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Efeito da administração intracerebroventricular de metilglioxal sobre a função astroglial no hipocampo e barreira hematoencefálica

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Continue a nadar, continue a nadar!

Dory – Procurando Nemo, 2003.

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À beleza da vida.

RESUMO

O metilglioxal (MG) é um composto dicarbonil, formado a partir de fontes exógenas e endógenas. A taxa de formação do composto depende do metabolismo e tipo celular e pelas condições fisiológicas, sendo, endogenamente, a via glicolítica, a mais importante fonte de produção. Níveis elevados de glicose estão relacionados ao acúmulo de MG no plasma e no líquido cefalorraquidiano (LCR), em doenças como o diabetes mellitus (DM) e a doença de Alzheimer (DA). A alta reatividade do MG leva a modificações de proteínas e outras biomoléculas, gerando produtos finais de glicação avançada (AGEs), apontados como mediadores nessas desordens neurodegenerativas. Além da glicação, outros efeitos resultantes de altos níveis de MG no sistema nervoso central (SNC) são envolvidas na direta modulação da neurotransmissão GABAérgica e glutamatérgica, com evidências sugerindo que os efeitos do MG podem ser relacionados a mudanças comportamentais e disfunção glial. No SNC, os astrócitos são mais suscetíveis ao dano para o estresse dicarbonílico, embora essas células apresentam um sistema glioxalase mais eficiente quando comparado com neurônios, sugerindo um aumento no dano funcional neuronal sob complicações diabéticas e em desordens neurodegenerativas. Em vista disso, o objetivo deste estudo foi avaliar a influência de altas concentrações de MG no SNC. Foi avaliado como a administração intracerebroventricular (ICV) de MG (3 μmol/μL) causa mudanças comportamentais como o declínio cognitivo e ansiedade bem como alterações bioquímicas no hipocampo, soro e LCR. Além disso, nós investigamos a integridade da barreira hematoencefálica (BHE) relacionada às funções astrocitárias no hipocampo. MG induziu, 12 h após a sua administração, um decréscimo na atividade locomotora no campo aberto e efeitos ansiolíticos nos ratos submetidos ao Teste do labirinto em cruz elevado. Subsequentemente, 36 h após a cirurgia ICV, a injeção de MG também induziu prejuízo cognitivo nas memórias de curto e longo prazo, avaliado pela tarefa do reconhecimento de objetos. Foi avaliado também a memória espacial de curto prazo, avaliada pelo teste do Y-maze. Setenta e duas horas após infusão do MG, a captação de glutamato, a atividade da glutamina sintetase e os níveis da glutationa foram diminuídos em relação ao grupo SHAM. Interessantemente, os níveis da proteína astrocitária, S100B, estavam aumentados no LCR, o que foi acompanhado pelo decréscimo na expressão do RNAm da S100B hipocampal, mas não nos níveis da proteína. Não houve mudanças no conteúdo do receptor para AGEs (RAGE). Além disso, nós observamos uma perda na integridade da BHE, avaliada pela entrada do corante azul de Evans blue no tecido cerebral e de albumina no LCR. Além destas alterações, houve decréscimo nos níveis da aquaporina-4 e conexina-43 no tecido hipocampal. A expressão gênica (RNAm) de dois importantes fatores de transcrição da resposta antioxidante, NfkB e Nrf2, não foram alterados no hipocampo. Contudo, a hemeoxigenase-1 (HO-1) foi aumentada no grupo tratado com o MG. Em conjunto, esses dados corroboram com a ideia de que os astrócitos, as principais células responsáveis pelo clearance do MG, são alvos na toxicidade do MG e que a disfunção da BHE induzida por este composto podem contribuir nas alterações cognitivas e comportamentais observadas nesses animais. Isso nos ajuda a melhorar nosso entendimento de como produtos derivados do metabolismo da glicose podem induzir a disfunções cognitivas observadas em pacientes diabéticos bem como em outras condições neurodegenerativas, e uma melhor definição do papel dos astrócitos nas doenças e terapêutica.

ABSTRACT

Methylglyoxal (MG) is a dicarbonyl compound, formed from exogenous and endogenous sources. The formation rate of compound depends on metabolism and cell type and physiological conditions, which the glycolytic pathway represents the most important endogenous production source. Elevated blood glucose levels are related to MG accumulation in plasma and cerebrospinal fluid (CSF) diabetes mellitus and Alzheimer's disease (AD). The high reactivity of MG leads to modification of proteins and other biomolecules, generating advanced glycation end products (AGEs) appointed as mediators in these neurodegenerative diseases. In addition to protein glycation, other effects resulting from high levels of MG in the central nervous system (CNS) may involve the direct modulation of GABAergic and glutamatergic neurotransmission, with evidence suggesting that the effects of MG may be related to behavioral changes and glial dysfunction. In the CNS, astrocytes are more susceptible to damage of dicarbonyl stress, although they have an improved glyoxalase system when compared to neurons, suggesting an increased neuronal functional damage under diabetic complications and neurodegenerative disorders. In view of this, the aim of this study was evaluating the direct influence of high concentration of MG in the CNS. It was evaluated how intracerebroventricular (ICV) administration (3 μmol/μL) cause behavioral changes, as cognitive decline and anxiety as well as biochemical alterations in the hippocampus, serum and CSF. Furthermore, we investigated the blood-brain barrier (BBB) integrity related to astrocytic functions in the hippocampus. MG induced, 12h after injection, a decrease in locomotor activity in the Open field (OF) and anxiolytic effects in rats submitted to elevated plus-maze (EPM). Subsequently, at 36 h after ICV surgery, MG injection also induced cognitive impairment in both short and long-term memory, as evaluated by novel object recognition tasks, and in short-term spatial memory, as evaluated by the Y-maze test. 72h after infusion of MG, hippocampal glutamate uptake decreased and glutamine synthetase activity and glutathione levels diminished. Interestingly, the astrocytic protein, S100B, was increased in the cerebrospinal fluid, accompanied by decreased hippocampal S100B mRNA expression, without any change in protein content and hippocampal contents of RAGE. In addition, we observed a loss of BBB integrity, as assessed by the entry of Evans dye into brain tissue and albumin in the CSF, as well as a decrease of aquaporin-4 and connexin-43 in hippocampal tissue. The mRNA expressions of two important transcription factors of antioxidant response - NfkB and Nrf2, were not changed. However, hemeoxigenase-1 was upregulated in MG-treated group. Taken together, this data corroborates with the idea that astrocytes, the main cells responsible for MG clearance, are targets of MG toxicity and that BBB dysfunction induced by this compound may contribute to behavioral and cognitive alterations observed in these animals. It helps us an improve our understanding of how this product of glucose metabolism can induce the brain dysfunction observed in diabetic patients, as well as in other neurodegenerative conditions, and further defines the role of astrocytes in disease and therapeutics.

LISTA DE ABREVIATURAS

ADHs - aldeído-desidrogenases

AG – Aminoguanidina

AGEs – Produtos finais de glicação avançada

AKRs - aldo-ceto redutases

AQP4 – Aquaporina 4

ARE – elemento-resposta antioxidante

ATP – Adenosina Trifosfato

BHE – Barreira hematoencefálica

BSA – Albumina sérica bovina

CEL – Carboxi-etil-lisina

CML – Carboxi-metil-lisina

Cx43 – Conexina 43

DA- Doença de Alzheimer

DM – Diabetes Mellitus

DM1 – Diabetes Mellitus tipo 1

DM2 – Diabetes Mellitus tipo 2

DNA – ácido deoxiribonucleico

EROs – espécies reativas de oxigênio

GABA – Ácido gama-aminobutírico

$GABA_AR$ – Receptor do ácido gama-aminobutírico tipo A

GFAP – Proteína glial fibrilar ácida

GLAST – Transportador Glutamato-Aspartato

Glo1 – Glioxalase 1

Glo2 – Glioxalase 2

GLT-1 – Transportador de glutamato tipo 1

GLUTs- Transportadores de glicose

GS – Glutamina sintetase

GSH – Glutationa reduzida

GSSG – Glutationa oxidada

HMGB1 – Proteína (High mobility group Box-1)

HO-1 – Hemi oxigenasse 1

ICV- Intracerebroventricular

IL-1β – Interleucina 1 beta

IRS-1 – Substrato do receptor de insulina

JNK - c-Jun N-terminal cinase

LCR – Líquido cefalorraquidiano

MG – Metilglioxal

MG-Hs – Hidroimidazolonas derivadas do MG

NfkB – Fator nuclear de cadeia κ de linfócitos B

NMDA – N-metil-D-aspartato

Nrf2 – Fator nuclear eritroide 2

RAGE – Receptor de produtos finais de glicação avançada

RNAm – ácido ribonucleico mensageiro

SGLTs – Transportadores de glicose dependentes de sódio

SNC – Sistema nervoso central

APRESENTAÇÃO

Esta tese está apresentada em seções organizadas da seguinte maneira: Introdução, Resultados (Capítulo I, Capítulo II), Discussão, Conclusões, Perspectivas, Referências Bibliográficas e Anexo. Na Introdução, são apresentados os temas estudados, a fundamentação teórica que nos levou ao desenvolvimento deste trabalho e os focos de estudo, descritos no item Objetivos.

A seção de Resultados está dividida em dois capítulos, de acordo com os diferentes focos de estudo. Em cada capítulo estão descritos os materiais e métodos utilizados bem como os resultados obtidos, sendo o primeiro capítulo o artigo científico publicado.

Capítulo I: Short-term alterations in behavior and astroglial function after intracerebroventricular infusion of methylglyoxal in rats. Este capítulo refere-se aos objetivos I a II.

Capítulo II: Acute methylglyoxal-induced damage in blood-brain barrier and hippocampal tissue**.** Este capítulo refere-se ao objetivo III.

A Discussão contém a argumentação bibliograficamente embasada da interpretação e da importância dos resultados obtidos nos dois capítulos. A seção conclusões aborda as hipóteses concluídas na tese. Nas perspectivas, estão elucidados os próximos passos para que as novas abordagens suscitadas pela tese possam ser investigadas a fim de colaborar para que o capítulo II seja publicado na forma de artigo científico, e complementar o entendimento acerca da linha de estudo. A seção referências bibliográficas lista elementos bibliográficos utilizados na redação da tese.

Nessa tese, foi incluso como anexo, resultados parciais referentes ao período da realização do doutorado sanduíche no departamento de Medicina Experimental da Universidade de Perúgia, Itália sob a orientação do professor Guglielmo Sorci. Todas as

atividades realizadas estavam vinculadas ao projeto: Toxicidade do MG mediando a via RAGE/Nrf2 em células gliais.

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PARTE I

1 INTRODUÇÃO

1.1 Metabolismo da glicose no sistema nervoso central

O sistema nervoso central (SNC) é caracterizado pelo requerimento de uma alta demanda energética. O principal substrato energético para o desempenho das funções é a glicose (Mergenthaler *et al.*, 2013). Embora o cérebro represente apenas 2% da massa corporal (Molina; Dimaio, 2012), é responsável por consumir aproximadamente 25% da energia derivada da glicose (Bordone *et al.*, 2019). Apesar de a concentração de glicose possa diferir em diferentes áreas do cérebro, com um acréscimo pós-prandial (Burdakov; Luckman; Verkhratsky, 2005; Thorens, 2011), os níveis basais fisiológicos são mantidos rigorosamente estáveis, permitindo a manutenção das atividades funcionais (Koekkoek; Mul; La fleur, 2017). A polaridade e concomitante hidrofilidade apresentada pela molécula de glicose exige a presença de transportadores específicos, como os transportadores de glicose (GLUTs) e/ou transportadores dependentes de sódio (SGLTs), para que esta possa atravessar a membrana plasmática celular (Szablewski, 2017). A distribuição e expressão das diferentes isoformas dos GLUTs e SGLTs são específicos de cada célula, e também dependentes das condições fisiológicas e do desenvolvimento (Patching, 2017). GLUT1 é a isoforma mais abundante nas células que formam a barreira hematoencefálica (BHE) (células endoteliais e plexos coroides), astrócitos e células ependimárias que revestem os ventrículos cerebrais (Gonçalves, 2019; Welcome and Mastorakis, 2018). Neurônios expressam principalmente GLUT3, embora alguns (no hipocampo, cerebelo e hipotálamo) expressam GLUT4 (transportador de glicose sensível a insulina) (Choeiri et al., 2002; Shah et al., 2012) e SGLT1 (no hipocampo e córtex cerebral) (Yu et al., 2010). Além disso, outros transportadores como o GLUT2, GLUT (6-8) e SGLT (1-2) tem sido observados nas células e unidades neuro vasculares da BHE (Patching, 2017).

Sob concentrações fisiológicas, a entrada de glicose dá-se através dos GLUTs (Lund-Andersen, 1979; Patching, 2017). A glicose derivada do sangue, atravessa células endoteliais da BHE, principalmente via GLUT1, alcançando o fluido intersticial e dessa forma é rapidamente absorvida por astrócitos, oligodendrócitos e micróglia, alcançando estruturas mais distantes (Harris et al., 2012; Navale and Paranjape, 2016; Patching, 2017). Os níveis intracelulares de glicose são mantidos pelo balanço entre as taxas de influxo, efluxo e metabolismo e, uma vez dentro das células a glicose é fosforilada pela enzima hexoquinase a glicose-6-fosfato, a qual pode ser então metabolizada por três vias principais: a glicólise, vias das pentoses e glicogênese (em astrócitos) (Bélanger et al., 2011; Pellerin, 2018). Dessa forma, intermediários dessas vias como aminoácidos (alanina, glicina, glutamina e serina) e moléculas neurotransmissoras/neuromoduladoras (aspartato, GABA, glutamato, lactato) são gerados, os quais podem regular atividades de diversas estruturas e funções celulares (Alle et al., 2009; Dienel, 2019; Mergenthaler et al., 2013; Pellerin and Magistretti, 1994; Serres et al., 2005; Zielke et al., 2009).

Existem inúmeros debates acerca das taxas de glicose utilizada como substrato nas interações metabólicas entre astrócitos e neurônios (Allaman et al., 2015; Bélanger et al., 2011; Juaristi et al., 2019). Alguns estudos defendem que os neurônios sustentam uma taxa mais alta de metabolismo oxidativo em comparação com as células gliais (Itoh et al., 2003; Lebon et al., 2002; Lopez-Fabuel et al., 2016) e a utilização de lactato como substrato energético, inclusive quando a glicose está disponível (Bouzier-Sore et al., 2006; Itoh et al., 2003). Porém, um estudo de (Dienel, 2019), considerando frações de volume/tipo de células, apresentou que essa a taxa de oxidação da glicose se assemelha ou até se apresenta mais alta em astrócitos. Somado a isso, é sabido que os astrócitos apresentam uma alta captação de glicose e taxa glicolítica (Bittner, 2010; Herrero-Mendez et al., 2009), a qual é catabolizada e liberada como ATP ou convertida a lactato

ao meio extracelular, dependendo do tipo e necessidade celular (Bouzier-Sore et al., 2006; Itoh et al., 2003; Lovatt et al., 2007; Serres et al., 2005) (Figura 1).

A via glicolítica não é inócua para as células, uma vez que gera inevitavelmente subprodutos altamente reativos como o metilglioxal (MG) (Allaman et al., 2015; Dienel, 2019). O acúmulo permanente de MG nas células é deletério, desde que esse composto é um dos mais potentes agentes glicantes (Rabbani and Thornalley, 2012; Thornalley, 2005; Thornalley and Rabbani, 2011a). Para manter-se em níveis baixos e não-tóxicos, a detoxificação do MG é realizada, majoritariamente pelo sistema de enzimas glioxalase (Rabbani, 2015).

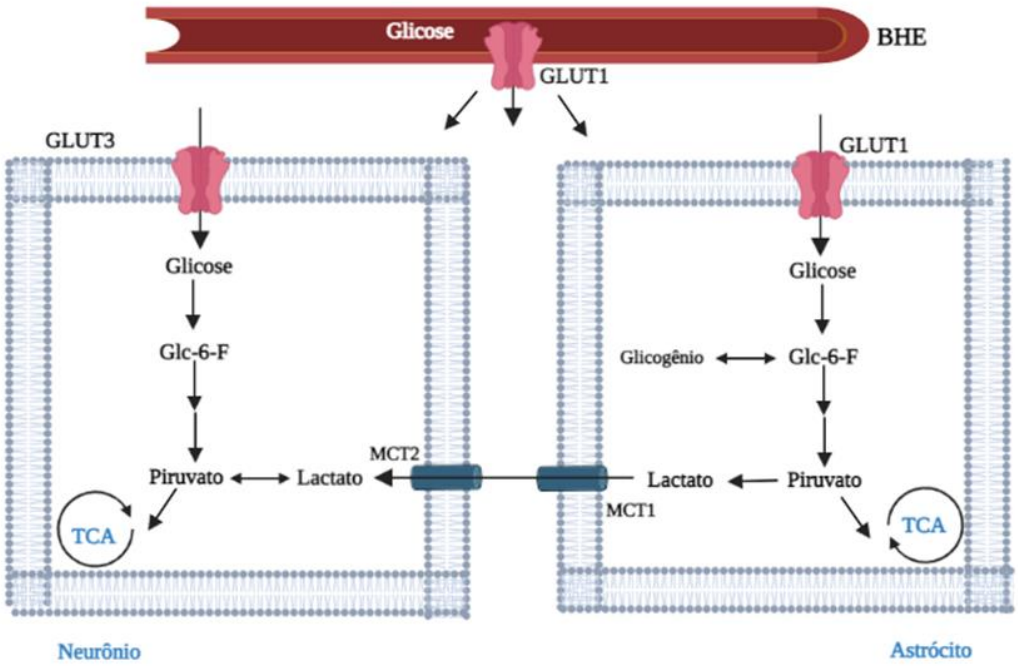


Figura 1. Metabolismo da glicose e geração de substratos astrócitos-neurônios. Adaptado de (Pellerin, 2018) e (Mergenthaler et al., 2013). Para a confecção da figura foi usado o programa Biorender.

1.2 A formação do MG

O MG é um composto dicarbonil com peso molecular de 72kDa, representado pela fórmula química $C_3H_4O_2$ (Phillips and Thornalley, 1993) (Figura 2). Pode ser originado de formas endógenas e exógenas (Kalapos, 2013). Endogenamente, é formado principalmente a partir de dois intermediários da via glicolítica, as triose-fosfato (diidroxiacetona-fosfato e o gliceraldeído-fosfato) através da fragmentação e eliminação espontânea (não enzimática), ou pela ação da triose-fosfato isomerase do grupo fosfato (Pompliano et al., 1990; Richard, 1993; Thornalley, 1996). Vale ressaltar também que o MG pode ser formado a partir de várias vias metabólicas nas quais as triose-fosfatos são intermediárias, como a gliconeogênese e a gliceroneogênese (Rabbani and Thornalley, 2015), através do metabolismo dos corpos cetônicos, com a ação das enzimas citocromo P450, mieloperoxidase e o catabolismo do aminoácido treonina (Phillips and Thornalley, 1993). O MG pode ser gerado também de forma não-enzimática pela lenta degradação da glicose e dos produtos de Amadori através da reação de Maillard (Thornalley, 1998), e em processos de peroxidação lipídica (Shibamoto, 2006; Vander Jagt and Hunsaker, 2003).

De forma exógena, a formação do MG via degradação de proteínas glicadas e monossacarídeos, ocorre em alimentos e bebidas durante o processamento e armazenamento prolongado (Nemet et al., 2006). A composição lipídica e presença de monossacarídeos (Nemet, 2006), além de fatores como temperatura e o tempo de preparação dos alimentos influenciam na maior formação do MG (Martins et al., 2003). O MG presente nos alimentos é rapidamente degradado e/ou reage com as proteínas antes da absorção no trato gastrointestinal e, portanto, parece não contribuir diretamente sobre os níveis totais no organismo (Degen et al., 2013; Nemet et al., 2006). Porém, seu papel através da dieta se dá indiretamente como precursor nos processos de glicação, com a

formação dos chamados produtos de glicação avançada (do inglês, AGEs). AGEs são formados durante o cozimento dos alimentos, e concentrações circulantes desses compostos no organismo podem influenciar em alterações metabólicas como a geração de inflamação, estresse oxidativo e uma propensão à resistência à insulina (Sandu et al., 2005; Uribarri et al., 2005).

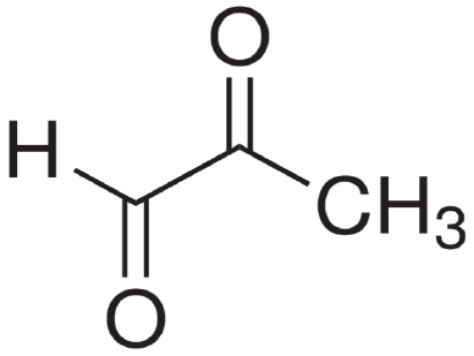


Figura 2. Fórmula estrutural do metilglioxal, $C_3H_4O_2$. Nome IUPAC: 2-Oxopropanal 72,06 g/mol. Adaptado de (www.pubchem.ncbi.nlm.nih.gov, 2021).

1.3 AGEs derivados do MG

Reações de glicação são processos endógenos que contribuem para modificações translacionais de proteínas ocorrendo através de complexas e sequenciais séries de reações chamadas coletivamente de reações de Maillard (Rabbani and Thornalley, 2012). A reação de Maillard é dividida em três estágios: inicial, intermediário e tardio (Lapolla et al., 2005). No estágio inicial, a glicose e/ou outros açúcares redutores reagem com grupos amino de várias biomoléculas formando base de Schiff. Através do rearranjo estrutural, essa base dá origem ao produto de Amadori, o qual *in vivo*, alcança o equilíbrio dinâmico (em 15-20 dias) acumulando-se em proteínas de vida curta e longa (Lapolla et al., 2005). A partir do estágio intermediário, o produto de Amadori através de reações de oxidação e desidratação podem degradar-se em vários compostos α-oxoaldeídico, entre eles o MG, glioxal e deoxyglucosonas (Rabbani and Thornalley, 2012). O estágio tardio

é representado por reações de oxidação, desidratação e ciclização destes compostos com grupos amino livres formando AGEs. Além disso, a formação de AGEs pode dar-se também através da via direta na qual níveis elevados de MG reagem com proteínas (Lapolla et al., 2005).

