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GUANIDINOACETATO INDUZ DÉFICIT DE MEMÓRIA E NEUROTOXICIDADE EM ESTRIADO DE RATOS: É A CREATINA UM BOM PROTETOR?

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Orientadora: Prof. Dra. Angela T.S. Wyse

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"For me, it is far better to grasp the Universe as it really is than to persist in delusion, however satisfying and reassuring."

Carl Sagan

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PARTE I

RESUMO

A deficiência de guanidinoacetato metiltransferase (GAMT) é uma rara doença metabólica do metabolismo da creatina que leva a níveis diminuídos de creatina e acúmulo de guanidinoacetato (GAA). Indivíduos afetados apresentam sintomas neurológicos, tais como: epilepsia não responsiva a medicamentos, memória e intelecto prejudicados, autismo, síndrome extrapiramidal, fala arrastada e hipotonia. Entretanto, a fisiopatologia desta doença ainda não é clara, sendo as propriedades neurotóxicas do GAA atribuídas principalmente à excitotoxicidade e estresse oxidativo. No presente estudo nós verificamos a toxicidade de uma injeção intraestriatal de GAA em parâmetros de metabolismo energético (atividades da succinato desidrogenase [SDH], complexo II e citocromo c oxidase [COX]; massa e potencial mitocondrial; e níveis de ATP), estresse oxidativo e inflamação (atividades das enzimas antioxidantes catalase [CAT] e superóxido dismutase [SOD], níveis de diclorofluoresceína [DCF], níveis de nitritos; e citocinas pró-inflamatórias [TNF-α, IL1-β and IL-6]), atividade e imunoconteúdo da acetilcolinesterase (AChE); atividade e imunoconteúdo da enzima Na^+,K^+-ATPase; captação de glutamato e imunoconteúdo de seus transportadores (GLAST e GLT-1); memória (via reconhecimento de objetos); assim como o papel da creatina como neuroprotetora. Ratos Wistar foram submetidos a um pré-tratamento com uma injeção intraperitoneal diária de creatina (50 mg/kg), ou salina por 7 dias. Ao completarem 60 dias os animais foram submetidos a uma cirurgia estereotáxica e divididos em quatro grupos: Controle (pré-tratamento com salina e injeção intraestriatal de salina), GAA (pré-tratamento com salina e injeção intraestriatal de 10 μM GAA [0.02 nmol/estriado]), GAA+Creatina (pré-tratamento com creatina e injeção intraestriatal de 10 μM GAA [0.02 nmol/striatum]), e Creatina (pré-tratamento com creatina e injeção intraestriatal de salina). Os experimentos foram realizados 30 minutos após a injeção. O GAA diminuiu a atividade da SDH, complexo II e COX, e os níveis de ATP, mas não afetou o potencial ou a massa mitocondrial. A creatina preveniu totalmente a diminuição da SDH e complexo II, mas preveniu apenas parcialmente a diminuição da COX e dos níveis ATP. O GAA aumentou os níveis de DCF e diminui as atividades das enzimas antioxidantes SOD e CAT. A creatina preveniu apenas a diminuição da CAT e aumento nos níveis de DCF. O GAA aumentou os níveis de citocinas pró-inflamatórias, nitritos e atividade da AChE, mas não alterou o imunoconteúdo da mesma. A creatina preveniu tais alterações, com exceção do aumento dos níveis de nitritos. O GAA diminuiu a captação de glutamato, não alterando o imunoconteúdo de seus transportadores. Além disso, o GAA diminuiu a atividade da Na^+,K^+-ATPase e levou a um aumento do imunoconteúdo de sua subunidade α3. Em relação à memória, o GAA prejudicou a performance no teste de reconhecimento de objetos. A creatina preveniu parcialmente a diminuição da captação de glutamato e na atividade da Na^+,K^+-ATPase, e preveniu totalmente a alteração de memória. Nossos resultados indicam que a toxicidade do GAA prejudica a cadeia transportadora de elétrons, levando a níveis mais baixos de ATP, estresse oxidativo e processos inflamatórios. Essas alterações estão associadas à diminuição na captação de glutamato e atividade da Na^+,K^+-ATPase, o que também contribui para a alteração comportamental. A creatina parece ter atuado como reserva energética, antioxidante e anti-inflamatória, já que foi capaz de prevenir quase todas alterações bioquímicas e comportamentais observadas neste estudo.

ABSTRACT

*G*uanidinoacetate methyltransferase (GAMT) deficiency is a rare metabolic disease of the metabolism of creatine that leads to a decrease in creatine levels associated with accumulation of guanidinoacetate (GAA) in brain, skeletal muscle, blood and other tissues. Affected individuals may present: intractable epilepsy, intellectual and memory impairment, autism, extra pyramidal syndrome, slurred speech and hypotonia. However, the pathophysiology of this disease is still unclear. In the present study, we investigate the toxicity of an intrastriatal injection of GAA on parameters of energy metabolism (activities of succinate dehydrogenase [SDH], complex II and citocrome c oxidase [COX]; mitochondrial mass and membrane potential; and ATP levels), oxidative stress and inflammation (activities of the antioxidant enzymes catalase [CAT] and superoxide dismutase [SOD]; dichlorofluorescein [DCF] levels; proinflammatory cytokines TNF-α, IL1-β and IL-6; and nitrite levels), acetycholinesterase (AChE) activity and immunocontent; Na^+, K^+-ATPase activity and immunocontent; glutamate uptake and immunocontent of its transporters (GLAST and GLT-1); memory (via object recognition test); as well as the role of creatine as a neuroprotector. Wistar rats underwent a pretreatment with a daily intraperitoneal injection of creatine (50 mg/kg), or saline for 7 days. Sixty-day-old animals then underwent stereotactic surgery and were divided into four groups: Control (pretreatment with saline and intrastriatal infusion of saline), GAA (pretreatment with saline and intrastriatal infusion of 10 μM of GAA [0.02 nmol/striatum]), GAA+Creatine (pretreatment with creatine and intrastriatal infusion of 10 μM of GAA [0.02 nmol/striatum]), and Creatine (pretreatment with creatine and intrastriatal infusion of saline). Thirty minutes after intrastriatal infusion experiments were carried out. GAA decreased SDH, complexes II and COX activities and ATP levels, but had no effect on mitochondrial mass/membrane potential. Creatine totally prevented SDH and complex II and partially prevented COX and ATP alterations. GAA decreased antioxidant enzymes activities and increased DCF levels. Creatine only prevented CAT activity and DCF alterations. GAA increased cytokines, nitrites levels and AChE activity, but had no effect on AChE immunocontent. Creatine prevented such effects, except nitrite levels. GAA decreased glutamate uptake with no effect on the immunocontent of its transporters, and Na^+,K^+-ATPase activity, as well as in the performance on the novel object recognition task. In addition, GAA increased the immunocontent of α3 subunit of Na^+,K^+-ATPase. Creatine partially prevented the changes in glutamate uptake and Na^+,K^+-ATPase activity, and completely prevented the memory impairment. Our findings indicate that GAA intrastriatal injection impairs the mitochondrial respiratory chain, leading to depleted ATP levels, redox imbalance and inflammatory processes. In addition, we have confirmed that glutamate uptake and Na^+,K^+-ATPase activity in the striatum of young adult rats are inhibited after GAA exposure, probably due to the depletion of ATP levels and attack from radical species, contributing for the memory impairment observed. These alterations may contribute to the neurological dysfunction presented by GAMT deficient patients. Creatine seems to act as an energy reservoir, antioxidant and anti-inflammatory agent, as well as a neuromodulator, since it was able to prevent almost every biochemical and behavioral alteration detected in this study.

LISTA DE ABREVIATURAS

ACh: acetilcolina

AChE: acetilcolinesterase

ADP: adenosina difosfato

AGAT: arginina-glicina amidinotransferase

AMPA: alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico

ATP: adenosina trifosfato

CAT: catalase

CK: creatina cinase

COX: citocromo c oxidase

DCF: diclorofluoresceína

EIM: erro inato do metabolismo

FADH2: dinucleótido de flavina e adenina (reduzido)

GAA: guanidinoacetato

GAMT: guanidinoacetato N-metiltransferase

GLAST 1: transportador de glutamato-aspartato 1

GLT1: transportador de glutamato 1

GPx: glutationa peroxidase

GSH: glutationa reduzida

GTP: guanosina trifosfato

iGluR: receptores ionotrópicos de glutamato

IL1-β: interleucina 1-β

IL-6: interleucina 6

iNOS: óxido nítrico sintase induzível

LTM: memórias de longa duração

mGluR: receptores metabotrópicos de glutamato

NADH: nicotinamida adenina dinucleotídeo (reduzido)

NMDA: N-metil-D-aspartato

PCr: fosfocreatina

SDH: succinato desidrogenase

SNC: sistema nervoso central

SOD: superóxido dismutase

STM: memórias de curta duração

TNF-α: fator de necrose tumoral alfa

vGLUT: transportadores vesiculares de glutamato

1. INTRODUÇÃO

1.1 Creatina

A creatina é um ácido orgânico nitrogenado naturalmente produzida pelo corpo humano a partir dos aminoácidos glicina e arginina. No primeiro passo da biossíntese, a reação é catalisada pela enzima arginina-glicina amidinotransferase (AGAT), formando guanidinoacetato (GAA), que é então metilado pela enzima guanidinoacetato N-metiltransferase (GAMT). A creatina assim gerada pode ser transportada para dentro e fora da célula através de seu transportador SLC6A8 (Barcelos et al. 2016). Uma vez dentro das células, aproximadamente dois terços da creatina são fosforilados pelas isoformas da enzima creatina cinase (CK) (Wallimann, Tokarska-Schlattner, e Schlattner 2011), originando a fosfocreatina (PCr). Essa molécula é uma reserva energética capaz de transferir reversivelmente seu grupo N-fosforil para a adenosina difosfato (ADP) quando este está em altas concentrações, regenerando, assim, adenosina trifosfato (ATP) (Saks et al. 2004, Sauer e Schlattner 2004, Wallimann et al. 1992).

A maior parte da creatina sintetizada endogenamente é produzida nos rins e fígado. No entanto, é importante mencionar que o cérebro possui seu próprio mecanismo para a síntese e manutenção dos níveis de creatina, com as enzimas AGAT e GAMT sendo expressas em todos os tipos celulares de maneira dissociada. Isso significa que para que a síntese de creatina ocorra no sistema nervoso central (SNC), o GAA deve ser transportado via SLC6A8 de células que expressam AGAT, para células que expressam GAMT (Braissant e Henry 2008, Braissant et al. 2010).

A manutenção dos níveis de creatina é fundamental para a função muscular e cerebral (Schlattner, Tokarska-Schlattner, e Wallimann 2006), possuindo ação

neuroprotetora em doenças neurodegenerativas (Bolaños et al. 2009, Wyss e Schulze 2002, Hersch et al. 2006, Pastula, Moore, e Bedlack 2012). Além de ajudar a prevenir a sobrecarga da cadeia transportadora de elétrons, reduzindo a geração de espécies reativas, o sistema creatina/PCr possui propriedades antioxidantes *per se,* o que estão interconectados com os efeitos anti-inflamatórios também atribuídos a esse sistema (Sestili et al. 2006, Young et al. 2010).

1.2 Erros inatos do metabolismo

Erros inatos do metabolismo (EIM) são doenças genéticas hereditárias, majoritariamente autossômicas recessivas, que se caracterizam pela síntese de uma proteína anômala, geralmente uma enzima, que apresentará atividade parcial ou totalmente reduzida. Essas alterações podem levar ao bloqueio de rotas metabólicas com consequente acúmulo de substrato, diminuição na síntese do produto ou até formação de produtos tóxicos por rotas metabólicas alternativas (Scriver et al. 2001).

Os EIM são considerados raros quando analisados individualmente, mas quando considerados todos os tipos possíveis, os cerca de 1000 EIM já descritos atingem um a cada mil nascidos vivos (Mak et al. 2013). A forma mais utilizada para classificar os EIM é separá-los de acordo com a área do metabolismo afetada (Scriver et al. 2001), como por exemplo: EIM de ácidos orgânicos, aminoácidos, glicídios, lipídios, glicosaminoglicanos, glicoproteínas, purinas e pirimidinas, enzimas eritrocitárias, metais, lipoproteínas, hormônios e proteínas plasmáticas, dentre outros.

A deficiência da GAMT foi o primeiro EIM detectado entre as síndromes da deficiência de creatina, sendo o foco deste trabalho.

1.3 Deficiência da Guanidinoacetato Metiltransferase (Deficiência de GAMT)

O N-amino-imino-metil-glicina, conhecido como GAA, pertence à classe dos compostos guanidínicos. Esses compostos são substâncias caracterizadas pela presença do grupo básico guanidino em sua estrutura (H_2N-C(=NH)-NH-), e exercem um importante papel biológico, incluindo a participação da arginina na síntese de uréia e da creatina na contração muscular (Wyss et al. 2007).

Em 1994, Stöckler e colaboradores descreveram um novo EIM que viria a ser considerado o primeiro EIM da creatina descrito na literatura (Stöckler et al. 1994). A nova doença recebeu o nome de acordo com o gene afetado, o qual codifica a enzima GAMT, sendo então chamada de Deficiência de GAMT. Essa doença metabólica é uma desordem genética hereditária autossômica recessiva que leva à diminuição nos níveis de creatina e excesso na concentração de GAA no sangue, músculo esquelético, cérebro e outros tecidos (Gordon 2010, Stöckler et al. 1996, Schulze et al. 2003). É uma doença muito rara, com apenas algumas centenas de casos reportados até o momento, sendo a maioria na Europa e no Oriente Médio (Desroches et al. 2015).

As alterações bioquímicas decorrentes desse EIM incluem excreção urinária contendo muito GAA e pouca creatina, altas concentrações de GAA no cérebro e em outros tecidos, e redução drástica de creatina no cérebro e nos músculos esqueléticos dos pacientes afetados (Stöckler et al. 1996, Schulze et al. 2003). Como a creatina consiste em um importante tampão energético, os sistemas altamente dependentes de um suporte contínuo de energia apresentam alterações significantes nos pacientes acometidos pela doença. Além da diminuição nos níveis de creatina, o SNC sofre com a neurotoxicidade provocada pelo acúmulo de GAA,

que ainda não é claramente compreendida. O GAA parece atuar como um agonista direto de receptores $GABA_{A,}$ levando a uma dessensibilização dos receptores quando em altas concentrações, contribuindo para os sintomas epilépticos apresentados pelos pacientes (Schulze et al. 2016). Outros sintomas se manifestam após o nascimento e podem incluir deficiências cognitivas e de memória, movimentos extrapiramidais involuntários, convulsões, hipotonia muscular, e até mesmo autismo (Stöckler et al. 1996, Arias-Dimas et al. 2006, Gordon 2010).

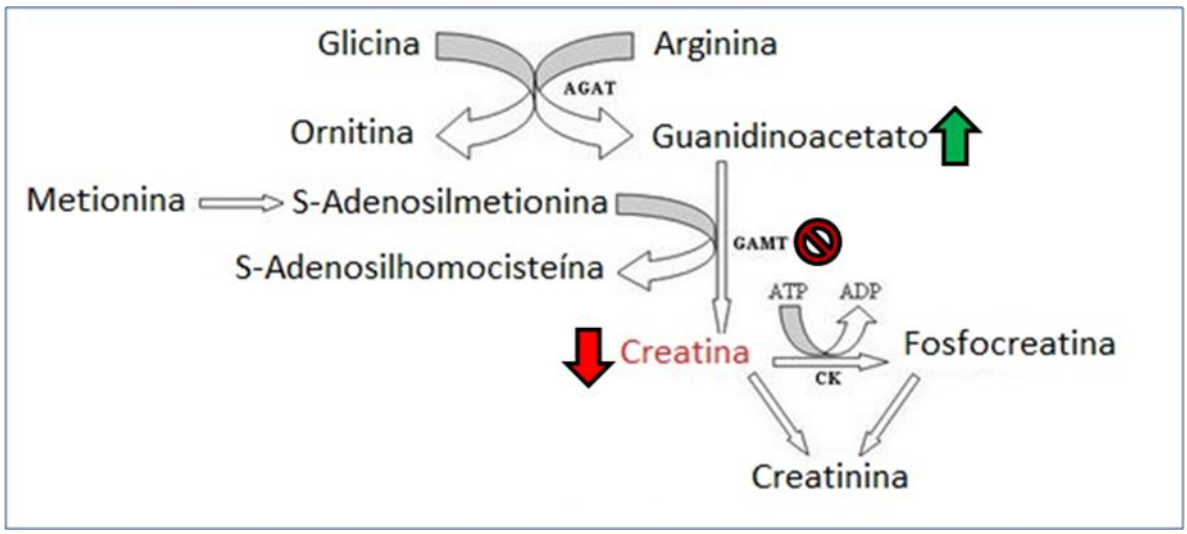

Figura 1. Bloqueio metabólico no metabolismo da creatina encontrado da deficiência de GAMT. Abreviações: AGAT, arginina: glicina-amidinotransferase; GAMT, guanidinoacetato metiltransferase; ATP, adenosina trifosfato; ADP, adenosina difosfato; CK, creatina quinase. Adaptado de Casey e Greenhaff, 2000.

O diagnóstico da deficiência de GAMT pode ser feito baseando-se na associação dos sintomas com uma série de testes que incluem: monitoramento da excreção de creatinina durante 24h, marcação de compostos guanidínicos presentes da urina através da reação de Sakaguchi, espectroscopia por ressonância magnética nuclear, ou até medida direta da atividade da GAMT em alguns tipos celulares (Schulze et al. 1997, Verhoeven et al. 2004). Uma vez confirmada a doença, o tratamento consiste em prevenir a depleção de creatina e reduzir as

concentrações de GAA. Estudos sugerem que uma diminuição na ingestão de um dos aminoácidos precursores do GAA, a arginina, combinada com suplementação de ornitina, pode prevenir as convulsões características da doença (Dhar et al. 2009, Stockler-Ipsiroglu et al. 2014). Entretanto, além dessas medidas, a suplementação oral contínua em altas doses (400 mg-2g/kg/ dia) de creatina mono-hidratada é imprescindível para a sobrevivência dos pacientes, podendo levar inclusive a um desenvolvimento normal se iniciada após o parto ou ainda na primeira infância (Stockler-Ipsiroglu et al. 2014, Akiyama et al. 2014, Mercimek-Mahmutoglu et al. 2012).

1.4 Metabolismo energético

Considerando que os pacientes afetados com a deficiência de GAMT apresentam diversas alterações neurológicas, um desbalanço do metabolismo mitocondrial parece desempenhar um papel importante nas mesmas, corroborando estudos do nosso grupo que já demonstraram que a injeção intraestriatal de GAA inibe a atividade de enzimas essenciais para a geração de energia no SNC, como complexo II da cadeia respiratória mitocondrial e a CK (Zugno, Scherer, et al. 2007, Zugno, Oliveira, et al. 2007).

