitante, a concentração intracelular de glutamato pode afetar a síntese dessa molécula antioxidante. Isso porque após o processo de captação, a concentração de glutamato dentro das células aumenta. Como consequência, através do seu transporte de volta para o meio extracelular, o glutamato direciona a captação de cistina através do trocador Xc^- pelas células astrogliais. A cistina consiste na forma oxidada do aminoácido cisteína, esse sim o precursor limitante da síntese de GSH, como ilustrado na figura 5 (Lewerenz et al., 2006; Robert et al., 2014).

Assim, o funcionamento adequado dos transportadores de glutamato também é importante para a manutenção das defesas antioxidantes no SNC.

A depleção da GSH no SNC também está associada à neurotoxicidade e à neuroinflamação . A diminuição do conteúdo de GSH em astrócitos pode favorecer uma resposta inflamatória astroglial, pois um ambiente pró-oxidante ativa diversas vias de sinalização que culminam com a síntese e liberação de citocinas pró-inflamatórias (Currais and Maher, 2013; M. Lee et al., 2010). Nesse sentido, nós demonstramos que a amônia estimulou a secreção de citocinas como TNFα, IL-1β, IL-6 e IL-18. O TNFα desempenha várias funções importantes no SNC, como a ativação de astrócitos e a gliotransmissão glutamatérgica (Santello et al., 2011). Além disso, o TNFα, juntamente com a IL-1β, iniciam uma cascata de ativação de outras citocinas, como IL-6 e IL-18 (Tanabe et al., 2010). Essas duas últimas citocinas também estão relacionadas ao processo inflamatório patológico no cérebro, sendo que a IL-18 é um membro da superfamília das IL-1, e está associada diretamente a doenças neurodegenerativas (Bossù et al., 2010). Estudos recentes têm demonstrado um aumento de citocinas pró-inflamatórias em pacientes com EH e, possivelmente, sua utilização como um marcador preditivo para o grau da doença (Butterworth, 2013, 2011a; Odeh et al., 2005).

O NFκB é um fator de transcrição considerado o principal mediador da produção de citocinas pró-inflamatórias (Baltimore, 2011) e, como esperado, a amônia induziu um aumento da sua ativação. Uma das vias a montante responsáveis pela ativação do NFκB é a ERK (Hua et al., 2002; E. O. Lee et al., 2010). Nós observamos que a inibição farmacológica da ERK bloqueou a ativação do NFκB e a indução da liberação das citocinas pró-inflamatórias avaliadas. Esses resultados, em conjunto, indicam uma provável via pelo qual a amônia possa estimular a resposta inflamatória: o estresse oxidativo induzido pela amônia pode ser responsável pela ativação da ERK que, por sua

vez, promove a translocação do NFκB para o núcleo, onde ele irá induzir a transcrição das citocinas. Esse fator de transcrição também atua regulando a síntese de outras proteínas, como a iNOS − responsável pela síntese de NO, amplificando a resposta inflamatória (Aktan, 2004; Wakabayashi et al., 2010).

Ainda, nós avaliamos a secreção da proteína S100B, tendo em vista os indícios da sua participação na resposta inflamatória. Como observado anteriormente em astrócitos e também em células astrogliais C6, a amônia aumentou a secreção dessa proteína (Bobermin et al., 2012; Leite et al., 2006). De maneira interessante, a secreção de S100B segue o mesmo perfil das citocinas pró-inflamatórias. Essa proteína, produzida e secretada predominantemente por astrócitos no SNC, pode ser utilizada em determinadas situações com um marcador do processo de ativação astroglial e seu aumento está relacionado a doenças neurológicas como Alzheimer e esquizofrenia (Petzold et al., 2003; Rothermundt et al., 2004; Steiner et al., 2011). Previamente, foi reportado um aumento dos níveis da S100B também no soro de pacientes com EH (Wiltfang et al., 1999). No entanto, nesse caso, outras fontes extracerebrais da proteína não podem ser descartadas, como o tecido adiposo (Gonçalves et al., 2008).

