UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROCIÊNCIAS
TESE DE DOUTORADO

Alterações neurogliais em hipocampo de ratos expostos à estreptozotocina intracerebroventricular: Estratégias de Neuroproteção

LETÍCIA RODRIGUES

Porto Alegre, julho de 2010.

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Dedicado à minha mãe...

Com eterna gratidão pelo que sou.

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O amor não é um fenômeno biológico eventual nem especial, é um fenômeno biológico cotidiano. Mais do que isto, o amor é um fenômeno biológico tão básico e cotidiano no humano, que frequentemente o negamos culturalmente criando limites na legitimidade da convivência, em função de outras emoções.

Humberto Maturana

...as teorias filosóficas não são libertadoras. Ao contrário, teorias filosóficas constituem domínios restritivos e imperativos, nos quais aqueles que as adotam negam a si mesmos e aos outros qualquer reflexão sobre os princípios, noções, valores, ou resultados desejados, em torno de cuja conservação elas são construídas ou projetadas...

Humberto Maturana

Sumário

Parte I

Abreviaturas

AG	aminoguanidina
AGE	produtos finais avançados de glicação
CA1	corno de Amon 1
BDNF	fator neurotrófico derivado do encéfalo
BHE	barreira hemato-encefálica
DA	doença de Alzheimer
DCF	diacetato de 2,7-diclofluoresceína
icv	intracerebroventricular
IGF	fator de crescimento semelhante à insulina
EI	esquiva inibitória
iNOS	enzima óxido nítrico sintase induzível
ip	intraperitoneal
FGF	fator de crescimento de fibroblastos
GFAP	proteína fibrilar glial ácida
GS	glutamina sintetase
GSK 3	glicogênio sintase cinase 3
GLUT	transportador de glicose
LAM	labirinto aquático de Morris
LCR	líquido cefalorraquidiano
NGF	fator de crescimento neural
NO	óxido nítrico
PI3K	fosfatidil inositol-3 cinase
PKB	protein cinase B/Akt
RAGE	receptor de produtos finais avançados de glicação
RI	receptor de insulina
SNC	sistema nervoso central
SRI	substrato do receptor de insulina
STZ	estreptozotocina

Resumo

Muitos trabalhos têm demonstrado que a deficiência cerebral de insulina ou um estado de resistência cerebral a esta são parte da fisiopatogenia da Doença de Alzheimer (DA). A estreptozotocina é uma substância que vem sendo utilizada, por administração intracerebroventricular, para produzir um modelo de doença de Alzheimer esporádica com características neuroquímicas e fisiopatológicas semelhantes à DA em roedores através da dessenbililização dos receptores de insulina, especialmente em hipocampo e córtex cerebral. Dessa forma, este trabalho tem como principal objetivo, avaliar as alterações neurogliais em hipocampo de ratos expostos à estreptozotocina intracerebroventricular, bem como o efeito de estratégias terapêuticas, através da medida da secreção da proteína S100B, medida de GFAP, GSH, GS, captação de glutamato, estresse oxidativo e nitrosativo e análise comportamental. Como resultado, encontramos declínio cognitivo, estresse oxidativo e nitrosativo e alterações astrocíticas hipocampais, especialmente relacionadas ao metabolismo do glutamato, à defesa antioxidante e à secreção da proteína S100B, a qual pode atuar tanto na sinalização neurônio-astrócito, em condições fisiológicas e patológicas, quanto em diversas doenças neurodegenerativas, incluindo a Doença de Alzheimer. Ainda, observou-se o efeito neuroprotetor do composto anti-glicação aminoguanidina, bem como do exercício físico em esteira sobre os animais expostos à estreptozotocina intracerebroventricular. Ambos mostraram-se eficazes na recuperação do déficit cognitivo causado pelo modelo, bem como das alterações astrocíticas e do estresse oxidativo/nitrosativo. Além disso, mostrou-se que a aminoguanidina e a corrida em esteira são importantes estratégias antioxidantes, podendo servir de agentes potenciais na busca de alternativas para o tratamento de doenças tão incapacitantes e incidentes como a Doença de Alzheimer. Por fim, este trabalho confirma o comprometimento cognitivo e o estresse oxidativo presentes no modelo, os quais se somam às alterações funcionais encontradas nos astrócitos. Tais alterações também estão presentes na DA, apontando a interação neuroglial como um importante alvo de estudo na doença e na busca por alternativas terapêuticas.

Abstract

Many studies have been shown that a brain´s insulin deficiency and/or a resistance state participate of the pathogenesis of Alzheimer's disease (AD). Streptozotocin is a drug that has been used via intracerebroventricular to produce a model of dementia in rodents with neurochemical and morphological characteristics similar to DA by desensitization of insulin receptors, especially in the hippocampus and cerebral cortex. In this way, the main aim of the present study is to investigate the neuroglial changes in hippocampus of rats submitted to intracerebroventricular streptozotocin, as well as therapeutic strategies by measuring secretion of S100B, content of GFAP, GSH and GS, glutamate uptake, oxidative and nitrosative stress as well behavioral analysis. The results show cognitive decline, oxidative/nitrosative stress and hippocampal astrocytic changes, particularly related to glutamate metabolism, antioxidant defense and secretion of S100B protein, which can act in the astrocyte-neuron signaling over physiological and several pathological disorders, including Alzheimer's disease. Besides, it was observed the neuroprotective effect of the anti-glycation compound aminoguanidine, as well as treadmill running on intracerebroventricular streptozotocin treated animals. Both were effective in the recovery of cognitive deficit caused by the model, as well as the astrocytic changes and oxidative/nitrosative stress. Moreover, it was shown that aminoguanidine and treadmill running are important antioxidant strategies, being potential agents in the search for alternatives to the treatment of debilitating and incidents disorders such as Alzheimer's disease.

Finally, this study confirms the cognitive impairment and oxidative stress of this model, associated to the alterations in the functioning of astrocytes. Such changes are also present in AD, pointed to the neuroglial interaction as an important target of study in this condition and in the search for alternative therapies.

Introdução

1. Demência

O termo demência abrange um conjunto de sintomas envolvendo principalmente perdas de memória e de raciocínio decorrentes do próprio envelhecimento do indivíduo ou associada a uma série de enfermidades, em sua maioria, neurodegenerativas. Tais sintomas ocorrem de uma maneira mais pronunciada do que se espera normalmente como consequência do envelhecimento, quando todas as funções celulares, de um modo geral, estão em declínio. Para fins de definição, pode-se conceituar demência como "disfunção crônica e progressiva da atividade cortical e/ou subcortical resultando em complexo declínio cognitivo" (Ritchie e Lovestone, 2002). Ou ainda, é a "perda substancial das habilidades intelectuais, memória em especial, de uma maneira severa a ponto de interferir com a vida social, profissional e emocional dos indivíduos" (Rademakers e Rovelet-Lecrux, 2009).

Na antiguidade, o declínio intelectual era considerado antes um problema social do que médico (Berrios, 1996); porém, hoje se pode diagnosticar um paciente com demência antes mesmo do comprometimento das funções intelectuais e das atividades de vida diária do mesmo, graças a técnicas de imageamento encefálico e marcadores periféricos (Kurz e Lautenschlager, 2010). Por outro lado, a confirmação diagnóstica da Doença de Alzheimer, o principal tipo de demência, só pode ser obtida com a análise morfológica *post mortem* do tecido encefálico.

Existem diversos tipos de demência, as quais podem ser primárias ou a causa base da própria demência; ou secundárias, quando decorrentes a determinadas patologias, tais como a Doença de Parkinson, a infecção pelo vírus HIV e até mesmo após traumatismo craniano (Levy e Chelune, 2007). Dentre os tipos mais incidentes,

estão as Demências Frontoparietais, a Demência Vascular e a Doença de Alzheimer (DA) (Shagam, 2009). A DA é de longe a forma mais prevalente de demência compreendendo de 50-70% de todos os casos, afetando 40% dos indivíduos com mais de 85 anos (Ferri *et al.*, 2005).

Dados epidemiológicos indicam um crescimento mundial da incidência de demência, particularmente do tipo Alzheimer, especialmente nos chamados países em desenvolvimento. Há uma estimativa de que haja 23,4 milhões de pessoas com algum tipo de demência no mundo, com um aumento em torno de 4,6 milhões ao ano. Além disso, como resultado do envelhecimento da população mundial, o número de pessoas afetadas duplica a cada 20 anos (Ferri *et al.*, 2005). É natural que, à medida que ocorre um aumento da expectativa de vida dos indivíduos, aumente a ocorrência de doenças associadas ao envelhecimento. Pode-se dizer que, de uma maneira ou de outra, todos estão ou estarão expostos às conseqüências da demência em suas vidas. Previsões apontam um crescimento astronômico dos casos de demência, especialmente DA, entre a população acima de sessenta anos em todo o mundo (Langa *et al.*, 2004; Haan e Wallace, 2004). Tal fato torna a doença um grave problema de saúde pública e um enorme custo para os governos, fazendo com que a busca por alternativas de terapia e prevenção seja extremamente relevante e emergencial.

1.1 Doença de Alzheimer

1.1.1 Características Clínicas e Fisiopatológicas:

Clinicamente, a DA é marcada por uma perda cognitiva progressiva e irreversível, com déficits importantes na capacidade dos indivíduos em formar novas memórias e lembrar acontecimentos recentes, entre outros distúrbios neuropsiquiátricos como alterações de humor e personalidade (Cummings *et al.*, 1994; Selkoe, 2001a).

Histopatologicamente, a doença caracteriza-se por uma extensa perda neuronal, com presença das chamadas placas senis formadas pelo depósito extracelular do peptídeo beta amilóide e por emaranhados neurofibrilares intracelulares resultantes da deposição anormal de uma proteína associada aos microtúbulos, a proteína tau, hiperfosforilada ou poliubiquitinada (Jalbert *et al.*, 2008; Duyckaerts *et al.*, 2009; Selkoe, 2001a; Selkoe, 2001b). Associado a estas características morfológicas em particular, ocorre um série de eventos que levam à disfunção neuronal, especialmente de neurônios colinérgicos, em áreas relacionadas à cognição e à formação de memórias, como o córtex pré-frontal e o hipocampo, juntamente com suas regiões associadas. Assim, cria-se um quadro de desequilíbrio neuroquímico onde se tem sinais de neurodegeneração, neuroinflamação, estresse oxidativo, aumento da sinalização pró-apoptótica, déficit colinérgico, excitotoxicidade glutamatérgica, disfunção mitocondrial e da homeostase do cálcio, com prejuízo da transmissão sináptica e do equilíbrio entre diferentes neurotransmissores (McGeer *et al.*, 1987; Selkoe, 2001a; Eikelenboom *et al.*, 2006).