Embora os níveis plasmáticos de MG são aproximadamente 50000 vezes menores, a reatividade para a glicação é de até 20000 vezes maior quando comparado com a glicose. Dessa forma, a formação dos adutos de proteínas através da glicação mediada pelo MG *in vivo* ocorre em um fluxo muito semelhante ao da glicação mediada pela glicose (Thornalley, 2005). Inúmeras proteínas como a hemoglobina, proteínas mitocondriais histonas ou moléculas como a GSH sofrem alterações estruturais e funcionais através da glicação mediada pelo MG (Bose, 2013; Galligan et al., 2018; Kalapos, 2008a; Morcos et al., 2008).

O principal aminoácido afetado pela glicação do MG é a arginina, resultando na perda da carga positiva com formação de hidroimidazolonas derivadas do MG (MG-Hs). Estas podem apresentar-se sob três formas estruturais (MG-H1), MG-H2 e MG-H3 (Rabbani and Thornalley, 2008; Xue et al., 2012). MG-H1 corresponde a 90% dos adutos (Rabbani and Thornalley, 2012), seguida por menores proporções formadas a partir da glicação da lisina pelo MG, a carboxi-etil-lisina (CEL) e a carboxi-metil-lisina (CML). Proteínas modificadas pela presença de resíduos de MG-H1 em sua estrutura são direcionadas para proteólise (Ahmed et al., 2005a; Dobler et al., 2006; Dudek et al., 2005; Hernebring et al., 2006). Elevadas concentrações de CEL e MG-H1 foram encontradas no LCR de pacientes com a doença de Alzheimer (DA) (Ahmed et al., 2005b) e no plasma e urina de indivíduos com diabetes mellitus (DM) (Ahmed et al., 2003; Beisswenger and Miller, 2013; Karachalias et al., 2010). Ratos nefrectomizados apresentaram um aumento de adutos livres MG-H1, com ausência no conteúdo de resíduos de MG-H1 em proteínas

plasmáticas, indicando que o *clearance* é realizado principalmente na excreção (Rabbani et al., 2007). Além disso, o MG pode reagir com a deoxiguanosina, formando derivados de imidazopurinona, contribuindo em processos para a glicação do ácido desoxirribonuclieco (DNA) (Schalkwijk and Stehouwer, 2020; Tamae et al., 2011); quando ligados DNA-MG formam um aduto chamado MGdG {metilglioxal-3-(2'-deoxiribosil) -6,7-dihidro-6,7-dihidroxi-6/7-metilimidazo-[2,3-b] purina-9(8) -um}, o qual foi associado com quebras na cadeia do DNA (Thornalley and Rabbani, 2011b). Ao mesmo tempo, *crosslinks* induzidos pelo MG entre a DNA polimerase e o DNA inibem a replicação do DNA (Murata-Kamiya and Kamiya, 2001). Além disso, o mal enovelamento de proteínas modificadas pelo MG são mais susceptíveis e direcionadas ao proteasoma para proteólise (Rabbani and Thornalley, 2018), processo que pode ocasionar mudanças na transcrição gênica, um aumento no apoptose e dano celular (Ramasamy et al., 2006).

1.3.1 Efeitos secundários da glicação

A extensão da glicação de proteínas pelos compostos carbonílicos em condições fisiológicas é baixa (1-5%), mas pode exacerbar-se durante o envelhecimento e em situações patológicas (Rabbani and Thornalley, 2015). A presença de proteínas glicadas na matriz celular e extracelular desencadeia disfunções mitocondriais, aumento de espécies reativas de oxigênio (EROs) (Morcos et al., 2008) e ativação de processos direcionadas a apoptose (Chan et al., 2007). Na BHE, a glicação induzida pelo MG acarreta prejuízos na funcionalidade e integridade nas células endoteliais (Beeri, 2011; Li et al., 2015; Rabbani and Thornalley, 2015), aumentando a secreção de citocinas como a IL-6 e IL-8, ocasionando uma maior permeabilidade da BHE e mudanças na morfologia celular (Hussain, 2016; Tóth et al., 2014a), bem como um decréscimo na expressão de

proteínas *tight junctions* como as ocludinas, claudinas e zona occludens-1 (ZO-1) (Li, 2013; Tóth et al., 2014b).

Os AGEs podem interagir com diferentes receptores celulares, como o receptor para os AGEs (*do inglês*, RAGE) (Ramasamy et al., 2011). Além de neurônios e micróglia, o RAGE é expresso na superfície celular de astrócitos (Gonzalez-Reyes and Rubiano, 2018). O receptor apresenta três domínios extracelulares (V, C1 e C2), uma hélice transmembrana e uma cauda citoplasmática curta (Palanissami and Paul, 2018). O domínio V do receptor pode se ligar a vários ligantes incluindo AGEs, proteínas intracelulares dependentes de cálcio, como a S100B, a proteína do grupo de alta mobilidade box 1, HMGB1, o peptídio β-amilóide e moléculas DAMPs (*do inglês, Damage-associated molecular patterns),* já a parte intracelular medeia a sinalização celular (Gonzalez-Reyes and Rubiano, 2018).

A interação AGEs-RAGE podem desencadear uma cascata de sinalização intracelular, como a geração de ERO via NADPH oxidase e também a ativação do fator de transcrição: fator nuclear kappa B (NfkB). Esse efeito gera um aumento da expressão de genes inflamatórios e a formação e liberação de citocinas pró-inflamatórias e ERO, assim como o aumento da expressão do próprio RAGE, amplificando dessa forma a sua resposta (Saleh et al., 2019). Devido ao grande número de interações/ligantes, o envolvimento do papel do RAGE em vários processos fisiológicos e patológicos é amplamente estudado.

1.4 O sistema glioxalase e o MG

No organismo dos eucariontes e procariontes, as células possuem diferentes mecanismos de detoxificação dos propagadores dicarbonil (Phillips and Thornalley, 1993; Vander Jagt and Hunsaker, 2003). O sistema glioxalase, presente no citosol da

maioria das células, desempenha o papel de defesa celular contra a glicação e estresse oxidativo provenientes da toxicidade do MG e de outros compostos dicarbonis (Kalapos, 2008a; Thornalley, 1993a). Este sistema de enzimas, catalisadas pela glioxalase 1 (Glo1) e a glioxalase 2 (Glo2), é dependente de glutationa reduzida (GSH). O MG reage com a GSH e forma hemitioacetal. Glo1 converte o hemitioacetal em S-D-lactoilglutationa, esta por sua vez, é utilizada como substrato para a Glo2 que catalisa a sua hidrólise levando à formação de uma molécula de D-lactato e regenerando a GSH em sua forma reduzida inicial (Figura 3) (Allaman et al., 2015; Rabbani and Thornalley, 2012; Thornalley, 1993b, 2005). A primeira etapa catalisada pela Glo1 é o passo limitante da reação e a eficácia no reciclamento de GSH nesse processo está intimamente relacionado com o status no estresse oxidativo celular. O transiente decréscimo na concentração de GSH pode levar ao aumento das taxas de glutationa oxidada (GSSG/GSH) e NADP/NAPPH (Dringen, 2000).

Estudos realizados *in vitro* e *in vivo* mostraram que, no SNC, os astrócitos apresentam uma maior resistência a toxicidade do MG devido a uma maior eficiência em ambas as enzimas que compõem o sistema glioxalase, bem como uma maior capacidade compensatória nos níveis de GSH quando comparado com os neurônios (Belanger et al., 2011; Dringen, 2000). A imunoreatividade para a Glo1 foi observada ao longo de todo filamento GFAP-positivo (Allaman et al., 2015) em células astrocitárias isoladas do córtex (Belanger et al., 2011), e uma expressão duplamente maior no RNAm da Glo1 em cultura de astrócitos isolados (Cahoy et al., 2008) quando comparado com neurônios e demais células no SNC. Estudos de viabilidade celular apresentaram que culturas neuronais hipocampais foram afetadas já em baixas concentrações de MG (de Arriba et al., 2007; Di Loreto et al., 2004, 2008), enquanto que a integridade celular de culturas de astrócitos ou linhagem C6, concentrações de até 1 mM não ocasionaram danos (Belanger

et al., 2011; Hansen et al., 2012). Interessantemente, em altas concentrações de MG em co-culturas com astrócitos, neurônios são protegidos da toxicidade do composto (Belanger et al., 2011).

Embora há uma grande eficiência e atividade da Glo1, devido a maior distribuição nos tecidos (Rabbani et al., 2016), outras enzimas podem ser envolvidas na detoxificação do MG nas células: as enzimas aldo-ceto redutases (AKRs) e as aldeído-desidrogenases (ADHs), formando hidroxi-acetona e piruvato, respectivamente, como produto da reação (Vander Jagt and Hunsaker, 2003) (Rabbani et al., 2016). Tem sido demonstrado que na ausência do sistema glioxalase, as AKRs compensariam efetivamente na prevenção ao acúmulo do MG (Schumacher et al., 2018). Somado a isso, recentemente, inúmeros estudos têm reportado o envolvimento da proteína DJ-1 (também conhecido como PARK7) na degradação do MG. Diferentemente do sistema glioxalase, a DJ-1 é capaz de neutralizar o efeito do MG quando este ainda se encontra conjugado com grupos tiois celulares (Andreeva et al., 2019; Jiang et al., 2021; Matsuda et al., 2017).

O controle do estresse oxidativo apresentado pelas enzimas responsáveis pela degradação do MG são reguladas através de fatores de transcrição como o Nrf2 (Fator nuclear eritroide 2*)*. O Nrf2, em condições fisiológicas basais, é mantido no citoplasma de forma inativa, ligado com Keap 1 (um complexo de proteínas associadas) (Xue et al., 2012). Sob estado oxidativo, Nrf2 se desliga do Keap 1 e se transloca ao núcleo, ligando-se ao elemento resposta-antioxidante (ARE) (presente em todos os genes regulados por Nrf2) (Liu et al., 2017), ou ao elemento de resposta nas regiões promotoras dos genes que codificam moléculas antioxidantes (Zoja et al., 2015).

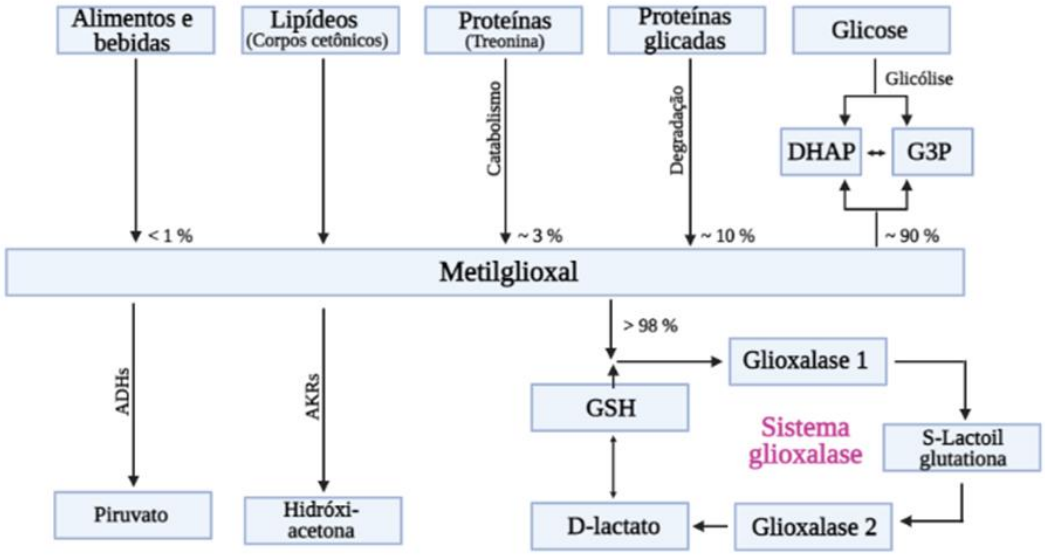


Figura 3. Vias metabólicas e fontes de formação e degradação do metilglioxal. Adaptado de (Bélanger et al., 2011) e (Schalkwijk and Stehouwer, 2020). Para a confecção da figura foi usado o programa Biorender.

1.5 Níveis de MG em condições fisiológicas e patológicas

Considerando a importância energética primária da glicólise na maioria dos tecidos, a formação de MG ocorre em todos as células e organismos (Angeloni et al., 2014; Kalapos, 2008b; Nemet et al., 2006). Embora a concentração de MG é dependente da especificidade do tecido, estima-se que 0.1-0.4% do fluxo glicolítico resulta na formação de MG (Kalapos, 2008b). Em humanos, a taxa de formação sistêmica é de 120µmol/kg/dia (Thornalley, 1998b). Células e tecidos apresentam concentrações de 2 - 4µM (Dobler et al., 2006; Nicolay et al., 2006), 100nm no plasma (Beisswenger et al., 1999; Nicolay et al., 2006) e níveis estimados entre 10 - 20µM no LCR (Kuhla et al., 2005). Isso significa que a formação periférica e até a circulação sistêmica do MG podem acessar e afetar o SNC, uma vez que é uma molécula capaz de se difundir através da membrana plasmática, portanto, capaz de permear também a BHE (Distler et al., 2012).

Em condições fisiológicas o MG se apresenta em três formas dinamicamente equilibradas: a forma não-hidratada, monoidratada e a diidratada, sendo a monoidratada a mais prevalente (McLellan and Thornalley, 1992; Nemet et al., 2004). A desidratação do MG é um passo limitante para definição no tempo de meia vida (aproximadamente 4 minutos) durante a permeabilização à membrana plasmática, que se dá através da difusão passiva (Rabbani and Thornalley, 2014a). Todavia, considerando o metabolismo do MG pelo sistema glioxalase, a meia vida seria de cerca de 10 minutos (Rabbani and Thornalley, 2014a). O MG formado nas células pode se difundir através do fluido intersticial para o plasma /ou células de outros tecidos (Rabbani and Thornalley, 2015). A maior parte do MG lábil (> 99%) está ligada reversivelmente a GSH e grupos tiois (Rabbani and Thornalley, 2014a). Ligações irreversíveis a proteínas no plasma ocorrem em 3,6 horas (Rabbani and Thornalley, 2015).

O MG em níveis fisiológicos (μM), age como um agonista parcial dos receptores do ácido γ-aminobutírico (GABA) do tipo A ($GABA_AR$), possuindo 1/3 da força de ativação de seu neurotransmissor clássico (Distler et al., 2012). Os receptores $GABA_A$ são membros de uma superfamília de canais iônicos ativados por ligantes, de estrutura pentamérica, permeável a cloreto (Cl^-) (Bormann, 2000; Chebib and Johnston, 2000). Esse aumento a permeabilidade ao íon aumenta a resposta sináptica inibitória à liberação pré-sináptica do principal ligante endógeno do receptor, o neurotransmissor GABA (Johnston, 2005; Scott and Aricescu, 2019). Efeitos inibitórios sobre a neurotransmissão diminuem a probabilidade da geração de um potencial de ação, devido a hiperpolarização pós-sináptica (Kullmann et al., 2005). O efeito do MG na ativação dos receptores $GABA_A$ se dá de forma parácrina, após se difundir pela membrana plasmática quando gerado intracelularmente por neurônios e pela glia (Nusser et al., 1999), uma vez que na fenda sináptica a concentração de GABA é centenas de vezes maior que a de MG. Em estudos

in vitro, alterações na concentração do MG ocasionaram bruscas mudanças no potencial e corrente da membrana celular neuronal, o que substancialmente alterou o comportamento (Distler et al., 2012).

Devido à alta reatividade apresentada pela molécula do MG, mimetizar os efeitos e manter os níveis desse composto em estudos, visando a concentrações patológicas ou fisiológicas é um grande desafio. Dessa forma, são encontrados na literatura inúmeros estudos que diferem na concentração do composto, nas vias de avaliação e os tempos de tratamento realizados, tanto *in vitro* (majoritariamente avaliados) quanto *in vivo.* Foi demonstrado que a citotoxicidade causada pelo MG é mediada pelo enfraquecimento do sistema de detoxificação e morte celular (Di Loreto et al., 2004, 2008). O MG induziu a morte das células progenitoras de neurogênese no hipocampo adulto de camundongos (Chun et al., 2016), bem como em diferentes linhagens celulares, cultura de neurônios e astrócitos e fatias de córtex, sobretudo em curtos intervalos de tempo (1h – 24h) (Chu et al., 2014; Hansen et al., 2017; Heimfarth et al., 2013). Em estudos com eletrofisiologia, foram avaliadas as concentrações de 0,01 a 100µM de MG, respectivamente em culturas de neurônios do hipocampo e do cerebelo (Distler et al., 2012), e fatias cerebrais de camundongos (10 µM) (Hambsch et al., 2010). Outras vias como a administração intraperitoneal também foram avaliadas, na qual doses entre 50 – 300mg/kg de peso corporal para ratos ou camundongos (Distler et al., 2012, 2013; Nigro et al., 2014), administração oral (600mg/kg de peso corpóreo) e administração intracerebroventricular (ICV), com injeções repetidas e únicas aplicadas em ratos (3 µM/µL) (Hansen et al., 2016a).

A produção de MG é acelerada em condições de hiperglicemia (Lee et al., 2009; Turk, 2010), por prejuízo na utilização da glicose e deficiência da enzima triose-fosfato isomerase (Ahmed et al., 2003). Dependendo do controle glicêmico, as concentrações

plasmáticas do MG e AGEs podem aumentar de 2-4 vezes em pacientes diabéticos (Lapolla et al., 2003; Rabbani and Thornalley, 2014b). Na DA, as concentrações de MG no LCR aumentaram até 2 vezes mais quando comparados com pacientes sem a doença (Kuhla et al., 2005) bem como o aumento do aduto MG-H1 (Ahmed et al., 2005c).

1.6 MG – Um biomarcador para Diabetes mellitus

O Diabetes mellitus (DM) é uma doença metabólica crônica, caracterizada por aumento nos níveis de glicose sanguínea (Zaccardi et al., 2016), classificada como diabetes tipo 1 (DM1) e diabetes tipo 2 (DM2), sendo a última a mais comum de todos os tipos de diabetes, abrangendo aproximadamente 90% de todos os casos (Koye et al., 2018; Zimmet et al., 2014). O DM1 é caracterizado pela falta de produção de insulina pelas células β-pancreáticas enquanto que o DM2, relacionado com a deficiência e/ou resistência à insulina dessas células (American Diabetes Association, 2020; Zarkogianni et al., 2015). A hiperglicemia crônica e a resistência à insulina são comumente associadas ao desenvolvimento de complicações vasculares, as quais levam a alterações em órgãos como os rins (nefropatia), retina (retinopatia) e nervos periféricos (neuropatia) (Donaghue et al., 2009).

Existem consideráveis evidências científicas que o significante aumento dos níveis de MG celular pela hiperglicemia contribuiria para o desenvolvimento dessas micro e macro complicações e outros fatores de risco como hipertensão, dislipidemia e obesidade (Schalkwijk and Stehouwer, 2020). Além disso, o MG contribui com a neuropatia diabética, já que modula a atividade de proteínas como o canal iônico de sódio dependente de voltagem, vinculado à hiperalgesia, Nav1.8, bem como age sobre o receptor de potencial transitório anquirina 1 (TRPA1) (Andersson et al., 2013; Bierhaus et al., 2012). Além disso, o MG acarretaria a predisposição a DM2 (Moraru et al., 2018), uma vez que

induz a resistência à insulina (Mey and Haus, 2018), desde que modifica estrutural e funcionalmente a molécula (Jia et al., 2006; Schalkwijk et al., 2008). Estudos anteriores demonstraram que o MG inibe a via do substrato da insulina (IRS)-1 e fosfoinositídeo 3-quinase (PI_3K), prejudicando a sinalização desempenhada pela molécula (Matafome et al., 2017; Nigro et al., 2014; Riboulet-Chavey et al., 2006). Dessa forma, o MG ocasiona uma diminuição na secreção da insulina sobretudo porque interage com as células β-pancreáticas e células endoteliais causando disfunções celulares (Bo et al., 2016; Fiory et al., 2011; Vulesevic et al., 2016) e geração de espécies reativas de oxigênio (Pi et al., 2007). Somado a isso, altos níveis de MG plasmáticos acarretaram um maior risco para diminuição na filtração renal (do inglês, eGFR) e albuminúria em pacientes DM1 e DM2 (Hanssen et al., 2017, 2018; Jensen et al., 2016) e, somado a isso, é sabido que praticamente todas as estruturas renais são susceptíveis ao acúmulo dos AGEs e assim, não apenas o efeito do MG *per se*, mas também, AGEs derivados como MG-H1 e CEL são indicadores precoces na progressão de lesões na nefropatia diabética (Beisswenger and Miller, 2013).

1.7 Relação entre o DM e o SNC

Ao lado das alterações ocasionadas em órgãos periféricos pela hiperglicemia, pacientes diabéticos também apresentam anormalidades estruturais e funcionais no SNC (Salas and De Strooper, 2019). Estudos sugeriram que pacientes diabéticos (DM1 e DM2) apresentaram declínio cognitivo (Cukierman et al., 2005; Kodl and Seaquist, 2008), principalmente associados a processos de aprendizagem e memória (Ryan, 1988). Além disso, foi proposto que o diabetes aumenta o risco no desenvolvimento de demência e especialmente demência do tipo Alzheimer (50-100% de incidência independentemente da origem étnica) (Baglietto-Vargas et al., 2016; Biessels and Despa, 2018), sobretudo

no DM2 (Biessels et al., 2006; Pugazhenthi et al., 2017; Tumminia et al., 2018). Abrangentes evidências mostram que no percurso inicial da DA, há um prejuízo na sinalização realizada pela insulina (de la Monte and Wands, 2005) e o consequente déficit na utilização da glicose (Mosconi et al., 2008, 2009), o que contribuiria para a progressão aos distúrbios na cognição (Dhamoon et al., 2009; Hoyer et al., 1991), e o desbalanço no metabolismo energético e na homeostase cerebral (Grünblatt et al., 2007; de la Monte, 2012; de la Monte and Wands, 2005; de la Monte et al., 2009). Modelos clínicos e experimentais de diabetes tem apresentado que a intolerância à glicose é associada a todas as causas de demência (Ohara, 2013) e aumento do risco quando comparado a indivíduos não-diabéticos (Cukierman et al., 2005; Sommerfield et al., 2004; Spauwen et al., 2013). Indivíduos DM2 com níveis aumentados de hemoglobina glicada apresentaram baixos *scores* para a aprendizagem e memória (Cukierman-Yaffe et al., 2009). Em virtude de todas essas similaridades características com o diabetes, a DA pode ser denominada como "diabetes tipo 3" ou "DM2 específica do encéfalo" (Sebastião et al., 2014; Steen et al., 2005).