O tecido cerebral depende de um fornecimento contínuo de energia para que ele possa desempenhar plenamente suas funções. Para isso utiliza os processos de glicólise e fosforilação oxidativa, processos acoplados responsáveis por gerar até 95% do ATP cerebral. A glicólise ocorre no citosol e tem como produto final o piruvato, que na presença de oxigênio é convertido em CO_2 e H_2O na mitocôndria através do ciclo de Krebs e da cadeia respiratória. O ciclo de Krebs forma três moléculas de nicotinamida adenina dinucleotídeo reduzidas (NADH), uma de dinucleótido de flavina e adenina reduzido ($FADH_2$) , duas de CO_2 e uma

de guanosina trifosfato (GTP). O NADH e $FADH_2$ funcionam como carreadores de elétrons para a cadeia respiratória, que consiste em quatro complexos proteicos com diferentes afinidades pelos elétrons: Complexo I (NADH: ubiquinona oxirredutase ou NADH desidrogenase), complexo II (succinato ubiquinona oxirredutase), complexo III (cicromo bc_1 ou ubiquinona: citocromo c oxirredutase) e complexo IV (citocromo c oxidase [COX]); além de elementos móveis que se localizam entre eles, como a coenzima Q e o citocromo c. Os elétrons são transferidos juntamente com prótons da matriz para o lado citosólico da membrana mitocondrial interna. Esse gradiente de prótons criado é usado como força-motriz para impulsionar a síntese de ATP através da F0F1-ATP sintase, completando o processo chamado de fosforilação oxidativa (Nelson e Cox 2014, Pierron et al. 2011).

Perturbações na função mitocondrial parecem estar intimamente relacionadas com a fisiopatologia de doenças neurodegenerativas como as doenças de Alzheimer, Parkinson, esclerose múltipla, entre outras (Bolaños et al. 2009, Falkowska et al. 2015). Tais alterações, quando revertidas, parecem levar a uma melhora no quadro neurodegenerativo (Liddell 2015).

1.5 Estresse Oxidativo

As espécies reativas possuem diversas funções fisiológicas, e são constantemente produzidas no organismo em níveis basais, principalmente durante o processo de respiração celular (Halliwell e Gutteridge 2007). O organismo possui diversos mecanismos para evitar que os efeitos das espécies reativas se tornem prejudiciais ao nosso corpo, entre eles estão as defesas enzimáticas (Superóxido Dismutase [SOD], Catalase [CAT] e Glutationa Peroxidase [GPx]) e as não-enzimáticas (Glutationa reduzida [GSH], vitaminas, entre outras) (Halliwell e

Gutteridge 2007). Entretanto, em algumas situações patológicas, pode haver um desequilíbrio entre a formação de espécies reativas e as defesas antioxidantes. A persistência desse desequilíbrio leva ao estresse oxidativo, um estado que pode desencadear danos em diferentes tipos de biomoléculas, incluindo DNA, lipídeos e proteínas (Halliwell e Whiteman 2004, Nelson e Cox 2014). Além disso, o estresse oxidativo pode contribuir para amplificação de respostas inflamatórias locais e sistêmicas, que por sua vez intensificarão o próprio estresse oxidativo (Dandekar, Mendez, e Zhang 2015).

1.6 Acetilcolinesterase e inflamação

A acetilcolina (ACh) é um neurotransmissor sintetizado pela enzima colina acetiltransferase a partir do acetato e da colina, sendo armazenada em vesículas nos neurônios pré-sinápticos. Ao ser liberada na fenda sináptica, a ACh liga-se a receptores muscarínicos ou nicotínicos localizados nas membranas pré e pós-sináptica. A acetilcolinesterase (AChE) é a enzima responsável pela degradação do excesso de ACh liberada na fenda, ajudando a co-regular a transmissão colinérgica (Geula and Darvesh 2004). Diversos estudos apontam que o aumento da atividade dessa enzima está relacionado à fisiopatologia de EIM e a outras doenças, incluindo as neurodegenerativas (Marques e Wyse 2016, Schweinberger e Wyse 2016, Sindi e Dodd 2015). Borlongan e colaboradores (Borlongan, Sumaya, e Moss 2005), demonstraram que inibidores da AChE permitem que o neurotransmissor ACh permaneça mais tempo na fenda sináptica, o que promove acentuada melhora no aprendizado e na memória de ratos isquêmicos.

Além da importância para a neurotransmissão, estudos observaram efeitos anti-inflamatórios após a estimulação do nervo vago (Borovikova et al. 2000), sugerindo a existência de uma via colinérgica anti-inflamatória que reage em

resposta à presença de estímulos pró-inflamatórios. Esta via é composta pelo nervo vago, pela ACh e pela subunidade α7 do receptor nicotínico de ACh (Pavlov et al. 2009), e pode ser ativada após um processo inflamatório sistêmico ou localizado que conte com a presença de citocinas pró-inflamatórias como fator de necrose tumoral alfa (TNF-α), interleucina 1β (IL1-β) e interleucina 6 (IL-6). As citocinas ativarão as fibras aferentes do nervo, fazendo com que o SNC libere sinais estimulando a produção de Ach pelo nervo vago, que por sua vez irá realizar um feedback negativo na produção das citocinas pró-inflamatórias pelo sistema imune inato (Gallowitsch-Puerta e Pavlov 2007).

A neuroinflamação, ou mais especificamente a ativação da microglia e dos astrócitos a um estado pró-inflamatório, é geralmente citada não como um iniciador, mas como um contribuidor patológico para a progressão de diversas doenças neurodegenerativas, e é constantemente associado ao acúmulo de uma molécula tóxica (Schain e Kreisl 2017). Um processo inflamatório descontrolado pode resultar na produção de fatores neurotóxicos que amplificam o processo neurodegenerativo, como as citocinas pró-inflamatórias TNF-α, IL1-β e IL-6, e nitritos (Pacher, Beckman, e Liaudet 2007, Glass et al. 2010).

Por desempenhar um papel fundamental no SNC e coordenar propriedades anti-inflamatórias que podem evitar um estado de estresse oxidativo, é importante que a atividade da AChE esteja regulada, já que poderia trazer consequências neurológicas indesejadas. Entretanto, estudos apontam que compostos guanidínicos, como o GAA, alteram a atividade dessa enzima (Delwing-de Lima et al. 2010), levando inclusive a alterações comportamentais (Zugno, Pereira, et al. 2008), o que pode estar associado com as alterações neurológicas presentes nos pacientes deficientes em GAMT.

1.7 Homeostase glutamatérgica

O estresse oxidativo e o aumento das citocinas pró-inflamatórias afetam astrócitos, oligodendrócitos e a microglia, exercendo alterações consideráveis nas concentrações extracelulares de glutamato (Sanacora e Banasr 2013, Lewerenz e Maher 2015). Essas alterações podem gerar consequências indesejadas, dado que o glutamato é o principal neurotransmissor excitatório do SNC e está virtualmente ligado a todas as atividades cerebrais (Sheldon e Robinson 2007).

O Glutamato é concentrado em vesículas no neurônio pré-sináptico majoritariamente pela ação dos transportadores vesiculares de glutamato (vGLUT) (Takamori 2006). Após a despolarização da membrana pré-sináptica, o glutamato é liberado na fenda onde se liga a receptores ionotrópicos (iGluR) e metabotrópicos (mGluR) de glutamato na membrana pós-sináptica. Os iGluRs são canais de íons e incluem os receptores de N-metil-D-aspartato (NMDA), os de alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiônico (AMPA) e os de cainato (Robinson 2006). Receptores AMPA e de cainato primariamente medeiam influxo de sódio, enquanto que receptores NMDA têm uma alta condutividade de cálcio, e sua ativação desempenha um papel importante na plasticidade sináptica e aprendizado (Miyamoto 2006). Os receptores mGluR, por sua vez, são acoplados à proteína G e induzem uma resposta pela ativação de segundos mensageiros intracelulares, como inositol trifosfato e adenosina 3',5'-monofosfato cíclico (Kantamneni 2015).

O glutamato excedente precisa ser importado de volta para o ambiente intracelular para ser reciclado. Essa recaptação é realizada por transportadores dependentes de Na+, sendo o transportador de glutamato-aspartato 1 (GLAST1), e o transportador de glutamato 1 (GLT1) os mais abundantes do SNC, sendo expressos principalmente em astrócitos e, em menor proporção, em alguns neurônios (Zhou e

Danbolt 2014). O glutamato, ao ser recaptado pelos transportadores, pode ser usado para a síntese de glutamina, que é liberada e, em seguida, captada pelos neurônios, onde é novamente convertida a glutamato. Alternativamente, o glutamato recaptado também pode ser convertido em α-cetoglutarato, que também pode ser usado para reciclagem de glutamato em neurônios, ou utilizado para produção de energia no ciclo dos ácidos tricarboxílicos (Zhou e Danbolt 2014).

A regulação do sistema glutamatérgico é complexa, e quando não funciona plenamente pode acarretar no processo patológico chamado de excitotoxicidade glutamatérgica, também comum em muitas doenças neurodegenerativas (Lewerenz e Maher 2015). Neste processo patológico há uma ativação excessiva de iGluRs com consequente aumento dos níveis de cálcio intracelular, resultando em perda de especificidade sináptica, estresse oxidativo e até morte neuronal (McCullumsmith e Sanacora 2015). Acredita-se que a neuroinflamação seja um grande contribuidor para a perda da homeostase glutamatérgica, diminuindo a capacidade dos transportadores gliais de recaptar esse neurotransmissor (Haroon, Miller, e Sanacora 2017). Esse efeito associado ao glutamato liberado pela microglia ativada e outras células imunes perpetua o processo neurodegenerativo. Além disso, o transporte de glutamato por seus transportadores também pode ser afetado pela atividade da enzima Na^+,K^+-ATPase, já que ele é dependente do gradiente de Na+ gerado por essa enzima (Rose et al. 2009).

1.8 Na^+,K^+-ATPase

A Na^+,K^+-ATPase é uma proteína integral de membrana que simultaneamente transporta três íons sódio (Na^+) para o compartimento extracelular e dois íons potássio (K^+) para o meio intracelular. A energia necessária para a troca iônica é derivada da hidrólise de uma molécula de ATP. A subunidade α (há quatro

isoformas: α_1, α_2, α_3 e α_4), responsável pela atividade catalítica da enzima, sofre fosforilação e transição conformacional acoplada à hidrólise de ATP e transporte dos íons Na^+ e K^+, sendo a isoforma α_3 altamente expressa nos núcleos da base (Bøttger et al. 2011). Nos neurônios, a restauração dos gradientes de Na^+ e K^+ pela isoforma α_3 é muito importante para a manutenção da excitabilidade neuronal e condução do potencial de ação em axônios mielinizados e para o transporte de neurotransmissores acoplados ao Na^+ (Benarroch 2011). A subunidade β (há 3 isoformas: β_1, β_2 e β_3) é uma proteína glicosilada de adesão intercelular necessária para direcionar a subunidade α para a membrana plasmática. Acredita-se que a subunidade γ regule a atividade da enzima (Geering 2008). A Na^+,K^+-ATPase é expressa em todas as células de mamíferos (Aperia 2007), e no cérebro ela se encontra em altas concentrações, consumindo 50-60% do ATP disponível (Erecińska e Silver 1994), sendo, portanto, de fundamental importância para a sobrevivência celular.

A Na^+,K^+-ATPase é suscetível ao ataque de radicais livres (Wang et al. 2003) e espécies reativas de oxigênio, que podem oxidar os grupos sulfidrilas presentes em altas concentrações no sítio ativo da enzima (Kurella, Tyulina, e Boldyrev 1999). Mudanças na atividade da Na^+,K^+-ATPase parecem desempenhar um papel importante na fisiopatologia de diversas desordens que afetam o SNC. A atividade dessa enzima está diminuída na enxaqueca (Suhail 2010), depressão (Zhang et al. 2012) desordens metabólicas (Schweinberger e Wyse 2016, Wyse e Netto 2011), bem como em doenças neurodegenerativas (Vitvitsky et al. 2012, Aperia 2007).

1.9 Memória

A memória é a capacidade de adquirir, armazenar e evocar informações que possam ser utilizadas posteriormente (Morris 2006). A formação de uma memória é

um processo extremamente complexo que vem sendo estudado há mais de cem anos, sendo que essa complexidade se deve, em partes, à existência de diferentes formas de memória, que utilizam distintos mecanismos bioquímicos e moleculares e podem ser armazenadas em distintas regiões cerebrais. Entretanto, sabe-se que ocorre a ativação do sistema glutamatérgico seguida de mobilização de segundos mensageiros com ativação de proteínas quinases (Izquierdo et al. 1998, Furini et al. 2013)

As memórias podem variar quanto ao seu tempo de duração, assim, quando perduram por poucos minutos ou horas, são denominadas de Memórias de Curta Duração (STM; sigla do inglês: *short-term memory*), e quando perduram por muitas horas, dias ou meses, são denominadas de Memórias de Longa Duração (LTM; sigla do inglês: *long-term memory*) (Izquierdo et al. 1998). Além disso, é importante destacar que, ao contrário da STM, a consolidação de uma LTM necessita da síntese de RNAm e proteínas relacionadas à plasticidade (Moncada et al. 2011).

O processo de aquisição, como já diz o nome, é a entrada de um dado por meio dos sensores externos, que é encaminhado aos sistemas neurais relativos à memória onde será armazenado por algum tempo que pode ser de segundos ou mesmo de anos pelo processo de consolidação. A consolidação consiste em um conjunto de reações bioquímicas e moleculares interdependentes que culminam em uma progressiva estabilização pós-aquisição da informação (Dudai 2012, Costa-Mattioli e Sonenberg 2008). Na evocação, memórias já estabilizadas retornam ao estado vulnerável e, para persistirem, necessitam passar por um novo processo de estabilização dependente de síntese de proteínas, chamado de reconsolidação (Myskiw et al. 2008).

Diversos métodos são utilizados a fim de avaliar a memória. Podem ser realizar testes para memória aversiva, memória espacial, memória de trabalho, dentre outras. Ennaceur e Delacour, em 1988, propuseram o paradigma de reconhecimento de objetos, baseado na tendência natural dos roedores em explorar mais os objetos novos do que os familiares. A utilização da tarefa de RO possui várias vantagens, mas vale destacar que não depende de sistema de recompensa; não há estímulos aversivos (como choque); não requer restrição a alimento ou água, e não precisa de um treinamento preliminar extenso já que o aprendizado se dá após uma única sessão.

2. OBJETIVOS

2.1 Objetivo geral

O objetivo geral do presente projeto foi estudar o efeito tóxico de uma injeção intraestriatal de GAA sobre parâmetros de metabolismo energético, estresse oxidativo, inflamação, atividade e imunoconteúdo das enzimas AChE e Na^+,K^+-ATPase, sistema glutamatérgico, bem como sobre o processo de aquisição da memória na tarefa de reconhecimento de objetos. O papel da creatina como neuroprotetora também foi avaliado.

2.2 Objetivos específicos

1) Realizar uma revisão da literatura sobre os efeitos da creatina como neuroprotetora;

2) Avaliar parâmetros do metabolismo energético, tais como: complexos da cadeia respiratória (succinato desidrogenase [SDH], complexo II e citocromo *c* oxidase), função mitocondrial (massa e potencial), e níveis de ATP em estriado de ratos submetidos à injeção intraestriatal de GAA;

3) Avaliar alguns parâmetros de estresse oxidativo e inflamação, tais como: atividade das enzimas antioxidantes (SOD e CAT), níveis de diclorofluoresceína (DCF), níveis de nitritos, e níveis de citocinas pró-inflamatórias (TNF-α, IL1-β e IL-6) em estriado de ratos submetidos à injeção intraestriatal de GAA;

4) Avaliar a atividade e imunoconteúdo da enzima AChE em estriado de ratos submetidos à injeção intraestriatal de GAA;

5) Avaliar a atividade da Na^+, K^+-ATPase e imunoconteúdo da subunidade α3 em estriado de ratos submetidos à injeção intraestriatal de GAA;

6) Avaliar a captação de glutamato e o imunoconteúdo dos seus transportadores (GLAST e GLT-1) em estriado de ratos submetidos à injeção intraestriatal de GAA;

6) Avaliar a memória/aprendizado de ratos submetidos à administração intraestriatal de GAA, utilizando as tarefas comportamentais de campo aberto e reconhecimento de objetos;

7) Investigar o papel neuroprotetor da creatina sobre as alterações bioquímicas e comportamentais encontradas.

PARTE II

3. PROCEDIMENTOS EXPERIMENTAIS E RESULTADOS

Esses itens serão apresentados na forma de artigos científicos.

3.1 Capítulo I - Artigo científico I

Creatine as a neuroprotector: an actor that can play many parts

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ABSTRACT

Creatine is a nitrogenous organic acid that plays a central role as an energy buffer in high energy demanding systems, including the muscular and the central nervous system. It can be acquired from diet or synthesized endogenously and its main destination is the system creatine/phosphocreatine that strengthens cellular energetics via a temporal and spatial energy buffer that can restore cellular ATP without a reliance on oxygen. This compound has been proposed to possess secondary roles, such as direct and indirect antioxidant, immunomodulatory agent and possible neuromodulator. However, these effects may be associated with its bioenergetic role in the mitochondria. Given the fundamental roles that creatine plays in the CNS, several preclinical and clinical studies have tested the potential that creatine has to treat degenerative disorders. However, although in vitro and in vivo animal models are highly encouraging, most clinical trials fail to reproduce positive results suggesting that the prophylactic use for neuroprotection in at-risk populations or patients is the most promising field. Nonetheless, the only clearly positive data of the creatine supplementation in human beings are related to the (rare) creatine deficiency syndromes. It seems critical that future studies must establish the best dosage regime to increase brain creatine in a way that can relate to animal studies, provide new ways for creatine to reach the brain and seek larger experimental groups with biomarkers for prediction of efficacy.

Key words: Creatine, Neuroprotection, Bioenergetics, Neurodegenerative Disease, Inborn Errors of Metabolism, Creatine supplementation

1. INTRODUCTION

Creatine is a nitrogenous organic acid firstly described in the early 19th century. It plays a central role as an energy buffer for systems throughout the body, particularly high energy demanding systems, like the muscular and the central nervous system (CNS) (Wyss and Kaddurah-Daouk 2000). An average adult has approximately 120g of creatine in his body. Since creatine is subject to spontaneous removal via a nonenzymatic chemical dehydration to creatinine, 2g must be acquired or synthesized every day in order to maintain this value constant (Casey and Greenhaff 2000). There is no main source of creatine in human metabolism since it is provided equally by dietary consumption and endogenous synthesis. The most relevant dietary sources of creatine is found in red meat, fish and to a lesser extent dairy products (Brosnan and Brosnan 2016). It is known that the small intestine expresses the Na+/Cl− creatine transporter (Peral et al. 2002).