A DA pode ser classificada em dois tipos: hereditária (tipo I), quando relacionada a diferentes genes como o da proteína precursora amilóide, da apolipopotreína E (apo E) ou das presenilinas; e esporádica (tipo II), a qual todos os indivíduos estão sujeitos à medida que envelhecem. Esta última está associada ao estilo de vida e a diversos fatores de risco (Hoyer, 2002b),os quais têm sido associados com um aumento na incidência da DA esporádica e sua fisiopatogenia. Esses fatores incluem distúrbios metabólicos como obesidade, hipercolesterolemia e, em especial, diabetes *mellitus* (de la Monte *et al.*, 2009; Qiu *et al.*, 2007; Hallschmid e Schultes, 2009); cardiovasculares como hipertensão e aterosclerose (Rocchi *et al.*, 2009) e até mesmo infecções (Holmes e Cotterell, 2009).

Um número crescente de investigações tem demonstrado a participação da insulina e do fator de crescimento semelhante à insulina 1 (IGF-1) no estabelecimento da DA, especialmente a do tipo esporádica. O declínio nas funções metabólicas e hormonais relacionado à idade, especialmente a diminuição das ações neurotróficas, da modulação neuronal e do próprio aporte energético são as conseqüências mais óbvias da disfunção no metabolismo da insulina encefálica (Gasparini e Xu, 2003). Além disso, a insulina e o IGF-1 têm ação direta no processamento e depuração do peptídeo beta amilóide (Carro *et al.*, 2002). Ao mesmo tempo, a ligação da insulina e do IGF-1 ao seu receptor também ativam respostas relacionadas à expressão gênica colinérgica e à inibição do estresse oxidativo e da apoptose (Sun *et al.*, 1993). Sabe-se também que durante o envelhecimento ocorre uma significativa diminuição na densidade dos receptores de insulina encefálica e dos níveis da própria insulina, havendo uma correlação entre esta e o declínio cognitivo observado em indivíduos idosos não diabéticos (Stolk *et al.*, 1997). E ainda, foram encontrados níveis mais baixos de insulina no LCR de pacientes diagnosticados com DA e níveis plasmáticos elevados (Craft *et al.*, 1998). Tais achados levaram um grupo de autores a propor um terceiro tipo de diabetes - "diabetes tipo 3" - para designar o estado metabólico, neuroquímico e molecular encontrado no encéfalo de pacientes com DA e também principal mecanismo patogênico da neurodegeneração causada pela mesma (Steen *et al.*, 2005; Rivera *et al.*, 2005; de la Monte e Wands, 2008). Entretanto, esta nova classificação ainda está em debate na literatura, por alguns autores que preferem reservar o termo "diabetes" somente à ação da insulina associada com deficiência na capacidade de manutenção da glicemia.

1.1.2 Doença de Alzheimer e glia.

Segundo estudos, na DA e em outras doenças neurodegenerativas relacionadas ao envelhecimento, o recrutamento e a ativação de células gliais ocorre antes mesmo de aparecerem os sinais patológicos e clínicos da doença (Mrak e Griffin, 2005; Strohmeyer e Rogers, 2001).

Em fases um pouco mais avançadas, estudos *in vitro* demonstram que depósitos do peptídeo beta amilóide ativam células da microglia, as células fagocíticas próprias do sistema nervoso. Estas células, na tentativa de combater as células neurais afetadas, acabam por causar danos ou a morte de neurônios através da liberação de óxido nítrico (NO) e espécies reativas de oxigênio (ERO) (Qin *et al.*, 2002). Espécies reativas de nitrogênio e oxigênio, como o NO, a ânion superóxido, radicais hidroxila e peróxido de hidrogênio, são produtos das reações metabólicas fisiológicas do organismo. Porém, quando a produção destes metabólitos e a atividade de enzimas e agentes antioxidantes está desequilibrada, tem-se o estabelecimento de estresse oxidativo.

Além da microglia, pode também ocorrer proliferação de astrócitos, a qual, entre outros fatores, está implicada com a produção excessiva de citocinas e mediadores inflamatórios (Klegeris e McGeer, 1997). Além disso, os astrócitos também aumentam a produção de NO e ERO, contribuindo com o dano oxidativo e, conseqüentemente, a ativação de mais astrócitos e agravamento do quadro inflamatório e a conseqüente deposição anormal de proteínas, como num círculo vicioso. Tal evento é denominado gliose reativa e é acompanhado de complexo remodelamento funcional e, às vezes, estrutural dos astrócitos (Liberto *et al.*, 2004; Pekny e Pekna, 2004).

1.1.2.1 Astrócitos nas doenças neurodegenerativas

Os astrócitos são as células gliais que constituem o terceiro elemento da chamada sinapse tripartida. Ao contrário do que se pensava inicialmente, sua função ultrapassa em muito a de simples célula suporte, ocorrendo uma importante intercomunicação entre astrócitos e estes com os neurônios (Allen e Barres, 2009; Araque *et al.*, 1999). As funções astrocíticas são muito amplas e incluem desde o controle da diferenciação e migração neuronal, à formação e manutenção da barreira hematoencefálica (BHE), tamponamento de potássio, captação e conversão do glutamato em glutamina, defesa antioxidante, homeostase do Ca^{2+} até a liberação de gliotrasmissiores e a própria modulação sináptica (Pellerin e Magistretti, 1994; Perea e Araque, 2005; Wang e Bordey, 2008). Como base de diversas doenças neurodegenerativas, incluindo DA, observa-se a participação ativa dos astrócitos, seja no sentido de combater as alterações fisiopatológicas ocorrentes nos neurônios através da produção e liberação de fatores neurotróficos ou controlando a excitoxicidade glutamatérgica, seja produzindo citocinas e tornando-se parte do agravamento destas alterações. Tais respostas tem se tornado alvo de diversas possibilidades terapêuticas nas doenças neurodegenerativas e neuroinflamatórias (Liberto et al., 2004).

1.1.2.2 Parâmetros para o estudo dos astrócitos

A proteína fibrilar glial ácida, GFAP (do inglês *glial fibrilar acidic protein*) é o principal filamento intermediário de astrócitos maduros do SNC. É considerada há décadas como um dos principais antígenos utilizados para a identificação e estudo do comportamento astrocítico (Eng *et al.*, 2000). É a principal, porém não a única, proteína com expressão aumentada na gliose reativa, respondendo rapidamente a diversos tipos de insulto ao SNC (Eddleston e Mucke, 1993).

Os astrócitos proveem um importante sistema antioxidante para o sistema nervoso através da produção e secreção de glutationa (GSH) (Dringen, 2000). Para a efetividade de tal papel antioxidante, é necessário um equilíbrio entre as formas oxidada (GSSG) e reduzida (GSH) da glutationa, bem como da quantidade sintetizada e do consumo e transporte para outras células (Hirrlinger e Dringen, 2010). A GSH age de maneira enzimática ou não enzimática na conversão do peróxido de hidrogênio em água, neutralizando esta espécie reativa.

A glutamina sintetase (GS) é uma enzima presente em diversos órgãos como rins, fígado, músculo esquelético, baço, coração e encéfalo, a qual utiliza ATP para converter glutamato e amônia em glutamina, evento crucial no SNC (Walton e Dodd, 2007). No SNC, está localizada essencialmente em astrócitos peri-sinápticos, estabelecendo um ciclo responsável pela reciclagem de glutamato paro o neurônio.

A S100B é uma proteína ligante de cálcio, pertencente a uma família de proteínas chamadas S100 (solúvel em 100% de sulfato de amônio), produzida - principalmente - e secretada - exclusivamente - por astrócitos. Ela possui ações parácrinas e autócrinas, tanto intra quanto extracelulares, sobre neurônios e sobre outros astrócitos (Rothermundt *et al.*, 2003). Dentre estas ações, estão a regulação da proliferação, diferenciação e morfologia celular, homeostase do Ca^{2+}, fosforilação e transcrição de proteínas, atividade enzimática e metabolismo (Donato *et al.*, 2009; Goncalves *et al.*, 2008). Tais efeitos são mediados, em parte, pela interação da S100B com o receptor para produtos finais avançados de glicação (RAGE), um receptor multiligante, envolvido na transdução de estímulos inflamatórios e de diversos fatores neurotróficos e neurotóxicos (Donato, 2003; Donato, 2001). Estudos *in vitro* demonstram que a proteína S100B pode também ter efeitos neurotróficos e neurotóxicos, dependendo da sua concentração e circunstância. Em concentrações pico

a nanomolares, estimula o crescimento de neuritos, promovendo a sobrevivência neuronal durante o desenvolvimento; enquanto que, em concentrações micromolares, desencadeia a liberação de citocinas pró-inflamatórias, podendo levar à morte neuronal por apoptose (Van Eldik e Wainwright, 2003). Estudos *in vivo* e com animais transgênicos mostram a importância da S100B para o crescimento neurítico e para processo de aprendizagem e memória (Mello e Souza *et al.*, 2000; Whitaker-Azmitia *et al.*, 1997).

1.1.2.3 S100B e Doença de Alzheimer

Em humanos, diversas condições patológicas e traumáticas investigadas têm demonstrado alterações da proteína S100B, especialmente no soro e no líquido cefalorraquidiano (LCR) de pacientes. Traumatismos cranianos, episódios isquêmicos, procedimentos cirúrgicos e distúrbios psiquiátricos provocaram aumento da proteína em soro e LCR em vários estudos (Andreazza *et al.*, 2007; Robson *et al.*, 2001; Bertsch *et al.*, 2001; Berger *et al.*, 2002; Romner *et al.*, 2000). Já em doenças neurodegenerativas, existe uma série de controvérsias. Alguns estudos mostram aumento de S100B em análise de tecido *post mortem* de pacientes com Síndrome de Down (Griffin *et al.*, 1998), Doença de Alzheimer (Van Eldik e Griffin, 1994) e esclerose lateral amiotrófica (ELA) (Migheli *et al.*, 1999) e em LCR nos estágio iniciais da Doença de Alzheimer (Peskind *et al.*, 2001). Por outro lado, também foi encontrado uma diminuição da S100B em soro de pacientes com DA, num estágio mais crônico da doença (Chaves *et al.*, 2010).