Além da hiperglicemia, diversos outros fatores também têm sido sugeridos e estudados como mediadores entre o diabetes e a demência, entre eles, o acúmulo de AGEs (Bornstein et al., 2014; Strachan et al., 2011; Yang and Song, 2013). Embora esteja claro que a DA e o diabetes compartilham um número de processos moleculares e mediadores que fundamentam a degeneração das respectivas desordens, os mecanismos envolvidos ainda não são exatamente claros. Dessa forma, já que o número de indivíduos com diabetes, prevalentemente DM2 continua a aumentar, e a população continua a envelhecer, é de grande importância aprofundar o conhecimento dessa sinergia entre DM, comprometimento cognitivo, idade e a DA.

1.8 MG, Diabetes e SNC

Nos últimos anos tem se aprofundado sobre os estudos da importância da regulação dos níveis de MG no SNC (Allaman et al., 2015; Angeloni et al., 2014; Distler et al., 2012; Kuhla et al., 2006, 2007). Elevados níveis séricos de MG (Beeri, 2011) e elevadas concentrações plasmáticas de AGEs derivados de alimentos (Cai et al., 2014; West, 2014), têm sido associados com um rápido declínio cognitivo em indivíduos idosos não dementes e camundongos. Outro estudo, conduzido por Srikanth e colegas, (Srikanth et al., 2013), avaliando pessoas idosas, demonstrou que níveis elevados de MG estavam associados com um decréscimo na memória, menor função executiva, e atrofia encefálica, caracterizada por um menor volume de matéria cinzenta dos idosos. Entre vários outros fatores, uma das explicações poderia estar relacionada com o decréscimo na atividade da Glo1 observado no cérebro de pessoas idosas quando comparado com indivíduos jovens (Kuhla et al., 2006). Esse prejuízo no metabolismo da Glo1 e consequente aumento nos níveis de MG séricos e no LCR tem sido associado também ao declínio cognitivo apresentado pelos pacientes com a DA quando comparado com indivíduos controles (Angeloni et al., 2014; Beeri, 2011; Chen et al.; Srikanth et al., 2013).

Em modelos experimentais de diabetes, os altos níveis de MG também estariam associados com prejuízos na aprendizagem e memória (Huang et al., 2012; Wartchow et al., 2020). A supressão dos efeitos do acréscimo nos níveis de MG pelo polifenol mangiferina preveniu o declínio cognitivo apresentado em ratos (Liu et al., 2013), e o uso de metformina no tratamento de diabéticos principalmente DM2, tem reduzido significativamente o MG plasmático acompanhada por um significativo aumento na atividade da Glo1 (Beisswenger et al., 1999; Kender et al., 2014). Um aumento nos níveis da Glo1 diminui significativamente os níveis de MG e AGEs no plasma de ratos diabéticos (Brouwers et al., 2011), bem como causou um decréscimo nos níveis de RAGE

e AGEs no hipocampo, com consequente melhora no dano cognitivo induzido pelo MG (Liu et al., 2013). Em adição a isso, a quantidade da proteína bem como o RNAm para Glo1 foi encontrada diminuída em vários órgãos, incluindo o cérebro, fígado e rins de ratos $Nrf2^{-/-}$, com um aumento de 100% nos níveis de MG e de seus adutos na urina (Xue et al., 2012).

Por outro lado, a regulação da expressão da Glo1 pelo MG tem sido relacionada a ansiedade (Distler et al., 2012; Hovatta et al., 2005; Kromer, 2005; Williams et al., 2009). Em roedores, o MG a curto prazo, tem sido reportado por apresentar efeitos ansiolíticos (Distler et al., 2012; Szczepanik et al., 2020) e doses diárias dessa molécula tem reduzido o comportamento do tipo-ansioso (Hambsch et al., 2010), com concomitante diminuição na atividade do receptor $GABA_A$ (Distler et al., 2012).

Os prejuízos cognitivos podem ser justificados pelo efeito do MG no hipocampo, já que esta estrutura cerebral é muito vulnerável à citotoxicidade celular ocasionada pelo composto (Kuhla et al., 2005; Schalkwijk and Stehouwer, 2020). Roedores submetidos a elevadas concentrações de MG, apresentaram déficit cognitivo em diferentes tipos de memória e em breves períodos de tempo (Hansen et al., 2016a; Lissner et al., 2021; Szczepanik et al., 2020). Na presença de MG, culturas neuronais de hipocampo apresentaram significativo aumento nos processos de apoptose celular com uma expressão aumentada na atividade de enzimas como a caspase-3 e Bax (Di Loreto et al., 2008; Heimfarth et al., 2013; Tajes, 2014), um descontrole no status oxidativo neuronal e depleção no conteúdo do GSH (Cai et al., 2002; Ighodaro, 2018), bem como aumento nos níveis dos marcadores inflamatórios (Vlassara et al., 2002; Weisman et al., 2006).

Além disso, foi proposto que a glicação acelera os processos de polimerização do β-amiloide (Aβ) e da hiperfosforilação da proteína tau, os quais são marcadores histopatológicos na DA (Li et al., 2012, 2013; Shuster et al., 2020). Na glicação do

peptídeo Aβ pelo MG, são geradas formas oligoméricas maiores contendo folhas-β (Li et al., 2013) e a formação de AGEs acelera a transição do peptídeo β-amilóide monomérico para sua forma oligomérica e, em seguida, gerando agregados ainda maiores (Taghavi et al., 2017). A toxicidade apresentada pelo peptídeo β-amiloide modificado pelo MG diminuiu a viabilidade celular (Angeloni et al., 2014). Estudos verificaram um acúmulo dos AGEs intra- e extracelularmente no cérebro durante o envelhecimento e na DA (Grillo and Colombatto, 2008; Münch et al., 2003). Nos pacientes com a DA, adutos de AGEs foram encontrados aumentados em 3 vezes em placas senis, quando comparados com controles pareados por idade (Vitek et al., 1994). Foi observado também uma colocalizarão com as placas e emaranhados neurofibrilares (Grillo and Colombatto, 2008; Muronetz et al., 2017) e um acúmulo dos produtos de Amadori nas principais proteínas presentes no LCR, incluindo a albumina (Shuvaev et al., 2001).

Conforme o exposto acima e segundo estudos prévios, e mesmo considerando que ainda existem várias perguntas inconclusivas e muitos estudos a serem realizados, o papel da desregulação nos níveis de MG fisiológicos são apontados como um dos principais mediadores nas complicações associadas à DA e diabetes (Krautwald and Münch, 2010; West, 2014).

2 OBJETIVOS

2.1 Objetivo geral

Avaliar os efeitos da administração ICV de MG sobre parâmetros comportamentais e neuroquímicos *in vivo.*

2.2 Objetivos específicos

I. Avaliar os efeitos comportamentais de MG (ICV) após 12, 36 e 60 horas em ratos Wistar:

- Atividade locomotora: campo aberto;
- Comportamento do tipo ansioso: labirinto em cruz elevado;
- Aprendizagem: habituação no campo aberto;
- Memória de reconhecimento: teste de reconhecimento de objetos;
- Memória espacial: teste do Y-maze.

II. Avaliar o efeito do MG sobre a função astroglial 72 h após a administração ICV:

- Na atividade da enzima glutamina sintetase (GS) e glioxalase 1 (Glo1) e conteúdo de glutationa reduzida (GSH) no hipocampo;
- No conteúdo dos transportadores de glutamato (GLAST e GLT-1), receptor de glutamato NMDA (subunidade GLUN1) e Glo1 no hipocampo;
- No conteúdo da proteína ligante de cálcio S100B (no hipocampo, LCR e soro) e na expressão gênica (RNAm, no hipocampo);
- No conteúdo da proteína ácida fibrilar glial (GFAP) hipocampal;
- Na expressão gênica (RNAm) da citocina interleucina 1β (IL-1β) hipocampal.

III. Avaliar o efeito do MG em possíveis alterações na BHE, particularmente sobre as funções astrocitárias 72 h após a sua administração ICV:

- No conteúdo de albumina (LCR/soro);

- Na permeabilidade da BHE através do corante Evans blue;

- No conteúdo das proteínas aquaporina 4 (AQP-4), Conexina-43 (Cx43) e receptor de produtos de glicação avançada (RAGE) no hipocampo;

- Na expressão gênica (RNAm) da proteína (High mobility group Box-1) (HMGB1), fator nuclear eritroide 2 (Nrf2), fator nuclear kB (NfkB), e Hemi oxigenase (HO)-1 hipocampais.

PARTE II

3 CAPÍTULO I

Article

Short-term alterations in behavior and astroglial function after intracerebroventricular infusion of methylglyoxal in rats

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ORIGINAL PAPER

Short-Term Alterations in Behavior and Astroglial Function After Intracerebroventricular Infusion of Methylglyoxal in Rats

Lílian Juliana Lissner[1] · Leticia Rodrigues[1] · Krista Minéia Wartchow[1] · Ederson Borba[1] · Larissa Daniele Bobermin[1] · Fernanda Urruth Fontella[1] · Fernanda Hansen[2] · André Quincozes-Santos[1] · Diogo Onofre Gomes Souza[1] · Carlos-Alberto Gonçalves[1]

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Abstract
Methylglyoxal (MG) is a by-product of glycolysis. In pathological conditions, particularly diabetes mellitus, this molecule is unbalanced, causing widespread protein glycation. In addition to protein glycation, other effects resulting from high levels of MG in the central nervous system may involve the direct modulation of GABAergic and glutamatergic neurotransmission, with evidence suggesting that the effects of MG may be related to behavioral changes and glial dysfunction. In order to evaluate the direct influence of MG on behavioral and biochemical parameters, we used a high intracerebroventricular final concentration (3 µM/µL) to assess acute effects on memory and locomotor behavior in rats, as well as the underlying alterations in glutamatergic and astroglial parameters. MG induced, 12 h after injection, a decrease in locomotor activity in the Open field and anxiolytic effects in rats submitted to elevated plus-maze. Subsequently, 36 h after surgery, MG injection also induced cognitive impairment in both short and long-term memory, as evaluated by novel object recognition task, and in short-term spatial memory, as evaluated by the Y-maze test. In addition, hippocampal glutamate uptake decreased and glutamine synthetase activity and glutathione levels diminished during seventy-two hours after infusion of MG. Interestingly, the astrocytic protein, S100B, was increased in the cerebrospinal fluid, accompanied by decreased hippocampal S100B mRNA expression, without any change in protein content. Taken together, these results may improve our understanding of how this product of glucose metabolism can induce the brain dysfunction observed in diabetic patients, as well as in other neurodegenerative conditions, and further defines the role of astrocytes in disease and therapeutics.

Keywords Methylglyoxal · Cognitive dysfunction · Anxiety-like behavior · Glutamatergic metabolism · S100B

Introduction

Glycation has been associated with diabetes mellitus (DM), Alzheimer's disease (AD) and other chronic diseases [1–4]. α-Dicarbonyl compounds (α-DCs) (methylglyoxal [MG], glyoxal and 3-deoxyglucosone) are the principal molecules that cause protein glycation, due to their extremely reactive arrangement that can promote the fast formation of adducts [5]. In general, high concentrations of α-DCs lead to the increased formation of advanced glycation end products (AGEs) [5]. MG is a byproduct of glycolysis, and associations of the peripheral concentration of this physiological compound with pathophysiologic processes have been extensively studied [5, 6]. Despite the existence of different detoxification systems for excess MG, such as the glyoxalase system [7] impairments caused by increased concentrations of MG and other α-DCs are almost inevitable in some diseases.

Elevated MG levels are commonly associated with various DM complications, including chronic renal disease [3, 8], a faster rate of memory decline [4] and anxiety-like behavior [9]. Moreover, it is known that MG can affect neurotransmission, altering glutamatergic [10, 11] and GABAergic synapses [9]. Nonetheless, there have been few studies of the effects of MG on behavior and its association with neuroglial functions [10, 12].

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Evidence suggests that the concentration of MG, in both the plasma and in cells, ranges from about 0.1–3 μM [5, 8]; however, these concentrations are 2–6 times higher in patients with DM [13]. Moreover, AD patients present an average MG concentration in the CSF of around 22 μM, which is far more significant than concentrations found in patients without AD [14]. Indeed, supraphysiological concentrations of MG have been used to study MG-related effects in *in vitro* and *in vivo* approaches [9–11, 15]. The challenge of mimicking the effects of MG using physiological or pathological concentrations is complex, especially considering MG reactivity and its rapid degradation in the organism. In order to simulate acute dicarbonyl stress, the use of high concentrations may aid in elucidating the mechanism of action of MG and its consequences, in experimental models.

Although there have been many efforts to elucidate the involvement of MG in DM complications and neurodegenerative diseases [14, 16], little is known about the *in vivo* acute effect on animal behavior and neuroglial functions. Studies have shown that repeated intracerebroventricular (ICV) administration of elevated concentrations of MG modulates anxiety-related behavior and neurochemical parameters in the rat brain [10, 15]. Herein, our focus was to investigate how an elevated MG concentration, directly infused in the lateral ventricle of rats, could acutely interfere with learning-memory processes, anxiety-related behavior and locomotor activity, and determine how astroglial modulation could be involved.

Experimental Procedure

Materials

Methylglyoxal (MG) solution (at 40%, in water), standard glutathione (GSH), Triton X-100, ophthaldialdehyde, S100B protein, anti-S100B (SH-B1), L-glutamate, o-phenylenediamine (OPD), meta-phosphoric acid, γ-glutamylhydroxamate acid and N-methyl-D-glucamine were purchased from Sigma-Aldrich (Saint Louis, MO, USA). L-[2,3-^{3}H] glutamate was purchased from Amersham International (United Kingdom). Polyclonal anti-S100B and polyclonal anti-GFAP were purchased from DAKO (São Paulo, SP, Brazil), anti-rabbit peroxidase linked antibody (GE Healthcare, Little Chalfont, United Kingdom). Polyclonal anti-GLAST and anti GLT-1 (Abcam, Cambridge, MA, USA), and polyclonal anti-glyoxalase 1 (Santa Cruz Biotechnology Inc. Dallas, Texas, USA). Mouse monoclonal anti-β-actin antibody (C4 clone), and mouse monoclonal anti-GluN1 (R1JHL clone) (Millipore, Darmstadt, Germany). All other chemicals were purchased from local commercial suppliers.

Animals

A total of seventy male Wistar rats (90-days old) were obtained from our breeding colony (Department of Biochemistry, UFRGS, Porto Alegre, Brazil). The rats were maintained under controlled light and environmental conditions (12 h light/12 h dark cycle, at a constant temperature of 22 ± 1 °C) and had free access to commercial chow and water. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), and all procedures were previously approved by the local Animal Care Ethical Committee (CEUA-UFRGS; project number 33663). All efforts were made to minimize animal suffering and reduce the number of animals used.

Surgical Procedure

MG was unilaterally infused in the lateral ventricle, as described in previous studies [10, 17]. The rats were divided into three groups: Naive (animals not submitted to surgical procedure), SHAM-operated and MG-treated. Briefly, rats were anesthetized with ketamine/xylazine (75 and 10 mg/kg, respectively, i.p) and positioned in a stereotaxic apparatus. A midline sagittal incision was made in the scalp and burr holes were drilled in the skull on the right side over the lateral ventricle. The right ventricle was accessed using the following coordinates: 0.9 mm posterior to the bregma; 1.5 mm lateral to the sagittal suture; 3.6 mm beneath the surface of the brain. Animals received 5 μL of MG ICV unilaterally with a final concentration of 3 μM/μL diluted in Hank's balanced salt solution (HBSS, pH 7.2); The SHAM group received an equal volume of HBSS. After the surgical procedure, animals were kept warm until recovery from anesthesia. From twelve hours after the ICV injection, the rats were submitted to behavioral assessments. The tasks were performed as follows: open field (OF) test and novel object-recognition task (NOR), elevated plus-maze (EPM) and Y-maze test. The behavioral tests were performed with different groups of animals, as follows: NOR was performed with different groups to those of the animals of the EPM and Y-maze test (performed together). Biochemical parameters were evaluated approximately seventy-two hours after ICV surgery.

Behavioral Tests

All behavioral experiments were performed at the active cycle of the animal (starting at 7 pm) and conducted in a sound-attenuated room under low-intensity light (12lx). The experimenters remained outside the room during the tests.

To avoid the presence of olfactory cues, all the testing apparatuses were cleaned with a 70% ethanol solution and dried with a paper towel after each trial.

Exposure to the Open Field (OF)

Motor activity was evaluated in the OF test. The OF apparatus consists of a square arena (60 × 60 cm) with dark wood and floor. The test consists of exposing the animals to this large arena where the central area is more aversive for rodents than the peripheral area and it is usually considered as an index of anxiety [18]. The rats were released in the corner of the arena to freely explore. The automated activity-monitoring system (Any-maze; Stoelting, Wood Dale, IL, USA) was used to measure the distance traveled over the arena and habituation. The distance traveled, as an index of general activity was registered twelve hours after surgery [10] modified.

Novel Object Recognition (NOR)

The cognitive performance was evaluated in the OF by the NOR task. This task comprised three distinct phases; habituation, training and discrimination devised by Ennaceur and Delacour [19] and adapted by Wartchow et al. [20]. The rats were placed in the corner of the arena to perform the task. In the habituation phase, the rats were allowed to explore the OF for ten minutes. Twenty-four hours after the habituation phase, the animals returned to the training trial: the rat was placed in the apparatus that contained two identical sample objects (A+A). After the end of the training trial, the rats were removed from the OF and kept in the home cage. One hour and twenty-four hours after the training trial, the rats returned to the apparatus to test short-term (STM) or long-term (LTM) memory, respectively. In the test session, the rat was returned to the OF arena that contained two objects; one object was identical to the training session and the other object was novel (A+B). For LTM, the object 'B' was replaced by a third one "C" (A+C), and the 'A' object was maintained the same. The time spent by the rats exploring each object was monitored with a video system placed in an adjacent room. Exploration of an object was defined as sniffing or touching the object with the nose. The recognition index was calculated based on time exploring the novel object×100/time exploring both objects [21–23].

Elevated Plus-Maze Test (EPM)

The elevated plus-maze test was used for evaluating anxiety-associated behavior [24] and was performed at twelve hours after surgery. The apparatus consisted of two opposing open arms (without walls) and two opposing closed arms (arms measured 45 × 10 cm), elevated 80 cm above the floor. On the day of the experiment, the animals were acclimated to the apparatus during one hour before the test. The rats were then placed individually on the central platform facing an open arm and were allowed to explore the maze for five minutes. Arm entry is defined as entering an arm with all four paws. The parameters analyzed here were the number of entries into the closed arms, entries into the open arms and the total time spent in each arm. The parameters of anxiety-like behavior are represented as percentage and calculated as follows: open arm (time exploring the open arm × 100/time exploring open and closed arms) or closed arm (time exploring the closed arm × 100/time exploring open and closed arms) [25].

Y-Maze

Thirty-six hours after surgery, the spatial memory of animals was investigated using the Y-maze paradigm. The apparatus consisted of three arms (50 × 10 × 20 cm and 120° apart) made of black wood placed in a room with visual cues on the walls. Y-maze testing consisted of two trials separated by an interval of one hour. In the first trial, 1 (of 3) arm was closed. The rats were placed at the end of one arm with their heads oriented in the opposite direction to the center of the maze and allowed to visit the 2 open arms for 5 min. In the second trial (one hour later), rats were placed back into the start arm of the maze with free access to all three arms for 5 min. The number of entries and the time spent in each arm were recorded. The index performance was calculated as follows: time exploring the novel arm × 100/time exploring the two other arms of the maze, except the arm in which the rat was first placed [26, 27].

Biochemical Analyses

Obtaining CSF, Serum and Hippocampal Samples

Animals were anesthetized and then positioned in a stereotaxic holder for CSF collection of 100 µL (approximately) from the cisterna magna. The puncture was performed using an insulin syringe (27 gauge X 1/2″ length). Rats were then removed from the stereotaxic apparatus and placed in a flat place; whole blood was obtained through an intracardiac puncture using a 0.37-mm diameter needle and was collected into clot activator tubes, centrifuged (3000 rpm, 10 min, 4 °C) to separate serum. The hippocampi were dissected and transverse of 0.3 mm were obtained using a McElwain Tissue Chopper. The samples were frozen at −80 °C until posterior analysis [28].

Glutamate Uptake Assay

Glutamate uptake was performed as previously described [29, 30]. Slices were incubated at 37 °C in HBSS and the assay was started by the addition of 0.1 mM L-glutamate and 0.66 µCi/mL L-[2,3-^{3}H] glutamate. Incubation was stopped after 5 min by removing the medium and rinsing the slices twice with ice-cold HBSS. The slices were then lysed in a 0.5 M NaOH solution. Sodium-independent uptake was determined using N-methyl-D-glucamine instead of NaCl. Sodium-dependent glutamate uptake (specific uptake) was obtained by subtracting the nonspecific uptake from the total uptake. Radioactivity was measured in a scintillation counter. Results were calculated as nmol/mg protein/min and were expressed as percentages of the SHAM.

CSF Glutamate Measurements by High Performance Liquid Chromatography (HPLC)

Glutamate levels in CSF were measured by HPLC, as previously described [31]. CSF samples were filtered and cell-free supernatant aliquots were used to quantify glutamate levels. The analysis was performed using a reverse phase column (Supelcosil LC-18, 250 mm × 4.6 mm × 5 µm, Supelco) in a Shimadzu liquid chromatograph (50 µL loop valve injection, 40 µL injection volume) and fluorescent detection after pre-column derivatization with *o*-phthalaldehyde. The mobile phase flowed at a rate of 1.4 mL/min and column temperature were 25 °C. Buffer compositions were as follows: Buffer A: 0.04 mol/L $NaH_2PO_4 \times H_2O$ buffer pH 5.5, containing 80% of methanol; B: 0.01 mol/L $NaH_2PO_4 \times H_2O$ buffer pH 5.5, containing 20% of methanol. The gradient profile was modified according to the content of buffer B in the mobile phase: 100% at 0.10 min, 90% at 15 min, 48% at 10 min, 100% at 60 min. Absorbance was read in a Shimadzu fluorescence detector. Samples of 20 µL were used and concentration was calculated in µM and was expressed as percentages of the SHAM.