Nonetheless, biosynthesis of creatine is a relative simple process that occurs mainly in the kidney and liver, involving a 2-step pathway with 2 enzymes: L-arginine: glycine amidinotransferase (AGAT) and *N*-guanidinoacetate methyltransferase (GAMT), along with one specific plasma membrane transporter, SLC6A8 (Barcelos et al. 2016). AGAT is present in the kidney, brain and pancreas (to a very smaller extent) of mammals (Braissant et al. 2001, da Silva et al. 2014). This enzyme is responsible for condensing the amino acids arginine and glycine, generating guanidinoacetate (GAA) and L-ornithine. This first reaction takes place predominantly in the mitochondria intermembrane space and in lower levels in the cytoplasm (Magri, Baldoni, and Grazi 1975). The second enzyme, GAMT is mostly present in the liver and brain of mammals, being the responsible for the transfer of a methyl group from S-adenosylmethionine (SAM) to GAA in order to produce creatine (Ogawa et al. 1988, da Silva et al. 2014). This reaction is responsible for the consumption of roughly 70% of available methyl groups in human bodies (Stead et al. 2006). Most of creatine that arises from this second step is produced in the liver and then secreted in the bloodstream by an unknown mechanism and distributed throughout the body, where it actively enters the cells using the specific creatine transporter, SLC6A8, a Na^+- and Cl^--dependent symporter (Magri, Baldoni, and Grazi 1975, Brosnan and Brosnan 2007, da Silva et al. 2014). Once inside the cells approximately two-thirds of the available creatine undergoes a reversible phosphorylation catalyzed by the enzyme

creatine kinase (CK), giving rise to phosphocreatine (PCr). CK has three cytosolic and two mitochondrial isoforms, but in most tissues a single cytosolic CK isoform is co-expressed with a single mitochondrial CK isoform (Wallimann, Tokarska-Schlattner, and Schlattner 2011, Koch, Pereira, and Machado 2014).

As previously pointed out, although most of endogenous creatine is produced in the liver, it has been shown that the brain has its own pathway for the synthesis and maintenance of creatine levels since SLC6A8 is present in low levels in the micro capillaries of the blood brain barrier (BBB) and it is not expressed by most perivascular astrocytes (Braissant 2012, Saunders et al. 2015). As a result, AGAT and GAMT are expressed in all CNS cell types (Braissant et al. 2001). Nonetheless, the expression of these two enzymes appears to occur in a dissociated fashion, with less than 20% of brain cells expressing both AGAT and GAMT. This means that for creatine synthesis to occur, GAA must be transported through SLC6A8 from AGAT to GAMT-expressing cells (Braissant and Henry 2008, Braissant et al. 2010).

After non-enzymatic dehydration and cyclization of creatine, creatinine is produced and it freely diffuses to the bloodstream where it will be eliminated in urine. Creatinine is frequently used as a marker of the renal function (Wyss and Schulze 2002). In order to compensate this loss creatine synthesis must be regulated physiologically, being that the major regulator is the activity of AGAT, which is the biosynthesis-initiating and rate-limiting step of creatine formation. There is down-regulation of AGAT in the kidney and in the developing brain caused by high levels of its product ornithine and the downstream end product creatine (Hanna-El-Daher et al. 2015). On the other hand, lower levels of creatine are able to stimulate a sustained increase in AGAT activity (Wyss and Kaddurah-Daouk 2000). SLC6A8 is regulated by creatine levels in the bloodstream in a time dependent manner, being that high levels of creatine cause a faster inhibition response when compared with the stimulation provoked by lower creatine levels. GAA is also able to inhibit this transporter (Wyss and Kaddurah-Daouk 2000).

2. CREATINE ROLES

Creatine has been used as a supplement and a potential adjuvant treatment for several disorders. The solid evidence available in the literature considers that the main function of creatine is by far energy buffer and transfer. In addition, studies raise a range of possible

secondary creatine functions and effects. However, molecular mechanisms remain a matter of debate and they may be related to bioenergetic role that creatine has in the mitochondria. The pleiotropic effects of creatine are summarized in Figure 1.

2.1 Creatine as an energy reservoir

The main destination of creatine is the system creatine/PCr which strengthens cellular energetics via a temporal and spatial energy buffer that can restore cellular ATP without a reliance on oxygen (Wallimann, Tokarska-Schlattner, and Schlattner 2011). Since the rate of ATP diffusion is insufficient to maintain the energy requirements within cells (de Graaf, van Kranenburg, and Nicolay 2000), PCr offers a solution for this problem because it has a higher diffusion capacity and can reversibly transfer its *N*-phosphoryl group to adenosine diphosphate (ADP). This happens when the concentration of this nucleotide rises inside the cell (Sauer and Schlattner 2004). Therefore, this temporal chemical energy pool in the cytosol is an efficient way to store energy not only in skeletal muscle (destiny of 90% of the body's creatine) but also in other organs and systems, like the CNS, which are highly dependent upon a fair amount of energy in order to properly perform its functions in human body (Schlattner, Tokarska-Schlattner, and Wallimann 2006).

Studies have shown that creatine supplementation protects against ATP depletion and delayed membrane depolarization in *in vitro* models using hippocampal slices and culture of cortical axons (Balestrino, Rebaudo, and Lunardi 1999, Shen and Goldberg 2012). Association of CK with ATP-providing or ATP-consuming processes may occur in order to make this buffer more efficient, in a process called metabolite channeling (Schlattner, Tokarska-Schlattner, and Wallimann 2006). This dynamic helps to prevent overload of the mitochondrial respiratory chain and accumulation of intracellular Ca^{2+} in rat brain, reducing generation of reactive species that have the power to cause oxidative stress and cytochrome C dissociation from the inner mitochondrial membrane thereby initiating early apoptotic triggering events (Meyer et al. 2006). It has been shown that creatine prevents or delays mitochondrial permeability transition pore opening in mitochondria from transgenic mice, an early event in apoptosis (Dolder et al. 2003). Chronic energy disruption also deteriorates cellular structure, in a level that may damage energy production processes as observed in several

neurodegenerative disorders, such as Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD) (van den Bogaard et al. 2011, Martin 2012). In the CNS, creatine plays essential functions in regenerating ATP for glutamate clearance during excitatory synaptic transmission (Oliet, Piet, and Poulain 2001). In this context, knock-out mice for CK isoforms showed behavioral abnormalities, including spatial learning impairment and defects in the formation and maintenance of hippocampal mossy fiber connections (Jost et al. 2002, Streijger et al. 2005). Nonetheless, creatine supplementation restored the corticomotor excitability and cognitive decline associated with hypoxia-induced oxygen deprivation in humans (Turner, Byblow, and Gant 2015) and in experimental models of brain injury (Scheff and Dhillon 2004, Sakellaris et al. 2006). Therefore, the creatine/PCr system appears to be of great value in disease states or situations where there is disruption of the cellular energy metabolism.

2.2 Secondary creatine functions and effects

In addition to the well-known effects of creatine as an enhancer of cellular energetics, there are several other properties that only in recent years have come to light, as it will be presented. It is important to highlight that the mechanistic basis of these properties are still unclear and much of the effects observed may be derived from the energy reservoir provided by creatine. Therefore, studies investigating molecular mechanisms of how creatine exert such secondary effects must be highly encouraged.

Creatine is in constant interaction with reactive species generated in higher levels by tissues with great energy production and consumption. On that account, creatine appears to be both a direct and an indirect antioxidant (Sakellaris et al. 2006) that does not necessarily act by increasing or preventing a drop of the activities of antioxidant enzymes (Guimarães-Ferreira et al. 2012). As one of the major sites of reactive species formation, the mitochondria must have defensive mechanisms to maintain its activity, and creatine may play a crucial role as a defender against such insults. In this context, studies have showed that creatine significantly protects rat mitochondrial DNA from oxidative damage in a dose dependent manner through an ADP-recycling mechanism (Meyer et al. 2006, Guidi et al. 2008). Therefore, creatine supplementation may play an important role in mitochondrial genome stability and cell viability, which can partially explain the enhancement in health and increased life-expectancy found in

rats supplemented with creatine (Bender et al. 2008). Direct antioxidant activity was first considered by Lawler and co-workers (2002), using a cellular experimental setting that showed that creatine protects against radicals such as superoxide anion ($O2^{-}$), peroxinitrite ($ONOO^{-}$), and 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical. From there, several experimental protocols tested the ability of creatine in preventing oxidative imbalance induced damage by UV in human epidermal cells (Lenz et al. 2005), by respiratory chain inhibitor rotenone in whole body homogenates of flies (Hosamani, Ramesh, and Muralidhara 2010), by acute exercise in muscle and plasma of rats (Deminice and Jordao 2012), high levels of GAA in striatum of rats (Kolling and Wyse 2010), and sedentary routine in different organs of rats (Stefani et al. 2014). Furthermore, Sestili and co-workers (2006) demonstrated that living cells in the presence of oxidants, creatine and Fe^{2+}, produced a molecule detected via mass spectrometry experiments with different molecular weight when compared with creatine. Based on this, it was possible to speculate that this finding represented a by-product of creatine oxidation derived from direct scavenging of radical species, rather than from spontaneous oxidation. In addition, there is an indirect antioxidant effect provided by creatine on differentiated mouse myotube cultures (C2C12) via up-regulation of peroxiredoxin-4 and thioredoxin-dependent peroxide reductase, two important antioxidant enzymes located in the cytoplasm and mitochondria, respectively (Young et al. 2010). This result opens the possibility to investigate whether this effect is reproducible in other cell types and highlights the superposition of multiple mechanisms that may explain the creatine´s antioxidant properties.

Besides creatine antioxidant effects, since the 1970s, studies have shown that creatine has an effect on the inflammatory response, which was observed in various experimentally induced inflammatory models (Khanna and Madan 1978, Madan and Khanna 1979). Although the mechanisms involved are not yet fully understood, in recent years the action of creatine as an immunomodulatory agent has gained more attention. The study reported by Nomura and co-workers (2003) examined the effect of creatine supplementation on a number of potent inflammatory mediators induced both locally and systemically in endothelial cell in vitro. The results showed that creatine inhibited endothelial permeability and neutrophil adhesion to endothelial cells by suppressing both ICAM-1 and E-selectin expressions on endothelial cells and adhesion molecule expression. Posterior studies showed that creatine has a positive effect

on the inflammatory response triggered by micro trauma in skeletal muscles after acute anaerobic sprint and strenuous exercise in human, diminishing levels of C-reactive protein, TNFα, INFα, IL-1β and PGE2 (Santos et al. 2004, Bassit, Curi, and Costa Rosa 2008, Deminice et al. 2013). Interestingly, creatine supplementation combined with these exercise protocols also inhibited the increase in CK and lactate dehydrogenase activity (Santos et al. 2004; Bassit et al. 2010), which indicates less muscle soreness and a decrease in skeletal cell injury. This effect on muscle integrity is one of the possible explanations for the effect of creatine on inflammatory markers since reducing muscle cell death might stop the inflammatory process as a whole (Bassit, Curi, and Costa Rosa 2008). A recent study also provided initial evidence that creatine supplementation prevents skeletal muscle atrophy provoked by tumors via attenuation of tumor-induced pro-inflammatory environment in rats (Cella et al, 2019). Based in *in vivo* studies, it was suggested that the decrease in experimentally induced inflammation was the result of creatine reducing the expression of Toll-Like Receptor (TLR) 2, a plasma membrane-bound protein that recognizes acylated bacterial lipoproteins on the macrophages, key cells involved in the early phases of the immune response (Leland, McDonald, and Drescher 2011). Additionally, a recent work with arginine (a precursor of creatine) suggest that much of the mechanism of how creatine acts upon inflammation may be evolutionarily conserved (Azeredo et al. 2015).

On the other hand, studies have surprisingly shown that creatine has an opposite effect on the airways, increasing collagen and elastic fibers deposition in airway walls, eosinophil infiltration and smooth muscle thickness in mice (Vieira et al. 2007, Vieira et al. 2009, Ferreira et al. 2010). These changes are probably mediated via the increase in the release of IL-4, IL-5 and IGF-1 by inflammatory cells, as discussed by Riesberg and co-workers (2016). Recent study showed that creatine increases IL-5 levels and the expression of P2×7 receptor in peribronchial leukocytes and epithelial cells in rats, worsening asthma pathology via purinergic signalling (Garcia et al. 2019). These results may also be partially explained by an increased availability of L-arginine after supplementation with creatine, leading to an increase in the synthesis of nitric oxide that may act either as a protective or a stimulatory factor regarding inflammation (De Gouw et al. 2001). Collectively, the data lead to the intriguing possibility that creatine boosts T helper cell type 2 (Th2) response after creatine supplementation (Vieira et al. 2007).

Regarding the CNS, creatine supplementation may increase cell hydration and membrane stabilization in mice (Wyss and Schulze 2002), both essential features for neuronal function and signal transduction. Moreover, researchers proposed that exocytotic creatine release would be electrically-evoked in an action potential-dependent process, being dependent from Ca^{2+}, inhibited by the Na^{+}-channel blocker tetrodotoxin and enhanced by the K^{+}-channel blocker 4-amino-pyridine, consistent with neurotransmitter behavior (Almeida et al. 2006). Creatine seems to affect GABAa receptors via competitive antagonism at the same time that stimulates glutamatergic transmission via NMDA receptors in the hippocampus (Koga et al. 2005, Royes et al. 2008; Joncquel-Chevalier Curt et al. 2015), which may be related to the spatial memory improvement observed in rats after intrahippocampal administration of creatine (Oliveira et al. 2008). Hot spots of expression of CK isoenzymes have been reported in hippocampal pyramidal cells, which are involved in learning and memory (Kaldis et al. 1996), suggesting that the CK/PCr-system plays an essential role for this group of cells. Such theory is supported by studies that showed that creatine supplementation improves the performance of complex central executive tasks during stress caused by sleep deprivation in humans (McMorris et al. 2007), and intelligence/working memory performance tests even in healthy volunteers (Watanabe, Kato, and Kato 2002, Rae et al. 2003). However, these studies are not unequivocal evidence supporting a particular neurotransmitter function since the highly energy-dependent release and recycling of neurotransmitters at the synapse and the bioenergetic function of creatine may explain these neurological findings. Therefore, more studies are needed to generate consensus towards the potential role of creatine as neurotransmitter, in particular by discovering a so far unknown specific postsynaptic creatine receptor.

3. CREATINE AND NEURODEGENERATIVE/NEUROMETABOLIC DISORDERS

As previously said, creatine is not only important to the muscular tissue, but also to other high energy demanding systems (Schlattner, Tokarska-Schlattner, and Wallimann 2006). The brain relies on an abundant and uninterrupted supply of energy substrates in order to allow electrical membrane potentials, action potential propagation, signaling activities and recycling of neurotransmitters. Disruption or imbalances in the supply of energy for such an important organ compromises its functions, leading to alterations that contribute for the pathogenesis and

progression of neurological and neurodegenerative conditions. These disorders are a group of acquired or inherited diseases that present a progressive loss of cells from one or multiple regions of the nervous system. A lot of work have been done to elucidate the mechanisms surrounding such diseases, but most knowledge still remains equivocal. Nonetheless, there are some biochemical features common in the development and progression of these otherwise different pathological states. Chronic energy disruption accompanied by degradation of mitochondrial/cell structure, apoptosis and oxidative stress are some of these fundamental biochemical processes, and they are in fact present in several neurodegenerative disorders such as PD, AD, HD, and amyotrophic lateral sclerosis (ALS) (Beal 2005, van den Bogaard et al. 2011, Martin 2012). Therefore, since creatine is a fundamental part of the CK/PCr system, it is expected that strategies to boost creatine levels in the brain have potential therapeutic value as it can help to replenish cellular ATP without a reliance on oxygen (Béard and Braissant 2010, Riesberg et al. 2016). One of the challenges of the supplementation with creatine is its difficulty to cross the BBB as previously cited (Braissant 2012). Nonetheless, studies have demonstrated that it is possible to modify brain creatine concentration with oral creatine monohydrate supplementation in different protocols (Dechent et al. 1999, Lyoo et al. 2003, Turner, Byblow, and Gant 2015), thus validating the rational behind the studies cited below.

3.1 Inborn errors of metabolism

Creatine deficiency syndromes, which can be caused by mutations in AGAT, GAMT and SLC6A8 genes, have been identified in humans (Stöckler et al. 1994, Item et al. 2001, Salomons et al. 2001). The alterations in the CNS caused by these inborn errors of metabolism are responsible for the most severe symptoms presented by these patients, like mental retardation, autism, brain atrophy, delays in speech acquisition or epilepsy. These symptoms are associated not only with the depletion of creatine, but also with the toxic accumulation of creatine precursors: arginine and GAA. Mutations in AGAT and GAMT can be treated efficiently with supplementation of creatine (Battini et al. 2006, Marques and Wyse 2016). However, the SLC6A8 deficiency has a less encouraging prognosis. In this deficiency, creatine appears to be able to cross the jejunum via paracellular movement (Orsenigo et al. 2005), providing some improvements on muscular, but none on neurological symptoms since there is no transport of

creatine occurring at the BBB (Arias et al. 2007; Valayannopoulos et al. 2012). Nonetheless, a very recent study has tried to overcome this problem by modifying the creatine molecule, creating di-acetyl creatine ethyl ester, a compound that should cross biological membranes independently of the transporter due to its very high lipophilicity. This compound was able to prevent electrophysiological failure and to increase intracellular creatine in hippocampal slices of mice (Adriano et al. 2018).

Our research group has studied the consequences of accumulation of GAA on rat brain, as well as the effect neuroprotector of creatine on GAA effects. We have observed that creatine is able to prevent several deleterious effects of GAA on energy metabolism and oxidative status, such as the decrease in the activities of complex II, Na^+,K^+-ATPase and creatine kinase, as well as the levels of thiobarbituric acid reactive substances, an index of lipid peroxidation (Kolling and Wyse 2010). In addition, these proprieties presented by creatine appear to be beneficial not only to models of creatine deficiencies but also to other experimental models of innate errors of metabolism, including homocystinuria and hyperprolinemia, where creatine was able to prevent memory impairment, lipid peroxidation, CK activity inhibition and imbalance of redox homeostasis (Kolling et al. 2014, Kolling et al. 2017, Wyse and Netto 2011).

3.2 Alzheimer's disease

AD is the most common form of progressive dementia, with patients presenting a loss of neurons (particularly of the cholinergic system) in cerebral cortex and specific subcortical regions. This neuronal loss is associated with deposits of extracellular plaques (amyloid-β peptide and cellular material) outside and around neurons, and deposits of intracellular neurofibrillary tangles (aggregation of the microtubule-associated protein tau in a hyperphosphorylated form). Mutations in amyloid precursor protein result in abnormalities in its processing, leading to such lesions (Blennow, de Leon, and Zetterberg 2006). At the molecular level, brain isoforms of CK have been shown to be significantly inactivated by oxidation in AD patients, and the depositions appear to be rich in creatine (Bürklen et al. 2006), which may lead to additional deleterious effects in the energetic state of neurons, exacerbating the neurodegenerative process. Creatine may exert neuroprotection by reducing protein aggregation since it was demonstrated that this compound can interfere in transglutaminase-

catalyzed protein aggregation in sedimentation experiments (Burguera and Love 2006). Furthermore, creatine supplementation has been shown to be an effective neuroprotector against amyloid-β neurotoxicity in hippocampal neurons cell culture (Brewer and Wallimann 2000) and recent studies demonstrated that amyloid-β addition to cell cultures of cortical neurons leads to internalization of NMDA-receptors (Snyder et al. 2005). In addition, since inflammation is considered a driving force in the pathogenesis of AD, the effects observed in cells may be, at least in part, due to the apparent anti-inflammatory properties presented by creatine (McGeer, Rogers, and McGeer 2016). However, AliMohammadi and co-workers (2015) showed that the supplementation with creatine had no effect on learning, memory retrieval, or neuron apoptosis in male Wistar rats submitted to amyloid-β injection. It is important to highlight that the situation is far more complex than it looks and more research is needed before the neuroprotective role of creatine in AD patients can be inferred. To date and to the best of our knowledge, no human trial of creatine associated with AD has been published.