Os efeitos protetores do resveratrol e do ácido lipoico

A EH é uma doença com alta taxa de mortalidade, cujo tratamento visa principalmente a causa periférica responsável pela sua precipitação (Leise et al., 2014; Waghray et al., 2014). Tendo em vista todas as alterações funcionais provocadas pela amônia em células astrogliais, torna-se relevante, também, a busca por moléculas que potencialmente atuem no SNC, favorecendo a recuperação da sua homeostase. Nas últimas décadas foram estudadas muitas propriedades biológicas de produtos naturais, e

muitos desses compostos apresentam um potencial terapêutico promissor, como o resveratrol e ácido lipoico.

Entre os inúmeros efeitos biológicos do resveratrol destacam-se as atividades antioxidante, anti-inflamatória, cardioprotetora, antitumoral e neuroprotetora (Bastianetto et al., 2014; Baur and Sinclair, 2006; Vang et al., 2011). No SNC, o resveratrol tem se mostrado eficaz em modelos de doenças neurodegenerativas, como Parkinson, Alzheimer e isquemia (Frozza et al., 2013; Khan et al., 2010; Sharma and Gupta, 2002; Simão et al., 2012b). Embora já existam diversos estudos mostrando que ele atua de forma direta e/ou indireta, modulando vias de sinalização celular, o seu mecanismo de ação em nível molecular necessita ser melhor explorado.

Em razão do seu papel fundamental na homeostasia do SNC, a modulação das funções gliais pode representar uma estratégia importante para o tratamento de patologias cerebrais. Nesse contexto, nosso grupo tem mostrado que o resveratrol é capaz de modular importantes funções gliais, como a captação de glutamato, a atividade da GS, os níveis de GSH e componentes da resposta inflamatória astroglial (Bellaver et al., 2014; dos Santos et al., 2006; Quincozes-Santos and Gottfried, 2011).

Nesse sentido, nós já havíamos observado que o resveratrol é capaz de prevenir diversos aspectos relacionados à toxicidade da amônia, como a produção de ERO e NO, a atividade da GS e a secreção de S100B e citocinas pró-inflamatórias (TNFα, IL-1β e IL-6). Além disso, observamos que o efeito protetor do resveratrol foi provavelmente dependente da redução dos níveis de NO e de um efeito inibitório sobre a ERK e a ativação do NFκB. Nesse mesmo estudo, nós também avaliamos o efeito de outros antioxidantes, como ácido ascórbico, trolox e L-NAME, frente ao dano induzido pela amônia. No entanto, eles não foram eficazes (Bobermin et al., 2012).

Nesta tese, investigamos o efeito protetor do resveratrol em aspectos relacionados à comunicação glutamatérgica, durante a exposição de células astrogliais C6 à amônia. Primeiramente, observamos que o resveratrol foi capaz de restaurar a expressão proteica de EAAC1 e a atividade dos transportadores de glutamato, na presença da amônia. Esses efeitos provavelmente sejam devidos a sua ação antioxidante. Em relação ao EAAC1, a oxidação do RNA pode ser uma das causas da redução do seu conteúdo proteico, como comentado anteriormente. Em trabalhos prévios, demonstramos que o resveratrol é capaz de prevenir a genotoxicidade frente a um insulto com H_2O_2 (Quincozes-Santos et al., 2007). Assim, o resveratrol pode prevenir a diminuição EAAC1 na presença da amônia evitando a oxidação do seu RNA. Cabe salientar que o resveratrol, *per se*, aumentou tanto a captação de glutamato quanto os níveis de EAAC1 nas células astrogliais C6.

O resveratrol também foi capaz de evitar o aumento da liberação de glutamato para o meio extracelular, indicando que o estresse oxidativo pode estar envolvido nesse efeito tóxico da amônia. De fato, a atividade do NKCC1 pode ser aumentada por oxidação, e alguns antioxidantes são capazes de prevenir sua ativação (Jayakumar et al., 2008). No entanto, o resveratrol, *per se*, não teve efeito sobre a liberação de glutamato. O resveratrol, portanto, é capaz de modular tanto a captação quanto a liberação de glutamato pelas células astrogliais, as quais representam um ponto importante na prevenção da excitotoxicidade mediada pela amônia.