Glutamine Synthetase (GS) Activity Assay

Enzymatic activity of glutamine synthetase (GS) was determined using the procedures described previously [32] with modifications. Briefly, slices were homogenized in 50 mM imidazole. Homogenates were incubated with (mM): 50 imidazole, 50 hydroxylamine, 100 L-glutamine, 25 sodium arsenate dibasic heptahydrate, 0.2 ADP, 2 manganese chloride, pH 6.2 for 15 min at 37 °C. The reactions were terminated by the addition of 0.2 mL of 0.37 M $FeCl_3$, 200 mM trichloroacetic acid, and 670 mM HCl. After centrifugation, the absorbance of the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamyl hydroxamate acid treated with ferric chloride reagent. GS activity was calculated as µmol/mg protein/h and was expressed as percentages of the SHAM.

Reduced Glutathione (GSH) Content Assay

GSH levels were measured as described [33]. Slices were homogenized in sodium phosphate buffer (0.1 M, pH8.0) containing 5 mM EDTA and protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with o-phthaldialdehyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was employed using standard GSH solutions at concentrations ranging from 0 to 500 µM. GSH concentrations were calculated as nmol/mg protein and were expressed as percentages of the SHAM.

Glyoxalase Activity (GLO1) Assay

Hippocampal slices were lysed and homogenized in phosphate buffered saline (PBS), pH 7.4. Subsequently, slices were centrifuged at 13,000 rpm for 15 min at 4 °C and the supernatant was used for enzymatic activity and protein content measurements. GLO1 activity was assayed according to [10]. A unit of GLO1 activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol of S-(D)-lactoylglutathione per minute. Specific activity was calculated in milliunits per milligram of protein (mU/mg protein) and was expressed as percentages of the SHAM.

Quantification of GFAP and S100B

Hippocampal, CSF and serum S100B contents were measured by an enzyme-linked immunosorbent assay (ELISA) [34]. Fifty µL of sample (previously diluted when necessary) plus 50 µL of Tris buffer were incubated for 2 h on a microtiter plate previously coated overnight with monoclonal anti-S100B (SH-B1) antibody. Polyclonal anti-S100 and peroxidase-conjugated anti-rabbit antibodies were incubated at the same time for 60 min at 37 °C. A colorimetric reaction with o-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng/mL. ELISA for hippocampal GFAP was performed as previously described [35] by coating the microtiter plate with 100 µL samples overnight at 4 °C. Incubation with a rabbit polyclonal anti-GFAP for 2 h was followed by incubation with a secondary antibody conjugated with peroxidase for 1 h, at room temperature; the standard GFAP curve ranged from 0.1 to 10 ng/mL. Data were calculated as ng/mg protein and were expressed as percentages of SHAM.

Immunohistochemical Staining of GFAP

Immunohistochemical staining of GFAP was performed according to [17]. The rats were anesthetized using ketamine/xylazine and perfused through the left cardiac ventricle with 200 mL of saline solution, followed by 200 mL of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and left for post-fixation in the same fixative solution at 4 °C for 2 h. The material was then cryoprotected by immersing the brain in 15% and 30% sucrose in phosphate buffer at 4 °C. The brains were sectioned (40 µm) on a cryostat (Leitz) and incubated with polyclonal anti-GFAP from rabbit (diluted 1:1000 in PBS-0.4% triton X-100 and 2% BSA) for 48 h at 4 °C. Subsequently, tissue sections were incubated with secondary anti-rabbit IgG Alexa Fluor 586 (A11011) (diluted 1:1000 in PBS) for 1 h at room temperature. Images were detected with the Olympus confocal microscope and analyzed in a computer with digital camera and Fluoviewer 3.1 FV1000 software.

Western Blotting Analysis of Glutamate Transporters (GLAST and GLT-1), Glutamate Receptor GLUN1 and GLO1

Equal amounts (20 µg) of proteins from each sample were boiled in a sample buffer [0.0625 M Tris–HCl pH 6.8, 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue] and separated by SDS-PAGE on 12% sodium dodecyl sulfate-polyacrylamide gel and electro transferred onto nitrocellulose membranes. The membranes were blocked overnight at 4 °C with 2% chicken egg albumin in Tris-buffered saline with Tween 20 (TTBS) and then incubated for 3 h at 4 °C with an anti-GLT-1 antibody (diluted 1:5000 in TTBS and 1% bovine standard albumin – BSA), or overnight with anti-GLAST, anti-GLUN1 and anti-GLO1 antibodies (diluted 1:5000 in TTBS and 1% BSA). Next, the membranes were incubated for 1h at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies (diluted 1:10,000). Chemiluminescent bands were detected by image analyzer (Image Quant LAS4000 from GE) and optical density was quantified using ImageJ software. The results were expressed as percentages of the SHAM group.

RNA Extraction and Quantitative RT-PCR for S100B and IL-1β

Total RNA was isolated from hippocampal slices using TRIzol Reagent (Invitrogen, Carlsbad, CA). The concentration and purity of the RNA were determined spectrophotometrically at a ratio of 260:280. Extracted RNA (1 µg) was submitted to complementary DNA (cDNA) synthesis using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific) in a 20 µL reaction, according to the manufacturer's instructions. Messenger RNA (mRNA) encoding S100B (#Rn04219408_m1) and IL-1β (#Rn00580432_m1) was quantified using the TaqMan real-time RT-PCR system, using inventory primers and probes purchased from Applied Biosystems [36]. Target mRNA levels were normalized to β-actin (#Rn00667869_m1) levels. Results were analyzed employing the $2^{-\Delta\Delta Ct}$ method [37] and expressed relative to the levels of the SHAM group.

Protein Determination

Protein content was measured by adapted Lowry's method using bovine serum albumin as standard [38].

Statistical Analysis

Data are reported as means ± standard errors and analyzed statistically by Student's *t*-test or one-way ANOVA, followed by Tukey's test. The NOR task was analyzed by a one-sample t-test to determine whether the recognition index was different from a chance performance (50%). Differences were considered significant when $p \leq 0.05$. All analyses were performed using the Prism 6.0 (GraphPad).

Results

Behavioral Effects

The experimental schedule used for all tests performed here is shown in Fig. 1. Initially, the present study investigated the acute effects of ICV-MG on the performance of rats in the OF task. The distance travelled was measured on day one (1), twelve hours after surgery, and was found to be diminished by MG injection, when compared to the SHAM group (Fig. 2a) ($F_{(2, 24)} = 10.27$, $p = 0.001$). Subsequently, the effect of MG administration was evaluated on cognitive performance using the training and discrimination phase in the NOR task. In the training phase, all groups evaluated did not present object preference/laterality, as indicated by the symmetric exploration of both familiar objects (Fig. 2b). In the discrimination phase, the MG group showed a decreased recognition index in both periods analyzed: STM (Fig. 2C) ($t_{(10)} = 0.286$, $p = 0.78$) and LTM (Fig. 2d) ($t_{(10)} = 0.665$, $p = 0.52$). Also, our study investigated the effects of MG injection on short-term spatial memory. The rats were exposed to a Y-maze task, thirty-six hours after surgery, and the number of entries and time spent in the novel arm were analyzed. There was a significant reduction in the time spent in the novel arm after MG infusion, suggesting an impairment in

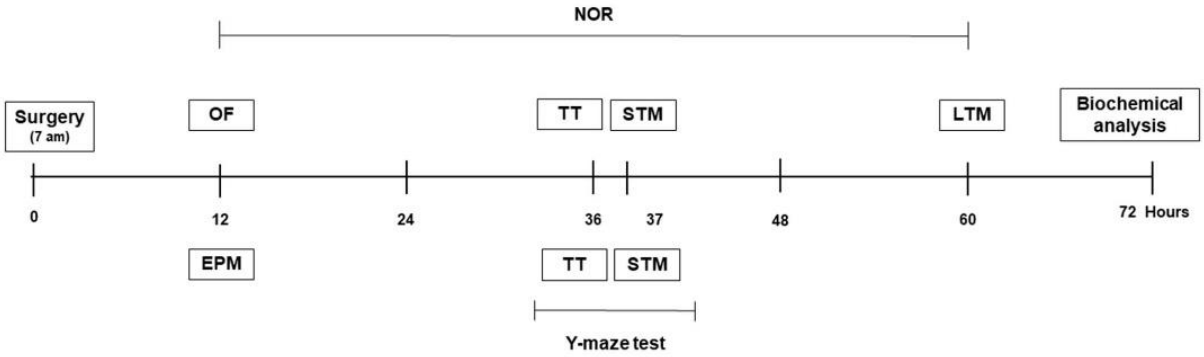


Fig. 1 Experimental timeline. Surgery corresponds to unilateral ICV infusion of MG (single injection of 5 µL with final concentration of 3 µM/µL or vehicle). Time is represented in hours after surgery. Behavioral tests were performed as follows: OF, NOR (training trial (TT), Short-time memory (STM) and Long-term memory (LTM)) of interval between each task, elevated plus maze (EPM) and Y-maze task. For biochemical analyses, cerebrospinal fluid, hippocampi and serum were harvested at seventy hours after the surgery

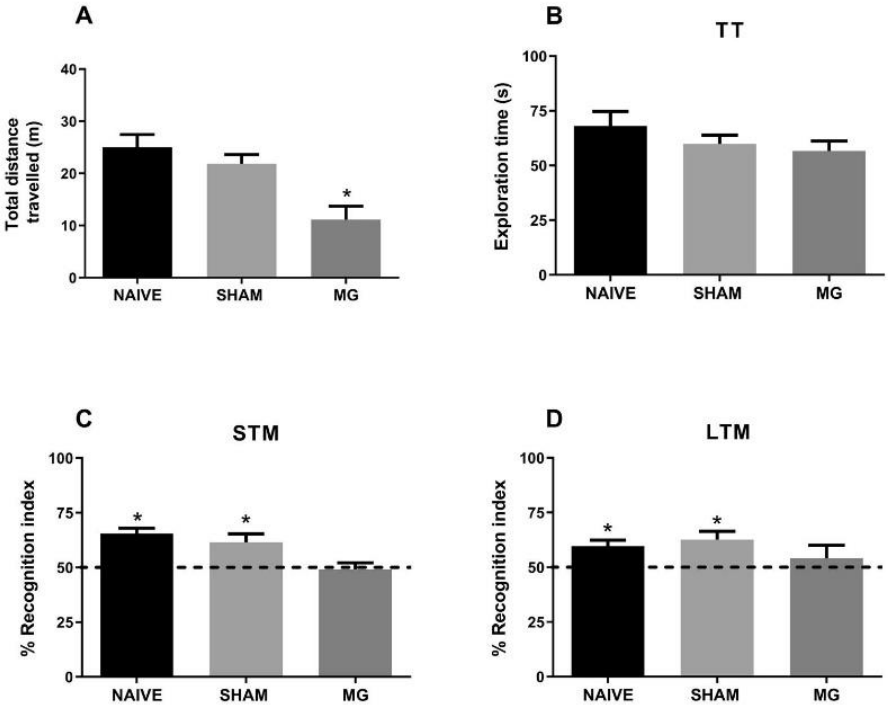


Fig. 2 Effect of ICV MG administration on locomotor activity and cognitive performance in the open field test. Locomotor activity was measured twelve hours after surgery (**a**) and cognitive performance was evaluated thirty-six hours by NOR test – TT (**b**), recognition index of STM (**c**) and LTM (**d**) after TT. Data are expressed as means ± SE (11–14 rats/group). The line in the graph indicates 50%. For graph A, * represents statistical difference from SHAM group (ANOVA followed by Tukey's test). For the other graphs, * represents statistical different group (one-sample *t*-test assuming $p < 0.05$, in which $p < 0.05$ versus chance level – 50% of novel object investigation in the discrimination phase)

spatial memory (Fig. 3a) ($F_{(2, 19)} = 6.11$, $p = 0.01$). Considering that MG is a $GABA_A$ agonist receptor, a reduction in anxiety-like behavior in rats in the EPM was expected. Data are presented in Table 1. Significant effects in the MG group were observed, as shown by the increase in time spent in the open arms ($F_{(2,17)} = 7.67$, $p = 0.0042$) and a decrease in entries in the closed arms ($F_{(2,20)} = 19.14$, $p < 0.0001$). These results indicate that MG presented an anxiolytic effect in this experimental design.

MG Infusion Decreased Glutamate Uptake in the Hippocampus

The glutamatergic system metabolism in hippocampal slices was investigated, approximately seventy-hours after ICV surgery. During neuronal activity, glutamate is released into the synaptic cleft and is rapidly taken up by excitatory amino acid transporters. Accordingly, the MG group presented a reduction of 25% in glutamate uptake (Fig. 4a, $p = 0.04$), when compared to SHAM. However, when we measured the

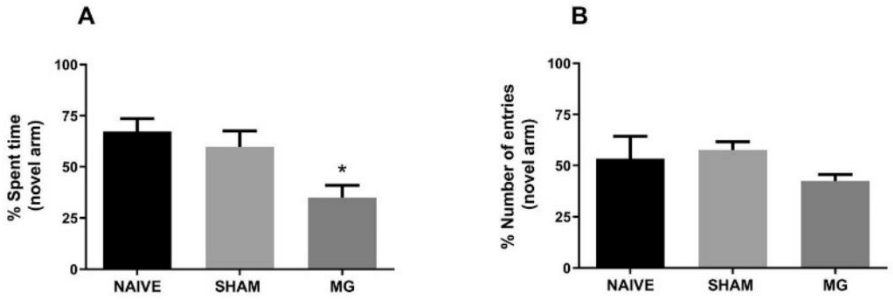


Fig. 3 Effect of ICV MG administration on spatial short-term memory, assessed in the Y-maze test. The percentage of total time spent in the novel arm (**a**) and the percentage of entries (**b**) in the novel arm during 5 min in the second trial are shown and were evaluated thirty-six hours after the ICV MG injection. Data are expressed as means ± SE (8 rats/group). *Represents statistical difference from SHAM group (ANOVA followed by Tukey's test, assuming $p < 0.05$)

Table 1 Effect of ICV MG administration on behavior in the elevated plus-maze test

Parameters	Naive	SHAM	Group MG
Time in open arms (s)	63.57 ± 18.60	67.78 ± 11.34	140.6 ± 40.68 *
Time in closed arms (s)	184.7 ± 11.48	158.9 ± 19.86	106.4 ± 42.26
Entries in open arms	5.714 ± 0.97	5.222 ± 0.85	4.429 ± 1.91
Entries in closed arms	11 ± 1.05	8.444 ± 1.25	1.571 ± 0.65 *
Percent time in open arms	29.36 ± 5.42	39.09 ± 8.56	79.45 ± 10.85 *
Percent time in closed arms	73.79 ± 5.55	58.99 ± 9.46	20.55 ± 10.85

Parameters were measured at twelve hours after surgery. Data are expressed as means ± SE (8 rats/group). *represents statistical difference from SHAM group (ANOVA followed by Tukey's test, assuming $p < 0.05$)

levels of glutamate in the CSF, no alterations were observed (Fig. 4b, $p = 0.80$). To determine whether the effect of MG on glutamate uptake was related to glutamate transporter levels, we compared the contents of GLAST and GLT-1 by Western blotting. However, no differences were observed in glutamate transporter immunocontent (Fig. 4c, $p = 0.19$; 4D, $p = 0.10$, respectively). Furthermore, the immunocontent of the GluN1 subunit of the NMDA glutamate receptor did not differ between groups (Fig. 4e, $p = 0.84$). With regard to GS activity, the enzyme that converts glutamate into glutamine, its hippocampal activity was diminished in response to MG ICV infusion (Fig. 4f, $p = 0.03$).

GSH Decreased by MG ICV Infusion

GSH content presented a decrease of approximately 60% following ICV infusion of MG, when compared to the SHAM group (Fig. 5a, $p < 0.0001$). Therefore, we investigated whether alterations in GLO1 activity or immunocontent could be involved in this effect, utilizing GSH as a cofactor, since GLO1 is a key enzyme in the glyoxalase system, participating in the detoxification of MG. No alterations in the activity of GLO1 or its immunocontent were observed following MG infusion, as indicated by Fig. 5b ($p = 0.43$) and Fig. 5c ($p = 0.88$), respectively.

Changes in Astroglial Markers After MG ICV Infusion

All the alterations in response to MG, observed so far (reduction in glutamate uptake and GSH content), are associated predominantly with astroglial functions. However, ELISA demonstrated that the levels of the major astroglial marker, GFAP, in the hippocampus were not significantly different in the MG group, when compared to the SHAM group (Fig. 6a, $p = 0.79$). Similar findings were demonstrated by immunohistochemistry for GFAP, which showed no apparent astrogliosis in CA1 during this short period of analysis (Fig. 6c). On the other hand, we observed an increase in the hippocampal expression of the pro-inflammatory cytokine, interleukin-1β (IL-1β) was increased in the MG group (Fig. 6b, $p = 0.02$), suggesting an acute inflammatory scenario in the hippocampus, without changes in GFAP. Concomitantly, it was observed a decrease in hippocampal S100B mRNA expression (another typical astroglial marker) in the MG group (Fig 7a, $p = 0.03$), with a significant increase in CSF S100B content in the MG group, compared to the SHAM group (Fig. 7c, $p = 0.01$). However, no changes in hippocampal S100B protein content (Fig. 7b, $p = 0.307$) or serum S100B levels (Fig. 7d, $p = 0.41$) were observed.

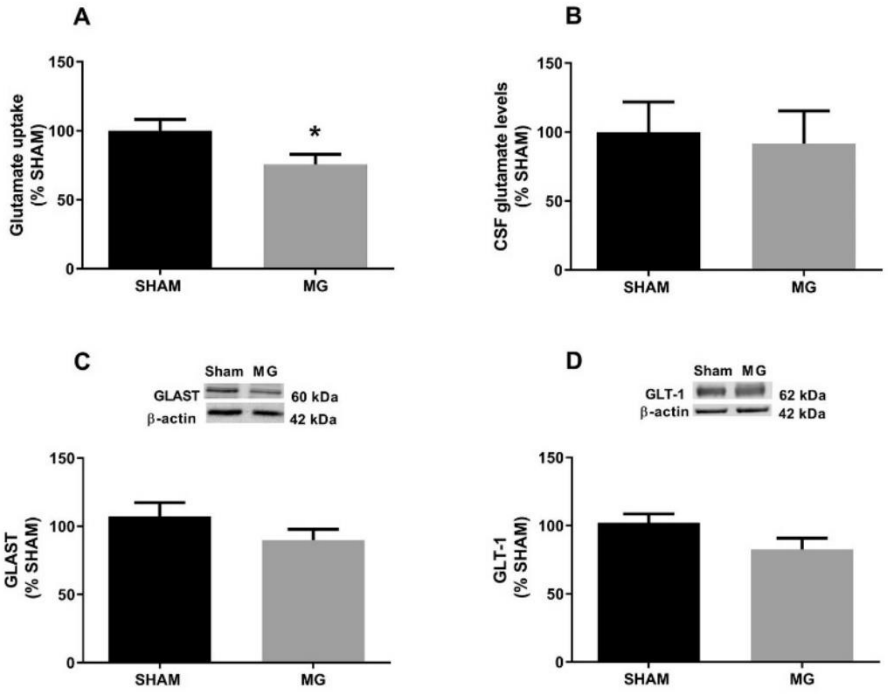


Fig. 4 Effect of ICV MG administration on the glutamatergic system in the hippocampus and cerebrospinal fluid. Glutamate uptake in hippocampal slices (N = 11) (**a**) and CSF levels of glutamate (N = 5) (**b**) were measured by HPLC seventy-two hours after surgery. Immunocontent of hippocampal glutamate transporters (GLAST and GLT1) (**c**, **d**) and NMDA receptor (GLUN1) (**e**), measured by Western blotting (N = 5 – 10) and GS activity (N = 13) (**f**). Data are expressed as means ± SE, assuming SHAM value as 100%. SHAM values of glutamate uptake and GS activity were 0.57 nmol/mg protein/min and 9.81 µmol/mg protein/h, in the hippocampus, respectively. *represents statistical difference from SHAM group (Student's *t*-test, assuming $p < 0.05$)

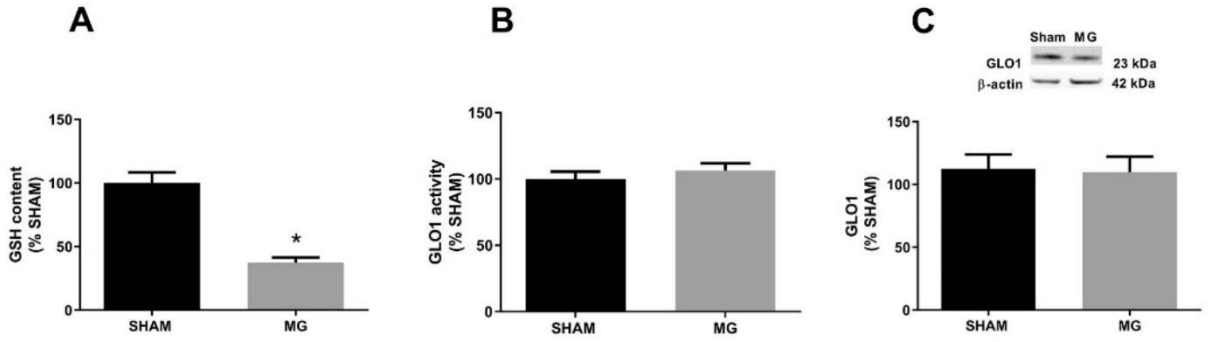


Fig. 5 Effect of ICV MG administration on GSH content, GLO1 activity and content in hippocampus. In (a), GSH content was measured by fluorometric method (N = 13), GLO1 activity by kinetic reaction (N = 13) (**b**) and GLO1 content was determined by Western blotting (N = 5) (**c**), at seventy-two hours after surgery. Data are expressed as means ± SE assuming SHAM value as 100%. SHAM values were: GSH, 19.16 nmol/mg protein; GLO1 activity, 215.15 mU/mg protein. *represents statistical difference from SHAM group (Student's *t*-test, assuming $p < 0.05$)