3.3 Amyotrophic lateral sclerosis

ALS is a term used to cover the spectrum of neurodegenerative syndromes characterized by progressive loss of motor neurons in the brain and spinal cord, that ultimately leads to muscular paralysis as these neurons degenerate and are replaced by gliosis (Wijesekera and Leigh 2009). Pathogenic mechanisms seem to include oxidative stress, glutamate excitotoxicity, impaired mitochondrial function, and aberrant protein folding (Turner and Talbot 2008). In addition, lower levels of cerebral ATP long before disease onset were identified in mouse models, and reduced CK activity has been reported in transgenic ALS mice (Wendt et al. 2002, Browne et al. 2006). Therefore, providing the cells with exogenous creatine supplementation might provide neuroprotective effects and constitute an effective treatment for ALS patients. Indeed, oral administration of creatine prevented neuronal loss in both the motor cortex and in substantia nigra, minimizing damage caused by reactive species, and producing a dose-dependent improvement in motor performance, as well as greater survival of transgenic mice for the SOD1G93A gene (Andreassen, Dedeoglu, et al. 2001, Dupuis et al. 2004). Shortly after these results were published, creatine treatment evolved to clinical trials. Despite its promising experimental animal models results, creatine tests in humans failed to reproduce

such effects. Creatine presented no effect on survival, motor function, or respiratory function of patients (Drory and Gross 2002, Shefner et al. 2004, Rosenfeld et al. 2008). A more recent systematic review also did not find any statistically significant effect (Pastula, Moore, and Bedlack 2012). Nonetheless, the discrepancy presented by these studies may be related to the period when the treatment starts. This period is of 40 days before onset of disease in mice, and an average of 500 days after onset of symptoms in patients (Beard and Braissant 2010). Futhermore, most animal models are based on the mutation in copper zinc superoxide dismutase presented by hereditary ALS, while sporadic ALS accounts for most cases and only a small percentage of the human patients have the same genetic defect found in these transgenic mice (Bender and Klopstock 2016). Given that a recent study showed that creatine enhances mitochondrial-mediated oligodendrocyte survival after demyelinating injury in mice (Chamberlain et al 2017), and that there is loss of oligodencrocyte in mouse models of ALS (Jones 2013), more work should be encouraged to shed light upon the initial discouraging clinical trials.

3.4 Parkinson's disease

PD is a progressive neurodegenerative disease with motor, nonmotor, and behavioral findings. There is no golden standard for diagnose, so family history and physical examination remain the most commonly used method. Some of the most common clinical symptoms presented by these patients are tremor, bradykinesia, postural imbalance, rigidity, speech impairments, blurred vision and constipation. It is histologically characterized by changes to the mesencephalic substantia nigra, which leads to a profound loss of dopaminergic input into the striatum and development of alpha-synuclein containing Lewy bodies in the surviving dopaminergic neurons of the region (Gazewood, Richards, and Clebak 2013, Johnson 2015). At the molecular level, mitochondrial electron transport system, in particular complex I, appears to play a part in the pathogenesis of PD (Alam and Schmidt 2002), suggesting mitochondrial imbalance and depletion ATP as one of the onset features leading to the development of the condition. Already in 1999, Matthews and co-workers demonstrated that creatine can prevent the loss of dopaminergic neurons in the substantia nigra caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration, the most widely used mouse model of PD. In

addition, co-treatment with cyclooxygenase 2 inhibitor rofecoxib and coenzyme Q10 seemed to enhance neuroprotective effects of creatine in transgenic mouse models (Klivenyi et al. 2004, Yang et al. 2009), suggesting that for better results a combined approach may be the best option. Creatine also improved dopaminergic cell survival against two in vitro model of PD using 1-methyl-4-phenylpyridinium ion (MPP+) or 6-hydroxydopamine (6-OHDA) on an organotypic tissue culture system (Andres, Ducray, Pérez-Bouza, et al. 2005). One of the main side effects of the treatment of PD with L-DOPA is the L-DOPA-induced dyskinesia (LID), characterized by abnormal involuntary movements. In order to study the effect of creatine on this feature, Valastro and co-workers (2009) induced PD in rats using 6-OHDA and submitted the animals to a diet with creatine. After 32 days on the diet, L-DOPA treatment began and signs for the development of abnormal involuntary movements were observed. The results showed that abnormal involuntary movements were considerably diminished in the creatine group, as well as biochemical markers associated with LID, indicating that creatine may be useful not only as a prevention strategy, but also to alleviate side-effects derived from traditional treatment.

Despite so many promising results, once again, when translated to the clinical trials, creatine results are not encouraging. Creatine was shown to result in improved patient mood, but the Unified PD rating scale remained unaltered (Bender et al. 2006). A 2014 systematic review on the subject concluded that all the clinical studies were made with small sample sizes and short duration, and that future well-designed randomized controlled trials with larger sample size and long-term follow-up were needed to assess creatine for PD (Xiao et al. 2014). However, a double-blinded, multicenter, long-term efficacy trial that recruited 1741 PD patients who were randomly treated with either creatine monohydrate (10 g d^{-1}) or placebo, showed no effect. Instead of an 8-year follow-up as intended, the study was interrupted because no differences were noted between creatine- and placebo-treated groups after 5 years (Kieburtz et al. 2015). Recently, metha-analysis studies have not supported the use of creatine for neuroprotection against PD, although the authors suggest that more correlated studies are still needed (Attia et al 2017; Mo et al 2017). Nonetheless, an improvement in upper-body strength of creatine supplemented PD patients (Hass et al 2007) has been reported as well as a decrease in cognitive decline of PD patients treated with coenzyme Q10 associated with creatine (Li et al. 2015). These findings could perhaps be a guide to determine whether an

earlier treatment would reproduce the beneficial effects observed in animal trials, since the Phase III trial was evaluated in patients with progressed stages of the disease.

3.5 Huntington's disease

HD is an autosomal dominant disorder caused by a CAG repeat expansion in exon 1 of the huntingtin gene, producing a mutant form of the Huntingtin protein (mHtt). It is characterized by some main symptoms that include progressive choreoathetotic movements, cognitive impairment and neuropsychiatric disturbance, leading ultimately to death after a mean survival time of 15–20 years (Quinn and Schrag 1998). Different from other neurodegenerative disorders that have multiple causes and factors involved, HD is caused by a mutation in a single gene. This makes it easier, at least in theory, to search for an effective treatment (Kim and Fung 2014). The exact mechanism by which mHtt leads to neuronal death (mostly GABAergic projections) is still unclear, but energy metabolism deficit has been observed and proposed as a possible factor. This impairment include reduced mitochondrial complex II and complex III activities, which leads to increased cerebral lactate levels and a reduced PCr/inorganic phosphate ratio in muscle (Grünewald and Beal 1999, Calabresi et al. 2001). Therefore, boosting intracellular energy stores may be a useful strategy to alleviate symptoms and neurodegenerative progression. In vitro models have shown that creatine protects GABAergic cells against 3-nitropropionic acid (3-NPA), an irreversible succinate dehydrogenase inhibitor, induced toxicity in striatal cultures (Andres, Ducray, Huber, et al. 2005). In rat models of brain injections of 3-NPA or malonate (a reversible succinate dehydrogenase inhibitor) oral supplementation with creatine resulted in smaller lesion sizes (Matthews et al. 1998), and creatine injected intraperitoneally protected against convulsive behavior and lactate production (Royes et al. 2006). Furthermore, in knock-out mouse models of HD, creatine administration resulted in greater survival, increased body weight, delayed motor symptoms, and considerably reduced brain lesion size with huntingtin-positive aggregates (Ferrante et al. 2000, Andreassen, Jenkins, et al. 2001). A drawback is that in both studies creatine supplementation started at a pre-symptomatic stage. However, a later study found similar results in post-symptomatic knock-out mice (Dedeoglu et al. 2003). Although well tolerated by patients, clinical trials have failed to improve Unified HD Rating Scale, an index used to assess cognitive, motor function,

and functional ability (Verbessem et al. 2003, Tabrizi et al. 2005). On the other hand, brain glutamate levels and serum 8-hydroxy-2′-deoxyguanosine (a marker of oxidative injury to DNA that is considerably elevated in HD) in patients were consistently decreased after a creatine enhanced diet, suggesting at least some efficacy of creatine treatment (Bender et al. 2005, Hersch et al. 2006). A 2014 study by Rosa and co-workers made use of higher doses of creatine to treat patients likely to develop HD since they had affected first degree relatives, or a pre-symptomatic gene mutation detected. At 6 and 18 months of creatine or placebo, neuroimaging was done to measure brain atrophy. Individuals in the creatine group had significantly less cortical and striatal atrophy compared to that observed in control group, suggesting that, if used in an ideal window, creatine may be able to delay disease progression and symptoms. Thus, a multicenter, randomized, double-blind, placebo-controlled study of up to 40 g daily of creatine monohydrate in participants with stage I and II HD treated for up to 4 years enrolled 553 participants but was halted for futility after the first interim analysis, providing contradictory data (Hersch et al. 2017).

4. CONCLUSION

Creatine has been widely used as an enhancer of muscular performance since the 1970s. In this review we addressed its possible effects and functions in human beings, as well as the results that creatine presented as an adjuvant treatment in preclinical models and clinical trials for several diseases. The solid evidence available in the literature considers that the main function of creatine is by far to allow fast regeneration of ATP, in ATP demanding sites, via CK activity. In addition, studies raise a range of possible secondary creatine functions and effects, including direct and indirect antioxidant activity, overall anti-inflammatory effects (with the exception of the airways), and possible neuromodulation of synapses. However, molecular mechanisms remain a matter of debate and they may be related to the bioenergetic role that creatine has in the mitochondria. It is a consensus that creatine supplementation is safe and has no serious collateral effects, as stated by the official position of the International Society of Sports Nutritition which has also refuted concerns surrounding renal toxicity (Kreider et al. 2017). In this context, and given the fundamental roles that creatine plays in the CNS, several preclinical and clinical studies have observed the potential that creatine has to treat

degenerative disorders. However, although *in vitro* and *in vivo* experimental models are highly encouraging, most clinical trials fail to reproduce positive results, suggesting that animal models are better to address biological aspects of a possible treatment than to predict clinical efficacy.

Indeed, very few, mainly small pilot studies reported promising functional or neurological improvements by creatine in human beings. Therefore, future studies should first try to establish the best dosage regime to increase brain creatine in a way that can relate to animal studies. In order to avoid the inevitable saturation of SLC6A8 and the poor permeability of the BBB for creatine, di-acetyl creatine ethyl ester, a compound that should cross biological membranes independently of the transporter due to its very high lipophilicity may represent a promising alternative (Adriano et al. 2018). Furthermore, available data suggests that a prophylactic use for neuroprotection in at-risk populations or patients is the most promising field. Therefore, future studies would benefit from biomarkers predictive of efficacy and determination whether baseline bioenergetics status is a significant variable in whether or not creatine supplementation works. To conclude, to this point the only clearly positive data on human creatine supplementation in neurodegenerative/metabolic diseases concern the (rare) creatine deficiency syndromes, when the enzymes responsible for creatine biosynthesis are impaired.

Conflicts of interest

The authors declare that they have no conflict of interest.

Legend

Fig 1 Creatine produced in the liver travels in the bloodstream, where it actively enters the cells using the specific creatine transporter, SLC6A8. These transporters are in low levels in the micro capillaries of the BBB, and are not expressed by the majority of perivascular astrocytes. Therefore, although the passage of creatine from the bloodstream to the CNS does occur, the CNS cells have their own pathway for the synthesis and maintenance of creatine levels. Once inside the cells, approximately two-thirds of the available creatine undergoes a reversible phosphorylation catalyzed by CK, giving rise to PCr, a temporal and spatial energy buffer that can restore cellular ATP without a reliance on oxygen, preventing overload of the mitochondrial respiratory chain, and generation of reactive species. In addition, studies raise a range of

possible secondary creatine functions and effects that are presented in the figure inside blue boxes: creatine appears to increase cell hydration and membrane stabilization, may have direct antioxidant activity, and a possible suppressor effect on inflammation (except in the airways, where it appears to exacerbate the response). Regarding the CNS, in addition to all previous effects, creatine may play a part as a neuromodulator. However, molecular mechanisms for all of these secondary functions remain a matter of debate and they may be related to creatine's bioenergetic role in mitochondria.

ABREVIATIONS

3-NPA: 3-nitropropionic acid

6-OHDA: 6-hydroxydopamine

AD: Alzheimer's disease

AGAT: L-arginine: glycine amidinotransferase

ADP: Adenosine diphosphate

AMPK: AMP-activated protein kinase

ALS: Amyotrophic lateral sclerosis

ATP: Adenosine triphosphate

BBB: Blood brain barrier

CK: Creatine kinase

CNS: Central nervous system

GAMT: *N*-guanidinoacetate methyltransferase

GAA: Guanidinoacetate

PCr: Phosphocreatine

SAM : S-adenosylmethionine

HD: Huntington's disease

LID: L-DOPA-induced dyskinesia

mHtt: Huntingtin protein

MPP+: 1-methyl-4-phenylpyridinium ion

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

PD: Parkinson's disease

Th2: T helper cell type 2

TLR: Toll-Like receptor

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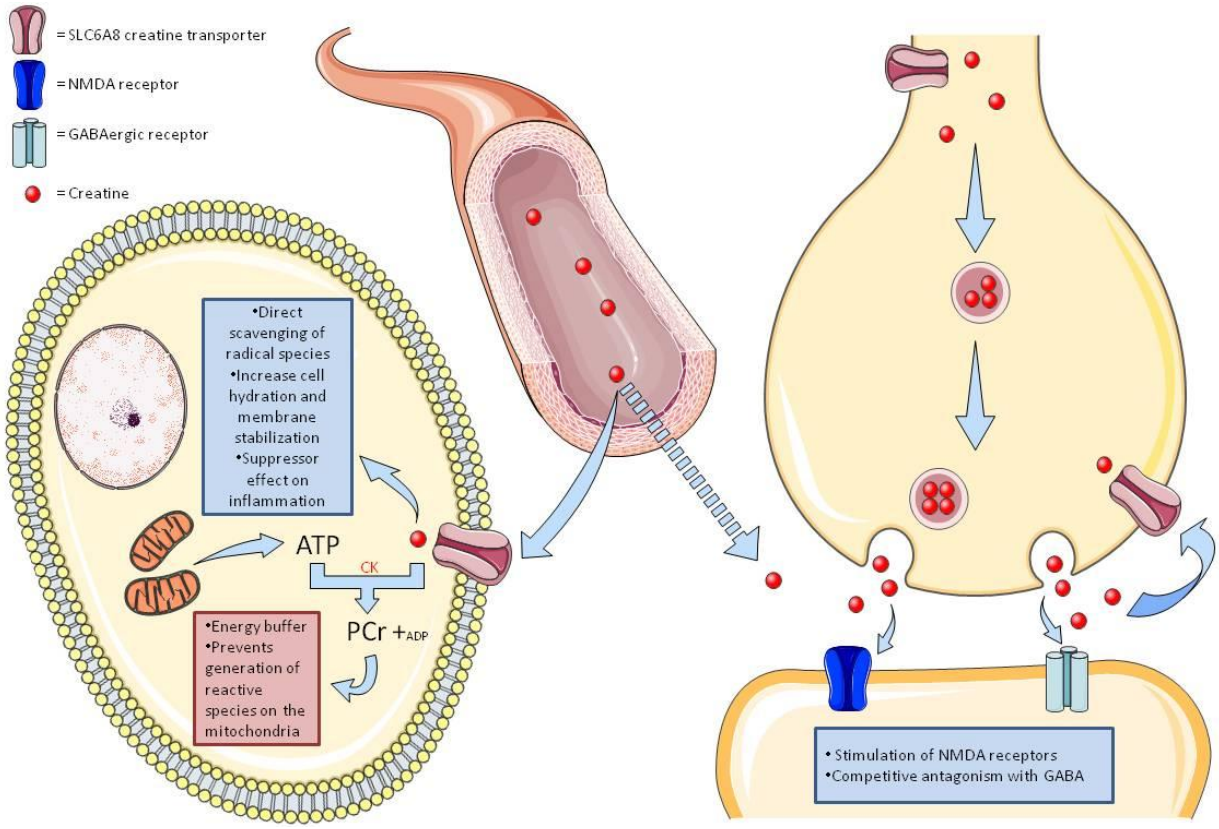

Fig 1 Creatine produced in the liver travels in the bloodstream, where it actively enters the cells using the specific creatine transporter, SLC6A8. These transporters are in low levels in the micro capillaries of the BBB, and are not expressed by the majority of perivascular astrocytes. Therefore, although the passage of creatine from the bloodstream to the CNS do occur, the CNS cells have their own pathway for the synthesis and maintenance of creatine levels. Once inside the cells, approximately two-thirds of the available creatine undergoes a reversible phosphorylation catalyzed by CK, giving rise to PCr, a temporal and spatial energy buffer that can restore cellular ATP without a reliance on oxygen, preventing overload of the mitochondrial respiratory chain, and generation of reactive species on the mitochondria. Furthermore, creatine appears to possess a number of secondary effects, such as increase cell hydration and membrane stabilization, direct antioxidant activity, and a suppressor effect on inflammation (except in the airways). Regarding the CNS, in addition to all previous effects, creatine may modulate GABAergic and glutamatergic receptors via competitive antagonism and stimulation of NMDA receptors, respectively.

3.2 Capítulo II - Artigo II

Cross-talk between guanidinoacetate neurotoxicity, memory and possible neuroprotective role of creatine

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Periódico: BBA - Molecular Basis of Disease

Status: Submetido

ABSTRACT

Guanidinoacetate Methyltransferase deficiency is an inborn error of metabolism that results in decreased creatine and increased guanidinoacetate (GAA) levels. Patients present neurological symptoms whose mechanisms are unclear. We investigated the effects of an intrastriatal administration of 10 μM of GAA (0.02nmol/striatum) on energy metabolism, redox state, inflammation, glutamate homeostasis, and activities/immunocontents of acetylcholinesterase and Na^+,K^+-ATPase, as well as on memory acquisition. The neuroprotective role of creatine was also investigated. Wistar rats were pretreated with creatine (50 mg/kg) or saline for 7 days. Sixty-day-old animals then underwent stereotactic surgery and were divided into four groups: Control, GAA, GAA+Creatine, and Creatine. Experiments were performed thirty minutes after intrastriatal infusion. GAA decreased SDH, complexes II and IV activities, and ATP levels, but had no effect on mitochondrial mass/membrane potential. Creatine totally prevented SDH and complex II, and partially prevented COX and ATP alterations. GAA increased dichlorofluorescein levels and decreased superoxide dismutase and catalase activities. Creatine only prevented catalase and dichlorofluorescein alterations. GAA increased cytokines, nitrites levels and acetylcholinesterase activity, but not its immunocontent. Creatine prevented such effects, except nitrite levels. GAA decreased glutamate uptake, but had no effect on the immunocontent of its transporters. GAA decreased Na^+,K^+-ATPase activity and increased the immunocontent of its α3 subunit. The performance on the novel object recognition task was also impaired. Creatine partially prevented the changes in glutamate uptake and Na^+,K^+-ATPase activity, and completely prevented the memory impairment. This study helps to elucidate the protective effects of creatine against the damage caused by GAA.