2. Modelo de Doença de Alzheimer esporádica por Infusão icv de Estreptozotocina

Existem diversos modelos animais classificados como não transgênicos, pois não envolvem manipulação dos genes envolvidos diretamente na DA, que visam mimetizar algumas das características neuroquímicas e comportamentais apresentadas na DA do tipo esporádica através de algum dos diversos caminhos que levam ao estabelecimento da mesma. Dentre estes, tem-se a infusão intracerebroventricular (ICV) direta do peptídeo beta amilóide (Lopes *et al.*, 2010), a destruição neurotóxica do núcleo basal magnocelular e suas eferências colinérgicas corticais (Swarowsky *et al.*, 2008), a oclusão permanente e bilateral das carótidas comuns, causando uma hipoperfusão cerebral crônica, evento também presente no estabelecimento da DA esporádica (Vicente *et al.*, 2007) e o modelo por infusão icv da estreptozotocina, nosso objeto de estudo, entre outros.

A estreptozotocina (STZ) é uma droga de ação antibiótica utilizada experimentalmente para induzir diabetes, principalmente em roedores. Para isso, é administrada por uma via sistêmica e age no pâncreas destruindo permanentemente as células beta pancreáticas produtoras de insulina, originando assim, um quadro de diabetes (Baydas *et al.*, 2003). No pâncreas, na dose em que a STZ é administrada (50-100 mg/Kg), o mecanismo de ação consiste na captação de STZ pelas células beta através de transportadores de glicose (GLUT) do tipo 2, conforme esquematizado na Figura 1. Por ser uma nitrosamida metilnitrosureia ligada a uma D-glicose, uma vez metabolizada pela célula, gera N-nitrosureido o qual causa a fragmentação do DNA celular, seguida da ativação de uma enzima de reparo, a poli-ADP-ribose-polimerase (PARP), a qual consome NAD+ e promove a formação de radicais livres que acabam promovendo a morte celular (Uchigata *et al.*, 1982; Szkudelski, 2001; Hosokawa *et al.*, 2001).

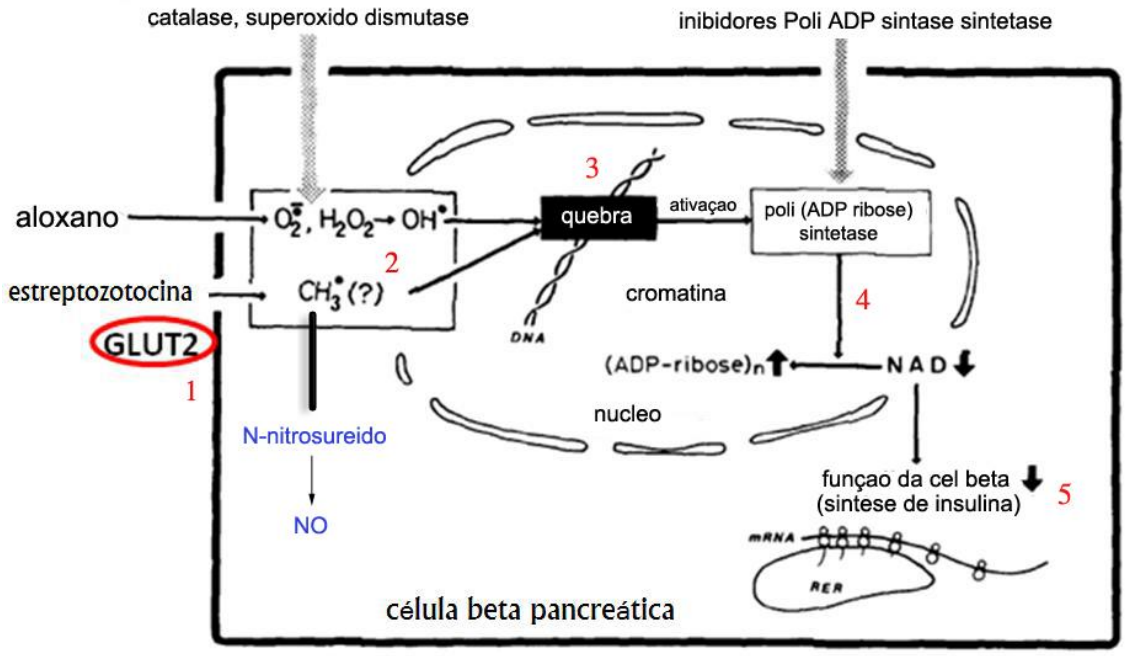

Figura 1: Mecanismo de ação da estreptozotocina (STZ) na célula beta pancreática. 1. captação pelo GLUT 2; 2. liberação de radicais livres (NO); 3. dano ao DNA; 4. ativação da PARP; 5. diminuição da síntese de insulina e morte celular. Adaptado de Uchigata et al, 1982

Quando administrada via icv, em dose bem menor que a utilizada para induzir o diabetes (1-3 mg/Kg), a STZ causa uma espécie de dessensibilização dos receptores de insulina (RI) e do IGF (ambos do tipo tirosina cinase) com consequente alterações bioquímicas e fisiopatológicas semelhantes às encontradas na demência do tipo Alzheimer. Por esta razão, tem sido amplamente aceita para produzir um modelo experimental para a DA (Weinstock e Shoham, 2004; Hoyer *et al.*, 2000; de la Monte, 2009). É importante ressaltar que a STZ não ultrapassa a BHE e a administração icv não causa comprometimento sistêmico (Duelli *et al.*, 1994; Lannert e Hoyer, 1998).

Diversos estudos indicam que a deficiência ou estado de resistência de insulina encefálica são características tanto do quadro tardio da DA do tipo esporádica (Hoyer, 1998; Hoyer, 2002a; de la Monte e Wands, 2005), quanto do próprio mecanismo

patogênico da doença (Steen *et al.*, 2005; Rivera *et al.*, 2005) com aumento do quadro de resistência conforme progride o estágio da DA (Rivera *et al.*, 2005).

O mecanismo pelo qual esta deficiência no funcionamento do receptor de insulina (RI), tanto no modelo experimental quanto na patogenia da DA, resulta nas características neuroquímicas e morfológicas da doença está diretamente relacionado com a transdução de sinal do RI (Grunblatt *et al.*, 2007; Hoyer, 2002b; de la Monte, 2009; Salkovic-Petrisic *et al.*, 2006) . De uma maneira resumida, quando a insulina se liga ao seu receptor (tanto central quanto periférico), este recruta seu substrato (SRI) no sítio de ancoramento, tornando-o fosforilado nos resíduos de tirosina. Este, por sua vez, torna-se capaz e recrutar várias moléculas sinalizadoras, dentre estas a enzima fosfatidilinositol-3 cinase (PI3K). Esta enzima fosforila o fosfoinotidide da membrana que, por sua vez, ancora a proteína cinase B (PKB), também conhecida como Akt, a qual promove a translocação do receptor de glicose GLUT 4 para a membrana plasmática, promovendo maior aporte de glicose nos tecidos dependentes de insulina. Por outro lado, a Akt também modula por fosforilação a atividade da enzima glicogênio sintase cinase 3 (GSK 3) a qual, dependendo de sua isoforma, regula os peptídios β-amilóides (isoforma α) e a fosforilação da proteína tau (isoform β) (Salkovic-Petrisic *et al.*, 2006; Salkovic-Petrisic e Hoyer, 2007).

Além disso, o GLUT2, por onde a droga parece atuar também no SNC, está localizado em áreas específicas do encéfalo, como as áreas límbicas e seus núcleos relacionados (Arluison *et al.*, 2004). Isto explica as semelhanças em alterações hipocampais encontradas entre o modelo e a DA. Este provável mecanismo de ação está ilustrado na Figura 2.

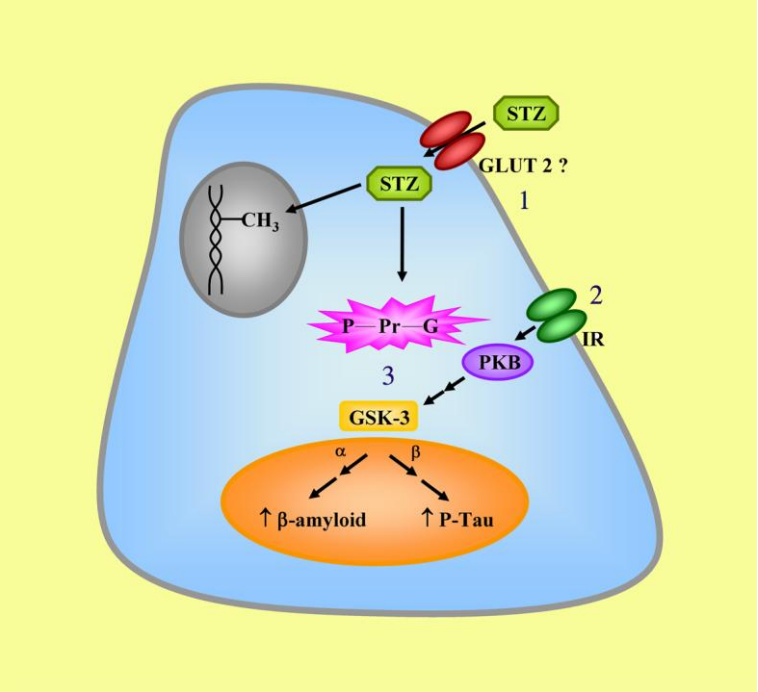

Figura 2: Provável mecanismo de ação da STZ na célula (neurônio e/ou astrócito). 1. Entrada através do transportador de glicose 2 (GLUT2) em neurônio ou astrócito, 2. Ação sobre o receptor de insulina (IR), 3. Consequente comprometimento da via da enzima glicogênio sintase cinase 3 (GSK 3) por desequilíbrio da fosforilação/glicação de proteínas no neurônio. Adaptado de Rodrigues et al, 2010; Handbook of Journal of Alzheimer Disease (Anexo I).