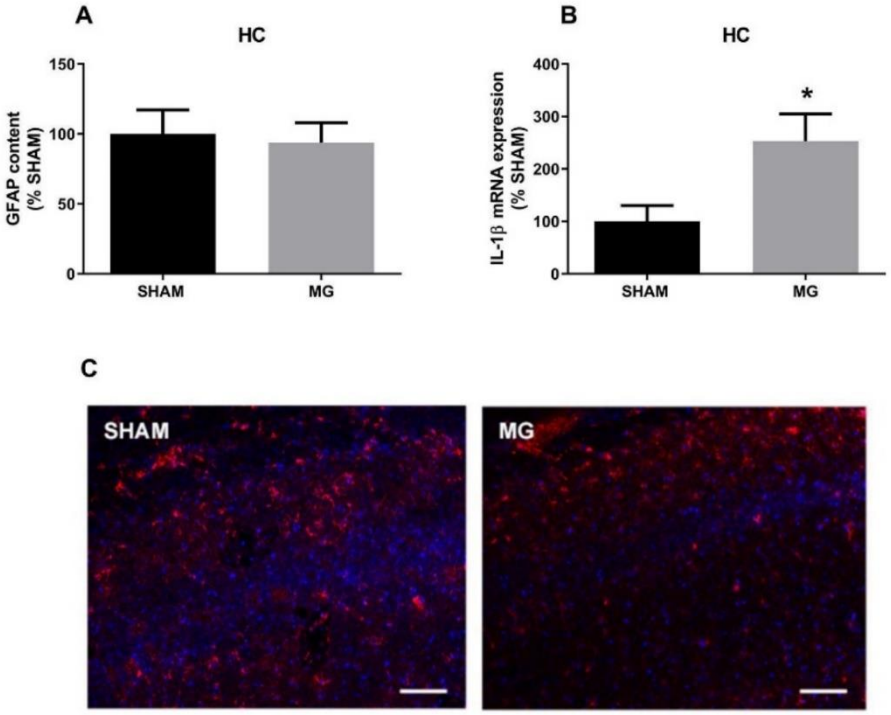


Fig. 6 Effect of ICV MG administration on GFAP content and IL-1β mRNA expression. Hippocampal slices of rats were dissected out at seventy-two hours after surgery and the following were determined; GFAP by ELISA (N = 13) (**a**), IL-1β mRNA expression (N = 8) (**b**) and immunohistochemistry for GFAP (**c**) magnified by 10X by confocal microscopy. Scale bars = 150 µm. Data are expressed as means ± SE, assuming SHAM value as 100%. SHAM values of GFAP: 35.25 nmol/mg. *represents statistical different from SHAM group (Student's *t*-test, assuming $p < 0.05$)

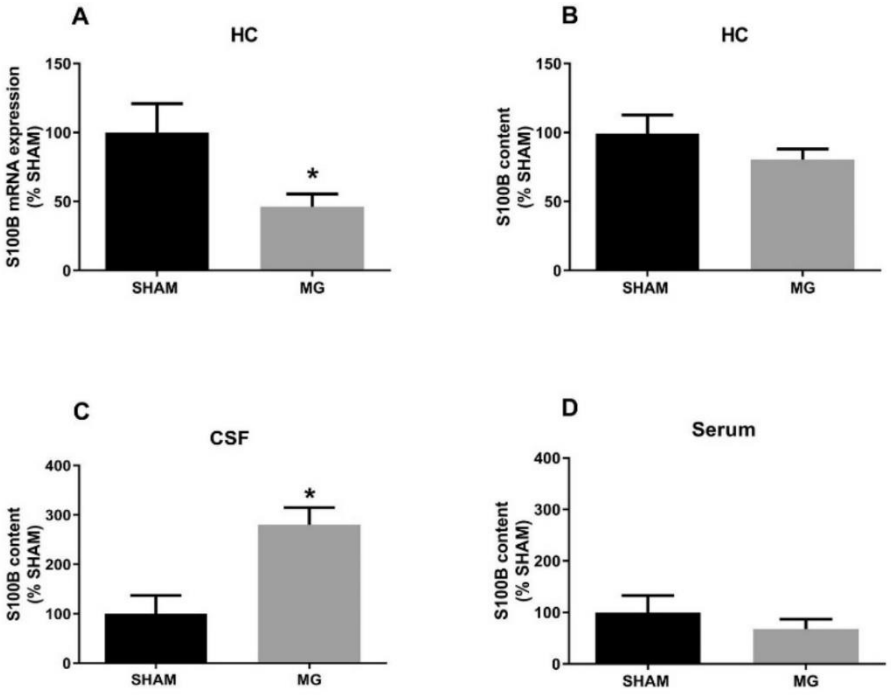


Fig. 7 Effect of ICV MG administration on the astroglial marker, S100B. Hippocampal slices of rats were dissected out at seventy-two hours after surgery and S100B mRNA expression (N = 8) (**a**) and S100B content (N = 13) (**b**) were evaluated. S100B levels in CSF (N = 5) (**c**) and serum (N = 8) (**d**) were also measured by ELISA. Data are expressed as means ± SE assuming SHAM as 100%. SHAM values of S100B content were 0.67 nmol/mg protein in the hippocampus, and 0.51 and 0.063 ng/mL protein in the CSF and serum, respectively. *represents statistical difference from SHAM group (Student's *t*-test, assuming $p < 0.05$)

Discussion

The dysfunction in glucose metabolism and generation of reactive intermediate metabolites as MG contribute to the metabolic complications observed in diabetic patients as well as to brain damage observed in neurodegenerative disorders, including Alzheimer's disease [39, 40]. In recent years, the relation between hyperglycemia in consequence of poor controlled diabetes and the development of cognitive deficiency as well as a greater propensity to the development of Alzheimer's disease has been extensively studied [41, 42]. Previous findings have shown that MG or MG-mediated glycation could be, at least in part, responsible for the faster cognitive decline presented in these disorders and also in older individuals [14, 43, 44]. Furthermore, MG can exacerbate cellular response, increasing oxidative stress and inflammation [45–48]. In the central nervous system, previous studies have suggested that astrocytes are more susceptible to damage of dicarbonyl stress caused by elevated levels of MG, once they are key players in neuroprotection and modulation of glutamatergic system [49].

The present study aimed to understand how an acute high concentration of methylglyoxal (administered by ICV infusion) modulates behavioral and neurochemical parameters in rats, with particular attention to astroglial function. Previous studies have shown that, at concentrations considered to be physiological (μM), MG acts as a partial agonist of $GABA_A$ receptors, and that acute elevated levels of MG induce locomotor depression and ataxia [9]. Accordingly, we found that a high concentration of MG (3 μM/μL) evoked a decrease in locomotor activity in the OF assessment, as seen by the reduction in the total distance travelled in the OF (twelve hours after surgery). Previous reports have also demonstrated similar effects on $GABA_A$ receptor activation [9, 15]. Concomitantly, our results indicated that acute MG infusion was able to generate anxiolytic-related behavior in rats (as evaluated by the plus maze task), as shown by the increase in time spent in the open arms. In addition to $GABA_A$ activation, MG levels can modulate GLO1 activity, the rate limiting step enzyme of the glyoxalase system [50]. In a chronic mouse model of inhibition of GLO1 expression, increased MG levels caused a decrease in anxiety-like behavior [51, 52]. MG has been also reported to show an acute anxiolytic effect [9, 12] and the daily injection of this molecule in rats reduced anxiety like-behavior [15]. Taken together, data suggest that anxiety behavior is dependent on the time of exposure and that acute MG administration affects this behavior.

For cognitive performance analysis, two different learning and memory tasks were performed. Firstly, in the NOR test, we found that MG-treated rats showed a decline in STM and LTM, consistent with the impairment in short-term spatial memory, evaluated by the Y-maze. Herein, we found that a cognitive deficit occurred in a brief period of time (thirty-seven hours after surgery) in different memory types. Our data corroborate those of previous studies, which suggest that MG induces a cognitive deficit within a short time [12]. Previous studies have demonstrated that MG accumulation, common in aged or diabetic patients, may be related to cognitive deficits [46]. A high serum level of MG was associated with a faster rate of cognitive decline [53, 54] and may contribute to the decline in executive function performance and memory in non-demented elderly patients [55]. In addition, reports have shown that MG cell treatment induces apoptosis in hippocampal neurons via an impairment of mitochondrial functions [45]. It should be noted that MG acts as a potent precursor for AGEs, which in turn are associated to cognitive dysfunction [56]. However, we did not investigate AGE/RAGE signaling, but considering the high dose and short protocol for administration, we suggest that both mechanisms - glycation and a direct effect on receptor - may be involved in the events observed.

The impairment of glutamate metabolism in astrocytes may be the basis of the cognitive deficit, induced by MG. In fact, MG decreased the glutamate uptake in hippocampal slices. This decrease was not accompanied by a decrease in the major astroglial glutamate transporters, GLAST and GLT-1, which is in agreement with other studies which indicated no differences in astrocyte glutamate transporter levels in an animal model of DM [57]. Aminoguanidine, an antiglycant compound, prevented the effect of MG on glutamate uptake in astrocyte cultures [11]. Therefore, the decrease in the activity of these transporters observed in hippocampal slices may be caused directly by glycation. Further investigating the glutamatergic system, we did not observe changes in the hippocampal content of GLUN1, a subunit of the NMDA glutamate receptor commonly involved in the glutamate excitotoxicity. This absence of alteration in GLUN1 does not exclude the possibility of excitotoxicity caused by a reduced astroglial glutamate uptake. However, a possible elevation of extracellular levels of glutamate was not confirmed by an increase in CSF glutamate. Such glutamate elevation in CSF would reinforce glutamate excitotoxicity, but its absence also does not exclude this possibility, since glutamate levels in the CSF do not reflect necessarily the extracellular levels of this neurotransmitter in the hippocampus.

Importantly, we observed a decrease in the activity of GS, the enzyme that converts glutamate to glutamine, which in turn is released to neuronal glutamate synthesis. GS in the brain tissue is exclusively glial and GS decrease impairs the glutamate-glutamine cycle and, consequently, glutamatergic

transmission [58, 59]. It seems clear that elevated MG affects astroglial function and that this, consequently, changes important systems of neurotransmission, which could also contribute to explain the memory impairment observed here.

The glyoxalase system, as discussed before, is responsible for preventing MG-induced dicarbonyl stress. This enzymatic pathway is dependent mainly on the endogenous antioxidant, GSH [50]. In our study, MG administration caused a depletion in GSH content, which was observed seventy hours after surgery. Nonetheless, GLO1 activity and protein expression were not significantly altered in the hippocampus. A previous study in astrocyte cultures demonstrated that GLO1 activity is increased at twenty-four hours after MG exposure [11]. We cannot rule out a fast and transient increment of GLO1 *in vivo*, but we only measured this enzyme seventy-hours after MG insult. GSH is an important antioxidant in the brain tissue, and is mainly produced and recycled by astrocytes [60]; as such, its decrease indicates astrocyte dysfunction; the decrease in GSH content observed may be explained, in part, by MG consumption, in turn forming S-D-lactoylglutathione, the substrate of GLO1. Thus, MG traps GSH. On the other hand, a decrease in GSH content may be explained by the decrease in glutamate uptake, since glutamate is a substrate for GSH synthesis [61]. Notably, GSH is exported by astrocytes to maintain GSH levels in neurons, via astrocyte-neuron shuttles for GSH. Moreover, extracellular GSH can also act as a neuromodulator of the glutamatergic system. In fact, at this point, it is necessary to consider that the action of MG (beyond protein glycation and the *per se* effect on GABA receptors) alters the concentration of two other neuroactive compounds. It causes an increase in D-lactate (the product of MG detoxification) and a decrease in GSH, which can affect glutamatergic, GABAergic and dopaminergic neurotransmission (see [6] for a review) and may also explain the cognitive deficit induced by MG here.

S100B is a calcium-binding protein that is predominantly expressed and secreted by astrocytes [62]. Extracellular S100B has been used as a marker of glial activation in response to injury stimuli [63]. Herein, we observed an increase in CSF S100B; however, the increase in CSF S100B was not accompanied by changes in serum S100B, confirming the idea that changes in CSF S100B are not necessarily related to serum S100B [64]. It is already known that CSF S100B is elevated in response to acute brain injury conditions [64, 65] and earlier stages of AD [66]. A decrease in hippocampal mRNA levels of S100B was also observed, without a corresponding decrease in S100B protein content. Changes in S100B mRNA do not necessarily accompany changes in the protein content [67], but note that we measured S100B parameters only at seventy-hours and, in further studies, other time points could allow us to better understand such variations. In addition to the increase in extracellular S100B, we observed an upregulation of the mRNA for interleukin-1β (IL-1β), suggesting an inflammatory process induced by MG, possibly by activation of RAGE. S100B both stimulates primary inflammatory cytokines (e.g. IL-1beta) and responds to them [68, 69]. Extracellular S100B functions as a neurotrophic cytokine and has, more recently, been considered an alarmin [70, 71].

In contrast, we did not find changes in GFAP hippocampal content (measured by ELISA), or in CA1 hippocampal region (evaluated by immunohistochemistry). Although there are only a few studies that show hippocampal GFAP alterations in MG-treated rats, this finding is in agreement with those of our previous study [10]. Conversely, MG administration has been shown to induce hippocampal astrocyte reactivity (GFAP and S100B increases), as reported by Chu et al. [72], however, this divergence could be explained by methodological differences, since authors evaluated astrocyte reactivity after chronic MG treatment (6 weeks after daily MG i.p doses). The cited study also reported an inflammatory response to MG, in which the c-Jun-N-terminal-kinase (JNK) pathway was strongly activated which may be related also to upregulation of IL-1β mRNA confirmed in this study.

Therefore, the ability of MG to affect astrocyte-neuron glutamatergic metabolism is quite clear. However, the precise mechanism by which this occurs deserves further investigation. MG-induced damage may be due to RAGE activation or to the direct glycation of cellular signaling pathways in brain tissue. This means that signaling pathways in neurons could be directly affected by MG or via RAGE [46]. In spite of this direct effect on neurons, our data indicate an important involvement of astrocytes in the brain injury induced by ICV administered MG. MG-induced brain damage was confirmed by cognitive deficit in behavioral tasks that depend on hippocampal functional integrity. In the hippocampal tissue, we found reduced glutamate uptake activity, as well as a decrease in GS activity and reduced levels of GSH. It is possible that MG directly, or via RAGE, caused astrocyte dysfunction, which in turn affected neuronal signaling and cognitive performance. Changes in glutamate uptake and GS activity, caused by elevated levels of MG, affected glutamatergic communication, and may alter the levels of other compounds that modulate synapses, such as D-lactate and GSH. Our data contribute to understanding MG toxicity and place astrocytes as targets and mediators of this toxicity.

It is important to mention some limitations of this study. Firstly, the MG concentration under pathological conditions (e.g. DM) is lower than that used in this study. However, this concentration was based on previous study that employed the administration of MG by ICV and seemed adequate for the investigation of the acute effect of this compound. Secondly, euthanasia was performed only seventy hours after the administration of MG. Shorter times would allow a better

correlation between neurochemical changes and behavioral changes. Finally, we used a commercial MG and did not analyze the purity of this compound. We cannot exclude the possibility that impurities may have contributed to the observed effects.

Conclusions

Acute ICV administration of MG impairs short- and long-term learning and memory processes, based on the NOR and Y-maze tasks, which depend on hippocampal functional integrity. In the hippocampal tissue, we found reduced glutamate uptake and GS activity, as well as reduced levels of GSH. In association with changes in S100B, these data suggest that astrocytes are targets of MG toxicity, despite displaying high activity of the glyoxalase system, which eliminates this metabolite. All these possibilities reinforce the role of astrocytes in neurotransmission and place them as targets of MG toxicity, suggesting their participation in neurodegenerative diseases such as DM and AD.

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Contribution Statement F.H., C.A.G. and L.J.L. designed the study. L.J.L., E.B., L.R., K.M.W., L.D.B., F.U.F., F.H performed laboratory experiments and collected data. L.J.L. and K.M.W performed statistical analyses. F.H., C.A.G., L.J.L, K.M.W., L.D.B and L.R. wrote the manuscript. All authors edited and approved the manuscript.

Disclosure of Potential Conflicts of Interest The authors declare that they have no conflicts of interest.

Data Availability The data used to support the findings of this study are available from the corresponding author upon request.

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4 CAPÍTULO II

Article

Acute methylglyoxal-induced damage in blood-brain barrier and hippocampal tissue

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Acute methylglyoxal-induced damage in blood-brain barrier and hippocampal tissue

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Abstract

Methylglyoxal (MG) is a reactive dicarbonyl compound formed mostly by the glycolytic pathway. Elevated blood glucose levels can cause MG accumulation in plasma and cerebrospinal fluid of diabetes mellitus and Alzheimer's disease patients, where the high reactivity of MG leads to modification of proteins and other biomolecules, generating advanced glycation end products (AGEs) appointed as mediators in these neurodegenerative diseases. Herein, we investigated the blood-brain barrier (BBB) integrity and astrocyte response in the hippocampus to acute insult induced by MG, intracerebroventricularly administered in rats. Seventy-two hours later, a loss of BBB integrity was observed, as assessed by the entry of Evans dye into brain tissue and albumin in the CSF, as well as a decrease of aquaporin-4 and connexin-43 in hippocampal tissue. MG did not induce changes in hippocampal contents of RAGE in this short time, but decrease expression of S100B, an astrocyte secreted protein that binds RAGE. The expressions of two important transcription factors of antioxidant response, NfkB and Nrf2, were not changed. However, hemeoxigenase-1 was upregulated in MG-treated group. This data corroborates with the idea that astrocytes, the main cells responsible for MG clearance, are targets of MG toxicity and that BBB dysfunction induced by this compound may contribute to behavioral and cognitive alterations observed in these animals.

1. Introduction

The blood-brain barrier (BBB) is a complex structure of cells, including endothelial cells, pericytes and astrocytes, which together, regulate transport of specific molecules into the brain, limiting the entry of blood-derived substances (e.g. immunoglobulins, albumin) (Hawkins and Davis 2005; Engelhardt and Sorokin 2009). Astrocytes contain proteins that are essential to ensure BBB functionality, such as aquaporin 4 (AQP4), which plays a critical role in the cellular water content and flow (Niermann et al. 2001; Badaut et al. 2014), and connexin 43 (CX43), which is a predominant gap junction protein in astrocytes, regulating the passage of ions and metabolites (Lapato and Tiwari-Woodruff 2018; Xing et al. 2019). Alterations in AQP4 and CX43 expression has been associated with several physiological and pathological conditions (Sáez et al. 2003; Zhang et al. 2015; Verkman et al. 2017; Mader and Brimberg 2019).

Methylglyoxal (MG) is a reactive dicarbonyl compound formed mainly by glycolytic pathway (Thornalley 1993; Angeloni et al. 2014). The chronic elevation of blood glucose results in MG accumulation in plasma and cerebrospinal levels that are encountered in patients with diabetes mellitus (DM) and Alzheimer's disease (AD) (Thornalley 1993; Kuhla et al. 2005). The high reactivity of MG leads to modification of proteins and other biomolecules, generating advanced glycated end products (AGEs) (Ahmed and Thornalley 2003; Rabbani and Thornalley 2008). Studies with diabetes models in rodents had presented that MG induces a brain endothelial cells dysfunction through barrier-protein glycation (Li et al. 2015), impairing the BBB function and integrity (Beeri 2011; Rabbani and Thornalley 2015; Hussain 2016), triggering inflammatory response, increment of permeability, cellular morphology changes (Tóth et al. 2014b; Hussain 2016) and disrupting gap junctions (Li 2013; Tóth et al. 2014a).

The cellular accumulation of AGEs alters protein machinery including nuclear factors (Pugliese 2008), contributes to abnormal formation of proteins cross-linking, which causes the functional changes typical of chronic diabetic complications (Sell et al. 1992). The activation of receptor for of AGEs (RAGE) leads to cellular signaling involving many pathways, including the nuclear factor kB (NfkB), mainly associated to inflammatory response (Piarulli et al. 2013; Saleh et al. 2019). Together with NfkB, the nuclear factor erythroid-2 related factor (Nrf2), regulates the inflammatory response, beyond the modulation of metabolic oxidative response (Bellezza et al. 2018). Nrf2 is a protein involved in the restoring of intracellular balance between oxidants and antioxidants after oxidative insult and regulates of numerous genes and enzymes (e.g heme-oxigenase 1, HO-1) (Minelli et al. 2009; Jian et al. 2011; Zhang et al. 2013; Choi et al. 2016; Wang 2019). Nrf2 deletion may contribute to the increased sensitivity to oxidative stress and inflammation (Jakel et al. 2007; Ichihara et al. 2010; Taki-Nakano et al. 2014; Zhao et al. 2019). It has been demonstrated that Nrf2 mediates signaling pathway against MG toxicity (de Oliveira et al. 2015) as well as prevents the damage caused by high concentrations of glucose in animal models of diabetes (Choi et al. 2016; Zhao et al. 2019).

There is evidence that chronic high levels of MG alter BBB and consequently contribute to brain changes observed in DM and neurodegenerative diseases (Fang et al. 2015, p. 201; Hussain 2016; Kim et al. 2020). However, few studies address the acute effect of MG, particularly *in vivo* on barrier integrity and neuroglial functions. In previous studies of our group, we observed that acute intracerebroventricular (ICV) administration of elevated concentrations of MG impairs short-and long-term learning and memory processes and glial function changes (Lissner et al. 2021). Seeking for a better understanding of this previous findings, herein, our focus was to investigate how high

concentration of MG applied by i.c.v. route affects the BBB function and integrity, and if this fact could modulate inflammatory pathways and cellular stress response in hippocampal astrocytes.

2. Materials and methods

2.1 Reagents

Methylglyoxal (MG) solution (40% in H_2O), Triton X-100, o-phthaldialdehyde, S100B protein, anti-S100B (SH-B1) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Polyclonal anti-S100B was purchased from DAKO (São Paulo, SP, Brazil) and Evans blue Dye (EBD) (Neon Comercial, São Paulo, Brazil). Immunoblots were performed using the following antibodies: Rabbit polyclonal (anti-AQP4 and anti-Cx43), mouse-monoclonal (anti- RAGE and anti-Synaptophysin), purchased form Millipore, Darmstadt, Germany and HRP-conjugated β-actin antibody (Proteintech, Rosemont, IL, USA). Peroxidase secondaries antibodies from GE (Little Chalfont, United Kingdom). All others chemicals were purchased from local commercial suppliers.