Key words: Guanidinoacetate; mitochondria; inflammation; memory recognition; creatine; neuroprotection.

1. Introduction

Guanidinoacetate (GAA) is highly involved in the metabolism of creatine, being its direct precursor [1]. Creatine can be phosphorylated by the creatine kinase (CK) isoforms to phosphocreatine (PCr), an important cellular energy buffer [2, 3]. *N*-guanidinoacetate methyltransferase (GAMT) deficiency is a rare metabolic disease of the metabolism of creatine that leads to a decrease in creatine levels associated with tissue accumulation of GAA, including brain, skeletal muscle, blood and other tissues [4, 5]. Symptoms include intractable epilepsy, intellectual and memory impairment, autism, extra pyramidal syndrome, slurred speech and hypotonia [6-8]

As the brain is a high energy demanding organ, alterations in its energy metabolism can lead to oxidative stress and other serious consequences [9, 10]. In this context, it has been shown that energy metabolism alterations are often correlated with several neurodegenerative diseases [11, 12]. Studies from our group demonstrated that the intrastriatal injection of GAA inhibited the activity of crucial enzymes for the generation of energy in the central nervous system (CNS), such as complex II and II-III of the mitochondrial respiratory chain [13], suggesting that oxidative stress is involved in the mechanisms underlying these changes [14]. Neuroinflammation, or more specifically, activation of the neuroimmune cells microglia and astrocytes into proinflammatory states, has been implicated not as an initiator, but as a pathological contributor for progression of several neurodegenerative diseases, and it is often caused by accumulation of a toxic molecule [15]. Uncontrolled inflammation may result in the production of neurotoxic factors that amplify underlying disease states, like the pro-inflammatory cytokines TNF-α, IL1β and IL6, as well as nitrites [16, 17]. Furthermore, the cholinergic system play a key role in learning and memory mechanisms, and more recently has been associated with the modulation of inflammatory pathways [18, 19]. Acetylcholinesterase (AChE) is the enzyme responsible for the degradation of acetylcholine (ACh) released in the synaptic cleft [20]; and an increase in its activity is related to neurodegenerative diseases and inborn errors of metabolism, including GAMT deficiency [21-23].

Cytokines and nitric oxide appear to affect astrocytes, oligodendrocytes, and microglia, exerting considerable effects on extracellular glutamate concentrations [24, 25]. Glutamate transport through its most frequent carriers (GLAST and GLT-1) is dependent on the Na^+ gradient provided by the enzyme Na^+,K^+-ATPase, thus it can be affected by fluctuations in the activity of this enzyme. However, GAA decreases membrane fluidity and reports from our laboratory have shown that

intrastriatal administration of GAA inhibits Na^+,K^+-ATPase activity embedded in the plasma membrane in the rat striatum [26]. Supplementation with creatine has been used as pharmacological treatment in GAMT deficiency for a long time [27, 28]. The system creatine/PCr provides an energy reservoir, protecting against adenosine triphosphate (ATP) depletion and delayed membrane depolarization [29, 30]. Moreover, creatine is also both a direct and an indirect antioxidant [31], an immunomodulatory agent [32, 33], and a possible modulator of GABAergic and glutamatergic receptors [34]. These creatine properties may have positive results not only to GAMT deficiency patients, but also to other innate errors of metabolism [35, 36].

In the present study, we extended our previous reports investigating the effects of an intrastriatal administration of GAA on some parameters of energy metabolism, mitochondrial function, redox state and inflammation, glutamate homeostasis, and activities and immunocontents of Na^+,K^+-ATPase and AChE, as well as the process of memory acquisition (via novel object recognition task). The neuroprotective role of creatine on the biochemical changes observed in this model was also investigated. Our hypothesis is that the alterations caused by GAA in the striatum energy metabolism are closely associated with oxidative insults and inflammatory processes, leading to alterations in the glutamatergic and synaptic homeostasis, and consequently relating with memory impairment. Since the creatine/PCr system is of great value in disease states or situations where there is disruption of the cellular energy metabolism [37, 38], we speculate that creatine might prevent such alterations. Striatum was used because patients with GAMT deficiency present basal ganglia abnormalities [39].

2. Materials and methods

2.1 Animals and reagents

Wistar rats weighing 180–200 g were obtained from the Central Animal House of the Department of Biochemistry, Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12h light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology following the Guide for Care and Use of Laboratory Animals and Arouca Law (11794/2008). The project was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil (number 29887). All efforts

were made to minimize the number of animals used and their suffering. The chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.2 Pretreatment with creatine

The animals used for experiments in this study were subjected to a pretreatment for 7 days, receiving a daily intraperitoneal injection of creatine (50 mg/kg), or saline [40]. During this pretreatment, stereotaxic surgery was performed in the animals in order to facilitate the administration of GAA, as described below.

2.3 Surgery and intrastriatal administration

Surgery and intrastriatal infusion was performed, according to Folbergrova and colleagues [41] and Zugno and colleagues [26]. Sixty-day-old animals were anesthetized with an intraperitoneal injection of equitesin solution contained thiopental (2,5 mg/kg i.p). The heads of the animals were fixed in a stereotaxic apparatus, the skin of the skull was removed and a 27-gauge 9-mm guide cannula was then placed above the striatum (AP: −0.5 mm; L: +/−2.5 mm; DV: −2.5 mm). The cannula was fixed with acrylic cement. The correct position of the needle was tested by 0.5 mL of methylene blue injection (4% in saline solution) and carrying out histological analysis. Experiments were performed at 48h after surgery. A 30-gauge cannula was fitted into the guide cannula and connected by a polyethylene tube to a 5 μL Hamilton micro syringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula towards the striatum. The animals were divided into four groups: Control (pretreatment with saline and intrastriatal infusion of saline), GAA (pretreatment with saline and intrastriatal infusion of 10 μM of GAA [0.02 nmol/striatum]), GAA+Creatine (pretreatment with creatine and intrastriatal infusion of 10 μM of GAA [0.02 nmol/striatum]), and Creatine (pretreatment with creatine and intrastriatal infusion of saline). The volume administered intrastriatally (saline or GAA solution) was 2 μL. Thirty minutes minutes after intrastriatal infusion, the rats were decapitated without anesthesia (for biochemical studies) or subjected to the behavioral assessment.

2.4 Assay of the activities of the mitochondrial respiratory chain complexes

Striata were homogenized (1:20, w/v) in SETH (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU mL−1 heparin) buffer, pH 7.4. The homogenates were centrifuged at 800×g for 10 min, and the supernatants were kept frozen until determinations. The activities of the respiratory chain complexes were calculated as nanomoles per minute per milligram of protein.

2.4.1 Complex II (Succinate: 2,6-Dichloroindophenol Oxireductase) Activity

Homogenates are following the decrement in absorbance due to the reduction of 2,6-dichloroindophenol at 600 nm with 700 nm as reference wavelength (ε = 19.1 mM−1 cm−1), in accordance to Fischer and colleagues [42]. The reaction mixture containing 40 mM potassium phosphate, pH 7.4, 16 mM succinate, and 8 µM 2,6-dichloroindophenol was pre-incubated with 40–80 µg homogenate protein for 20 min at 30 °C. After, 4 mM sodium azide and 7 µM rotenone were added. After adding 40 µM 2,6-dichloroindophenol, the reaction initiated and was monitored for 5 min. All samples were run in triplicate.

2.4.2 Succinate dehydrogenase (SDH) activity

SDH activity was measured as described by Fischer and colleagues [42]. Samples were frozen and thawed three times to break mitochondrial membranes. The enzymatic activity was determined following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol at 600 nm with 700 nm as reference wavelength ($\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$) in the presence of phenazine methasulfate. The reaction mixture containing 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8 µM 2,6-dichloroindophenol was preincubated with 40–80 µg homogenate protein for 20 min at 30 °C. Then, 4 mM sodium azide, 7 µM rotenone, and 40 µM 2,6-dichloroindophenol were added. After adding 1 mM phenazine methasulfate, the reaction initiated and was monitored for 5 min. All samples were run in triplicate.

2.4.3 Cytochrome c oxidase (COX) activity

COX activity was measured according to Rustin and colleagues [43]. The activity of this enzyme was determined at 25 °C for 10 min by following the decrease in absorbance due to oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\varepsilon = 19.1\ mM^{-1} \times cm^{-1}$). The reaction buffer consisted of 10 mM potassium phosphate, pH 7.0, 0.6 mM *n*-dodecyl-β-D-maltoside, 2–4 µg homogenate protein. Reaction initiated after addition of 0.7 µg reduced cytochrome *c*. All samples were run in triplicate.

2.5 ATP Levels Assay

Striata were immediately dissected and frozen in liquid nitrogen. Each striatum was weighed and homogenized in 1 mL of 0.1 M NaOH (to inactivate cellular ATPases activity). Samples were assayed using the ATPlite Luminescence ATP detection assay system (Perkin-Elmer, Waltham, MA, USA) according to Witt and colleagues [44]. The measurement of

chemiluminescence was performed using a Perkin-Elmer Microbeta Microplate Scintillation Analyzer. ATP concentrations were calculated from a standard curve, normalized against wet tissue weights in grams and expressed as micromoles per gram of tissue. All samples were run in triplicate.

2.6 Mitochondrial Mass and Mitochondrial Membrane Potential Measurements

Striata were mechanically dissociated in PBS containing collagenase to yield digestion to a density of about 200,000 cells/ml. The dissociated contents were then filtered into sterile 50-mL Falcon tubes through a 40-μm nylon cell strainer and kept on ice until mitochondrial staining. Dissociated cells were stained with 100 nM MitoTracker Green and 100 nM MitoTracker Red (diluted from 1 mM stock solutions in dimethylsulfoxide) for 45 min at 37 °C (and in the dark), according to the method described by Keij and colleagues [45] and Pendergrass and colleagues [46] with some modifications, to determine mitochondrial mass and mitochondrial membrane potential, respectively. Immediately after staining, cell suspensions were analyzed on a FACSCalibur flow cytometer, using red (670 nm long pass) and green (530 nm/30) filters. Controls stained with a single dye were used to set compensation. For each sample, 10,000 events corresponding to intact cells (as gated in FSC versus SSC plots) were analyzed. All flow cytometric acquisition and analyses were performed using CELLQuest Pro data acquisition and FlowJo software.

2.7 Oxidative stress parameters

2.7.1 Tissue Preparation

The striatum was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl, to determine the oxidative stress parameters. The homogenate was centrifuged at 750 × *g* for 10 min at 4 °C; the pellet was discarded and the supernatant was immediately separated and used for the measurements.

2.7.2 Superoxide dismutase (SOD) assay

SOD activity assay is based on the auto-oxidation ability of pyrogallol, a process highly dependent on superoxide, which is the substrate for SOD [47]. The inhibition of this compound autoxidation occurs in the presence of SOD, whose activity is then indirectly assayed at 420 nm using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA, USA). A calibration curve was performed with purified SOD as standard in order to calculate the

activity of SOD present in the samples. The results were reported as units per mg of protein. All samples were run in triplicate.

2.7.3 Catalase (CAT) assay

CAT activity was assayed using SpectraMax M5/M5 Microplate Reader. The method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1–0.3 mg protein/mL [48]. One CAT unit is defined as one μmol of hydrogen peroxide consumed per minute and the specific activity is calculated as CAT units/mg protein. All samples were run in triplicate.

2.7.4 2′,7′-dichlorofluorescein (H_2DCF) oxidation assay

Reactive species production were measured second to LeBel and colleagues [49] method, based on the oxidation of H_2DCF. Samples (60 μL) were incubated for 30 min at 37 °C in the dark with 240 μL of 100 μM 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) solution in a 96 wells plate. H_2DCF-DA is cleaved by cellular esterases and the resultant H_2DCF is eventually oxidized by reactive species presenting in samples. The last reaction produces the fluorescent compound dichlorofluorescein (DCF) which was measured at 488 nm excitation and 525 nm emission and the results were represented by nmol DCF/mg protein. All samples were run in triplicate.

2.8 Cytokines Levels Measurements

The samples were homogenized (1:5, w/v) in phosphate buffered saline (PBS, pH 7.4). The homogenates were centrifuged at 800×g for 10 min at 4 °C, and the supernatants were used for analysis. TNF-α, IL-1β and IL-6 levels in the striata were measured by kit rat high-sensitivity enzyme-linked immunoabsorbent assays (ELISA) with commercially available kits (Sigma-Aldrich®). All samples were run in triplicate.

2.9 Nitrite Assay

The striatum was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. The homogenate was centrifuged at 750 × *g* for 10 min at 4 °C. 100 μL of supernatant was mixed with 100 μL Griess reagent (1:1 mixture of 1 % sulfanilamide in 5 % phosphoric acid and 0.1 % naphthylethylenediamine dihydrochloride in water) and incubated in 96-well plates for 10 min at room temperature [50, 51]. The absorbance was measured on a microplate reader at a wavelength of 543 nm. Nitrite concentration was calculated using sodium nitrite standards. All samples were run in triplicate.

2.10 AChE activity assay

AChE activity was determined by the method of Ellman and colleagues [52]. For AChE assay, striatum was homogenized in 10 volumes (1:10, w/v) of 0.1 mM potassium phosphate buffer, pH 7.5, and centrifuged for 10 min at 1000 × g, being the supernatants used for the enzymatic AChE analyses. Hydrolysis rate was measured at ACh (S) concentration of 0.8 mM in 1 ml assay solutions with 100 mM phosphate buffer (pH 7.5) and 1.0 mM DTNB. Fifty microliters of striatum homogenate was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) at 25°C. All samples were run in triplicate.

2.11 Glutamate uptake

Glutamate uptake was performed according to previous reports [53, 54]. The uptake assay was carried out by adding 0.66 μCi ml^{-1} L- [^{3}H] glutamate and 100 μM unlabeled glutamate (final concentration) in 20 μl Hank's balanced salt solution (HBSS) at 37°C. Incubation proceeded at 35°C and was stopped after 3 min by two ice-cold washes with 1 ml HBSS, immediately followed by addition of 0.5N NaOH, which was kept overnight. Incorporated radioactivity was measured using a scintillation counter (Wallac 1400). Sodium-independent uptake was determined in parallel assays using N-methyl-D-glucamine instead of sodium chloride. This uptake was substracted from the total uptake to obtain the sodium-dependent uptake. All experiments were performed in triplicate.

2.12 Na^+,K^+-ATPase Activity Assay

The samples were homogenized (1:10, w/v) in 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.5. Homogenates were centrifuged at 1000×g for 10 min at 4 °C. Supernatants were taken for Na^+,K^+-ATPase activity assay. Reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM MgCl2, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 μL. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of ATP to a final concentration of 3.0 mM and was incubated for 20 min. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays [55]. Inorganic phosphate (Pi) released was measured by the method of Chan and colleagues [56] and enzyme-specific activity was expressed as nanomoles Pi per minute per milligram of protein. All samples were run un triplicate.

2.13 Behavioral procedures

Behavioral procedures were performed between 10 a.m. to 3 p.m. in a controlled light and sound room, by a researcher blind to the animal's experimental condition. GAA was injected 30min before the training session, in order to evaluate the process of memory acquisition. The test session was performed 1h after the training to assess short-term memory. One day before the training session, all animals were habituated to walk freely in the empty arena for 5 min. The arena used was a black wooden box (50 × 50 × 50 cm). In the training session, two identical objects (objects A1 and A2) were placed equidistant from the sidewalls. In this chamber, each animal performed a trial of 5 min. After each trial, the apparatus was cleaned to alleviate olfactory cues. In the second trial, the test session, one of the objects (object A2) was substituted by a new and different one (object B). An experimenter registered the time of object exploration, i.e., touching it with paws or exploring it by olfaction with direct contact of the snout [57]. The object discrimination index was calculated in the test session, as follows: the difference in exploration time divided by the total time spent exploring the two objects {[(B − A1)/(A1 + B)] where B is the new object and A1 is the familiar object}. Rats without memory impairment explore the new object for more time when compared with the old one [58].

2.14 Western Blot Analysis

Western blotting was performed as described by Biasibetti-Brendler and colleagues [59]. The striatum was homogenized in 200 µL of a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, and 4% SDS. For electrophoresis analysis, samples were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, and 20 mM Tris–HCl, pH 6.8 and boiled for 3 min. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE (30 µg/lane of total protein) and transferred (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48-mM Trizma, 39-mM glycine, 20% methanol, and 0.25% SDS). The blot was then washed twice for 5 min in 0.05% Tween-20 Tris-buffered saline (T-TBS) and twice for 5 min with Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by a 2-h incubation in blocking solution (TBS plus 5% bovine serum albumin, fraction V). Then, membranes were washed for 10 min with TBS and incubated with primary antibody overnight at 4 °C: 1:1000 rabbit anti-AChE, 1:1000 mouse anti-Na^+,K^+-ATPase (α3 subunit), 1:1000 rabbit anti-GLAST and rabbit anti-GLT-1 glutamate transporters, and 1:2000 mouse anti-β-actin. The blot was then washed twice for 5 min with T-TBS and twice for 5 min with TBS,

followed by an incubation for 2 h in antibody solution containing peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-rabbit IgG diluted 1:2000. The blot was then washed twice for 5 min with T-TBS and twice for 5 min with TBS, and then it was developed using a chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate, Millipore) and detected by ImageQuant LAS 4000 (GE Healthcare Life Sciences). Band intensities were analyzed using Image J software (developed at the US National Institutes of Health and available on the website http://rsb.info.nih.gov/nih-image/). Band intensity was normalized to β-actin as a loading control to assess protein levels.

2.15 Protein determination

Protein concentration was measured by the method of Lowry and colleagues [60] using bovine serum albumin as standard for most methods. For AChE and Na^+,K^+-ATPase activities, proteins were measured by the Comassie Blue method according to Bradford and colleagues [61].

2.16 Statistical analysis

The parametric data for four groups were analyzed by two-way analysis of variance (ANOVA) followed by post hoc Tukey test when F-test was significant. Values of $p<0.05$ were considered statistically significant. All analyzes and graphics were performed using GraphPad Prism 6.0 software program in a compatible computer.

3. Results

In the present study we addressed the ability of creatine in preventing the deleterious action of GAA in striatum of rats. Firstly, we evaluated the effect of GAA intrastriatal injection on cellular energy metabolism as shown in Table 1. The results showed that GAA decreased the activities of complex II ($F(3,29) = 17.08$ $p<0.05$), SDH ($F(3,29) = 18.14$ $p<0.05$) and COX ($F(3,21) = 16.69$ $p<0.05$). Creatine totally prevented the impairment in complex II ($F(3,29) = 4.181$ $p<0.05$) and SDH ($F(3,29) = 4.305$ $p<0.05$), but had only a partial prevention of COX ($F(3,21) = 4.977$ $p<0.05$). ATP levels were decreased in rats submitted to an intrastriatal injection of GAA ($F(3,18) = 12.7$ $p<0.05$), and creatine was able to partially prevent such effect ($F(3,18) = 4.918$ $p<0.05$). To study if the enzymatic effect were due physiological changes in the mitochondria, we assess mitochondrial mass and mitochondrial membrane potential, but these parameters remained unaltered when compared to the control group ($p>0.05$).