Nós também observamos que o resveratrol preveniu a diminuição da atividade da enzima GS induzida pela amônia. É importante lembrar que essa enzima é sensível ao estresse oxidativo/nitrosativo (Matés et al., 2002) e, dessa forma, a atividade antioxidante do resveratrol pode ter um impacto positivo. Um declínio da atividade da GS tem sido observado em várias doenças neurológicas. Devido ao papel da GS na conversão do glutamato à glutamina, mudanças na sua atividade podem afetar a concentração de

glutamato intracelular nas células astrogliais afetando, consequentemente, tanto a captação quanto a liberação de glutamato (Butterworth, 2014; Shaked et al., 2002). Nesse contexto, a GS pode mediar um efeito protetor do resveratrol no SNC, pelo seu papel importante tanto no metabolismo do glutamato quanto na detoxificação da amônia.

O resveratrol também aumentou os níveis intracelulares de GSH e preveniu a redução provocada pela amônia. A manutenção da glutationa no seu estado reduzido (ou seja, GSH) é controlada por diversos fatores como a sua biossíntese, sua reciclagem através da enzima glutationa redutase e sua utilização como antioxidante diretamente ou como substrato da glutationa peroxidase (Choi and Gruetter, 2012). Assim, os efeitos do resveratrol podem ser decorrentes de uma diminuição da oxidação da GSH, de um aumento da reciclagem através da glutationa redutase ou pela modulação da síntese de GSH nas células (Chen et al., 2013), contribuindo para a atenuação dos impactos negativos da toxicidade da amônia, a qual apresenta um forte componente oxidativo.

Outro efeito importante do resveratrol foi a modulação da atividade da Na^+K^+-ATPase. O resveratrol foi capaz de proteger o déficit causado pela amônia e também de aumentar, *per se*, a atividade dessa enzima. Trabalhos já existentes demonstram que outros antioxidantes são capazes de modular a Na^+K^+-ATPase (Ferreira et al., 2011; Stefanello et al., 2011). Além disso, outro estudo mostrou que o resveratrol preveniu a queda da atividade dessa enzima em um modelo *in vivo* de isquemia global, devido ao seu efeito antioxidante (Simão et al., 2011). Dessa forma, a modulação positiva do resveratrol na atividade da Na^+K^+-ATPase, em conjunto com os outros parâmetros analisados, representa um aspecto importante para seu efeito benéfico no SNC.

Os efeitos biológicos do resveratrol também podem ser dependentes da sua relação com alvos celulares, os quais atuariam como controladores a jusante do resveratrol e mediadores dos eventos celulares gerados por ele. Um dos alvos candidatos

é a proteína heme oxigenase 1 (HO1), uma enzima que faz parte do mecanismo de defesa celular em situações de dano oxidativo (Bastianetto et al., 2014; Syapin, 2008). Em relação às HO, elas podem apresentar duas isoformas principais: a induzível (HO1) e a constitutiva (HO2). Essas enzimas são etapas limitantes da via de degradação do grupamento pró-oxidante heme em monóxido de carbono (CO) e biliverdina /bilirrubina – as quais são moléculas biologicamente ativas e que podem atuar como antioxidantes (Haines et al., 2012; Syapin, 2008). O grupamento heme livre pode ser originado de fontes extracelulares, como pela degradação da hemoglobina, e de fontes intracelulares, a partir do metabolismo de proteínas que contenham o heme em sua estrutura. Durante situações de excitotoxicidade, por exemplo, ocorre um aumento da produção de heme em sua forma livre, o qual não pode ser reciclado e é, então, degradado pela HO (Doré et al., 1999; Sakata et al., 2010).

Nós verificamos que os efeitos protetores do resveratrol podem ser mediados por essa proteína e/ou seus metabólitos, uma vez que quando as células foram pré-incubadas com ZnPP IX, um inibidor potente e seletivo da HO, os efeitos benéficos do resveratrol sobre parâmetros avaliados (captação de glutamato, atividade da GS e níveis de GSH) foram abolidos, tanto na presença quanto na ausência de amônia. Similarmente, outros polifenóis (como a catequina e a curcumina), também exercem seus efeitos biológicos através da HO1 (Bastianetto and Quirion, 2010).