O mau funcionamento do RI resulta também em redução do metabolismo energético cerebral com conseqüente estresse oxidativo, o que se reflete em disfunções cognitivas devido, entre outros fatores, à inibição da formação de ATP e acetil-CoA (Ishrat *et al.*, 2006). Trabalhos demonstram que a injeção icv de SZT em dose subdiabetogênica reduz de 10 a 30% os níveis de glicose no córtex cerebral e hipocampo, com diminuição significativa das principais enzimas da via glicolítica (Plaschke e Hoyer, 1993). Além disso, estudos feitos por Duelli e colaboradores (Duelli *et al.*, 1994) mostraram redução na utilização da glicose em 17 de 35 áreas encefálicas estudadas. Também foram relatados comprometimentos de aprendizagem e memória, bem como redução dos níveis da enzima colina acetiltransferase (devido à falta de acetil-CoA), também no hipocampo, o que interfere na neurotransmissão colinérgica

(Ishrat et al., 2006; Sharma e Gupta, 2001; Blokland e Jolles, 1993; Lannert e Hoyer, 1998). Estudos em pacientes com DA através de técnicas de imageamento do encéfalo como a tomografia por emissão de pósitrons, demonstraram uma significativa redução do metabolismo da glicose em diversas áreas encefálicas, especialmente nos córtices frontal e parietotemporal, corroborando com o modelo proposto (Mielke *et al.*, 1992; Kumar *et al.*, 1993).

3. Aminoguanidina

Compostos guanidínicos endógenos tais como o ácido guanidinoacético, a arginina, a creatinina, a guanidina, entre outros, estão presentes no encéfalo de diversos mamíferos. As estruturas de guanidina, metilguanidina e aminoguanidina estão representados na Figura 3.

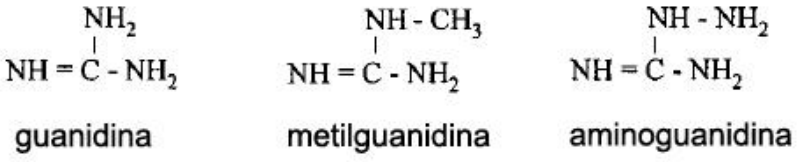

Figura 3. Estrutura química de compostos guanidínicos simples e da aminoguanidina.

Compostos guanidínicos estão envolvidos em eventos que vão desde a indução de convulsões, produção de radicais livres até a geração de energia e proteção contra morte celular e apoptose (Hiramatsu, 2003). A aminoguanidina, comercialmente chamada Pimagedina®, é um composto guanidídico sintético que tem recebido maior atenção pelo seu potencial como droga de ação anti-glicação. Ela atua reagindo com os grupos carbonila de açúcares reduzidos ou com o 3-diacilglicerol, prevenindo a formação de produtos finais avançados de glicação (do inglês *advanced glycation end products* ou AGE (Ahmed, 2005).

3.1 Aminoguanidina e AGE

Produtos finais de glicação avançada (AGE) são formados a partir de uma série de reações não enzimáticas chamadas reações de Maillard. Tais reações ocorrem entre os grupamentos carbonila de açúcares reduzidos e os grupamentos amino de proteínas, ácidos nucleicos e fosfolipídeos, formando bases de Schiff. Em seguida, ocorre um rearranjo do tipo Amadori e subsequentes reações oxidativas (glicoxidação) induzidas por espécies reativas de oxigênio e nitrogênio (Reddy e Beyaz, 2006). Sabe-se que a formação de AGE é um dos mecanismos no desenvolvimento do diabetes e de doenças neurodegenerativas como a Doença de Alzheimer (Ahmed *et al.*, 2005; Sasaki *et al.*, 1998; Monnier, 2003).

A aminoguanidina é uma substância altamente reativa, inibidora da reação de Maillard, que sequestra compostos tóxicos 1,2-dicarbonil através da formação de correspondentes menos tóxicos 1,2,4-triazinas. A aminoguanidina também age no combate ao estresse oxidativo através da ligação e neutralização do peroxinitrito (Giardino *et al.*, 1998), e como agente quelante de íons de metais de transição (Stadler *et al.*, 2005). Além disso, tem outras ações; tais como a inibição da enzima aldose redutase (Kumari *et al.*, 1991).

3.2 Ações terapêuticas da aminoguanidina:

Clinicamente, sabe-se que a aminoguanidina previne o estresse tecidual crônico decorrente da formação de AGE, e, por esse motivo, tem sido testada no tratamento do diabetes (Makita *et al.*, 1992; Bolton *et al.*, 2004). Porém, na forma como vem sendo apresentada, tem causado uma série de efeitos colaterais na fase III das triagens clínicas, como, por exemplo, a deficiência de vitamina B6 pelo sequestro do piridoxal (Bolton *et al.*, 2004).

No sistema nervoso periférico, existem trabalhos experimentais que mostram a ação da aminoguanidina em animais diabéticos, no sentido de atenuar ou reverter a neuropatia periférica, aumentando a velocidade da condução nervosa em nervos sensoriais e motores, além de prevenir alterações funcionais e estruturais (Yagihashi *et al.*, 1992; Cameron *et al.*, 1992). Quanto aos efeitos no sistema nervoso central (SNC), alguns estudos apontam para as propriedades neuroprotetoras da aminoguanidina em modelos animais de danos corticais e isquemia (Cash *et al.*, 2001; Yamanaka *et al.*, 1995). Os mecanismos de tal efeito não estão ainda totalmente esclarecidos, porém se sabe que além de diminuir a formação de AGE e outras ações citadas acima, este composto atua como inibidor seletivo da enzima óxido nítrico sintase induzível (iNos) (Hiramatsu, 2003; Iadecola *et al.*, 1995).

4. Exercício físico

Muitos estudos têm demonstrado os efeitos de diferentes tipos de exercício físico sobre o SNC em humanos (Young, 1979; Baker *et al.*, 2010; Larson *et al.*, 2006) e em outros animais (Collins *et al.*, 2009; Ding *et al.*, 2005; Ding *et al.*, 2004; Cotman e Berchtold, 2007). Dentre os principais efeitos da atividade física sobre o SNC está a ação sobre o sistema cardiovascular, com consequente melhora cognitiva e principalmente liberação de neurotransmissores e fatores de crescimento, que fornece o substrato funcional para se entender estruturalmente tal melhora cognitiva. Diversos trabalhos demonstraram a liberação de várias substâncias após exercício físico de diferentes modalidades. Dentre estas substâncias, tem-se o fator de crescimento de fibroblastos (FGF) (Gomez-Pinilla *et al.*, 1998), a serotonina (Chen *et al.*, 2008; Blomstrand *et al.*, 1989), a sinapsina I e o fator neurotrófico semelhante à insulina (IGF) (Ploughman *et al.*, 2005), as proteínas de choque térmico, juntamente com proteínas pré

e pós sinápticas (Hu *et al.*, 2009), as integrinas (Ding *et al.*, 2006), o fator de crescimento neural (NGF) (Ding et al., 2004) e, principalmente, o fator neurotrófico derivado do encéfalo (BDNF) (Ding et al., 2004; Neeper *et al.*, 1995; Russo-Neustadt *et al.*, 2000; Berchtold *et al.*, 2005). Além disso, tem-se observado, como consequência do exercício físico, ocorrência de neurogênese hipocampal no adulto (Uda *et al.*, 2006), aumento de *c-fos* e de histonas acetiladas, indicando aumento na expressão gênica hipocampal (Collins *et al.*, 2009) e da angiogênese (Ding *et al.*, 2004; Kleim *et al.*, 2002).

Outro efeito obtido com o exercício físico, especialmente o moderado, é o aprimoramento da ação antioxidante tecidual (Mallikarjuna *et al.*, 2009). Sabe-se que o exercício físico crônico ou exaustivo causa um aumento do estresse oxidativo em diversos órgãos, inclusive no encéfalo (Aksu *et al.*, 2009; Liu *et al.*, 2000). Por outro lado, muitos resultados são ainda controversos, os tipos e protocolos de exercício são variados, com alguns trabalhos mostrando que mesmo o exercício crônico pode ser benéfico para as defesas antioxidantes (Teixeira *et al.*, 2008). Entretanto, o número de trabalhos comprovando a atividade antioxidante do exercício físico moderado, especialmente a corrida em esteira é bastante expressivo (Cechetti *et al.*, 2008; Salim *et al.*, 2010).

As duas principais modalidades de corrida a qual os animais se submetem em laboratório são o exercício voluntário em roda de correr e a corrida em esteira. A primeira é interessante por ser voluntária, porém a segunda é semelhante ao ocorrido com os seres humanos, pois ocorre durante um determinado intervalo de tempo, uma vez ao dia, como normalmente são os programas de exercício possíveis na vida diária do indivíduo comum.

Em relação à demência, uma série de estudos vem demonstrando os efeitos benéficos de programas de exercício físico tanto na prevenção quanto no tratamento (Colcombe e Kramer, 2003). Em modelos animais de doença de Alzheimer, tem-se encontrado melhora do declínio cognitivo, especialmente em modelos transgênicos (Nichol *et al.*, 2007; Lazarov *et al.*, 2005; Nichol *et al.*, 2008; Adlard *et al.*, 2005; Wolf *et al.*, 2006).

Dessa forma, o estudo do provável papel neuroprotetor da aminoguanidina e do exercício físico, através da análise de parâmetros bioquímicos e histológicos no encéfalo de ratos submetidos ao modelo de STZ é de fundamental importância para a compreensão dos benefícios desencadeados por estas duas formas de intervenção clínica, além da melhor compreensão do modelo de demência e do seu paralelo com a Doença de Alzheimer.

Objetivos

Objetivo Geral

Avaliar o estado cognitivo, bem como parâmetros bioquímicos e histológicos relacionados à astrócitos em hipocampo de ratos submetidos ao modelo de doença de Alzheimer esporádica induzido pela administração intracerebroventricular de estreptozotocina e assim poder investigar o provável papel neuroprotetor da aminoguanidina e do exercício físico sobre tais parâmetros.

Objetivos específicos:

1. Padronizar o modelo de demência que mimetiza as características fisiopatológicas da Doença de Alzheimer do tipo esporádica através da administração intracerebroventricular de estreptozotocina.
2. Avaliar o déficit cognitivo em animais submetidos ao modelo de doença de Alzheimer esporádica por infusão intracerebroventricular de estreptozotocina.
3. Avaliar o estresse oxidativo e nitrosativo.
4. Avaliar o metabolismo glutamatérgico.
5. Medir o imunoconteúdo de S100B e GFAP no tecido hipocampal, bem como o conteúdo de S100B secretado em LCR e em fatias hipocampais agudas.
6. Avaliar o efeito da aminoguanidina sobre os parâmetros acima.
7. Avaliar o efeito da corrida em esteira sobre os parâmetros acima.