2.2 Animals

Male Wistar rats (90-days old) were obtained from our breeding colony (Department of Biochemistry, UFRGS, Porto Alegre, Brazil). The rats were maintained under controlled light and environmental conditions (12 h light/12 h dark cycle at a constant temperature of 22 ± 1°C) and had free access to commercial chow and water. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), and all procedures were previously approved by the local Animal Care Ethical Committee

(CEUA-UFRGS; project number 33663). All efforts were made to minimize animal suffering and reduce the number of animals used.

2.3 Surgical procedure

MG was unilaterally infused in the lateral ventricle as described in previous studies (Rodrigues et al. 2019; Lissner et al. 2021). The rats were separated in two groups: SHAM operated and MG-treated. Briefly, rats were anesthetized with ketamine/xylazine (75 and 10 mg/kg, respectively, i.p) and positioned in stereotaxic apparatus. A midline sagittal incision was made in the scalp and burr holes were drilled in the skull on right sides over the lateral ventricle. The right ventricle was accessed using the following coordinates: 0.9 mm posterior to the bregma; 1.5 mm lateral to the sagittal suture; 3.6 mm beneath the surface of the brain. Animals received 5μL of MG ICV unilaterally with a final concentration of 3μmol/μL; the SHAM group received an equal volume of Hank's balanced salt solution – HBSS, pH 7.2. After the surgical procedure, animals were kept warm until recovery from anesthesia. Approximately seventy-two hours after MG injection, rats were euthanized, and biochemical parameters were evaluated. The experimental schedule is shown in Fig. 1.

2.4 Biochemical analysis

2.4.1 Obtaining cerebrospinal fluid, serum, and hippocampal slices

Animals were anesthetized and then positioned in a stereotaxic holder for CSF collection of 100 μL (approximately) from the cisterna magna. The puncture was performed using an insulin syringe (27gauge x 1/2" length). Rats were then removed from the stereotaxic apparatus and placed in a flat place; whole blood was obtained through an

intracardiac puncture using a 0.37-mm diameter needle and was collected into clot activator tubes, centrifuged (3000 rpm, 10 min, 4°C) to separate serum. The hippocampi were dissected, and transverse of 0.3 mm were obtained using a McElwain Tissue Chopper. The samples were frozen -80°C until posterior analysis (Netto et al. 2006).

2.4.2 Determination of CSF/Serum Albumin Ratio

The CSF/serum albumin ratio was analyzed using the bromocresol green assay kit from Doles (Goiânia, Brazil) as described to (Durgawale et al. 2005), modified. 10 µL of serum or 50 µL of CSF was mixed with 2 mL of color reagent. The samples were incubated for 10 min at 37°C, and the absorbance was measured at 546 nm against a reagent blank.

2.4.3 In vivo EBD injection and dye extraction

BBB permeability was evaluated according to the distribution in the rat brain of EBD administered at a dose of 2 mg/kg by an intracarotid injection (Manaenko et al. 2011; Wang and Lai 2015). After a two-hours period to enable uniform EBD distribution, the rats were anesthetized using ketamine/xylazine and were perfused through the left cardiac ventricle with 200 mL of saline solution. The brains were removed, weighed and the content of the dye extracted from each brain was determined with a spectrophotometer (at 620 nm). The quantitative calculation of the dye content was based on (Wang and Lai 2015).

2.4.4 Western blot analysis

Proteins from hippocampal sample were homogenized in a sample buffer [62.5 mM Tris–HCl pH 6.8, 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 10% (v/v) glycerol,

0.002% (w/v) bromophenol blue] and separated by SDS-PAGE on 12% sodium dodecyl sulfate polyacrylamide and electro transferred onto nitrocellulose membranes. Membranes were blocked overnight at 4°C with 2% chicken egg albumin in Tris-buffered saline with Tween 20 (TTBS). After incubation overnight at 4°C with primary antibodies (anti-AQP4, anti-Cx43, anti-RAGE and anti-Syn at dilution 1:5000), the membranes were incubated for 1 h at room temperature and exposed to horseradish peroxidase-linked anti-IgG antibodies. Chemiluminescent bands were detected by image analyzer (Image Quant LAS4000 from GE) and optical density was quantified using ImageJ software. The results were expressed as percentages of SHAM.

2.4.5 RNA extraction and quantitative RT-PCR

Total RNA was isolated from hippocampal slices using TRIzol Reagent (Invitrogen, Carlsbad, CA). The concentration and purity of the RNA were determined spectrophotometrically at a ratio of 260:280. 1 μg of extracted RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific) in a 20 μL reaction according to manufacturer's instructions. The messenger RNA (mRNA) encoding HMGB1 (#Rn02377062_g1), HO-1 (#Rn01536933_m1), Nrf2 (#Rn00582415_m1) and p50 NfkB (#Rn01399572_m1) were quantified using the TaqMan real-time RT-PCR system using inventory primers and probes purchased from Applied Biosystems (Bobermin et al. 2019). Target mRNA levels were normalized to β-actin (#Rn00667869_m1) levels. Results were analyzed employing the $2^{-\Delta\Delta Ct}$ method (Bobermin et al. 2020, p. 20) adapted from (Livak and Schmittgen 2001) and expressed relative to the levels of SHAM group.

2.5 Protein determination

Protein content was measured by Lowry's method using bovine serum albumin as standard (Peterson 1977).

3. Statistical analysis

Data are reported as means ± standard errors and analyzed statistically by Student's *t*-test. Differences were considered significant when $p \leq 0.05$. All analyses were performed using the Prism 6.0 (GraphPad).

4. Results

4.1 MG infusion disrupted BBB permeability and increased albumin levels

Initially we evaluated the effect of MG-ICV on functional permeability of BBB using the Evans blue staining assay and the CSF/serum albumin ratio. The concentration of EBD extracted of total brain was increased in the MG group (Fig. 2A, $p = 0.001$) and concomitantly, the CSF/serum albumin ratio was increased drastically in MG-treated group when compared with SHAM group (Fig. 2B, $p<0.001$).

4.2 Decreased of AQP4 and Cx43 levels in MG-treated group

To identify alterations in astroglial proteins related to BBB, we measured the levels of AQP4 and Cx43 by western blotting. In addition, we examined the synaptophysin levels, a protein expressed in synaptic vesicles, related to synaptic connectivity. Here, it was observed a decrease of AQP4 (Fig. 3A, $p = 0.04$) and CX43 (Fig. 3B, $p = 0.01$) levels without differences in the synaptophysin content (Fig. 3C).

4.3 RAGE signaling is altered in MG-treated group

In order to understand if RAGE signaling is involved, we measured the contents of RAGE and S100B, a RAGE ligand secreted by astrocytes. No changes were observed in RAGE (Fig 4A, p =0.89) or S100B (Fig 4B, p = 0.19). Then we evaluated mRNA levels of S100B and HMGB1, another RAGE ligand. Interestingly, in MG-treated we observed an up-regulation of mRNA levels for S100B (Fig 4C, p =0.04), but not for HMGB1 (Fig 4D, p = 0.16).

4.4 MG exposure increased HO-1 mRNA expression in the hippocampus

Then we investigated hippocampal inflammatory pathways possibly affected by MG exposure. No changes were observed in mRNA levels for NfkB (Fig 5A, p =0.74) and Nfr2 (Fig 5B, p = 0.82) in hippocampal tissue of animals exposed to MG. However, it was observed an increase of mRNA levels for HO-1 in the MG-group (Fig. 5C, p = 0.03).

5. Discussion

The carbonyl stress is triggered by accumulation of reactive species as MG (Ramasamy et al. 2006) and the risk of glycation levels in cardiovascular and cerebrovascular system of diabetic and Alzheimer's diseases patients are elevated (Lapolla et al. 2003; Vander Jagt and Hunsaker 2003; Tóth et al. 2014a). Previous studies demonstrated that, in hyperglycemic conditions, MG induces endothelial barrier microvascular dysfunction (Li 2013; Hussain 2016; Irshad et al. 2019), a reduced integrity and increased BBB permeability (Tóth et al. 2014b, a) and contributes to a higher progression of vascular complications and cerebrovascular injury (Fang et al. 2015). Vascular alterations in diabetes are linked with breakdown of BBB functioning (Hawkins

et al. 2007) and these alterations contribute functional and structurally with brain changes and cognitive impairment observed in patients and experimental models (Serlin et al. 2011; Zanotto et al. 2017). Previously we showed that the acute insult with MG affects astroglial activity (Lissner et al. 2021) and considering the involvement of these cells in BBB, we decided to evaluate this possibility more closely.

In fact, we observed an acute BBB rupture measured by a higher EBD concentration in brain tissue and an increased CSF/serum albumin ratio in MG group. It is known that long-term incubation with high MG concentration leads a dramatic change in the permeability of BBB endothelial cells, possibly due to glycation of tight junction proteins (Hussain 2016). It has been showed that MG binds specific basic sites of proteins causing endothelial cell detachment in brain tissue (McDonald et al. 2009). Although we have not characterized the glycated proteins of BBB in this acute MG-induced model of damage, it is clear the functional impairment in BBB, characterized by the greater permeability of serum albumin and Evans dye.

A comment needs to be made here. In the previous work we showed that levels of S100B, a calcium-binding protein secreted by astrocytes, are increased in the CSF but not in the serum of animals submitted to ICV administration of MG (Lissner et al. 2021). Therefore, even if there is a clear loss of the integrity of the BBB, the S100B does not flow from the CSF to the serum in this acute model. This is especially important considering that the serum increase in S100B by many authors is attributed to the loss of BBB integrity and these data contradict this idea.

The BBB disruption may affect the balance of water and ion homeostasis as well as to impair the intercellular communication in astrocytic cells (Chu 2016; Hussain 2016). Astroglial impairment induced by MG has been described in cell cultures (Chu 2016; Hansen et al. 2017) and hippocampal slices directly exposed (Hansen et al. 2016a) and

hippocampal slices after ICV administration (Hansen et al. 2016b). Considering the importance of astrocytes to BBB, we investigated whether glial parameters related to the activity of this barrier are altered in this model of acute injury produced by MG.

Our results showed that the MG-treated group exhibited a decrease in the hippocampal AQP4 and Cx43 levels. Cx43 is an important connexin for BBB functionality because it is involved in the gap communication between endothelial cells and between astrocytes (Kovács et al. 2012; Johnson et al. 2018). AGE-albumin treatment of aortic endothelial cells reduced gap communications (Wang et al. 2011). Our data suggest the impairment of gap communication dependent on Cx43 caused by MG exposure, affects BBB functionality and astroglial syncytium in the hippocampus.

Moreover, we found a decrease of hippocampal AQP4. This water channel is highly expressed in astrocytes, including in the endfoots surrounding endothelium in the BBB. Although we do not know whether these proteins are direct targets of MG-mediated glycation, these data show, for the first time, the acute impairment of BBB cell connectivity to this glucose derivative. Changes in CX43 and AQP4 reveal an acute failure in BBB functionality due to MG exposure, which could compromise aqueous and ionic homeostasis. On the other hand, based on the unchanged levels of synaptophysin, the neuronal connectivity at this initial stage does not appear to be affected. However, altered glutamate clearance played by astrocytes and a direct effect of MG on synaptic receptors could explain behavioral and cognitive changes induced by this compound (Liu et al. 2013; Hansen et al. 2016b; Lissner et al. 2021).

MG *per se* and/or AGEs induce oxidative stress and inflammatory pathways activation through interaction with RAGE (Kalapos 2008; Ramasamy et al. 2011; Liu et al. 2013; Choi et al. 2016; MacLean et al. 2019). Exacerbated expression of RAGE has been implicated in many neurodegenerative disorders, which can be activated by AGEs

as well as the ligands HMGB1 and S100B (Sagheddu et al. 2018; MacLean et al. 2019). In this acute model of brain lesion, elevated extracellular levels S100B are observed in MG-treated animals and could contribute to RAGE activation (Lissner et al. 2021). However, the immune contents of S100B as well as RAGE are not altered in the hippocampal tissue in these animals. On the other hand, mRNA levels for S100B (but not HMGB1) are reduced in the hippocampus, confirming an astroglial commitment due to MG exposure. At this moment, we can think but do not know if this reduction in S100B gene expression is related to the reduction of protein secretion in hippocampal slices exposed directly to MG (Hansen et al. 2016b). Considering such changes, therefore, it is possible to conceive an alteration in the S100B / RAGE communication in this acute model, which could be occurring in situations of hyperglycemic peaks.

The oxidative stress caused by MG, which can be evaluated by GSH depletion (Chen 2010; Lissner et al. 2021), activates several defense and damage pathways (Di Loreto et al. 2008). We investigated the levels of two main transcription factors possibly activated in this condition – NfkB and Nfr2, as well as the HO-1, an enzyme related to neuroprotection (Nitti et al. 2018). Activation of Nfr2 has been associated on modulation of antioxidant and detoxifying enzymes expression, including HO-1 (Syapin 2009; Calkins et al. 2009; Niture et al. 2014) and its absence is related to cellular damage by increase of oxidative stress molecules and inflammation (Ichihara et al. 2010; Taki-Nakano et al. 2014). Oxidative stress environment can activate also NfkB and concomitantly releasing proinflammatory cytokines (Patel and Santani 2009). We observed no changes in expression of Nrf2 and NfkB under acute MG exposure. However, the HO-1 mRNA expression was increased. These data suggest that this up-regulation of HO-1 would be a protection response against the acute insult generate by MG. These data confirm the importance of HO-1 against MG toxicity and point out that

other transcription mechanisms, in addition to Nfr2 and NF-kB, must be involved in the most initial antioxidant response (Nitti et al. 2018).

It is important to mention some limitations of this study. Firstly, the MG concentration under pathological conditions (e.g. diabetes), is lower than that used in this study. However, this concentration was based on previous studies of ICV administration, and it seemed appropriate to study an acute MG insult in BBB. Secondly, euthanasia was performed seventy-two hours after the administration of MG and shorter times would allow a better temporal correlation between functional and neurochemical changes. However, in 72 hours it is possible to disregard the free circulating presence of MG since the endogenous metabolism by glyoxalase system is approximately 10 minutes. Finally, we used a commercial MG and did not analyze the purity of this compound. We cannot exclude the possibility that impurities may have contributed to the observed effects.

6. Conclusions

Our findings demonstrated that elevated levels of MG, here administered by ICV infusion, were able to induce loss of BBB integrity in short time, measured by increment of permeability and decrease of AQP4 and Cx43 levels. This reinforce the idea that astrocytes, the main cells responsible for MG clearance, are targets of this compound and that BBB dysfunction may contribute to behavioral and cognitive alterations observed in these animals. The decrease of mRNA for S100B emphasizes the glial susceptibility to damage caused by MG. The defense against the acute MG-induced oxidative stress involves an upregulation of HO-1, whose initial expression does not seem to involve concomitant Nfr2 and/or NfkB expression.

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Figure legends

Figure 1

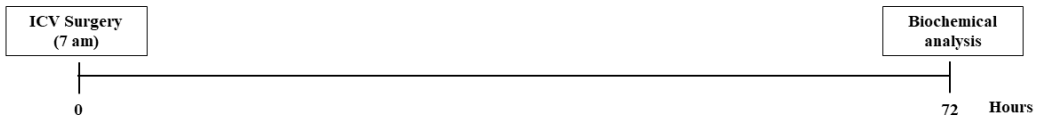


Figure 1. **Experimental timeline.** Surgery corresponds to unilateral ICV infusion of MG (single injection of 5µL with final concentration of 3µM/µL or vehicle). Time is represented in hours after surgery. Cerebrospinal fluid, hippocampi and serum were harvested and biochemical analyses are performed seventy hours after the surgery.

Figure 2

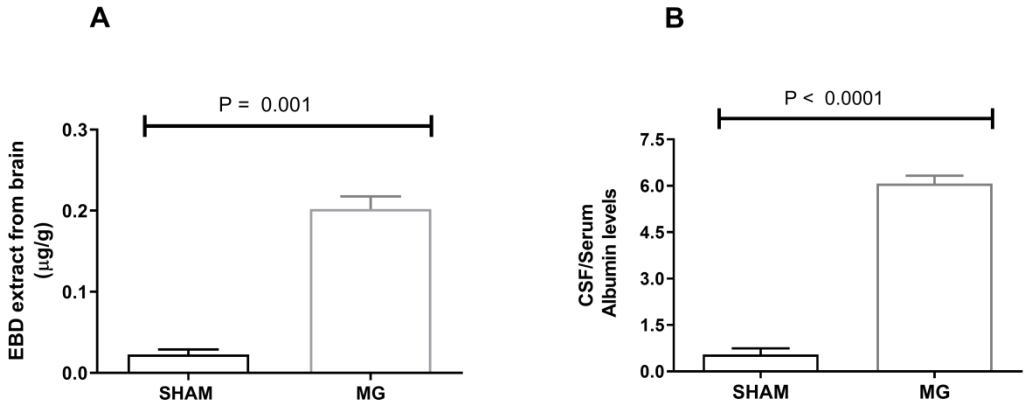


Figure 2. Effect of ICV administration of MG on function and integrity of BBB. It was measuring seventy-two hours after surgery. The concentration of EBD was extracted from total brain and measured by spectroscopy (absorbance at 620nm) (A) and CSF/serum albumin ratio was measured by bromocresol green assay kit (B). Data are expressed as means ± S.E.M (N = 4-6 animals per group). Data were analyzed by Student's *t*-test assuming $p<0.05$ and the statistical significance represents difference from the SHAM group.

Figure 3

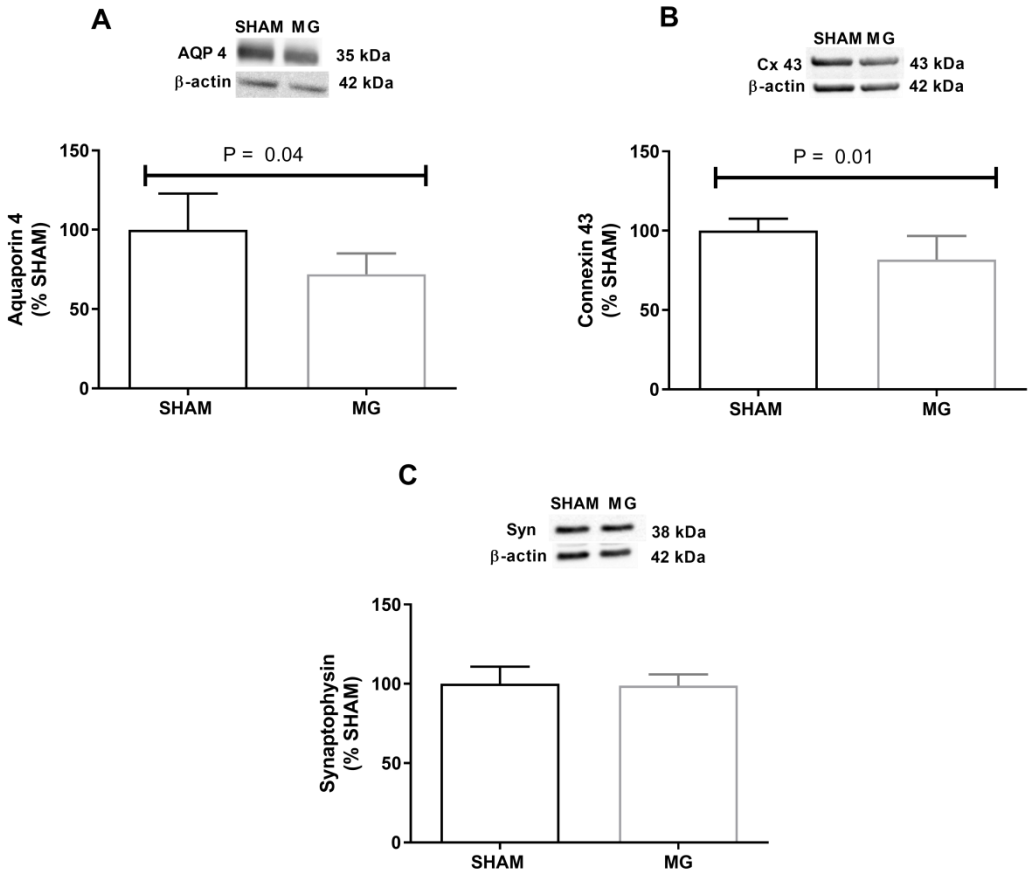


Figure 3. Effect of ICV administration of MG on the astroglial connectivity. It was measuring seventy-two hours after surgery. Hippocampal slices of rats were dissected out and immunocontents of hippocampal Aquaporin 4 (AQP4), Conexin 43 (Cx43) and Synaptophysin (A, B and C, respectively) was determined by western blotting and normalized by actin and assuming SHAM value as 100%. Data are expressed as means ± S.E.M (N = 3-11 animals per group). Data were analyzed by Student's *t*-test assuming $p<0.05$ and the statistical significance represents difference from the SHAM group.