Nesse sentido, Sakata et al sugeriram que a HO1 seria uma importante candidata pela qual o resveratrol poderia exercer uma sinalização celular endógena, que levaria a uma resistência ao estresse oxidativo e, consequentemente, à neuroproteção (Sakata et al., 2010). Além disso, a produção de HO1 é regulada pelo fator de transcrição Nrf2 (do inglês *nuclear-related fator 2*), o qual leva à síntese de enzimas do sistema de defesa antioxidante celular. A HO1 também atua como sequestradora de NO inibe a síntese da

iNOS, contribuindo para o controle do estresse oxidativo/nitrosativo (Wakabayashi et al., 2010). Nosso grupo também demonstrou que o resveratrol é capaz de aumentar a expressão da HO1 em células astrogliais C6 sob condição pró-oxidante, além de diminuir a expressão da iNOS, mostrando que a HO1 está intimamente relacionada com os efeitos protetores do resveratrol (Quincozes-Santos et al., 2013).

Em conjunto, esses dados mostram que o resveratrol pode modular diversas etapas envolvidas no metabolismo glutamatérgico nas células astrogliais, contribuindo para a prevenção de um processo de excitotoxicidade e para a detoxificação da amônia. Além disso, contribuindo para o entendimento dos mecanismos de ação do resveratrol, observamos uma importante participação da HO1 nos seus efeitos.

Outra molécula que tem chamado atenção devido seus papéis antioxidante e anti-inflamatório é o ácido lipoico. Seu potencial terapêutico tem sido evidenciado em diversas situações, como doenças neurodegenerativas, neuropatia, isquemia, síndrome metabólica e diabetes (Gorąca et al., 2011; Maczurek et al., 2008; Ziegler, 2009). Em um trabalho prévio, nosso grupo também demonstrou que o ácido lipoico é capaz de modular a funcionalidade astroglial *in vitro* (Kleinkauf-Rocha et al., 2013). Baseado nisso, nós também avaliamos um potencial efeito protetor do ácido lipoico em células astrogliais expostas à amônia.

Em relação à captação de glutamato, o ácido lipoico preveniu a diminuição induzida pela amônia e, *per se*, aumentou a atividade. Da mesma forma que o resveratrol, uma das maneiras pelas quais o ácido lipoico pode modular a captação de glutamato é através da prevenção da oxidação dos transportadores glutamatérgicos, bem como do NKCC1. Nós também investigamos um possível papel da proteína cinase C (PKC) no efeito do ácido lipoico sobre a captação de glutamato, pois a ativação dessa proteína pode regular a expressão de EAAC1 (Davis et al., 1998). Quando as células foram incubadas

com o inibidor da PKC (Bis II), os efeitos positivos do ácido lipoico sobre a captação de glutamato foram perdidos, tanto na presença quanto na ausência de amônia, indicando que essa via de sinalização está envolvida na ação do ácido lipoico.

A diminuição da atividade da GS induzida pela amônia também foi prevenida pelo ácido lipoico, o qual também apresentou um efeito *per se* sobre a GS, provavelmente secundário ao seu efeito antioxidante. Extrapolando nossos dados para o cérebro, o aumento da síntese de glutamina nos astrócitos poderia estar relacionado com um aumento de GSH neuronal, em razão do ciclo glutamato-glutamina, que transporta o glutamato de volta para os neurônios na forma de glutamina. Nos neurônios, a glutamina pode ser convertida em glutamato, o qual é precursor para a síntese de GSH (Matés et al., 2002).