Parte II

Capítulo I

Hippocampal alterations in rats submitted to streptozotocin-induced dementia model are prevented by aminoguanidine

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Hippocampal Alterations in Rats Submitted to Streptozotocin-Induced Dementia Model are Prevented by Aminoguanidine

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Abstract. Although the exact cause of Alzheimer's disease remains elusive, many possible risk factors and pathological alterations have been used in the elaboration of *in vitro* and *in vivo* models of this disease in rodents, including intracerebral infusion of streptozotocin (STZ). Using this model, we evaluated spatial cognitive deficit and neurochemical hippocampal alterations, particularly astroglial protein markers such as glial fibrillary acidic protein (GFAP) and S100B, glutathione content, nitric oxide production, and cerebrospinal fluid (CSF) S100B. In addition, prevention of these alterations by aminoguanidine administration was evaluated. Results confirm a spatial cognitive deficit and nitrative stress in this dementia model as well as specific astroglial alterations, particularly S100B accumulation in the hippocampus and decreased CSF S100B. The hippocampal astroglial activation occurred independently of the significant alteration in GFAP content. Moreover, all these alterations were completely prevented by aminoguanidine administration, confirming the neuroprotective potential of this compound, but suggesting that nitrative stress and/or glycation may be underlying these alterations. These findings contribute to the understanding of diseases accompanied by cognitive deficits and the STZ-model of dementia.

Keywords: Aminoguanidine, astrocyte, hippocampus, nitrosative stress, S100B, streptozotocin

INTRODUCTION

Dementia is a serious and growing public health problem that is pathologically characterized by a progressive decline in cognitive function that affects cortical and/or subcortical brain structures beyond what might be expected from normal aging. The condition affects about 5% of the elderly population of over 65 years old and 25% of those over 80 years old, where Alzheimer's disease (AD) represents more than 50% of cases [1].

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Although the exact cause of AD remains elusive, many possible risk factors and pathological alterations have been used in the elaboration of *in vitro* and *in vivo* models of this disease. Non-transgenic animal models include chronic cerebral hypoperfusion, intracerebroventricular (ICV) infusion of amyloid-β peptide [2] or streptozotocin (STZ) [3], and lesion of nucleus basalis magnocellularis [4]. ICV infusion of STZ provides a relevant animal model of chronic brain dysfunction that is characterized by long-term and progressive deficits in learning, memory, and cognitive behavior, indicated by decreases in working and reference memory, along with a permanent and ongoing cerebral energy deficit [5,6].

Astrocytes are intimately associated with neurons and the importance of these glial cells in brain disor-

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ders, including AD, has been strongly suggested [7]. Astrocytes, beyond energetic support [8], are involved in the brain antioxidant defense and secretion of neurotrophic factors. Glial activation in response to injury stimuli commonly involves changes in glial fibrillary acidic protein (GFAP), S100B protein, synthesis and release of glutathione and glutamate uptake and metabolism. Therefore, the evaluation of glial activation in STZ-induced and other models of dementia is extremely useful to understand the role of astrocytes in these diseases, as well as to identify possible molecular therapeutic targets.

GFAP is a specific astrocyte marker and its increase is taken as a sign of astrogliosis, observed in many conditions of brain injury (see [9] for a review). A cortical astrogliosis, evaluated by immunohistochemistry for GFAP, has been observed in a STZ-induced model of dementia [10]. S100B is a calcium-binding protein found in brain tissue, predominantly in astrocytes, where it regulates protein phosphorylation of cytoskeleton components and transcriptional factors [11]. Moreover, S100B is secreted and, extracellularly, plays a trophic role in neuronal and glial cells. Cerebrospinal fluid (CSF) and serum S100B levels have been used as marker of brain insult [12]. Although many cognitive tasks dependent on hippocampal integrity, only a few studies have broached glial activation in this brain region.

Many putatively neuroprotective compounds have been evaluated in the STZ-induced model of dementia, including 17-β-esteroid [13], antioxidants [14–16], calcium channels blockers [17], and drugs acting on glucose uptake [18]. However, no reports are available on drugs that reduce protein glycation (e.g., aminoguanidine) due to the lower glucose utilization observed in cerebral cortex and all subfields of hippocampus [19]. Aminoguanidine has been proposed as a neuroprotective agent in many conditions of brain injury (e.g. [20]) and is apparently effective against many systemic alterations observed in STZ-induced diabetes (e.g. [21]). The complete activity of this compound is not known, but its inhibition of inducible nitric oxide (NO) synthase and inhibition of glycation helps to explain many actions [22].

In this study, our aim was to evaluate spatial cognitive deficit and hippocampal alterations in rats submitted to the STZ-induced dementia model, particularly with regard to astroglial protein markers (GFAP and S100B), glutathione content, NO production, and CSF S100B. The ability of aminoguanidine administration to prevent these alterations was also studied.

MATERIAL AND METHODS

Chemicals

Sodium carbonate, barbital, albumin, Tween-20, streptozotocin, aminoguanidine hemisulfate, glutamylhydroxamate, sodium nitrate, nitrate reductase, 3,3-diaminobenzidine (DAB), o-phenylenediamine, and monoclonal anti-S100B antibody were purchased from Sigma. Anti-S100 antibody conjugated with peroxidase and anti-GFAP antibody were from Dako. Peroxidase- and fluorescein-conjugated secondary antibodies were from Amersham and Calbiochem, respectively.

Animals

Forty-seven male Wistar rats (90 days old, weighing 250–320 g) were obtained from our breeding colony (at the Department of Biochemistry, Universidade Federal do Rio Grande do Sul), and were maintained under controlled light and environmental conditions (12 hour light/12 hour dark cycle at a constant temperature of 22 $\pm$ 1°C) with free access to food and water. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and following the regulations of the local animal house authorities.

Rats were divided into 4 groups: sham (N = 11), sham-aminoguanidine (N = 12), STZ (N = 12), and STZ-aminoguanidine (N = 12). Two set of experiments were carried out, using 5 or 7 rats of each group in each set, respectively. After behavioral tasks, rats were anaesthetized, as subsequently described, for CSF puncture, immunohistochemistry and hematoxylin-eosin staining (first set of experiments), or for brain slice preparation (second set of experiments), aiming to evaluate S100B and GFAP contents, S100B secretion, NO, and glutathione contents.

Surgical procedure

Streptozotocin was ICV infused based on previous work [15]. Briefly, on the day of the surgery animals were anesthetized with ketamine/xylazine (75 and 10 mg/Kg, respectively, i.p.) and placed in a stereotaxic apparatus. A midline sagittal incision was made in the scalp. Burr holes were drilled in the skull on both the sides over the lateral ventricles. The lateral ventricles were accessed using the following coordinates [23]: 0.9

mm posterior to bregma; 1.5 mm lateral to sagittal suture; 3.6 mm beneath the surface of brain. Rats received a single bilateral infusion of 5 μL STZ (3 mg/Kg) or vehicle (Hank's balanced salt solution – HBSS – containing in mM: 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 10 glucose, in pH 7.4) using a 5 μL Hamilton syringe. After the surgical procedure, rats were placed on a heating pad to maintain body temperature at 37.5 $\pm$ 0.5°C and were kept there until recovery from anesthesia. The animals were submitted to behavioral tasks and biochemical analysis 2–3 weeks after SZT-injection.

The STZ/aminoguanidine and aminoguanidine groups also received aminoguanidine hemisulfate (100 mg/Kg i.p.) dissolved in HBSS on the 2nd and 4th day after ICV-infusion of STZ. There are different schedules for aminoguanidine administration. Our protocol was based on (i) short-term administration, avoiding long-term procedures, which are stressful "per se" and putatively, could affect the behavioral tasks; (ii) administration after (not together with) surgical procedure and STZ injection, aiming to avoid a potential interference; and (iii) a potentially neuroprotective dose (e.g. [20]).

Cognitive evaluation

Two weeks after surgery, rats were submitted to training in the Morris water maze [24,25]. The apparatus consisted of a circular pool (180 cm diameter, 60 cm high) filled with water (depth 30 cm; 24 $\pm$ 1 °C), placed in a room with consistently located spatial cues. An escape platform (10 cm diameter) was placed in the middle of one of the quadrants, 1.5 cm below the water surface, equidistant from the sidewall and the middle of the pool. The platform provided the only escape from the water and was located in the same quadrant every trial. Four different starting positions were equally spaced around the perimeter of the pool. On each training day, all four start positions were used once in a random sequence, i.e., four training trials per day. A trial began by placing the animal in the water facing the wall of the pool at one of the starting points. If the animal failed to escape within 60 s it was gently conducted to the platform by the experimenter. The rat was allowed to stay there for 20 s. The inter-trial interval was 10 min. After each trial, the rats were dried, and returned to their cages at the end of the session. Animals were trained for 5 days. 24 h after the last training session, the rats were submitted to a test session (three weeks after surgery). Before this session, the submerged platform was removed. The retention test consisted of placing the animals in the water for 1 min. The number of crossings over the original position of the platform and time spent in the target quadrant compared to the opposite quadrant were measured.

Obtaining CSF and hippocampal samples

Animals were anesthetized as described above and then positioned in a stereotaxic holder and CSF was obtained by cisterna magna puncture using an insulin syringe (27 gauge $\times$ 1/2" length). CSF was frozen (−20°C) until further analysis [26]. The animals were killed by decapitation, and the brains were removed and placed in cold saline medium with the following composition (in mM): 120 NaCl; 2 KCl; 1 CaCl2; 1 $MgSO_4$; 25 HEPES; 1 KH_2PO_4 and 10 glucose, adjusted to pH 7.4 and previously aerated with O_2. The hippocampi were dissected and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper. Slices were then frozen (−20°C) (for measurement of GFAP and S100B) or transferred immediately to 24-well culture plates, each well containing 0.3 mL of saline medium for measuring S100B secretion, as described before [27].

Quantification of S100B and GFAP

S100B content in the hippocampus and CSF was measured by ELISA [28]. Briefly, 50 μL of sample plus 50 μL of Tris buffer were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B (SH-B1). Polyclonal anti-S100B was incubated for 30 min and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min. A colorimetric reaction with o-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.025 to 2.5 ng/mL. ELISA for GFAP [29] was carried out by coating the microtiter plate with 100 μL samples containing 30 μg of protein for 48 h at 4 °C. Incubation with a rabbit polyclonal anti-GFAP for 2 h was followed by incubation with a secondary antibody conjugated with peroxidase for 1h, at room temperature; the standard GFAP curve ranged from 0.1 to 10 ng/mL.