Since mitochondria is the main site for generation of reactive species, and that redox imbalance may originate from the energetic disturbances like the ones that we observed [62], our next step was to evaluate if GAA and this imbalance in the enzymes of the mitochondrial respiratory chain could lead to alterations in oxidative stress parameters and if creatine could prevent them. GAA led to a decrease in SOD ($F(3,28) = 29.36$ $p<0.05$) and CAT ($F(3,28) = 7.650$ $p<0.05$) activity, but creatine was only able to prevent the decrease in CAT activity ($F(3,28) = 6.745$ $p<0.05$). Furthermore, the reactive species production was increased in rats that suffered GAA intrastriatal infusion when compared with control group, as indicated by the increase of DCF levels ($F(3,21) = 5.071$ $p<0.05$). Creatine was able to prevent such alteration ($F(3,21) = 12.21$ $p<0.05$). These results are summarized in Table 2.

Changes in redox homeostasis are highly interconnected with inflammatory processes, so we measured several inflammatory markers in order to investigate if this was the case in our acute model, as can be seen in Table 3. GAA increased the levels of cytokines TNF-α ($F(3,18) = 8.680$ $p<0.05$), IL1β ($F(3,20) = 5.351$ $p<0.05$) and IL6 ($F(3,20) = 15.05$ $p<0.05$), and creatine completely prevented these alterations ($F(3,18) = 6.264$ $p<0.05$; $F(3,20) = 8.390$ $p<0.05$; $F(3,20) = 5.932$ $p<0.05$, respectively). GAA also increased nitrite content ($F(3,18) = 54.56$ $p<0.05$), which was not prevented by creatine ($p>0.05$).

In addition, we evaluated the effect of GAA intrastriatal injection on AChE activity and immunocontent. GAA infusion increased AChE activity ($F(3,23) = 10.264$ $p<0.05$) and creatine prevented this effect ($F(3,23) = 5.743$ $p<0.05$) (Figure 1a). However, GAA had no effect on AChE immunocontent ($p > 0.05$) (Figure 1b).

Having established the overall energetic, redox and inflammatory state, we went on to see possible alterations in the glutamatergic system or in the Na^+,K^+-ATPase activity. Figure 2a shows that GAA intrastriatal injection decreased glutamate uptake in the striatum ($F(3,23) = 12.78$ $p<0.05$) when compared to the control group, and that creatine was able to partially prevent this alteration ($F(3,23) = 4.359$ $p<0.05$). Immunocontents of GLAST and GLT-1 transporters were also evaluated, and the results showed that GAA did not alter the immunocontents of these transporters ($p>0.05$), as shown in Figure 2b and 2c.

Regarding Na^+,K^+-ATPase activity, Figure 3a shows that GAA significantly inhibited this enzyme ($F(3,20) = 8.645$ $p<0.05$), and creatine was able to provide only a partial prevention ($F(3,20) =$

7.007 $p<0.05$). Examination of Na^+,K^+-ATPase α3 subunit by immunoblot also revealed that this protein content was increased in the striatum of rats submitted to the intrastriatal injection of GAA ($F(3,24) = 31.40$ $p<0.05$) and that creatine did not prevent this increase, as can be seen in Figure 3b.

Finally, we addressed whether the decreased glutamate uptake and the alterations in crucial enzymes of the CNS could lead to memory deficits as the ones observed in patients, and if creatine would be able to successfully prevent them. In the object recognition test, control rats spent less time on the familiar object in the test session (Figure 4B) when compared to the training (Figure 4A), as highlighted by the discrimination index (Figure 4C). Therefore, the time exploring the novel object increased in the control group while the same did not happened in animals that received intrastriatal infusion of GAA ($F(3,34) = 9.328$ $p<0.05$). Creatine per se exerts no effect on this parameter, but when associated with GAA it was able to prevent the alterations ($F(3,34) = 4.345$ $p<0.05$).

4. Discussion

Neurological symptoms are common and variable among individuals with GAMT deficiency, but the pathophysiology of this disease is still unclear. Previous work from our laboratory had already found alterations in redox state and in energy metabolism in rats submitted to an intrastriatal injection of GAA [13, 14, 63]. In the present study, we expanded previous reports by verifying creatine's preventive potential on these effects and on inflammation, as well as the effect on a non-aversive memory test.

The brain is a high energy demanding organ, and any alterations in its energy metabolism can lead to oxidative stress and other serious consequences [10]. Chronic energy disruption also deteriorates cellular structure in a level that may damage energy production processes as observed in several neurodegenerative disorders [11, 12]. Intrastriatal injection of GAA led to decreased activity in the enzymes of mitochondrial respiratory chain (Complex II, SDH and COX). The incapacity of this chain to support the cell with a sufficient amount of energy can promote accumulation of intracellular Ca^{2+}, thus generating reactive species that have the power to cause oxidative stress and cytochrome C dissociation from the inner mitochondrial membrane [64, 65]. The increase in reactive species generation, indicated by the increase in DCF levels may lead to alterations of components of the plasma membrane in which enzymes are anchored and decrease of sulphydryl groups, important for enzymatic activity [66]. This becomes even more of a problem when the antioxidant enzymes SOD and CAT present diminished activity, like in our model. These impairments are probably due to

reactive species causing a site-specific amino acid modification [67]. These results suggest that GAA provokes an antioxidant imbalance in striatum. This can cause further impairment in the electron transport chain complexes since they are included in the group of enzymes vulnerable to free radicals attack [68]. COX catalyzes a rate-limiting step, being responsible for transferring electrons from cytochrome *c* to molecular oxygen [69]. In this context, the inhibition of this enzyme may lead to an incomplete reduction of oxygen, and a consequent increase in the formation of free radicals, which makes COX a special target for oxidative damage [70]. The role that COX has as a key player in the formation of ROS may be the cause why creatine was not able to fully restore its functionality. To verify if the enzymatic changes were due to changes in the mitochondrial morphology or biology we tested mitochondrial mass and mitochondrial membrane potential, but these parameters remained unaltered when compared to the control group. This indicates that the interval of time between the injection and the death of the animals was insufficient to induce profound changes in the mitochondrial network structure. Nonetheless, the observed activity decline of the enzymes of the mitochondrial respiratory chain was able to generate a drop in ATP levels in the striatum. Taking all this into account, we suggest that high levels of GAA affects brain bioenergetics and ROS generation.

Creatine completely restored complex II and SDH activities, and partially restored the decrease in COX activity and ATP levels. The main destination of supplemented creatine is the system creatine/PCr which strengthens cellular energetics via a temporal and spatial energy buffer that can restore cellular ATP and protects against energy depletion and delayed membrane depolarization [29, 30]. Additionally, it has been shown that creatine prevents or delays mitochondrial permeability transition pore opening, an early event in apoptosis [71]. Therefore, the boost of creatine provided by our pre-treatment have potential therapeutic value as it can replenish cellular ATP without a reliance on oxygen, helping to prevent the overflow of the mitochondrial respiratory chain [72, 73]. In addition to the effects of creatine as an enhancer of cellular energetics, one of the major roles of creatine is also as both a direct and an indirect antioxidant [38]. Creatine can prevent oxidative imbalance induced by several experimental protocols, such as induced damage by respiratory chain inhibitor rotenone [74], UV [75], acute exercise [76], and sedentary routine [77]. In this study, we have observed that creatine was able to prevent the decrease in CAT, but not in SOD. Nonetheless, DCF levels were normalized by creatine, and this may be due to the fact that creatine not necessarily acts as an antioxidant by increasing or preventing drops on the activities of antioxidant enzymes [78]. It has

been demonstrated that creatine can have a direct effect as antioxidant, which can be observed in living cells exposed to many oxidative agents [79]. Still in the context of the energy metabolism alterations, studies showed that creatine significantly protects mitochondrial DNA from oxidative damage in a dose dependent manner [64, 80].

Since neuroinflammation is closely related to oxidative stress and that both have been implicated as a pathological contributor in several neurodegenerative diseases [81-83], we have measured several inflammatory markers. All pro-inflammatory cytokines observed (TNF-α IL1β and IL6) had their levels increased in rats that suffered the GAA intrastriatal injection. TNF-α is the key initiator of immune-mediated inflammation in several organs, including the brain [84]. It acts synergistically with IL-1β to induce IL-6 expression [85]. It is worth to mention that recent studies have shown that TNFα leads to the activation of an unknown downstream tyrosine kinase that phosphorylates COX on Tyr304 leading to strong enzyme inhibition, like the one observed in this study [86]. Therefore, this excessive production of proinflammatory cytokines and free radicals can be interpreted as an indicator of uncontrolled activation of microglia, and it may be suggested that GAA is a toxic metabolite with potential to induce neuroinflammation. This is reinforced by the fact that GAA increased nitrite content, implying activation of iNOS. Of note, it is known that activation of toll-like receptors in microglia and also in astrocytes leads to excessive production of nitric oxide via iNOS [87]. In addition, ACh is a well-known neurotransmitter for signal transduction and also plays a crucial role in regulating immune and inflammatory reactions [18, 88, 89]. Glial cells have the ability to produce ACh that appears to be responsible for the regulation of microglial activation via nicotinic receptors [90, 91]. Our results confirmed that GAA increases AChE activity in the striatum of adult rats, suggesting a reduction in the ACh levels, which contributes to a proinflammatory state. This is supported by studies that have shown that AChE inhibition reduces microglial production of TNF-α in a hypoxia model [92] and that AChE inhibitors suppress systemic cytokine levels during endotoxemia [93]. Furthermore, the cholinergic system plays a crucial role in cognitive function, including memory [94]. Previous studies have shown that cholinergic neurotransmission modulates aversive conditioning [95]. Our results showed that GAA significantly increased AChE activity, implying that GAA accumulation affects memory processing, and that AChE probably plays a role in this alteration. The immunocontent of this enzyme remained unaltered.

Although the mechanisms involved are not yet fully understood, in recent years the action of creatine as not only an antioxidant, but also as an immunomodulatory agent has gained increasing attention. Creatine can inhibit endothelial permeability, neutrophil adhesion to endothelial cells, and adhesion molecule expression, as well as diminish the increase in the levels of some inflammatory markers like C-reactive protein, TNFα, INFα, IL-1β and PGE_2 [33, 96-98]. Additionally, a recent work with arginine (a precursor of creatine) suggested that much of the mechanism of how creatine acts in modulating inflammation may be evolutionarily conserved [99].

Our studies are in agreement with previous reports that showed that Na^+,K^+-ATPase, an essential enzyme for the maintenance of brain functions [100], is diminished by an intrastriatal injection of GAA, which also may play a role in the memory impairment observed in this study [101]. The inhibition of Na^+,K^+-ATPase activity was inversely correlated to the immunocontent of its α3 subunit, a subunit highly expressed in the striatum [102]. This result suggests that the GAA-induced decrease in Na^+,K^+-ATPase activity did not occur by altering the overall number of molecules of this enzyme, but is a post-translational inhibition. It is possible that the inhibition of this enzyme is associated with alteration of components of the plasma membrane where Na^+,K^+-ATPase is anchored [66]. Furthermore, the active site of Na^+,K^+-ATPase presents several cysteine residues, which makes it an easy target for irreversible oxidation of SH-groups, causing protein degradation [14, 103]. The capacity of creatine to partially prevent such alterations may be due its antioxidant capacity. However, since Na^+,K^+-ATPase relies on high amounts of ATP in order to maintain resting potential in neurons, the inability of creatine to fully restore ATP levels may be one of the reasons why it was not able to fully restore this enzyme activity. Besides, the up-regulation in translation with consequent increase in the amount of the enzyme probably indicates the development of an adaptive compensatory mechanism. We also evaluated glutamate uptake and immunocontent of the most important glutamate transporters for extracellular glutamate clearance, GLAST and GLT-1, in the striatum of rats submitted to the injection of GAA. Since no alterations in the immunocontent of these transporters were observed, we suggest that the drop in glutamate clearance was due to alterations in the function of the transporters induced by GAA, and not due to a decrease in the number of transporters available at the synaptic cleft. It is possible that glutamate transport through its carriers is being affected by fluctuations of Na^+,K^+-ATPase activity since glutamate clearance is dependent on the Na^+ gradient provided by this enzyme [104]. Consequently, the decline in Na^+,K^+-ATPase activity in rat striatum

could be linked to the reduction of glutamate uptake observed in our work. In addition, glutamate transport can also be impaired by oxidative stress [105]. Prolonged impairment of astrocytic functions could increase the vulnerability of dopaminergic neurons in the substantia nigra, accelerating their degeneration, which may play a role in some of the motor symptoms presented by GAMT deficiency patients [106]. In the context of inflammation, astrocytes present receptors for several immune-derived molecules including cytokines, chemokines, complement, and acute-phase proteins. Once activated by cytokines, astrocytes are able to secrete a considerable number of innate immune inflammatory mediators including several complement cascade proteins and further cytokines like the ones that we found increased in this study [107-109]. This activation is frequently related with impaired glutamate clearance and oxidative stress, both of which contribute to excitotoxicity. Lower clearance and excessive liberation of glutamate by glial cells during immune activation may lead to glutamate boost and promote abnormal extrasynaptic signaling through ionotropic and metabotropic glutamate receptors. These alterations can ultimately cause severe synaptic dysfunction [110, 111]. This synaptic dysfunction mediated by glutamate may play a part in the memory impairment observed in our study, since cortico inputs to and from the basal ganglia play a role in cognitive function, such as working memory and attention [112].

In the CNS, creatine develops a fundamental role in the regeneration of ATP for glutamate clearance over excitatory synaptic transmission [113]. In this context, knock-out mice for CK isoforms showed behavioral abnormalities, including spatial learning impairment and alterations in the arrangement and preservation of hippocampal mossy fiber connections [114, 115]. This may explain the partial prevention that creatine had on glutamate uptake in the striatum. Nonetheless, studies suggest that creatine may play a part as a neurotransmitter or neuromodulator, regulating GABAergic and glutamatergic receptors [34, 116]. Therefore, creatine may act not only preventing some of the biochemical alterations found in this study, but could also be a modulator of synapses, which would help to prevent the memory impairment in the object recognition test.

5. Conclusion

In summary, in the present study we demonstrated that GAA intrastriatal injection impairs the mitochondrial respiratory chain, leading to depleted ATP levels, redox imbalance and inflammatory processes. In addition, we have confirmed that AChE activity, glutamate uptake and Na^+,K^+-ATPase

activity in the striatum of young adult rats are inhibited after GAA exposure, probably due to the depletion of ATP levels and attack from radical species, contributing for the memory impairment observed. Nonetheless, imunnocontent of AChE, and glutamate transporters GLAST and GLT-1 were not affected by GAA, while immunocontent of α3 subunit of Na^+,K^+-ATPase was increased, indicating that this enzyme underwent an adaptive compensatory mechanism. Creatine appeared to act as an energy reservoir, antioxidant and anti-inflammatory agent, as well as a neuromodulator, since it was able to prevent almost every biochemical alteration detected in this study, as shown by Figure 5. Creatine is a safe approach to improve the quality of life of GAMT deficiency patients, and our work may have put some light upon how creatine acts in the presence of high concentrations of GAA in the CNS, specifically in the striatum.

6. Compliance with Ethical Standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The ethical standards followed the official governmental guidelines issued by the Brazilian Federal of Societies for Experimental Biology, following the Guide for Care and Use of Laboratory Animals and Arouca Law (Law no. 11.794/2008) and the experimental protocol was approved by the University's Ethics Committee (CEUA) under the project #29887.

7. Conflict of Interest

The authors declare that they have no conflict of interest.

8. Abbreviation list

ACh: acetylcholine

AChE: achetylcholinesterase

ANOVA: analysis of variance

ATP: adenosine triphosphate

CAT: catalase

CK: creatine kinase

CNS: central nervous system

COX: cytochrome c oxidase

DCF: dichlorofluorescein

GAA: guanidinoacetate

GAMT: *N*-guanidinoacetate methyltransferase

H_2DCF: 2′,7′-dichlorofluorescein:

H_2DCF-DA: 2′,7′-dichlorofluorescein diacetate

HBSS: Hank's balanced salt solution

IEM: inborn error of metabolism

iNOS: inducible nitric oxide synthase

PCr: phosphocreatine

Pi: inorganic phosphate

RNS: reactive nitrogen species

ROS: reactive oxygen species

SDH: succinate dehydrogenase

SOD: superoxide dismutase assay

TBARS: thiobarbituric acid reactive substances

TBS: tris-buffered saline

T-TBS: tween-20 tris-buffered saline

9. Reference list

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Table 1. Effect of the GAA intrastriatal injection on cellular energy metabolism.

	Control	GAA	GAA+Creatine	Creatine
Complex II activity (nmol/min/mg protein)	150.89 ± 39.02	65.51± 18.41*	104.68 ± 29.18#	129.44 ± 42.19
SDH activity (nmol/min/mg protein)	7.35 ± 1.65	4.12 ± 0.97*	6.36 ± 2.07#	7.47 ± 1.33
COX activity (nmol/min/mg protein)	150.89 ± 39.02	66.51 ± 18.41*	104.68 ± 29.18	129.44 ± 42.19
ATP levels (mmol/g tissue)	3.07 ± 0.60	1.92 ± 0.19*	2.37 ± 0.53	2.64 ± 0.32

Results are expressed as means ± standard deviation for 6–9 animals in each group. *$p<0.05$ when compared with control group; #$p<0.05$ compared to GAA group (two-way ANOVA followed by Tukey's post hoc test)

Table 2. Effect of the GAA intrastriatal injection on redox state..

	Control	GAA	GAA+Creatine	Creatine
SOD activity (units/mg protein)	16.19 ± 0.88	12.76 ±1.78*	12.82 ± 1.71*	16.05 ± 1.91
CAT activity (units/mg protein)	2.70 ± 0.80	1.22 ± 0.43*	2.35 ± 0.62#	2.4 ± 0.95
DCF levels (nmol/mg protein)	1525.52 ± 385.41	2215.07 ± 199.03*	1440.57 ± 240.33	1589.60 ± 278.84

Results are expressed as means ± standard deviation for 6–9 animals in each group. *$p<0.05$ when compared with control group; #$p<0.05$ compared to GAA group (two-way ANOVA followed by Tukey's post hoc test)

Table 3. Effect of the GAA intrastriatal injection on inflammatory markers.