O ácido lipoico também recuperou completamente o conteúdo intracelular de GSH nas células tratadas com amônia e, *per se*, induziu um aumento nos níveis de GSH. O ácido lipoico possui um efeito bem descrito na recuperação de antioxidantes, dentre os quais a da glutationa, regenerando-a da sua forma oxidada (GSSG) à reduzida (GSH). Além disso, o ácido lipoico pode estimular a síntese de GSH através do aumento dos níveis intracelulares de cisteína, pela estimulação da captação de cistina pelo trocador Xc^- (Packer and Cadenas, 2011). O ácido lipoico também pode induzir a atividade do Nrf2, o qual facilita a síntese de GSH e também induz a expressão da HO1 (Koriyama et al., 2013; Suh et al., 2004). Assim, a modulação do conteúdo de GSH e de outros sistemas antioxidantes celulares reforça a atividade antioxidante intrínseca do ácido lipoico, e pode contribuir para seus efeitos neuroprotetores.

Como mencionado anteriormente, a depleção de GSH em células gliais induz um aumento significativo na produção de citocinas inflamatórias, ambos característicos da toxicidade da amônia (Leise et al., 2014; Waghray et al., 2014). Nesse contexto, o ácido

lipoico, além de modular os níveis de GSH, foi capaz de regular negativamente a liberação de citocinas estimulada pela amônia. Neste trabalho, nós também testamos a hipótese de que a regulação das citocinas pelo ácido lipoico pudesse ser mediada pelo NFκB e proteínas como a ERK e HO1. Nós observamos que o ácido lipoico diminuiu a ativação do NFκB e potencializou o efeito do inibidor da ERK, indicando que essa via esteja envolvida no efeito anti-inflamatório do ácido lipoico. Além disso, a HO1 pode inibir a ativação do NFκB e nós verificamos que na presença do inibidor da HO1, o ácido lipoico perdeu seu efeito positivo sobre a ativação do NFκB nas células gliais. Ainda dentro de um contexto inflamatório, nós verificamos o efeito do ácido lipoico sobre a secreção da proteína S100B. Como discutido anteriormente, a S100B teve um perfil de aumento semelhante às outras citocinas pró-inflamatórias e o ácido lipoico também foi capaz de prevenir esse efeito. Assim, a soma das atividades antioxidante e anti-inflamatória do ácido lipoico nas células astrogliais poderia torna-lo uma um potencial agente farmacológico durante a hiperamonemia.

Em relação à diminuição do conteúdo de GSH e ao aumento da liberação de citocinas pró-inflamatórias induzidos pela amônia, nós também testamos o papel protetor do NAC, um antioxidante clássico já usado clinicamente em diversas situações patológicas, inclusive na EH (Butterworth, 2011b; Vaquero and Butterworth, 2007). Um estudo *in vivo* mostrou um efeito anti-inflamatório e neuroprotetor do NAC em um modelo de dano hepático (Bémeur et al., 2010). Nessa situação, o NAC poderia agir como uma molécula antioxidante e anti-inflamatória tanto em nível periférico quanto central, resultando numa redução da progressão da EH.

É importante destacar que o principal efeito biológico do NAC consiste em aumentar a síntese intracelular de GSH, uma vez que é um precursor acetilado da cisteína. No entanto, os efeitos protetores do NAC independentes da síntese de GSH

ainda são pouco conhecidos. Nós observamos que o NAC, além de aumentar a síntese de GSH, também participou da modulação da ativação do NFκB de maneira dependente de ERK e HO, contribuindo para a elucidação dos seus mecanismos de ação moleculares.

A alteração do estado redox induzida pela amônia em células neuronais e o efeito neuroprotetor dos antioxidantes

Apesar da toxicidade da amônia no SNC estar bastante relacionada a um dano astroglial, e o dano neuronal ser considerado secundário a esse tamponamento inadequado do microambiente cerebral, algumas evidências mostram que a amônia pode exercer efeitos tóxicos diretos sobre as células neuronais (Chen et al., 2014; Klejman et al., 2005). Assim, no quarto capítulo desta tese, nós investigamos um possível papel protetor do resveratrol e do ácido lipoico em neurônios.

Primeiramente, nós investigamos o efeito da amônia, em diferentes concentrações (1−10 mM) por um período de 24 h, na linhagem SH-SY5Y. Apenas a concentração mais alta de amônia (10 mM) causou uma redução da viabilidade celular. No entanto, a produção de ERO e conteúdo de GSH não foram afetados durante a exposição destas células à amônia. Esses resultados podem ser devidos a uma menor suscetibilidade dessas células a estímulos tóxicos, devido às suas propriedades bioquímicas.