Immunohistochemistry for GFAP

Rats were anesthetized using ketamine/xylazine and were perfused through the left cardiac ventricle with 200 ml of saline solution, followed by 20 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and left for post-fixation in the same fixative solution at 4°C for 2 h. After this, the material was cryoprotected by immersing the brain in 30% sucrose in phosphate buffer at 4°C [4]. The brains were sectioned (50 μm) on a cryostat (Leitz) and sections were treated in 10% methanol and 3% H_2O_2 for 30 min. The sections were then preincubated in 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 for 30 min and incubated with polyclonal anti-GFAP from rabbit, diluted 1:200 in 2% BSA in PBS-Triton X-100, for 48 h at room temperature. After washing several times, tissue sections were incubated in a fluoresceine-conjugated anti-rabbit IgG, diluted 1:200 in PBS, at room temperature for 2 h. Afterwards, the sections were mounted on slides with Fluor Save® and covered with coverslips. Images were viewed with a Nikon microscope and images transferred to a computer with a digital camera. Slices from the same brains used for GPAP immunohistochemistry were also stained with hematoxylin-eosin.

Glutathione and NO contents

The glutathione content was determined as described before [30]. Briefly, hippocampal slices were homogenized in sodium phosphate buffer (0.1 M, pH 8.0) containing 5 mM EDTA and protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with *o*-phthaldialdehyde (1 mg/mL of methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard glutathione solutions (0–500 μM). Glutahione concentrations were expressed as nmol/mg protein. NO metabolites, NO_3^- (nitrate) and NO_2^- (nitrite), were determined according to [31]. Briefly, homogenates from hippocampal slices were mixed with 25% trichloroacetic and centrifuged at 1800 g for 10 min. The supernatant was immediately neutralized with 2 M potassium bicarbonate. NO_3^- was reduced NO_2^- by nitrate reductase. The total NO_2^- in the incubation was measured by a colorimetric assay at 540 nm, based on the Griess reaction. A standard curve was performed using sodium nitrate (0–80 μM). Results were expressed as μM of nitrite.

Statistical analysis

Parametric data from the experiments are presented as means $\pm$ standard error and statistically evaluated by two-way analysis of variance, followed by the Tuckey's test, assuming $p < 0.05$. The escape latency parameter in the water maze task was evaluated by repeated measures analysis of variance, assuming $p < 0.05$.

RESULTS

Behavioral effects

The Morris water maze was used to evaluate reference memory in the four groups: sham, sham/aminoguanidine, STZ, and STZ/aminoguanidine. There was a decline in the average time to find the platform (escape latency) from day 2 onwards in the sham group (Fig. 1A) ($F(3, 23) = 4.429$, $p = 0.035$). In addition, STZ rats spent less time in the target quadrant, as compared to the sham group (Fig. 1B) ($F(3, 26) = 3.999$, $p = 0.049$). The performance of STZ/aminoguanidine was not different from the sham group, based on the average time to find the platform ($p = 0.896$), and time spent in the target quadrant (Fig. 1B; $F(3, 26) = 0.718$, $p = 0.668$). The number of crossings over the platform location was significantly lower in the STZ group, compared to the other experimental groups ($F(3, 26) = 4.500$, $p = 0.023$) (Fig. 1C). Notice that aminoguanidine "per se" had no effect on analyzed behavior.

Changes in S100B and GFAP contents

A significant increase in S100B immunocontent ($F(3, 23) = 4.942$, $p = 0.019$) of the hippocampus was observed in STZ-treated rats (Fig. 2A) and this increase was not found in the STZ/AG group ($p = 0.899$); this effect in STZ group was not found in cerebral cortex and aminoguanidine by itself did not change S100B and GFAP contents in both brain regions (data not shown). Hippocampal GFAP content was apparently higher in the STZ group, however this increase was not statistically significant ($F(3, 23) = 0.444$, $p = 0.724$; Fig. 2B) nor was it significantly higher in the cerebral cortex (data not shown).

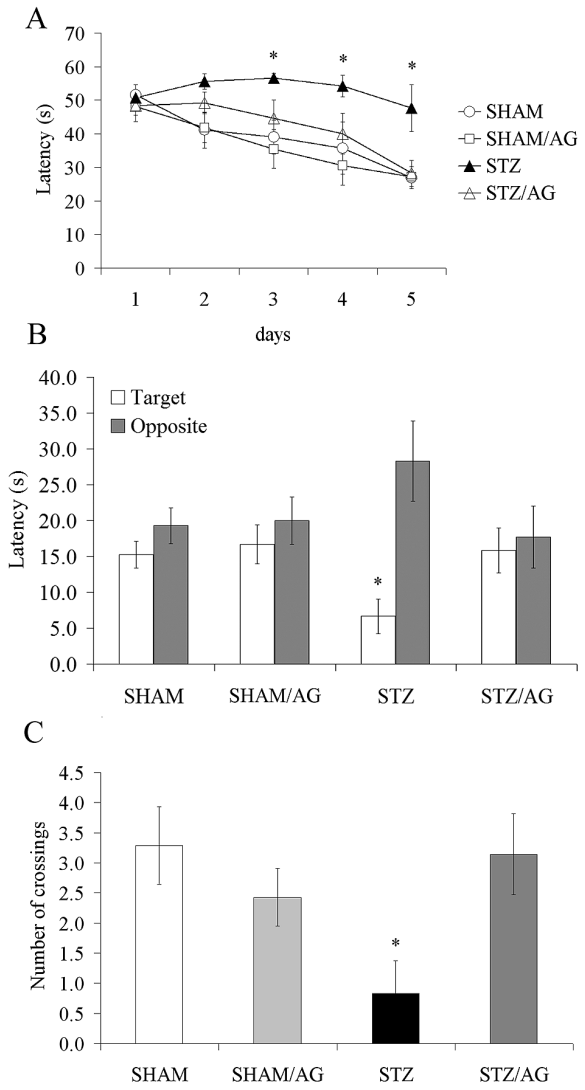

Fig. 1. Cognitive performance of rats submitted to ICV-STZ injection evaluated in the water maze. (**A**) Performance in the reference memory protocol, based on escape latency. Each line represents the mean ± standard error. * Significant differences were detected from day 2 onwards when compared to the control group (N = 7, repeated measures analysis of variance, $p < 0.05$); (**B**) Memory in the probe trial of reference memory, as measured by time spent (in s) in the target quadrant. Values are mean ± standard error. *Significantly different from the control group (N = 7, two-way ANOVA followed by Tukey's test, $p < 0.05$); (**C**) Number of crossings over the platform position. Values are mean ± standard error. *Significantly different from control group (N = 7, two-way ANOVA followed by Tukey's test, $p < 0.05$).

Immunohistochemistry for GFAP

In order to evaluate hippocampal astroglial changes observed in rats submitted to ICV-injection of STZ, we carried out a GFAP immunohistochemistry study. The

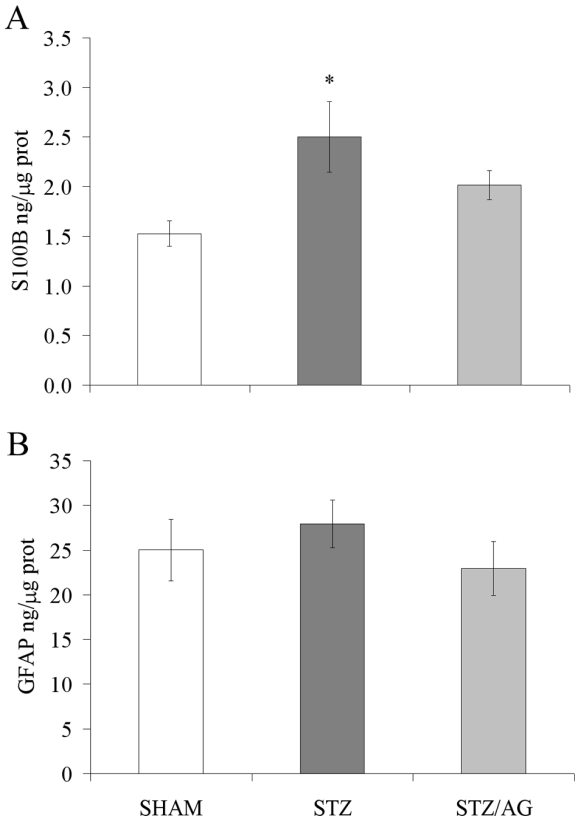

Fig. 2. GFAP and S100B content in hippocampus of rats submitted to ICV- STZ injection. Adult rats were submitted to ICV injection of STZ. Hippocampi and cerebral cortex were dissected out and the contents of S100B (panel A) and GFAP (panel B) were measured by ELISA in the third week. Values are mean ± standard error of 7 rats in each group. * Significantly different from control (two-way ANOVA, $p < 0.05$).

photomicrographs of astrocyte GFAP immunoreactive (ir) did not indicate signs of astrogliosis in CA1 (Fig. 3) or CA3 and DG (data not shown). We also observed neuron nuclei in the pyramidal layer of the hippocampus using hematoxylin and eosin staining. There is a clear impairment of marked nucleus density (asterisk) in the STZ group, when compared to the sham group, indicating neuronal loss provoked by ICV-injection of STZ.

Alterations in CSF S100B

Interestingly, a significant decrease in CSF S100B was observed in the STZ treated group as compared to the sham group, and this difference was prevented by aminoguanidine administration (Fig. 4A) ($F(3, 15) = 3.804$, $p = 0.040$). CSF S100B in sham group was not different to that of the sham-aminoguanidine group

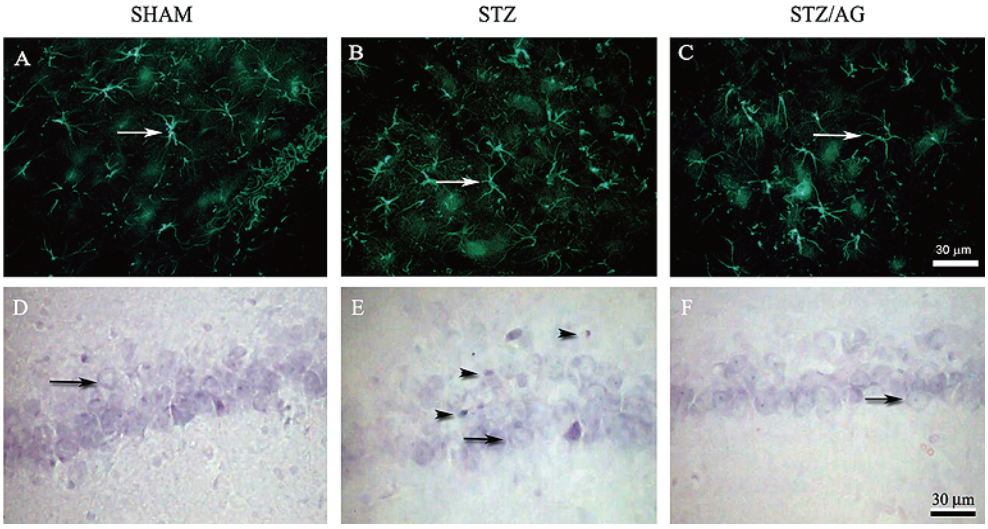

Fig. 3. Immunohistochemistry for GFAP and histological analysis of the hippocampi from rats submitted to ICV-STZ injection. Photomicrographs showing GFAP immunoreactive cells (ir) in the hippocampus of sham (panel A), STZ (panel B), and STZ/aminoguanidine (AG) (panel C) groups. No differences were observed in GFAP-ir in the radiatum layer of CA1 region (white arrows) between groups. Hippocampal sections show a higher density of hematoxylin-eosin stained nuclei of pyramidal layer in sham group (panel D) than in STZ group (panel E). In panel E, black arrow and arrow-heads indicate normal and pyknotic nuclei, respectively. Scale bars are indicated in panels C and F. (Colours are visible in the online version of the article at www.iospress.nl.)