	Control	GAA	GAA+Creatine	Creatine
TNF-α levels (pg/mg protein)	10.6 ± 1.1	17.6 ± 5.5*	11.5 ± 0.2#	10.9 ± 2.5
IL-1β levels (pg/mg protein)	182.64 ± 70.93	293.43 ± 60.08*	157.05 ± 23.14#	169.46 ± 63.24
IL6 levels (pg/mg protein)	36.55 ± 5.74	59.08 ± 8.97*	42.74 ± 9.88#	37.59 ± 8.95
Nitrite levels (µmol/mg protein)	15.19 ± 2.08	23.07 ± 3.20*	21.22 ± 1.60	14.95 ± 0.59

Results are expressed as means ± standard deviation for 6–9 animals in each group. *$p<0.05$ when compared with control group; #$p<0.05$ compared to GAA group (two-way ANOVA followed by Tukey's post hoc test)

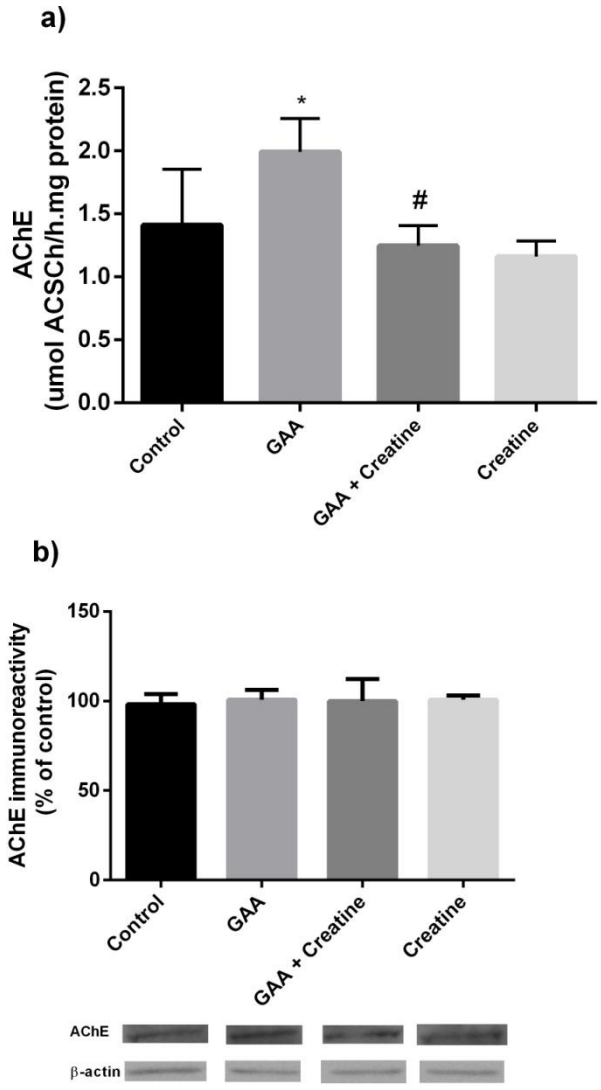

Fig.1 Effect of GAA intrastriatal infusion and/or creatine administration on AChE activity (a) and on immunocontent (b) in homogenates of the striatum of 60-day-old rats. Results are expressed as means ± standard deviation for 6–9 animals in each group. *p<0.05 when compared with control group; #p<0.05 compared to GAA group (two-way ANOVA followed by Tukey's post hoc test)

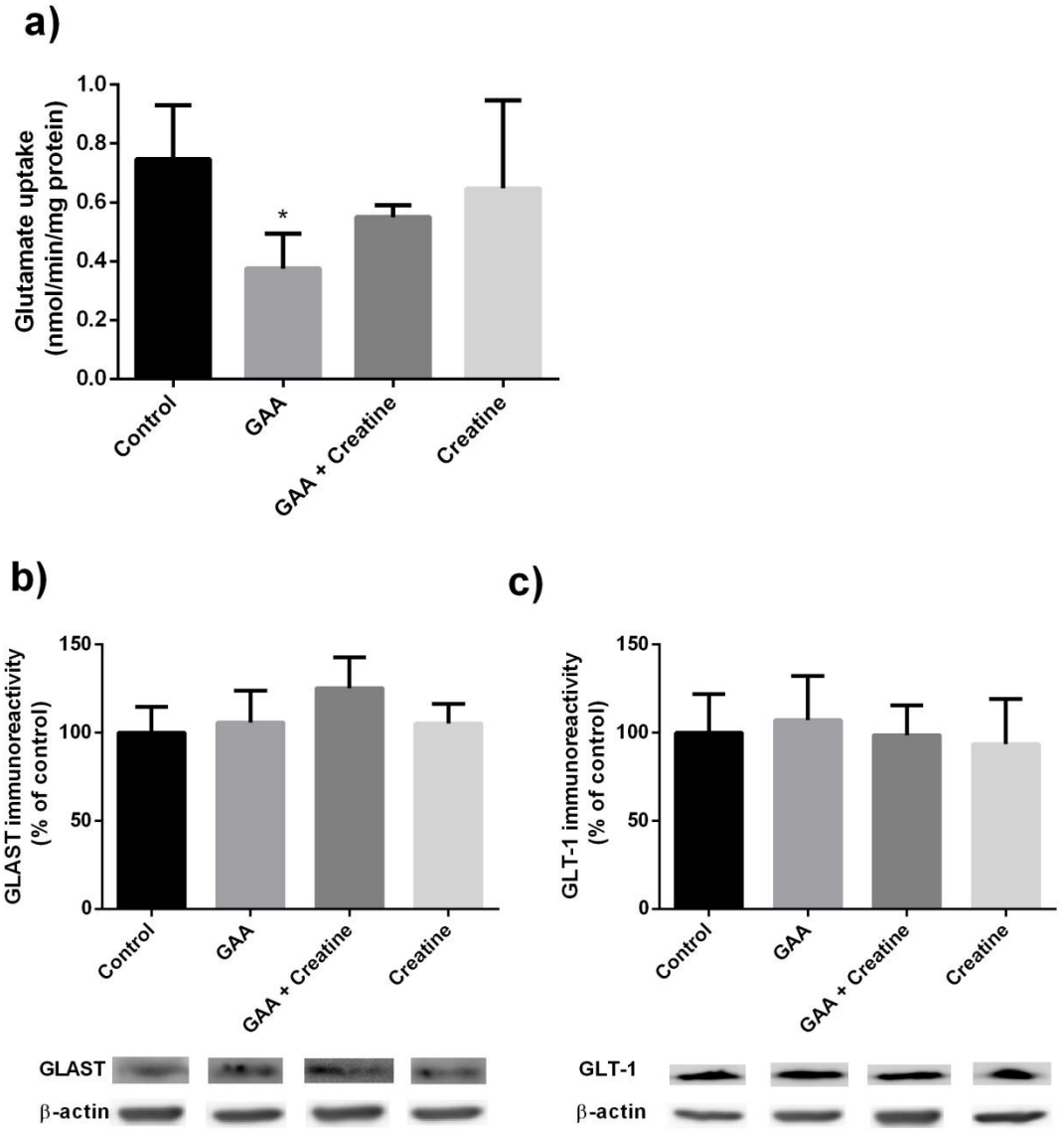

Fig.2 Effect of GAA intrastriatal infusion and/or creatine administration on glutamate uptake (a) and on glutamate transporters' immunocontent of GLAST (b) and GLT-1 (c) in homogenates of the striatum of 60-day-old rats. Results are expressed as means ± standard deviation for 6–9 animals in each group. *p<0.05 when compared with control group (two-way ANOVA followed by Tukey's post hoc test)

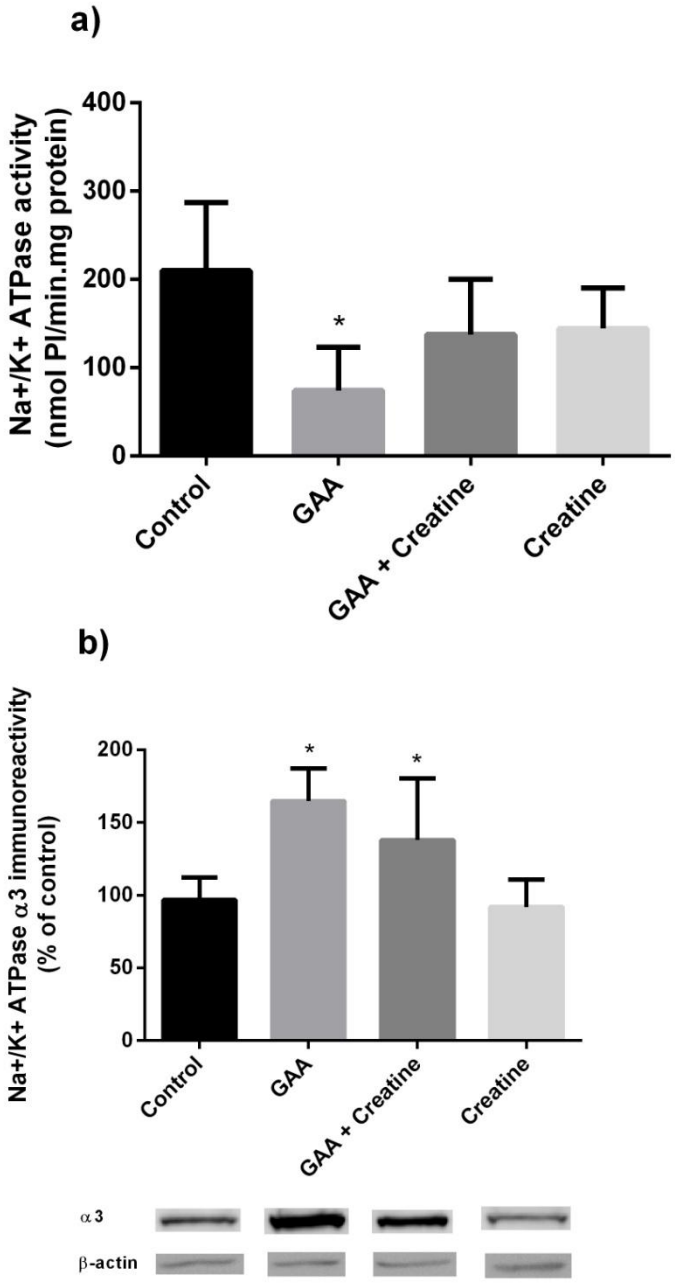

Fig.3 Effect of GAA intrastriatal infusion and/or creatine administration on Na^+,K^+-ATPase activity (a) and on immunocontent of its α3 subunit (b) in homogenates of the striatum of 60-day-old rats. Results are expressed as means ± standard deviation for 6–9 animals in each group. *p<0.05 when compared with control group (two-way ANOVA followed by Tukey's post hoc test)

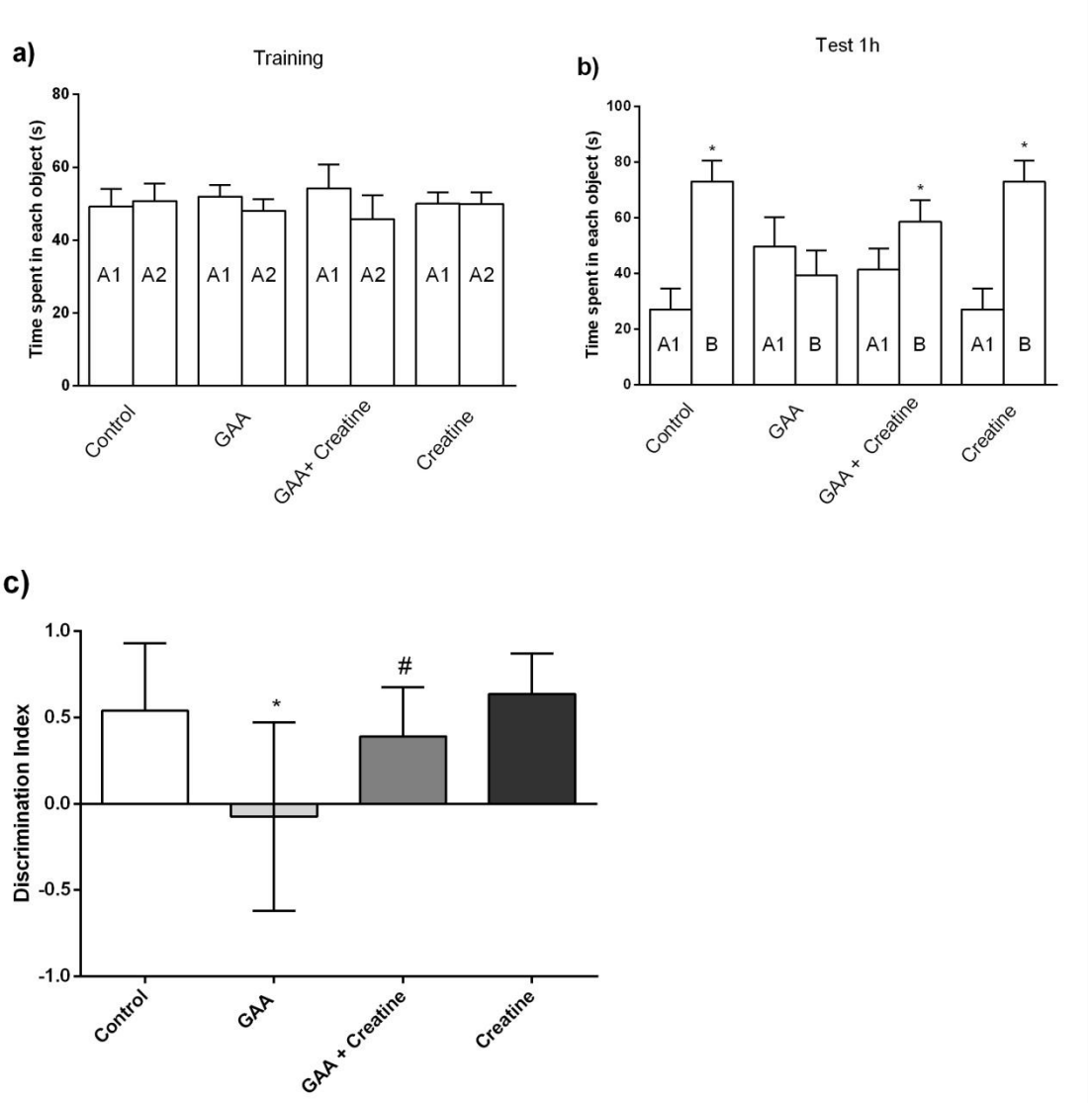

Fig.4 Effect of GAA intrastriatal infusion and/or creatine administration on the novel object recognition test. (a) Results in the training session (b) Results in the test session (c) Discrimination index. Data are expressed as mean±SD for 8-12 animals in each group.*p<0.05 between objects A1 and B for graph b; *p<0.05 when compared with control group; #p<0.05 compared to GAA group for graph c(two-way ANOVA followed by Tukey's post hoc test)

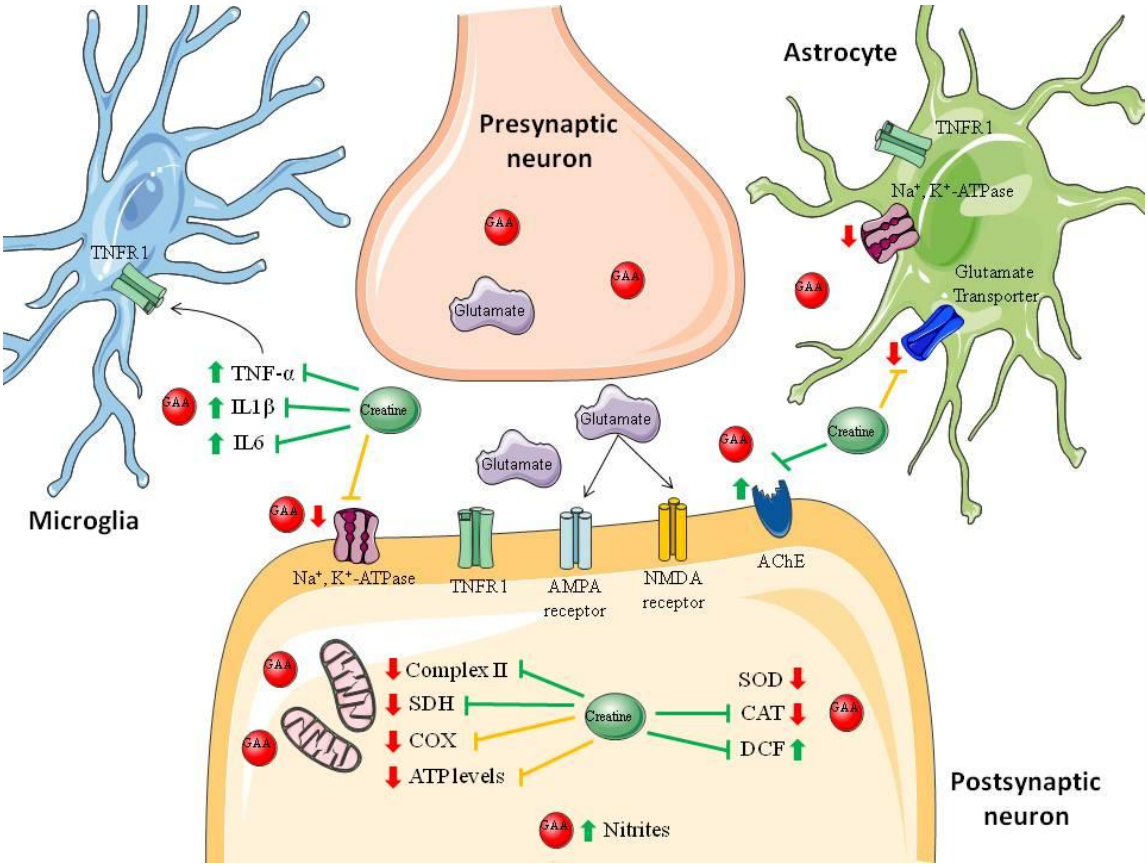

Fig.5 Summary of the effects of GAA and creatine. Accumulation of GAA may exert its actions mainly by four possible pathomechanisms, namely energy deficit, oxidative stress, inflammation and excitotoxicity. GAA inhibit key enzymes of the mitochondrial respiratory chain, leading to diminished ATP levels while increasing the generation of reactive oxygen species and reducing tissue antioxidant defenses. This processes appear to be highly interconnected inflammation, with increase in nitrite levels, release of pro-inflammatory cytokines and the inhibition of AChE. TNF-α can interact with its receptor TNFR1 in order to amplify the inflammatory response with the release of more cytokines by the microglia, more glutamate by the astrocytes, or to indirectly stimulate AMPA and NMDA receptors in neurons. Since GAA also inhibits glutamate reuptake by its astrocytes transporters, it is possible that GAA cause excitotoxicity via overstimulation of NMDA receptors. Furthermore, Na^+,K^+-ATPase, a key enzyme for neuronal function, is impaired by GAA probably via oxidative stress and ATP depletion, while the immunocontent of its α3 subunit is increased, indicating an adaptive compensatory mechanism. Creatine had no effect on nitrite levels or SOD activity. Even so, creatine appears to be a strong neuroprotector, completing preventing almost every alteration caused by GAA, with the exception of partial preventions on Na^+,K^+-ATPase activity, COX activity, drop in ATP levels and on the inhibition of glutamate uptake

PARTE III

4. DISCUSSÃO

Pacientes com deficiência de GAMT apresentam níveis diminuídos de creatina, e GAA em excesso no músculo esquelético, sangue, cérebro e outros tecidos (Stöckler et al. 1994, Gordon 2010). Os sintomas neurológicos nestes pacientes são abundantes, frequentes, e distintos, incluindo: epilepsia não responsiva a medicamentos, movimentos extrapiramidais involuntários, memória e intelecto debilitados, fala arrastada, hipotonia muscular e até mesmo autismo (Schulze et al. 2001, Hanna-El-Daher et al. 2015, Marques e Wyse 2016). Considerando a variabilidade de sintomas apresentados, a fisiopatologia desta doença ainda não é clara o suficiente. Estudos prévios do nosso grupo já mostraram alterações no estado redox e no metabolismo energético em ratos submetidos à injeção intraestriatal de GAA (Zugno, Stefanello, et al. 2008, Zugno, Oliveira, et al. 2007, Zugno, Scherer, et al. 2007). No presente estudo, nós estendemos a investigação para verificar o efeito do GAA sobre parâmetros de metabolismo energético, estresse oxidativo, inflamação, atividade e imunoconteúdo das enzimas AChE e Na^+,K^+-ATPase, sistema glutamatérgico, bem como sobre o processo de aquisição da memória na tarefa de reconhecimento de objetos. Também investigamos a influência da creatina nesses efeitos.