Figure 4

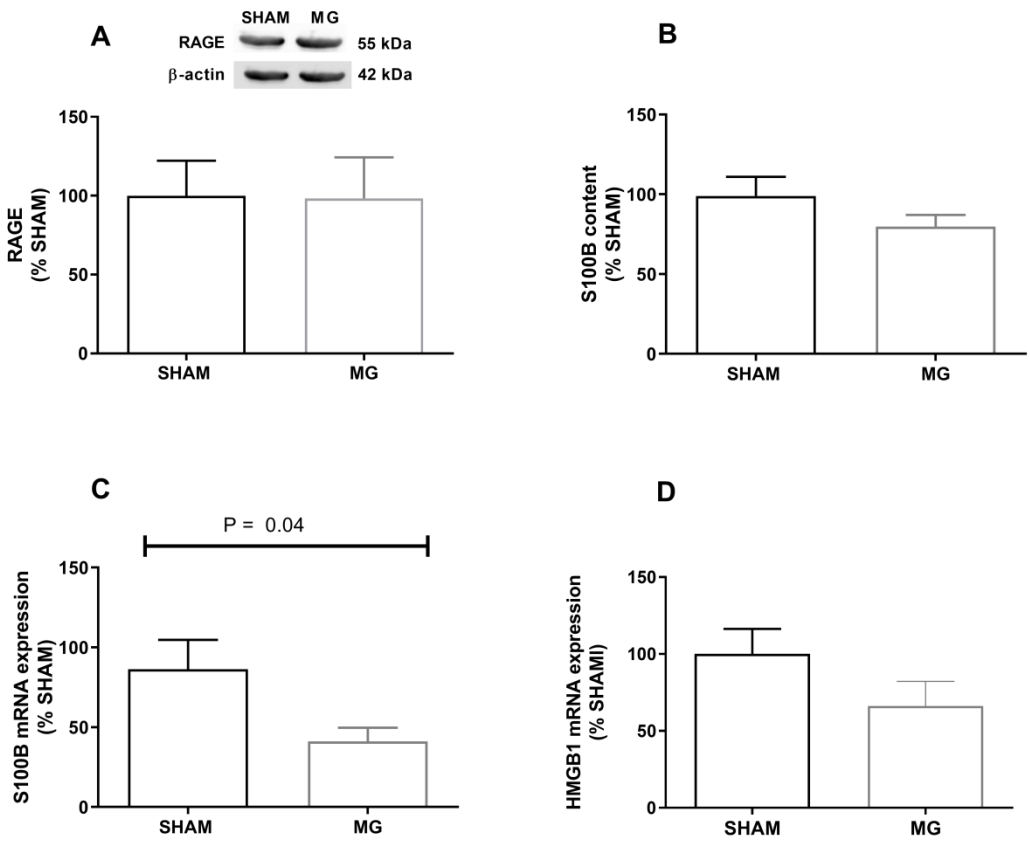


Figure 4. Effect of ICV administration of the RAGE signaling. It was measuring seventy-two hours after surgery. Hippocampal slices of rats were dissected out and the S100B content, measured by ELISA (A), S100B (B) and HMGB1 (C) mRNA expression and immunocontents of RAGE (D) determined by western blotting and normalized by actin. Data are expressed as means ± S.E.M (N = 5- 13 animals per group) assuming SHAM as 100%. SHAM value of S100B content was 0.67 nmol/mg protein in hippocampus. Data were analyzed by Student's *t*-test. assuming $p< 0.05$ and the statistical significance represents difference from the SHAM group.

Figure 5

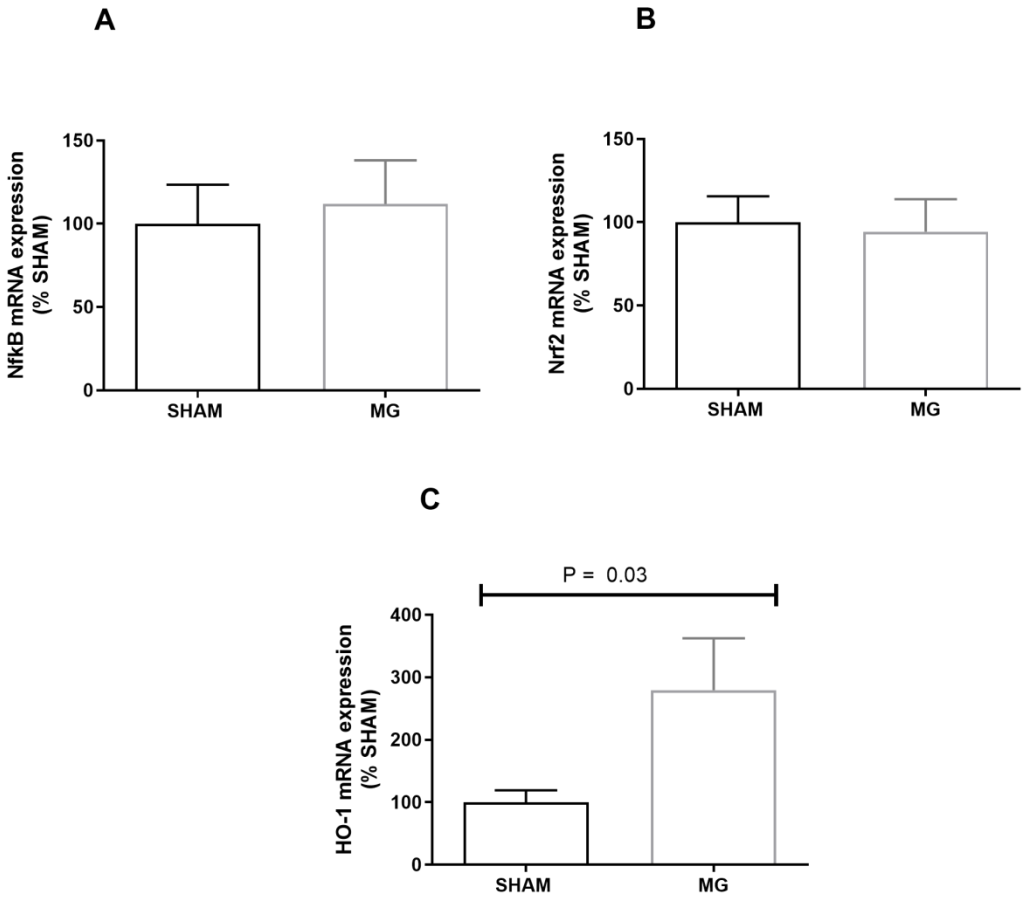


Figure 5. Effect of ICV administration of MG on the Nrf2/HO-1 pathway. It was measuring seventy-two hours after surgery. Hippocampal slices of rats were dissected out and mRNA expression of NfkB (A), Nrf2 (B) and HO-1 (C) were measured. Data are expressed as means ± S.E.M (N = 5- 8 animals per group assuming SHAM value as 100%. Data were analyzed by Student's *t*-test. assuming $p < 0.05$ and the statistical significance represents difference from the SHAM group.

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PARTE III

5 DISCUSSÃO

Conforme dados de órgãos mundiais da saúde, há um estimado aumento da prevalência em escala global, de DM e doenças neurodegenerativas como a DA nos próximos anos (Alzheimer's Association, 2016; American Diabetes Association, 2020) . Embora muitos grupos de pesquisa têm aprofundados os estudos acerca dos processos envolvidos na etiologia e patogenia dessas desordens, um total entendimento ainda não é completamente elucidado. Nos últimos anos, abrangentes evidências, baseadas em estudos clínicos e experimentais indicam que disfunções no metabolismo da glicose e gerações de intermediários metabólicos reativos como o MG contribuem para as complicações metabólicas observadas em pacientes diabéticos e, às disfunções cerebrais observadas em doenças neurodegenerativas como a DA (Schalkwijk and Stehouwer, 2020; Yang and Song, 2013). Além disso, tem sido extensivamente estudado a relação entre hiperglicemia no DM não-tratado e a incidência de demência, principalmente demência do tipo Alzheimer (Biessels and Despa, 2018; Kopf and Frölich, 2009). Estudos anteriores têm demonstrado também que, em condições de hiperglicemia, o MG induz a disfunções microvasculares de células endoteliais da BHE (Hussain, 2016; Irshad et al., 2019; Li, 2013), reduzindo a integridade e permeabilidade celular (Tóth et al., 2014b, 2014a). Dessa forma, podemos afirmar que o MG *per se* ou mediando glicação pode ser, ao menos em parte, responsável pelo rápido declínio cognitivo, bem como às exacerbadas respostas celulares geradas, como o estresse oxidativo celular e ativação de processos inflamatórios (Angeloni et al., 2014; Di Loreto et al., 2004, 2008; Kuhla et al., 2005; Liu et al., 2013; Rabbani and Thornalley, 2008; Sena et al., 2012).

O SNC, devido à alta demanda energética proveniente da glicose, apresenta uma maior susceptibilidade a toxicidade induzida pelo MG, colocando sobretudo os astrócitos, como o alvo principal devido ao papel na neuroproteção neuroglial, representado pelo

alto metabolismo no sistema de enzimas glioxalases (responsáveis pela detoxificação celular do MG) (Allaman et al., 2015; Bélanger et al., 2011).

Na presente tese, foram obtidos diversos resultados gerando dois artigos científicos. Os resultados foram apresentados em dois capítulos, ambos estudando como altas concentrações de MG a curto prazo, induzem alterações comportamentais e astrogliais (Capítulo 1), e como parâmetros gliais relacionados a funcionalidade da BHE estariam sendo modulados (Capítulo 2) em ratos Wistar machos adultos. A discussão referente a esses achados, segue abaixo.

Avaliação dos efeitos do MG em parâmetros comportamentais 12, 36 e 60 horas após a administração ICV

Para a realização deste estudo, o MG foi administrado ICV na concentração de 3μmol/μL em três diferentes grupos: Naive (ratos não submetidos ao procedimento cirúrgico), SHAM-operados e grupo MG. Após diferentes intervalos de tempo (12, 36 e 60 horas) tarefas de comportamento, relacionados a aprendizagem e memória, e a ansiedade bem como a atividade locomotora foram avaliados.

Doze horas após a cirurgia, a atividade locomotora e o comportamento do tipo-ansioso foram avaliados no teste do campo aberto e EPM, respectivamente. A performance cognitiva, tanto para a memória de reconhecimento quanto para a memória espacial, foi avaliada 36h após administração do MG.

A hipótese do nosso trabalho se baseia na busca pelo melhor entendimento de achados de estudos anteriores do nosso grupo, no qual Hansen et al (Hansen et al., 2016a) observou que a administração ICV de MG modularia comportamentos relacionados a ansiedade e processos de aprendizagem e memória em ratos, a longo prazo (quatro semanas). Embora os níveis de MG usados no estudo sejam muito maiores do que aqueles apresentados em complicações diabéticas, nós propormos reproduzir como esse aumento preponderante de MG endogenamente formado no encéfalo, inferiria em alterações comportamentais, abrangendo a ansiedade e processos da aprendizagem e memória, a curto prazo, e translacioná-lo aos efeitos, de forma crônica. Adicionado a isso, embora existam na literatura vários estudos buscando entender o envolvimento do MG nas complicações do DM e em doenças neurodegenerativas, a maioria dos estudos sobre o efeito do MG, a curto prazo, não são *in vivo.*

Além disso, nossa hipótese é respaldada por evidências indicando que o acúmulo de MG, comum na idade e em pacientes diabéticos têm sido relacionados com um rápido

declínio cognitivo, o qual contribui para déficits no processamento de informações e atenção e aumento da atrofia encefálica em idosos não-dementes (Di Loreto et al., 2008; Srikanth et al., 2013; West, 2014). O aumento de AGEs circulantes provenientes da dieta também estão associados ao declínio cognitivo apresentado em modelos experimentais e clínicos (Cai et al., 2014; West et al., 2014).

Em relação a performance cognitiva, duas diferentes tarefas para a avaliação da aprendizagem e memória foram realizadas. Primeiramente, no teste de reconhecimento de objetos, nós observamos que os ratos tratados com o MG apresentaram déficits cognitivos em memórias de curta e longa duração, consistentes com o prejuízo na memória espacial de curto prazo avaliada no teste do Y-maze. Embora haja poucos estudos na literatura que avaliam o efeito cognitivos do MG, nossos achados são respaldados por estudos anteriores que sugerem que o MG induz a déficits cognitivos em breves intervalos de tempo (3.6 horas – 11 dias) após a administração (Szczepanik et al., 2020), e que um aumento nos níveis de MG pode ser indicativo de lesão das células cerebrais iniciada antes do comprometimento cognitivo ser clinicamente evidente (Beeri, 2011). Dessa forma, é possível inferir que a elevação nos níveis de MG no encéfalo, afetaria já em curto intervalo de tempo processos de aprendizagem e memória e sendo assim, a susceptibilidade hipocampal à citotoxicidade do composto. Estudos anteriores têm demonstrado que o MG, *in vitro*, causou diminuição da neurogênese bem como o enfraquecimento da capacidade de detoxificação e morte celular apoptótica nos neurônios hipocampais de ratos (Chun et al., 2016; Di Loreto et al., 2004, 2008). Neste trabalho, embora não avaliado a formação de AGEs, é sabido que o MG se liga irreversivelmente a proteínas plasmáticas em 3,6 horas (Rabbani and Thornalley, 2015) o qual reforçaria nossa hipótese que o MG *per se*, ou mediando processos de glicação estaria envolvido nos prejuízos apresentados nos processos cognitivos.

Em adição, nossos resultados indicaram que a infusão aguda do MG foi capaz de gerar comportamento ansiolítico nos ratos (avaliados pelo *Plus maze*), representado pelo aumento da permanência nos braços abertos do aparato. Isso sugere a importância na modulação dos níveis do MG no SNC, já que em concentrações fisiológicas o composto age como um agonista parcial de receptores $GABA_A$ (do inglês, $GABA_AR$) (Distler et al., 2012; McMurray, 2016). A observação do efeito ansiolítico em ratos induzidos pelo MG, a curto prazo, anteriormente já tem sido reportada (Distler et al., 2012; Szczepanik et al., 2020) bem como a redução no comportamento do tipo ansioso através injeções diárias de MG (Hambsch et al., 2010). Concomitantemente, nós observamos que no grupo MG, os ratos apresentaram um decréscimo na atividade locomotora medida 12 horas após a administração ICV, no campo aberto. Isto está de acordo com estudos na literatura que demonstraram que a ativação de $GABA_AR$ é caracterizado por efeitos agudos como decréscimo na atividade locomotora, ataxia e hipotermia (Distler et al., 2012). Embora nós não avaliamos pontualmente o efeito do MG na atividade locomotora nas diferentes tarefas comportamentais usadas nesse trabalho, e nos demais intervalos de tempo, vale ressaltar aqui que esse prejuízo locomotor não foi capaz de afetar os níveis de exploração importantes para a avaliação da memória, na fase treino do NOR, 36 horas após a administração do MG, já que os índices de exploração do grupo MG, durante a fase treino foram satisfatórios. Somado a isso, como nós avaliamos o efeito do MG em um pequeno intervalo de tempo, foi adicionado um grupo Naive para excluir o viés da cirurgia nos testes comportamentais. Esses achados fortalecem a ideia de que altas concentrações de MG são capazes de induzir a ativação de $GABA_AR$, representada pela alta sinalização inibitória, o que por sua vez, pode influenciar diferentes efeitos fisiológicos e comportamentais (Chua and Chebib, 2017).

Avaliação dos efeitos do MG na BHE e sobre a função neuroglial, 72 horas após a administração ICV

Nessa tese nós analisamos parâmetros gliais 72 horas após a administração do MG e investigamos, sobretudo se a funcionalidade da BHE estaria alterada nesse modelo de injúria aguda ao MG.

Observamos que a concentração de MG (3μmol/μl) foi capaz de diminuir a captação de glutamato nas fatias de hipocampo. Esse decréscimo não foi acompanhado por mudanças na quantidade dos transportadores, GLAST e GLT-1, o qual está de acordo com achados indicando que não há diferenças nos níveis dos transportadores em um modelo de DM (Coleman et al., 2004). Portanto, nossos resultados sugerem que o MG modularia essas mudanças através da glicação. O comprometimento da captação de glutamato, aumentaria os níveis do neurotransmissor na fenda sináptica e desse modo, geraria disfunção glutamatérgica. Um estudo anterior do nosso grupo, Hansen, 2017 (Hansen et al., 2017), culturas de asctrócitos incubadas com MG e Aminoguadinina (AG), um potente agente anti-glicante, demonstrou uma reversão na captação de glutamato. Outro estudo também demonstrou que o MG foi capaz de diminuir a captação de glutamato através da inativação do transportador de glutamato GLT-1, atribuído a formação do aduto GLT-1 – CML (Kawaguchi et al., 2005). A seguir, nós avaliamos se o MG induziu a mudanças no conteúdo hipocampal do GLUN1, uma subunidade do receptor de glutamato, NMDA, comumente envolvida na excitoxicidade glutamatérgica. Nenhuma mudança foi observada, porém esta ausência não exclui a possibilidade de relação com a excitoxicidade causada pela reduzida captação de glutamato astroglial. Estudos anteriorestêm apontado uma ativação do receptor após o tratamento com o MG (Garcia de Arriba et al., 2006), alterações na transmissão glutamatérgica em receptores de glutamato em modelo experimental de diabetes (Gardoni et al., 2002). A

excitotoxicidade pode ser reforçada também pelo aumento nos níveis de glutamato no LCR. Neste trabalho, embora esse aumento não foi confirmado, nós não excluímos a possibilidade de excitotoxicidade, já que os níveis de glutamato no LCR não refletem necessariamente os níveis extracelulares desse neurotransmissor no hipocampo.

Para prevenir excitotoxicidade após a neurotransmissão, o glutamato é rapidamente removido da fenda sináptica e transformado em glutamina pela enzima específica de astrócitos, a glutamina sintesase (GS), e posteriormente liberada para o meio extracelular e captada pelos neurônios para a síntese de glutamato novamente (Bak et al., 2006). O decréscimo na atividade da GS está relacionado ao prejuízo no ciclo glutamato-glutamina e consequentemente, na neurotransmissão glutamatérgica (Coulter and Eid, 2012; Rose et al., 2013). Importantemente, nós observamos um decréscimo na atividade da GS. Esses resultados permitem esclarecer que elevados níveis de MG afetam a função astroglial e consequentemente, alterações na sinalização glutamatérgica. Todos esses achados contribuem inclusive para a base da explicação dos déficits cognitivos observado nos ratos tratados com o MG, já que o glutamato é um importante neurotransmissor na sinalização de vários processos, incluindo o processo de aprendizagem e memória.

Como já mencionado nessa tese, o sistema glioxalase (composto pela Glo1 e Glo2) é o principal sistema de detoxificação na prevenção do estresse dicarbonílico induzido pelo MG, o qual é dependente da glutationa reduzida como cofator (GSH) (Rabbani and Thornalley, 2014b). No presente estudo, o grupo MG apresentou um decréscimo no conteúdo da GSH, sem alterações na atividade e conteúdo da Glo1. Estudos anteriores observaram um aumento da atividade da Glo1 após 24 horas de exposição ao MG (Hansen et al., 2012, 2017). Contudo, aqui, nós medimos apenas o efeito do insulto ao MG, 72 horas após a administração, e não descartamos a possibilidade de um rápido e transiente incremento da Glo1. A GSH é um importante antioxidante

endógeno, principalmente produzido e reciclado pelos astrócitos (Dringen, 2000), decréscimos nos níveis dessa molécula indicam disfunção astrocitária. GSH é exportado pelos astrócitos para manter os níveis de GSH nos neurônios. Além disso, a GSH extracelular pode agir com um neuromodulador no sistema glutamatérgico. A ação do MG (além da glicação e o efeito em receptores GABA e outros moduladores sinápticos como a GSH), aqui descritos, podem estar relacionados a um aumento na formação de moléculas como D-Lactato, contribuindo assim para alterações na neurotransmissão glutamatérgica, GABAérgica e sobretudo dopaminérgica (Gonçalves, 2019), os quais também podem contribuir também na explicação do porquê ratos tratados com MG apresentam déficits em processos relacionados a memória e aprendizado.

Recentes estudos têm sugerido o vasto e crítico papel da Glo1 no comportamento, principalmente no que se refere a regulação nos níveis celulares de MG (Distler et al., 2012; Hambsch et al., 2010). Em modelos experimentais, muitas evidências tem associado a expressão da Glo1 a ansiedade (Benton et al., 2012; Distler and Palmer, 2012; Reiner-Benaim et al., 2007), na qual a sobreexpressão do gene da Glo1 aumentaria o comportamento do tipo ansioso em ratos devido à redução no conteúdo de MG ((Distler et al., 2012; Hovatta et al., 2005; McMurray, 2016). Além disso, foi reportado que polimorfismos ao gene da Glo1 estariam envolvidos em outras desordens psiquiátricas como a depressão, autismo, esquizofrenia e dores neuropáticas (Benton et al., 2012; Bierhaus et al., 2012; Loos et al., 2009).

Como parte desse trabalho, foi avaliado também o efeito do MG na função astrocitária, representada pela expressão e conteúdo da S100B e GFAP. Na DA e outras doenças neurodegenerativas, o prejuízo na função e atividade astroglial ocorre mesmo antes dos sinais patológicos e clínicos da doença (Fakhoury, 2018; Fuller et al., 2010). A S100B é uma proteína ligante de cálcio, encontrada em altos níveis no SNC, expressa e

secretada majoritariamente por astrócitos no SNC (Gonçalves, 2008) e perifericamente pelo tecido adiposo (Haimoto et al., 1987; Michetti et al., 1983). No SNC, a S100B está localizada em grande parte nas células da glia e pode atuar como fator neurotrófico ou como uma citocina inflamatória por meio de sua ligação com o RAGE (Donato et al., 2009). Níveis extracelulares elevados têm sido reconhecidos como marcadores da ativação glial em resposta a injúrias no SNC (Steiner et al., 2007). Aqui, nós observamos um aumento do conteúdo da S100B no LCR, o qual não foi acompanhado por alterações no soro, confirmando com estudos anteriores na qual essa não correlação também foi observada (Guerra et al., 2011). É sabido que níveis elevados de S100B no LCR são respostas a condições agudas de injúria neuronal (Guerra et al., 2011; dos Santos et al., 2017), e observados em estágios iniciais da DA (Peskind et al., 2001). Aqui, um decréscimo nos níveis de RNAm hipocampal da S100B foram também observados sem uma correspondente diminuição no conteúdo da S100B, mas entende-se que alterações gênicas para a S100B não necessariamente acompanham mudanças no conteúdo proteico (Zimmer et al., 1997). Nós medimos os parâmetros relacionados a expressão gênica e da proteína S100B apenas 72 horas após a administração do MG, e talvez, caso avaliássemos outros intervalos de tempo poderiam nos fazer entender melhor tais variações. Somado ao aumento da S100B extracelular, nós um aumento nos níveis de RNAm da IL-1β. Níveis extracelulares elevados da S100B se apresentam como citocinas neurotróficas e mais recentemente, consideradas como alarminas (Bianchi, 2007; Xu et al., 2016). A secreção da S100B é seguida pela liberação de citocinas primárias como a IL-1β e, sobretudo, é modulada por estas (de Souza et al., 2009, 2013).