(data not shown). Hippocampal slice preparations from the four groups were used to evaluate *in vitro* S100B secretion (Fig. 4B). No significant changes in basal S100B secretion were found at 1 h ($F(3, 11) = 0.242$, $p = 0.742$).

Oxidative stress in the hippocampus

Glutathione content and NO production (based on nitrite content) were used as parameters to evaluate a possible hippocampal oxidative stress (Fig. 5). Glutathione content was lower in STZ-treated rats and aminoguanidine prevented this decrease (Fig. 5A) ($F(3, 29) = 19.680$, $p = 0.006$). Interestingly, aminoguanidine "per se" induced an increase in glutathione content ($p = 0.004$). Conversely, in the sham-aminoguanidine group, no changes in hippocampal NO content were observed (Fig. 5B) ($p = 0.229$). NO content was higher in the STZ group than in the control group and STZ-treated rats that received aminoguanidine exhibited a lower NO content than the other two groups ($F(3, 17) = 22.510$, $p = 0.003$).

DISCUSSION

Based on glucose metabolism abnormalities observed in sporadic AD [32], the ICV-injected STZ model of dementia has been widely used. This model presents long-term and progressive deficits in learning, memory, and cognitive behavior, along with a permanent and ongoing cerebral energy deficit [5], and the neuroprotective activities of 17-β-estradiol, melatonin, resveratrol, and lercanidipine have been evaluated in this model [13–15,17]. Our data suggest a neuroprotective effect of aminoguanidine in the STZ-induced model of dementia. All parameters evaluated in the water-maze task, which is widely recognized to evaluate hippocampal integrity, were impaired in the STZ group and prevented in the STZ/AG group. Notice that aminoguanidine by itself did not alter cognitive behavior, S100B and GFAP contents, nor NO content. However, it was able to induce an increase in glutathione content.

Although the mechanism of neural damage caused by STZ is not well understood, oxidative stress is associated with such damage and could be, at least in part, underlying the injury [32]. Astrocytes are strongly involved in the brain antioxidant defense, particularly producing and secreting glutathione [8]. Many studies have demonstrated the decrease in glutathione in ICV-injected STZ rats (e.g. [15]). This decrease is reverted by antioxidants such as resveratrol [14] and coenzyme Q [16] as well as some drugs used in diabetes mellitus treatment such as pioglitazone [18] and pitavastatin [33]. Aminoguanidine was also able to pre-

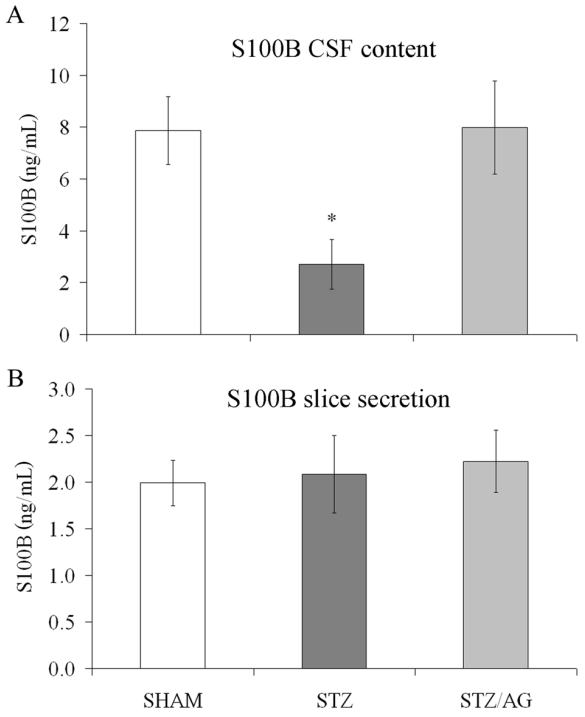

Fig. 4. S100B levels in the cerebrospinal fluid and secreted by hippocampal slices of rats submitted to ICV-STZ injection. Seven adult rats were submitted to ICV-injection of STZ. (A) Three weeks later, cerebrospinal fluid (CSF) was collected by cisterna magna puncture. S100B content was measured by ELISA. (B) Hippocampi were dissected out and chopped into 0.3 mm slices for measurement of S100B secretion. Values are mean ± standard error of 5 rats in each group. * Significantly different from control (two-way ANOVA, $p < 0.05$).

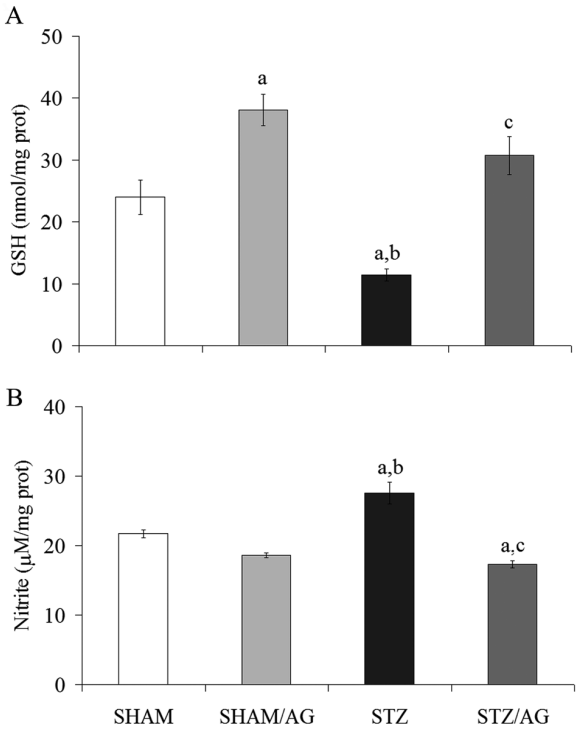

Fig. 5. Glutathione and NO levels in the hippocampus of rats submitted to ICV- STZ injection. Adult rats were submitted to ICV injection of STZ. Three weeks later, hippocampi were dissected out and homogenized for measurement of glutathione (in panel A) or NO (in panel B, measuring nitrate content). Values are mean ± standard error of 7 rats in each group. Two-way ANOVA, followed by Tukey's test, $p < 0.05$: [a], differs from sham group; [b], differs from sham/aminoguanidine group (AG); [c], differs from STZ group.

vent the glutathione decrease in hippocampus, induced by STZ, and was able to prevent the increase in NO. This increase in NO is agreement with a recent observation of nitrative stress, observed in ICV-injected STZ rats [10].

There are few studies regarding GFAP hippocampal alterations in STZ-treated rodents. In diabetes mellitus models, both increases [34,35] and decreases in GFAP [36] have been described in hippocampus. Recently, scattered reactive astrocytes were reported in the CA1 hippocampal region in ICV-injected STZ rats [10]. We did not, herein, find any signs of hippocampal astrogliosis, as indicated by GFAP content (measured by ELISA) and confirmed by immunohistochemistry in different hippocampal regions. This discrepancy could be due to methodological differences.

Interestingly, we found a significant increment in hippocampal S100B in the STZ group that was prevented by aminoguanidine administration. This finding reinforces the concept of astrogliosis, not necessarily accompanied by changes in GFAP content. S100B is a calcium-binding protein predominantly expressed and secreted by astrocytes in vertebrate brain [37,38]. Intracellularly, S100B binds to many protein targets, possibly modulating cytoskeleton plasticity, cell proliferation, and astrocyte energy metabolism (see [11] for a review). S100B alterations could contribute to the down-regulation of the glycolytic pathway in ICV-injected STZ of rats [32], particularly since some glycolytic enzymes are putative targets of S100B [11]. In agreement, high levels of brain tissue S100B have been observed in neurodegenerative disorders, including AD [39]. It is important to mention that S100B is able to stimulate the protein phosphatase calcineurin [40], which appears to be involved in the inflammatory activation of astrocytes in transgenic AD models [41].

On the other hand, the present study showed that CSF S100B is lower in the STZ group and aminoguanidine administration prevented this decrease. Assuming that extracellular S100B has neurotrophic activity [11,38],

this reduction could indicate impairment in astroglial function in some brain regions in STZ-treated rats. In agreement, rats exposed to chronic cerebral hypoperfusion also exhibited lower levels of CSF 100B [42]. No change was observed in the *ex-vivo* basal secretion of S100B secretion in hippocampal slices of ICV-injected STZ rats, contrasting with the idea that elevated intracellular concentrations of S100B result in increased S100B secretion or, conversely, that augmented intracellular S100B occurs as a consequence of the lower S100B secretion. This reinforces the idea of two independent events: intracellular S100B accumulation and S100B secretion in astrocytes [38]; however, further studies involving S100B-stimulated secretion in different brain regions are necessary to clarify this hypothesis.

In the present study, aminoguanidine was able to prevent, predictably, NO production and, interestingly, glutathione decrease, intracellular S100B accumulation, and CSF S100B decrease in the hippocampus of ICV-injected STZ rats. However, no direct effect of this compound was analyzed on neuronal death, microglia activation, or amyloid-β deposition described in this model [43,44], which, in further studies, should be investigated to evaluate the neuroprotective effect of aminoguanidine (and other advanced glycation end product inhibitors).