Então, nós avaliamos os efeitos da amônia em culturas primárias de neurônios granulares cerebelares. Diferente do observado em células SH-SY5Y, a redução da viabilidade celular ocorreu já na concentração de 5 mM, a mesma utilizada para avaliação dos parâmetros astrogliais nos capítulos anteriores. Além disso, a maior concentração de amônia (10 mM) provocou alterações na morfologia celular, observadas através de microscopia de contraste de fase. Em relação ao controle, as células expostas à amônia

apresentaram um reduzido número de neuritos, além de corpos e processos celulares desintegrados.

Para os parâmetros que foram analisados posteriormente foram utilizadas apenas as concentrações menores de amônia (1 e 2 mM), as quais não alteraram a viabilidade, mas mesmo assim poderiam causar disfunções que, em última instância, levariam à morte celular. Nessa situação, nós observamos um aumento da produção de ERO e uma redução do conteúdo intracelular de GSH. Ou seja, a amônia, independentemente do dano astroglial, pode induzir estresse oxidativo nas células neuronais. Um trabalho anterior, também em cultura primária de neurônios, havia demonstrado um efeito neurotóxico da amônia induzindo morte celular, a qual foi protegida por uma suplementação exógena de GSH nas células, mostrando a participação da depleção da GSH nesse efeito (Klejman et al., 2005).

Buscando prevenir o estresse oxidativo neuronal induzido pela amônia, nós investigamos os efeitos do resveratrol e do ácido lipoico. Para isso, primeiramente, foi avaliado o efeito de diferentes concentrações desses antioxidantes *per se* sobre a viabilidade celular (resultados não mostrados na tese). Ao contrário do que acontece nas células astrogliais, o resveratrol na concentração de 100 μM provoca uma redução na viabilidade dos neurônios. Então, a partir dos resultados obtidos, nós elegemos a concentração de 10 μM de resveratrol para avaliarmos seus efeitos neuroprotetores. Porém, em relação ao ácido lipoico, nós utilizamos a mesma concentração testada nas células astrogliais (10 μM), uma vez que ela não induziu alterações na viabilidade dos neurônios.

Ainda em relação às concentrações dos antioxidantes que causam efeitos neuroprotetores, elas podem variar entre os trabalhos dependendo do tipo celular utilizado. Além disso, tanto o resveratrol quanto o ácido lipoico podem provocar efeitos

opostos em alguns parâmetros biológicos. Previamente, nós observamos que o resveratrol (acima de 100 μM) e o ácido lipoico (a partir de 500 μM) podem exercer efeitos pró-oxidantes em células astrogliais C6, além de reduzir a viabilidade celular, os quais foram dependentes de suas concentrações e do estado redox celular (dos Santos et al, 2006; Kleinkauf-Rocha et al, 2013; Quincozes-Santos et al, 2009). Estes resultados estão de acordo com outros estudos, que descrevem propriedades pró-oxidantes de moléculas conhecidas por sua ação antioxidante, dependendo da concentração e do tipo celular usado (de la Lastra & Villegas, 2007; Gutteridge & Halliwell, 2010; Halliwell, 1996). É importante salientar que nós não observamos efeitos tóxicos ou pró-oxidantes do resveratrol e do ácido lipoico nas concentrações e condições experimentais utilizadas nesta tese, tanto nas células astrogliais C6 quanto nas células neuronais.

O resveratrol e o ácido lipoico foram capazes de prevenir tanto a produção de ERO quanto a diminuição dos níveis de GSH, exercendo um importante efeito antioxidante nos neurônios. Além disso, em condições basais (ou seja, na ausência da amônia), esses dois antioxidantes também apresentaram um efeito benéfico, diminuindo a produção de ERO e aumentando o conteúdo de GSH nas células, nas concentrações em que foram testados.