A exacerbada resposta do RAGE tem sido implicada em muitas doenças neurodegenerativas. A ativação desse receptor pode ser realizada por diferentes ligantes além da S100B, como os AGEs e a HMGB1 (MacLean et al., 2019; Sagheddu et al.,

2018). Neste trabalho, nós não observamos alterações no imunoconteúdo do receptor RAGE e na expressão gênica para a HMGB1 hipocampais. Considerando tais mudanças, portanto, é possível conceber uma alteração na comunicação S100B/RAGE neste modelo agudo, o que poderia estar ocorrendo em situações de picos hiperglicêmicos. Por outro lado, nós não observamos diferenças no conteúdo hipocampal da GFAP (medido por ELISA), e na região CA1 do hipocampo (avaliada por imunofluorescencia). Elevações nos níveis de GFAP são relacionados a sinais de astrogliose, envolvidos em condições severas de injúria cerebral (Brenner, 2014; Wang et al., 2018). Embora existam poucos estudos avaliando o efeito do MG *in vivo*, a curto prazo e alterações na GFAP, nossos achados estão de acordo com outros de nossos estudos anteriores (Hansen et al., 2016a). A administração do MG tem sido acompanhada de aumento na reatividade astrocitária hipocampal (aumento nos níveis de GFAP e S100B), como reportado por Chu et al. (Chu, 2016). Porém, o protocolo seguido pelo grupo do Chu avaliou o efeito crônico do MG seis semanas após a administração (doses diárias, i.p.), o que poderia ser a explicação na divergência para os resultados aqui expostos. Diferenças metodológicas nos modelos animais, heterogeneidade das células gliais, tempo das análises e ensaios para a medida de GFAP podem caracterizar as discrepâncias nos resultados em estudos experimentais e clínicos para a DM vistos na literatura (Amin et al., 2013; El-Akabawy and El-Kholy, 2014; de Senna et al., 2011). Também é importante enfatizarmos que, embora a GFAP seja o marcador mais comum de astrogliose, a ativação astroglial não necessariamente envolve aumento no conteúdo desta proteína, já que as células astrocitárias podem estar com sua morfologia alterada e não ter uma maior produção de GFAP (Liberto et al., 2004). Neste estudo(Chu, 2016) foi observado também uma resposta de ativação na via de regulação a inflamação c-Jun N-terminal cinase (*JNK)*, a qual pode estar relacionada a um aumento nos níveis de IL-1β, já descrita aqui anteriormente.

O estresse oxidativo causado pelo MG, o qual pode ser observado pela depleção do GSH (Chen, 2010; Lissner et al., 2021), ativa várias vias anti- e pró-oxidantes (Di Loreto et al., 2008). Nós investigamos a expressão dos dois maiores fatores de transcrição, o Nrf2 e o fator nuclear kB (NfkB), possivelmente ativados nessa condição, bem como a Heme oxigenase -1 (HO-1).

A ativação do Nrf2 tem sido associado na modulação de enzimas responsáveis pela detoxificação celular e antioxidantes, incluindo a HO-1 (Calkins et al., 2009; Niture et al., 2014; Syapin, 2009). Desregulação na via regulatória Keap1-Nfr2-ARE é implicado em diversas doenças, incluindo a DA e estudos com ratos *knockout* para o gene do Nrf2 apresentaram uma maior propensão ao dano celular ocasionado pelo aumento do estresse oxidativo e inflamação (Ichihara et al., 2010; Jakel et al., 2007; Taki-Nakano et al., 2014; Zhao et al., 2019). Estudos anteriores tem demonstrado que o Nrf2 mediaria vias de sinalização contra a toxicidade induzida pelo MG (de Oliveira et al., 2015) e preveniria o dano causado por altas concentrações de glicose em um modelo experimental de diabetes (Choi et al., 2016; Zhao et al., 2019). Além disso, um aumento no status oxidativo celular pode contribuir na ativação do NfkB (Patel and Santani, 2009). Em nosso trabalho, nós não observamos mudanças na expressão do Nrf2 e NfkB, contudo a expressão gênica para a HO-1 foi aumentada. Nossos achados sugerem que este aumento nos níveis HO-1 poderia estar relacionado como uma resposta de proteção ao insulto agudo causado pelo MG. Esses dados confirmam a importância da HO-1 contra a toxicidade induzida pelo MG e ressalta que outras vias importantes na regulação/síntese/degradação do Nrf2 e NfkB poderiam estar envolvidos na resposta antioxidante inicial (Nitti et al., 2018).

Em resumo, os resultados que nós obtivemos demonstraram a habilidade do MG em interferir na homeostase cerebral através do prejuízo na remoção do glutamato extracelular. O efeito direto do MG causou disfunção astrocitária, o qual afeta também a sinalização neuronal e consequente a performance cognitiva. A excitotoxicidade glutamatérgica tem sido relacionada na DA e também no DM (Anderson and Swanson, 2000), representada pela perda de neurônios e elevada ativação de receptores pós sinápticos (Scott et al., 2011). Em associação com alterações na proteína S100B, estes dados reforçam o papel dos astrócitos como alvos na toxicidade induzida pelo MG.

Os astrócitos, dentre as inúmeras funções apresentadas, pode-se citar devido a sua proximidade aos vasos sanguíneos e as células endoteliais, a participação na manutenção e integridade da BHE (Abbott, 2002; Abbott et al., 2006). A BHE é uma complexa estrutura celular envolvida na regulação do transporte de específicas moléculas para a distribuição dentro do SNC, limitando a entrada de substâncias tóxicas provenientes do sangue (Engelhardt and Sorokin, 2009; Hawkins and Davis, 2005), ajuda na manutenção da demanda metabólica e homeostase cerebral (Andreone et al., 2015). Funcionalmente, a BHE é composta por uma monocamada fina de células endoteliais, que compreendem os capilares da vasculatura encefálica, as quais estão em contato com células vasculares (pericitos e células do músculo liso), os astrócitos, que cercam os capilares com os pés astrocitários; e os neurônios que inervam diretamente a microcirculação (Haddad-Tóvolli et al., 2017). Juntas, essas células formam a unidade neurovascular (Banks, 2016; Chow and Gu, 2015; Haddad-Tóvolli et al., 2017).

A BHE constitui uma permeabilidade seletiva, falta de fenestrações e atividade pinocitótica baixa (Shah et al., 2012). O transporte de moléculas através da BHE é regulado por difusão passiva e facilitada (e.g., albumina, immunoglobulinas) ou por transporte ativo (e.g., glicose) (Tumani et al., 2017). A perda da integridade da BHE

expõe o encéfalo a substâncias potencialmente danosas como, por exemplo, componentes plasmáticos, moléculas imunológicas, células, íons, aminoácidos, proteínas e outras macromoléculas que podem perturbar a homeostase encefálica e levar à disfunção e degeneração neuronal (Daneman and Prat, 2015; Obermeier et al., 2013). Além disso, a BHE é uma estrutura heterogênea e em certas regiões encefálicas está mais vulnerável ao dano oxidativo e ao desacoplamento neurovascular (VanGilder et al., 2009).

Especificamente, a estreita proximidade entre os pés astrocitários e as células endoteliais se dá através de proteínas transmembranas de ancoragem, como a Aquaporin 4 (AQP4), a qual juntamente com o canal de potássio Kir4.1, permitem um crítico controle no transporte de água para fora do parênquima cerebral por aumentar o fluxo de água transmembrana nos astrócitos e no tamponamento do potássio (Badaut et al., 2014; Niermann et al., 2001). A AQP4 é a aquaporina mais abundante no encéfalo, e um dos 13 tipos encontrados em mamíferos, que representam uma família de proteínas integrais de membranas (Vella et al., 2015). A distribuição astrocitária da AQP4 pode diferir significativamente nas estruturas cerebrais, apresentando-se no hipocampo, corpo caloso, cerebelo, hipotálamo e tronco cerebral (Badaut et al., 2000; Hsu et al., 2011). Essa diferença na distribuição se dá devido às múltiplas funções fisiológicas apresentadas pela AQP4, que além do controle na homeostase da água participa na migração celular, difusão de gases, solutos e substâncias potencialmente danosas ao SNC e na migração astrocitária durante a astrogliose bem como em processos de neuroinflamação (Badaut et al., 2014; Verkman et al., 2017).

Ao mesmo tempo, astrócitos formam uma rede fortemente interconectada via junções GAP (Xing et al., 2019). As junções GAP são canais especializados de comunicação entre células no SNC. Nos astrócitos, encontra-se predominantemente a conexina 43 (Cx43), abundante nos pés astrocitários circundando os vasos sanguíneos na

BHE, e em processos nos quais há o envolvimento dos astrócitos circundando as sinapses (Chew et al., 2010). A Cx43 é responsável na regulação da entrada e saída de íons e metabólitos, o qual é crítico em processos de tamponamento do íon K^+ e glutamato, propagação do íon cálcio (Ca^{2+}) e na plasticidade sináptica (Lapato and Tiwari-Woodruff, 2018; Li et al., 2014).

Nós demonstramos que um insulto agudo com o MG afetaria a atividade astroglial e, considerando o envolvimento dessas células na BHE, foi decidido avaliar o efeito do MG na funcionalidade da barreira. Foi observado um indicativo de ruptura da BHE representada pela alta concentração do corante de Evans blue no encéfalo seguido por um aumento na albumina, indicada pela taxa LCR/Soro nos ratos tratados com o MG. A albumina, devido ao alto peso molecular não atravessa a BHE em condições fisiológicas (Banks, 2015; Jacob and Alexander, 2014), e mudanças no gradiente de concentração entre o LCR e o plasma indicam dano a barreira (Banks et al., 2000). Somado a isso, embora a idade contribua para a diminuição no turnover das proteínas no LCR, aumentadas taxas de albumina no LCR/soro podem ser relacionados com o aumento na permeabilidade da BHE encontrada em pacientes com a DA e fatores de risco vasculares (Chen, 2011; Silverberg et al., 2001; Yamazaki and Kanekiyo, 2017). Estudos anteriores têm demonstrado um aumento na permeabilidade da BHE quando células endoteliais passaram por longas incubações com altas concentrações de MG (Hussain, 2016). Também foi observado mudanças morfológicas de proteínas *tight junctions* incubadas com o composto (Hussain, 2016; Li, 2013), mediados por processos de glicação (Kim et al., 2020; Tóth et al., 2014a).

As células endoteliais são envoltas por uma complexa estrutura de proteínas, a matriz extracelular, e, devido a essa composição, elevados níveis de agentes glicantes como o MG são prejudiciais a integridade dessas células (Hussain, 2016). O MG

modificaria os resíduos de arginina de sítios específicos formando hidroimidazolonas, o que acarretaria mudanças estruturais drásticas nas células endoteliais (McDonald et al., 2009). Embora nós não caracterizamos a glicação nas proteínas da BHE neste trabalho, podemos inferir claramente que há um comprometimento da funcionalidade da BHE causada pelo MG.

Como explanado anteriormente, foi observado níveis aumentados de S100B no LCR, mas não no soro de animais submetidos à administração ICV de MG (Lissner et al., 2021). Portanto, mesmo que haja uma perda evidente da integridade da BHE, a S100B não flui do LCR para o soro neste modelo agudo. Isso é especialmente importante considerando que o aumento sérico de S100B por muitos autores é atribuído à perda da integridade da BHE e esses dados contradizem essa ideia.

O dano a BHE pode afetar também outros membros que compõe a unidade vascular. As consequências podem gerar a desregulação no tamponamento do potássio celular, na circulação do LCR, absorção do fluido intersticial e na entrada de macromoléculas ou influxo iônico (Obermeier et al., 2013; Tumani et al., 2017). Considerando a importância dos astrócitos para a BHE, nós investigamos se parâmetros gliais importantes para a funcionalidade da barreira estariam alterados em nosso modelo de injúria aguda ocasionada pelo MG.

Nossos resultados demonstraram que o grupo tratado com MG apresentou um decréscimo na quantidade da proteína hipocampal, Cx43. Embora existam poucos estudos avaliando o efeito do MG na Cx43, Hansen et al. (Hansen et al., 2016b) demonstrou que as junções GAP são importantes para proteger a toxicidade mediada por MG em cultura de astrócitos, e que a altas concentrações do AGE-BSA em células endoteliais aórticas reduziram expressão e quantidade da Cx43 (Wang et al., 2011). Dessa forma, nossos dados sugerem que a exposição ao MG causou um prejuízo na comunicação celular

desempenhada pela Cx43, afetando a funcionalidade da BHE e a "rede" de comunicação astrocitária no hipocampo.

Além disso, nós observamos um decréscimo no conteúdo da AQP4 hipocampal. Embora não saibamos se essas proteínas são alvos diretos na glicação mediada pelo MG, estes dados apresentam em primeira instância, um prejuízo na conectividade da BHE devido a exposição ao MG, o qual poderia comprometer a homeostase iônica e aquosa. Por outro lado, o conteúdo de sinaptofisina inalterado demonstra que a conectividade neuronal não parece estar afetada neste estágio inicial. Contudo, alterações no *clearance* do glutamato, realizado por astrócitos e um direto efeito do MG nos receptores sinápticos poderia explicar o prejuízo cognitivo e comportamental induzido pelo composto (Hansen et al., 2016a; Lissner et al., 2021; Liu et al., 2013).

No presente trabalho, foi avaliado como uma administração aguda ICV de uma alta concentração de MG poderia afetar a BHE, modular parâmetros comportamentais como processos de aprendizagem e memória, e ansiedade, bem como funções bioquímicas astrogliais. Embora mais estudos sejam necessários, todos esses achados contribuem para um melhor entendimento acerca do papel do MG nas complicações observadas no DM e em doenças neurodegenerativas. Esses dados reforçam também o papel dos astrócitos, as principais células responsáveis pelo *clearance* do MG como alvos na toxicidade induzida pelo composto e que disfunções na BHE podem contribuir para as alterações cognitivas, comportamentais e bioquímicas observadas nesses animais.

6. CONCLUSÃO

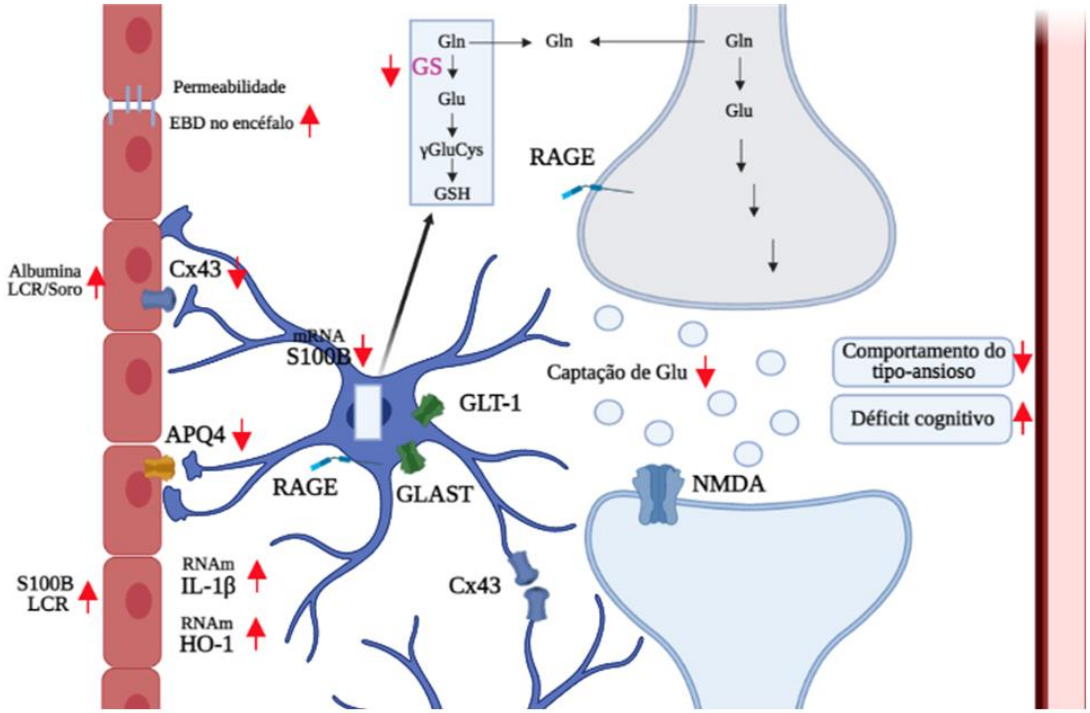


Figura 4. Representação esquemática dos principais resultados obtidos nesta tese. Para a confecção da figura foi usado o programa Biorender.

Os resultados apresentados nesta tese confirmaram claramente que altas concentrações de MG interferem diretamente na funcionalidade do SNC. Elevados níveis de MG são associados com inúmeras complicações no DM e prejudicam processos de aprendizagem e memória em diferentes estudos experimentais e clínicos. Além disso, o MG tem sido reportado por afetar a neurotransmissão, alterando neurotransmissores como o glutamato e o GABA. Aqui, o MG, *per se* ou mediando glicação, comprometeu funções astrocitárias e disfunção na BHE os quais podem contribuir nas alterações das funções cognitivas, tanto na memória de reconhecimento quanto na memória espacial, bem como uma diminuição no efeito ansioso observados em ratos tratados com o MG.

7. PERSPECTIVAS

- Aperfeiçoar as metodologias de dosagem de AGEs para tecidos cerebrais principalmente hipocampo;
- Aprofundar estudos acerca das modificações específicas de proteínas ocasionadas pelo MG, como reações de glicação, oxidação e nitração nesse modelo e *in vitro*;
- Investigar os mecanismos pelos quais o MG afetaria o sistema glutamatérgico;
- Investigar o efeito do MG em outras proteínas específicas da BHE, incluindo processos de glicação.

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9. ANEXO

O desenvolvimento das atividades durante o doutorado sanduíche, no período de fevereiro de 2020 a dezembro de 2020, foi realizado no Laboratório de Miologia molecular e celular, do Departamento de Medicina Experimental da Universidade de Perugia, sob orientação do Professor Dr. Gugliermo Sorci. A interação com o grupo de pesquisa começou a partir da parceria científica com o agora professor aposentado Rosário Donato, desde 2004.

O grupo de pesquisa investiga, com particular atenção, o papel do RAGE e da proteína S100B na biologia das células precursoras musculares, regeneração e atrofia muscular (incluindo sarcopenia e caquexia cancerosa), e distrofia muscular, visando estudos principalmente acerca da Distrofia Muscular de Duchenne (DMD). Além disso, a estrutura do laboratório consiste em uma ampla estrutura com disposição de várias técnicas que utilizam biologia molecular, imunofluorescência, citoquímica e imunocitoquímica, cultural celular, PCR e RT-PCR, ELISA, Western Blotting bem como a disposição animais de laboratório, entre outros.

A DMD é uma doença neuromuscular causada por uma mutação em um gene localizado no cromossomo X, na qual a distrofina é sintetizada (Doorenweerd, 2020; Frigeri et al., 2002). A distrofina é um dos componentes essenciais no complexo de proteínas associados a ela, localizados no sarcolema, responsável pela integridade funcional e estrutural das mio fibras durante a contração (Sagheddu et al., 2018). A ausência da proteína é caracterizada pela progressiva degeneração muscular e inflamação crônica e, ao lado disso, prejuízos cognitivos e sintomas neuropsiquiátricos são prevalentes (Naidoo and Anthony, 2020; Sagheddu et al., 2018). O RAGE é envolvido na miogênesis e inflamação, ausente em músculos adultos saudáveis, mas re-expressos

em mioblastos, regenerando fibras e ativando células do sistema imune durante injúrias musculares agudas e certas miopatias (Sagheddu et al., 2018).

Dessa forma, objetivando aprofundar os conhecimentos acerca do papel do RAGE e seus ligantes, principalmente S100B e HMGB1, foram avaliados o perfil desses ligantes bem como vias de regulação a resposta inflamatória hipocampais em camundongos duplo mutantes, mdx e mdx/Ager$^{-/-}$, com idade avançada. Os dados apresentados aqui, são referentes apenas ao perfil da S100B e HMGB1 neste estudo, visto que ainda estamos trabalhando para o aperfeiçoamento de todos os resultados adquiridos.

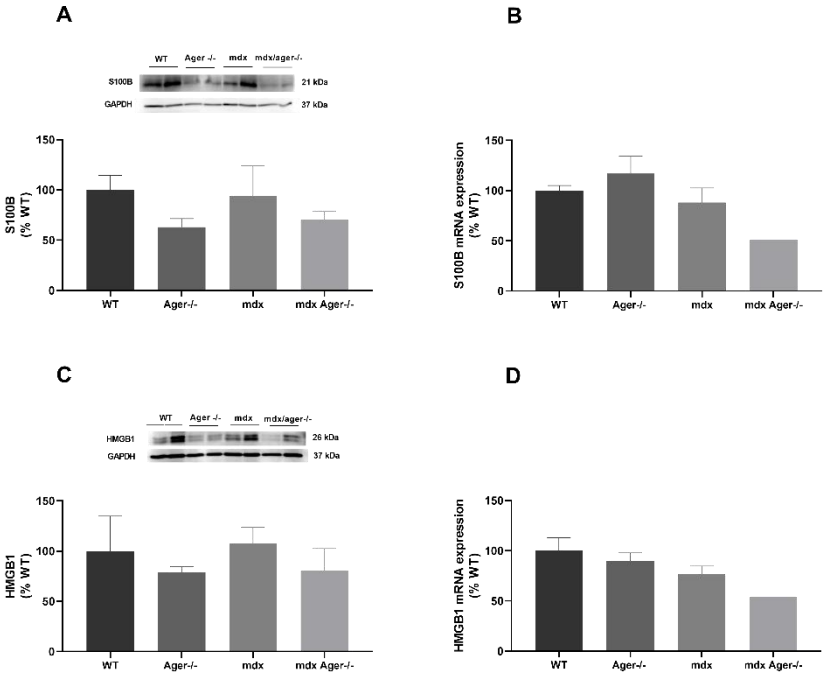


Figure 1. Profile of S100B and HMGB1 in mdx mice, an experimental of DMD, and double mutant, mdx/Ager$^{-/-}$ mouse lacking dystrophin and RAGE. Hippocampal slices of rats were dissected out and immunocontents of hippocampal S100B and HMGB1 (A and C, respectively) was determined by western blotting and normalized by GAPDH, assuming wild type (WT) value as 100%. It was measured also the mRNA expression of these two proteins (B and D, respectively). Data are expressed as means ± S.E.M (N = 2-3 animals per group). Data were analyzed by one-way ANOVA. Tukey's post hoc test was performed assuming $p \leq 0.05$ and the statistical significance represents difference from the WT group.

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