Apesar de representar cerca de 2% do peso corporal de um ser humano, o cérebro consome cerca de 20% da energia proveniente da glicose, e qualquer alteração em seu metabolismo energético pode levar a um estado de estresse oxidativo e outras sérias consequências (Falkowska et al. 2015). Uma diminuição crônica na energia disponível pode deteriorar a maquinaria celular em um nível em que a formação de ATP é comprometida, como observado em diversas doenças neurodegenerativas (van den Bogaard et al. 2011, Martin 2012). No presente

trabalho foi verificado que a injeção intraestriatal de GAA diminuiu na atividade de enzimas da cadeia respiratória mitocondrial (Complexo II, SDH e COX). A incapacidade dessa cadeia em suprir a demanda celular por energia pode promover o acúmulo intracelular de Ca^{2+}, gerando espécies reativas que tem o poder de causar estresse oxidativo, e, possivelmente, a dissociação do citocromo C da membrana mitocondrial interna, iniciando eventos apoptóticos (Meyer et al. 2006, Hariharan et al. 2014).

O aumento das espécies reativas, indicado pelo aumento dos níveis de DCF após injeção intraestriatal de GAA, pode alterar grupos que são essenciais para a atividade enzimática, tais como grupos sulfidrilas ou componentes da membrana plasmática onde enzimas estão ancoradas (Wolosker, Panizzutti, e Engelender 1996). Isso pode se tornar um problema ainda maior em nosso modelo pois as atividades das enzimas antioxidantes SOD e CAT estão diminuídas, provavelmente devido às espécies reativas causando uma modificação específica de aminoácidos no sítio ativo (Singh, Jain, e Kaur 2004). Esses resultados sugerem que o GAA provoca um desbalanço oxidativo no estriado de ratos, demonstrando que as defesas antioxidantes não foram suficientemente efetivas. Isso pode contribuir para prejuízos mais profundos na formação de energia, já que as enzimas da cadeia transportadora de elétrons estão incluídas no grupo de enzimas vulneráveis ao ataque de radicais livres (Dudkina et al. 2008).

A COX catalisa um passo limitante da formação de energia na cadeia respiratória, sendo a responsável por transferir elétrons do citocromo c para o oxigênio molecular. Assim, a inibição dessa enzima pode levar a redução incompleta do oxigênio, e, consequentemente, a um aumento da formação de radicais livres, o que faz a COX um alvo fácil de ataque por parte destas espécies reativas (Bose et

al. 1992). O papel desempenhado pela COX pode ser um dos motivos pelos quais a creatina não conseguiu restaurar totalmente sua atividade. Para verificar se essas mudanças na atividade enzimática eram causadas por mudanças na morfologia ou biologia da população de mitocôndrias presente no estriado, nós avaliamos a massa e o potencial de membrana mitocondrial, mas ambos os parâmetros permaneceram inalterados neste estudo, sugerindo que o intervalo de tempo entre a injeção e a morte dos animais não foi suficiente para induzir mudanças profundas na estrutura da rede de mitocôndrias. Entretanto, o declínio da atividade enzimática foi suficiente para levar a uma diminuição dos níveis de ATP presente nas amostras. Levando esses dados em consideração, nós sugerimos que altos níveis de GAA afetam a bioenergética cerebral e a formação de espécies reativas.

A creatina foi capaz de prevenir totalmente o declínio da atividade do complexo II e da SDH, e prevenir parcialmente a diminuição na atividade da COX e dos níveis de ATP. Como anteriormente mencionado, o principal destino da creatina é o sistema creatina/PCr, que fortalece a bioenergética celular ao prover um tampão espacial e temporal que pode restaurar ATP sem necessitar de oxigênio, protegendo a célula contra a depleção de energia, e despolarização persistente (Balestrino, Rebaudo, e Lunardi 1999, Shen e Goldberg 2012). Isso ocorre não apenas no músculo esquelético, mas também provavelmente esteja ocorrendo em nosso modelo, no SNC, onde apesar de existirem limitações para a difusão da creatina através da barreira hematoencefálica (Braissant 2012), estudos já demonstraram que é possível alterar a concentração de creatina cerebral com suplementação de creatina em diferente modelos (Dechent et al. 1999, Lyoo et al. 2003, Turner, Byblow, e Gant 2015). Ademais, já foi demonstrado que a creatina retarda ou até impede a abertura do poro de transição de permeabilidade mitocondrial, um evento

que acontece no começo do processo apoptótico (Dolder et al. 2003), sendo ainda capaz de restaurar a excitabilidade corticomotora e declínio cognitivo observado em situações com hipóxia induzida em humanos (Turner, Byblow, e Gant 2015). Consequentemente, o aumento nos níveis de creatina proporcionado pelo nosso pré-tratamento tem potencial terapêutico, dado que parece regenerar o ATP sem necessitar de oxigênio, ajudando a prevenir a sobrecarda da cadeira respiratória mitocondrial (Béard e Braissant 2010, Riesberg et al. 2016).

Além dos efeitos do sistema creatina/PCr como reserva energética, outra importante função da creatina é como antioxidante, tanto de maneira direta quanto indireta (Sakellaris et al. 2006). Neste estudo nós observamos que a creatina foi capaz de prevenir o declínio da atividade da enzima antioxidante CAT, mas não da SOD. Ainda assim, os níveis de DCF foram normalizados com o pré-tratamento com creatina, e isso provavelmente se deve ao fato da ação antioxidante apresentada pela creatina não necessariamente estar associada com a prevenção da diminuição da atividade enzimática, nem a indução de aumento da mesma (Guimarães-Ferreira et al. 2012). Estudos já demonstraram que a creatina pode ter efeitos antioxidantes diretos em células vivas expostas a vários agentes oxidantes (Sestili et al. 2006). Ainda no contexto das alterações bioenergéticas, estudos mostraram que a creatina também protege significativamente o DNA mitocondrial do dano oxidativo de uma maneira dose dependente (Meyer et al. 2006, Guidi et al. 2008).

Já que a neuroinflamação está associada com o estresse oxidativo, e que ambos são considerados fatores de risco para diversas doenças neurodegenerativas e neurometabólicas (Raha et al. 2017, Appel et al. 2011, Sapp et al. 2001, Scherer et al. 2014), no presente estudo foram realizados testes com o objetivo de detectar marcadores inflamatórios em ratos submetidos ao nosso modelo. Todas as citocinas

pró-inflamatórias observadas (TNF-α, IL1-β e IL-6) apresentaram níveis elevados em ratos que receberam uma injeção intraestriatal de GAA. O TNF-α é o iniciador chave da inflamação em diversos órgãos, incluindo o cérebro (Feldmann e Maini 2003), agindo sinergicamente com IL-1β para induzir a expressão de IL-6 (Lucas, Rothwell, e Gibson 2006). É relevante mencionar que estudos recentes mostraram que o TNF-α leva à ativação de uma via em que tirosina cinases fosforilam a COX no resíduo Tyr304, o que leva a uma forte inibição da enzima como a que nós observamos em nosso estudo (Lee e Hüttemann 2014). Essa excessiva produção de citocinas pró-inflamatórias e espécies reativas podem ser interpretados como um indicador de ativação descontrolada da microglia. Portanto, sugerimos que o GAA é um metabólito tóxico com potencial de induzir neuroinflamação. Isso é reforçado pelo fato do GAA ter aumentado os níveis de nitritos, o que implica uma possível ativação das isoformas da enzima óxido nítrico sintase, inclusive a óxido nítrico sintase induzível (iNOS), que pode ser expressa para induzir a produção de óxido nítrico após a ativação de receptores do tipo toll presentes na microglia e astrócitos (Sun et al. 2018).

Além disso, o neurotransmissor ACh também desempenha um papel crucial como regulador da resposta imune e inflamatória (Scherer et al. 2014, Hachisu et al. 2015, Kalkman e Feuerbach 2016). As células da glia têm a habilidade de produzir ACh para regular a ativação da própria microglia através de receptores nicotínicos (Wessler et al. 1997, Shytle et al. 2004). Nossos resultados confirmaram que o GAA aumenta a atividade da enzima AChE no estriado de ratos adultos, sugerindo uma redução nos níveis de ACh disponíveis, o que contribuiria para um estado pró-inflamatório. Isso é embasado em estudos que demonstraram que a inibição da AChE reduz a produção microglial de TNF-α em modelos de hipóxia (Wang, Zhang,

e Tang 2010), e que inibidores dessa enzima suprimem os níveis sistêmicos de citocinas durante endotoxemia (Pavlov et al. 2009). Ademais, o sistema colinérgico também desempenha um papel crucial na função cognitiva, incluindo o processamento de novas memórias (Mushtaq et al. 2014). Estudos prévios mostraram que a neurotransmissão colinérgica modula condicionamento aversivo, por exemplo (Tinsley, Quinn, e Fanselow 2004). O aumento da atividade da AChE encontrado em nosso estudo pode desempenhar um papel nas alterações de memória encontradas. O imunoconteúdo dessa enzima permaneceu inalterado. Apesar dos mecanismos envolvidos ainda não serem completamente compreendidos, estudos sobre a ação da creatina como agente imunomodulador vêm ganhando crescente atenção. A creatina apresenta um efeito supressor da inflamação ao nível celular, inibindo a permeabilidade endotelial, a adesão dos neutrófilos ao endotélio (suprimindo ICAM-1 e selectinas), e expressão de outras moléculas de adesão (Nomura et al. 2003). Além disso, a creatina pode diminuir os níveis de alguns marcadores inflamatórios como proteína C reativa, TNF-α, INFα, IL-1β e PGE_2 (Santos et al. 2004, Bassit, Curi, e Costa Rosa 2008, Deminice et al. 2013). Um recente trabalho com arginina (precursora da creatina) sugeriu que muito do mecanismo pelo qual a creatina age modulando a inflamação pode ser conservado evolutivamente(Azeredo et al. 2015).

Nossos estudos também mostraram que a atividade da Na^+,K^+-ATPase, uma enzima essencial para a manutenção das funções cerebrais (Pierozan et al. 2018), é diminuída após injeção intraestriatal de GAA, o que também pode desempenhar um papel nas alterações de memória descritas a seguir. A inibição da atividade da Na^+,K^+-ATPase foi inversamente correlacionada ao imunoconteúdo da subunidade α3, subunidade altamente expressa no estriado (Bøttger et al. 2011). Este resultado

sugere que a inibição induzida por GAA não ocorre por alteração no número total de moléculas da enzima, mas sim por alguma inibição pós-translacional. É possível que a inibição dessa enzima esteja associada com a alteração de componentes da membrana plasmática em que ela está ancorada (Wolosker, Panizzutti, e Engelender 1996), ou com a diminuição de grupamentos sulfidrilas induzida por GAA, já que a Na^+,K^+-ATPase possui muitos resíduos de cisteína em seu sítio ativo, e a oxidação irreversível de grupamentos-SH pode causar inativação (Zugno, Stefanello, et al. 2008, Dergousova et al. 2017). Nós sugerimos que a habilidade da creatina em prevenir parcialmente a diminuição na atividade da enzima Na^+,K^+-ATPase possa ser mediada por sua capacidade antioxidante. Entretanto, já que a Na^+,K^+-ATPase necessita de um alto suprimento de energia para repolarizar as células, a inabilidade da creatina em restaurar totalmente os níveis de ATP pode ser uma das razões pela qual ela também não conseguiu restaurar totalmente a atividade dessa enzima. Além disso, o aumento da translação com consequente aumento da quantidade de enzima disponível indica o desenvolvimento de um mecanismo adaptativo compensatório.

Nosso estudo também avaliou a captação de glutamato e o imunoconteúdo de seus principais transportadores, GLAST e GLT1, no estriado de ratos submetidos à injeção intraestriatal de GAA. Os resultados encontrados indicam que a diminuição da captação de glutamato induzida por GAA não envolve uma mudança quantitativa nos transportadores, mas pode ser devido a possíveis alterações na funcionalidade dos mesmos, já que não observamos alterações em seus imunoconteúdos. O transporte de glutamato pelos seus transportadores pode ser afetado pela atividade da Na+,K+-ATPase, já que ele é dependente do gradiente de Na+ gerado por essa enzima (Rose et al. 2009). Consequentemente, a diminuição da atividade da Na^+,K^+-

ATPase no estriado de ratos pode estar associado à diminuição da captação de glutamato observado neste estudo. Ademais, o transporte de glutamato por seus transportadores também pode ser afetado pelo estresse oxidativo (Pedersen, Cashman, e Mattson 1999). Uma alteração prolongada nas funções astrocitárias pode aumentar a vulnerabilidade de neurônios dopaminérgicos, acelerando sua degeneração e podendo contribuir para os sintomas motores apresentados por pacientes com deficiência da GAMT (Kuter et al. 2018).

No contexto da inflamação, astrócitos expressam receptores para muitas moléculas imunomoduladoras, como citocinas, quimiocinas, sistema complemento, e proteínas de fase aguda. Após ativação por IL1-β, os astrócitos podem amplificar a resposta liberando ainda mais desses mediadores, como as citocinas que mensuramos em nosso estudo (Johnstone, Gearing, e Miller 1999, Santello e Volterra 2012, Fischer et al. 2014). Esse estado também está relacionado com diminuição na recaptação do glutamato e estresse oxidativo, ambos fatores contribuintes para excitotoxicidade.

Nossa hipótese consiste na ideia de que níveis aumentados de citocinas, através de receptores TNFR1, desencadeiam um feedback positivo em sua própria liberação por parte da microglia ativada e dos astrócitos. As citocinas, em níveis cada vez maiores, estimulam a liberação de glutamato da própria microglia e dos astrócitos. Essa liberação de glutamato, associada com a falha na recaptação, estimula ainda mais o aumento nos níveis de citocinas, além de estimular receptores NMDA e AMPA de maneira crônica. Além disso, níveis elevados de citocinas diminuem a expressão de receptores GABAa inibitórios na membrana de neurônios pós-sinápticos, também através do receptor TNFR1. Todos esses fatores associados podem levar a um aumento tóxico de Ca+, estresse oxidativo e morte neuronal.

Portanto, é possível supor que assim como em outras doenças neurodegenerativas, a imunidade inata e adaptativa criam um ambiente autossustentável onde a inflamação persiste, levando a neuroinflamação crônica (McCullumsmith e Sanacora 2015, Haroon, Miller, e Sanacora 2017). Essa disfunção sináptica mediada por glutamato pode desempenhar um papel importante na alteração de memória aqui observada, já que a comunicação neuronal entre o córtex e os núcleos da base desempenha um papel crucial na função cognitiva, incluindo memória de trabalho e atenção (Asif-Malik et al. 2017).

No SNC, a creatina desempenha funções essenciais na regeneração de ATP para a recaptação de glutamato durante a transmissão sináptica (Oliet, Piet, e Poulain 2001). Nesse contexto, ratos *knock-out* para as isoformas da CK mostraram alterações de memória e comportamentais, como aprendizado espacial defeituoso e defeitos na formação e manutenção das conexões de fibras musgosas do hipocampo (Jost et al. 2002, Streijger et al. 2005). Isso pode explicar a prevenção parcial que a creatina proporcionou na diminuição da recaptação de glutamato após injeção intraestriatal de GAA. Além disso, a creatina vem sendo cada vez mais considerada como um possível neurotransmissor capaz de modular receptores GABAérgicos e glutamatérgicos (Almeida et al. 2006), (Joncquel-Chevalier Curt et al. 2015). Essa teoria encontra suporte em estudos que demonstraram que o sinaptossoma de ratos expressa o transportador de creatine SLC6A8 (Peral, Vázquez-Carretero, e Ilundain 2010), sugerindo a existência de um sistema de recaptação na membrana terminal do axônio.

Ademais, estudos mostraram que a suplementação com creatina melhora a performance na execução de tarefas complexas durante estresse causado por privação de sono (McMorris et al. 2007), e inteligência/memória de trabalho em

indivíduos saudáveis (Watanabe, Kato, e Kato 2002, Rae et al. 2003). A creatina parece agir não apenas prevenindo as alterações bioquímicas encontradas nesse estudo, mas também modulando diretamente sinapses, o que ajuda a entender a prevenção provida por ela durante o teste de reconhecimento de objetos.

Em resumo, nosso estudo demonstrou que uma injeção intraestriatal de GAA altera parâmetros de metabolismo energético, estresse oxidativo, inflamação, atividade e imunoconteúdo da Na^+,K^+-ATPase, sistema glutamatérgico, bem como a aquisição da memória na tarefa de reconhecimento de objetos. A creatina parece agir como neuroprotetora.

5. CONCLUSÕES

Os resultados do presente trabalho mostraram que a injeção intraestriatal de GAA levou aos seguintes efeitos:

Prejuízo da cadeia transportadora de elétrons, levando à diminuição nos níveis de ATP, estresse oxidativo, e processos inflamatórios.

Diminuição da captação de glutamato e a atividade da enzima Na^+,K^+-ATPase em estriado de ratos, provavelmente devido à depleção energética e ataque de espécies reativas.

O imunoconteúdo da AChE e dos tranportadores de glutamato GLAST e GLT-1 não foi afetado pelo GAA, enquanto que o imunoconteúdo da subunidade α3 da Na^+,K^+-ATPase encontra-se aumentado, indicando um mecanismo adaptativo compensatório.

Prejuízo do processo de aquisição da memória, diminuindo a performance no teste de reconhecimento de objetos.

A creatina parece agir como reserva energética, antioxidante, e anti-inflamatório, assim como neuromodularora, já que foi capaz de prevenir quase todas as alterações bioquímicas observadas em nosso estudo.

Em conclusão, nosso estudo demonstrou que uma injeção intraestriatal de GAA altera parâmetros de metabolismo energético, estresse oxidativo, inflamação, atividade e imunoconteúdo da Na^+,K^+-ATPase, sistema glutamatérgico, bem como a aquisição da memória na tarefa de reconhecimento de objetos. A creatina parece agir como neuroprotetora, sendo uma abordagem segura para melhorar a qualidade de vida de pacientes com deficiência de GAMT, e nosso trabalho ajuda a elucidar a maneira com que a creatina age na presença de altas concentrações de GAA no SNC, especificamente no estriado.

6. PERSPECTIVAS

Avaliar os efeitos do GAA sobre:

1) Imunoconteúdo de outras moléculas relacionadas com a inflamação e aprendizado, como iNOS, IBA1, NF-κB, Nrf2 and BDNF;

2) Níveis de Ach;

3) Ativação microglial;

4) Vias de sinalização relacionadas à sobrevivência celular.

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