Assim como nas células astrogliais, nós observamos o envolvimento da proteína HO1 nos efeitos protetores do resveratrol e do ácido lipoico, uma vez que a inibição dessa via impediu que eles exercessem seu papel protetor frente ao dano por amônia e, também, em condições basais. Esses dados são consistentes com o trabalho de Sakata et al, que mostraram que o resveratrol aumentou os níveis de HO1 em neurônios expostos a concentrações tóxicas de glutamato (Sakata et al., 2010). Dessa forma, os efeitos desses antioxidantes também em células neuronais frente a uma situação de dano reforçam os seus papéis neuroprotetores.

Considerações finais

A análise dos resultados apresentados nessa tese mostra que o resveratrol e o ácido lipoico modulam importantes funções gliais, as quais estão prejudicadas numa situação de hiperamonemia. Além disso, essas moléculas também são capazes de proteger neurônios do dano oxidativo induzido pela amônia. Assim, os efeitos protetores do resveratrol e do ácido lipoico no SNC parecem ser mediados não somente por suas ações diretas sobre os neurônios, mas também pela modulação da atividade glial. Ainda, os seus efeitos sobre células astrogliais e neuronais parecem compartilhar alguns mecanismos, como o da HO1.

Por fim, este estudo *in vitro* propõe que o resveratrol e o ácido lipoico podem representar potenciais agentes terapêuticos para o SNC frente a danos oxidativos e inflamatórios, como os induzidos pela amônia, por exercer efeitos benéficos nos dois principais tipos celulares que o compõe.

CONCLUSÕES:

- ✓ Importantes parâmetros bioquímicos astrogliais e neuronais são alterados pela amônia;
- ✓ Observamos que o resveratrol e o ácido lipoico exercem efeitos protetores tanto sobre as células astrogliais quanto sobre as células neuronais frente ao dano induzido por amônia;
- ✓ A HO1 está envolvida nos efeitos protetores do resveratrol e do ácido lipoico;
- ✓ A modulação da funcionalidade astroglial parece ser um elemento importante para os efeitos neuroprotetores do resveratrol e do ácido lipoico;
- ✓ O resveratrol e o ácido lipoico podem representar potenciais agentes terapêuticos para o SNC durante situações patológicas, como na EH.

PERSPECTIVAS:

- Investigar a ação do resveratrol e do ácido lipoico sobre possíveis danos oxidativos a biomoléculas induzidos pela amônia, como a peroxidação lipídica e danos ao DNA e RNA;
- Avaliar os efeitos do resveratrol e do ácido lipoico sobre a reatividade antioxidante total das células astrogliais e neuronais expostas à amônia, bem como sobre a atividade de enzimas antioxidantes SOD e catalase;
- Ampliar o entendimento dos efeitos do resveratrol e do ácido lipoico sobre o metabolismo da glutationa em células astrogliais e neuronais expostas à amônia, avaliando tanto a razão GSH/GSSG intra e extracelular, bem como a atividade de enzimas que participam do seu metabolismo, como a glutationa peroxidase, glutationa redutase e glutationa S-trasferase. Além disso, avaliar a expressão e atividade das enzimas responsáveis por sua síntese, como a glutamato cisteína ligase e a glutationa sintetase, e os possíveis mecanismos regulatórios envolvidos;
- Confirmar o papel da enzima HO1 nos efeitos protetores do resveratrol e do ácido lipoico, através do silenciamento dessa enzima *in vitro*, utilizando a técnica de RNA de interferência;
- Avaliar os mecanismos de indução da HO1 pelo resveratrol e pelo ácido lipoico, como a ativação do fator de transcrição Nrf2 e do co-ativador de transcrição gênica PGC-1α;
- Ampliar o entendimento da atividade anti-inflamatória do resveratrol e do ácido lipoico durante a exposição das células astrogliais à amônia, avaliando seus efeitos sobre a síntese de outras moléculas pró-inflamatórias, como a prostaglandina E2, e a expressão e ativação de proteínas como ciclooxigenases e fatores de transcrição (AP-1, CREB e STATs);

- Verificar os efeitos protetores do resveratrol e do ácido lipoico sobre células gliais e neuronais em um modelo de hiperamonemia *in vivo*.

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