Moreover, it remains unclear as to how this compound "per se" increased hippocampal glutathione content. Long-term administration of aminoguanidine prevented apoptosis in retina glial cells of diabetic rats, but did not alter intracellular glutathione content [45]. On the other hand, acute administration of aminoguanidine was able to increase gastric glutathione content of rats submitted to brain ischemia [46]. In astrocyte cultures, aminoguanidine prevented cell death induced by 1-methyl-4-phenylpyridinium, apparently involving changes in glutathione content, but a direct effect of aminoguanidine on glutathione levels was not reported [47]. Therefore, at this moment, it is difficult to know whether aminoguanidine's prevention of the glutathione decrease in STZ-treated rats, under our conditions, is only due to an opposite and independent effect.

In summary, our results in ICV-injected STZ rats confirm the spatial cognitive deficit and nitrative stress in this model and demonstrate, for the first time to our knowledge, some astroglial alterations, particularly S100B accumulation in the hippocampus and the decrease in CSF S100B. Such alterations were prevented by aminoguanidine administration, confirming the potential neuroprotective of this compound. Findings contribute to understanding diseases accompanied by cognitive deficits and the STZ model of dementia.

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Capítulo II

S100B secretion in rat hippocampal slices is altered in streptozotocin model of dementia

Artigo em preparação

S100B secretion in rat hippocampal slices is altered in streptozotocin model of dementia

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Abstract

There are several animal models available for studying the mechanisms of dementia, particularly trying to mimic sporadic type Alzheimer's disease (AD). The intracerebroventricular infusion of streptozotocin (STZ) is one of these models and useful to evaluate neuroprotective effects on cognitive decline, oxidative stress and astrogliosis. We investigated astroglial parameters: S100B secretion, glutamate uptake and glutamine synthetase in the hippocampus from rats submitted to STZ model of dementia, as well as we evaluated cognitive behavior and oxidative stress in this brain region, considering that many cognitive tasks are dependent on hippocampal integrity. Our results indicate that STZ model of dementia, confirmed by cognitive behavior impairment, is accompanied by changes in hippocampal glutamine synthetase activity and in the S100B secretion modulated by high K^+. A chronic elevation of extracellular levels of S100B in the hippocampus of STZ rats could contribute to understand the hippocampal impairment in this model. Moreover, the neuroprotective activity of aminoguanidine was confirmed by its ability to prevent those STZ-induced alterations. Finally, these results emphasize the role of astrocytes in dementias, as well as the importance of this cell as targets as therapeutic targets in brain diseases

Introduction

Dementia is described as a progressive decline in cognitive function that affects cortical and/or subcortical brain structures beyond what might be expected from normal aging. It presents a growing incidence and cost for the care of patients all over the world and Alzheimer's disease (AD) represents more than 50% of cases [1, 2].

There are several animal models available for studying the mechanisms of sporadic type AD [3]. The intracerebroventricular infusion of streptozotocin (icv-STZ) is one of these models and it has been largely used to mimic the main characteristics of AD, including cognitive decline, oxidative stress and astrogliosis [4-7].

Although specific and characteristic intracellular alterations in AD (i.e. neurofibrillary tangles) are observed in neurons, growing evidence suggest that astrocytes, which are in close morphological and functional association with neurons, have an important role in the development of AD [8]. Astrocytes are key elements in the brain metabolism of glutamate; they are particularly responsible for removing glutamate from the synaptic cleft and for the synthesis of glutamine, which is sent back to the neuron to renew glutamate stocks [9]. In fact, glutamate uptake and glutamine synthetase activities have been proposed to evaluate astroglial function in AD [10].

S100B is a calcium-binding protein found in brain tissue, predominantly in astrocytes, where it regulates protein phosphorylation of cytoskeleton components and transcriptional factors (see [11] for a review). Moreover, S100B is secreted and, extracellularly, it plays a trophic role in neuronal and glial cells. The role of this protein on cognitive parameters has been investigated [12-14]. Cerebrospinal fluid (CSF) and serum S100B levels have been used as marker of astroglial activation, including AD [15, 16]. We observed a decreased CSF amount of this protein in two models of dementia: chronic cerebral hypoperfusion [17]

and icv-STZ [18]. In STZ model, aminoguanidine, an anti-glycation compound, was able to prevent this decrease.

In this work, we investigated S100B secretion, glutamate uptake and glutamine synthetase activity in the hippocampus from rats submitted to STZ-model of dementia, as well as we evaluated cognitive behavior and oxidative stress in this brain region, considering that many cognitive tasks are dependent on hippocampal integrity and many signals of hippocampal impairment are observed in AD. The neuroprotective activity of aminoguanidine also was investigated.

Material and methods

Animals. Forty male Wistar rats (90–days old, weighing 250-320 g) were obtained from our breeding colony (at the Department of Biochemistry, Universidade Federal do Rio Grande do Sul), and were maintained under controlled light and environmental conditions (12 hour light/12 hour dark cycle at a constant temperature of 22 ±1°C) with free access to food and water. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and following the regulations of the local animal house authorities.

Chemicals. Sodium carbonate, barbital, albumin, Tween-20, streptozotocin, aminoguanidine hemisulfate, glutamylhydroxamate, 3,3-diaminobenzidine (DAB), o-phenylenediamine (OPD), γ-glutamylhydroxamate, DCFDA and monoclonal anti-S100B antibody were purchased from Sigma. Anti-S100 antibody conjugated with peroxidase was from Dako. Peroxidase secondary antibodies and L-[2,3-3 ^{H}] glutamic acid were from Amersham.

Surgical procedure for STZ model and aminoguanidine administration. Streptozotocin was ICV infused as described before [18]. The animals were divided into 4 groups: sham, sham/aminoguanidine, STZ and STZ/aminoguanidine. On the day of the surgery, the animals were anesthetized with ketamine/xylazine (75 and 10 mg/Kg, respectively, i.p.) and placed in a stereotaxic apparatus. The lateral ventricles were accessed using the following coordinates (Paxinos, 1997): 0.9 mm posterior to bregma; 1.5 mm lateral to saggital suture; 3.6 mm beneath the surface of brain. The animals received a bilateral infusion of 5 μL STZ (3 mg/Kg) or vehicle (Hank's balanced salt solution - HBSS - containing in mM: 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 10 glucose, in pH 7.4) using a 10 μL Hamilton syringe. After the surgical procedure, rats were placed on a heating pad to maintain body temperature at 37.5 ± 0.5 °C, and were kept there until recovery from anesthesia. The sham/aminoguanidine and STZ/aminoguanidine groups also received aminoguanidine hemisulfate (100 mg/Kg i.p.) dissolved in HBSS on the 2^{nd} and 4^{th} day after ICV-infusion of STZ (Rodrigues et al, 2009). The animals were submitted to behavioral tasks and biochemical analysis 2-3 weeks after SZT-injection.

Inhibitory avoidance task. The step-down inhibitory avoidance task was carried out in an automatically operated, brightly illuminated box, in which the left extreme of the grid (42.0 cm×25.0 cm grid of parallel 0.1 cm caliber stainless steel bars spaced 1.0 cm apart) was covered by a 7.0cm wide, 5.0 cm high formica-covered platform. Animals were placed 21 days after surgery on the platform and their latency to step-down and place their four paws on the grid was measured. In the training session, immediately upon stepping down, the animals received a 0.5 mA, 3.0 s scrambled footshock. The test was carried out 24 h after

training for accessing long-term memory [13]. In the test session, no footshock was given, and a ceiling of 180 s was imposed on the step-down latency. The latency to step-down in the test session was taken as the measure of memory for the task.

S100B secretion assay in acute hippocampal slices. S100B secretion assays were performed as described before [19]. Briefly the animals were killed by decapitation, the brains were removed and placed in cold saline medium with the following composition (in mM): 120 NaCl; 2 KCl; 1 $CaCl_2$; 1 $MgSO_4$; 25 HEPES; 1 KH_2PO_4 and 10 glucose, adjusted to pH 7.4 and previously aerated with O_2. The hippocampi were dissected and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper. Slices were then transferred immediately into 24-well culture plates, each well containing 0.3 ml of physiological medium and only one slice. The medium was changed every 15 min with fresh saline medium at room temperature for equilibration (maintained at 25ºC). Removed medium was stored (at -20ºC) until S100B measurement. Following a 120-min equilibration period, the medium was removed and replaced with basal or 30 mM KCl (high K^+) media for 60 min at 30ºC in a warm plate. Thirty microliters of media were collected for S100B measurement.

Quantification of S100B. S100B content in the medium obtained from acutely incubated hippocampal slices was measured by ELISA [20]. In summary, 50 μL of sample plus 50 μL of Tris buffer were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B (SH-B1). Polyclonal anti-S100B was incubated for 30 min and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min. A colorimetric reaction with o-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.025 to 2.5 ng/mL.

Glutamine synthetase activity. The enzymatic assay was performed as previously described [21]. Briefly, one hippocampal slice homogenate (0.1mL) was added to 0.1 mL of reaction mixture containing (in mM): 10 $MgCl_2$; 50 L-glutamate; 100 imidazole-HCl buffer (pH 7.4); 10 2-mercaptoethanol; 50 hydroxilamine-HCl; 10 ATP and incubated for 15 min at 37ºC. The reaction was stopped by the addiction of 0.4 mL of a solution containing (in mM): 370 ferric chloride; 670 HCl; 200 trichloroacetic acid. After centrifugation, the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of g-glutamylhydroxamate treated with ferric chloride reagent.

Glutamate uptake assay. Slices obtained as described above were immediately transferred into 24-well culture plates, each well containing 0.3 mL of physiological medium and only one slice. Glutamate uptake was performed as previously described [19]. Briefly, media were replaced by Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 1.11 glucose, in pH 7.2. The assay was started by the addition of 0.1 mM L-glutamate and 0.66 μCi/mL L-[2,3-3^H] glutamate. Incubation was stopped after 5 min by removal of the medium and rinsing the slices twice with ice-cold HBSS. Slices were then lysed in a solution containing 0.1 M NaOH and 0.01% SDS. Sodium-independent uptake was determined using N-methyl-D-glucamine instead of sodium chloride. Sodium-dependent glutamate uptake was obtained by subtracting the non-specific uptake from the specific uptake. Radioactivity was measured with a scintillation counter.

Measurement of reactive oxygen species. Intracellular ROS production was detected using the non-fluorescent cell permeating compound, 2'–7'-dichlorofluorescein diacetate (DCF-DA). Samples homogenized in sodium phosphate buffer, pH 7.4 with 140 mM KCL were treated with DCF-DA (10 μM) for 30 min at 37°C. The fluorescence was measured in a

plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm, as described previously [22]. Values are obtained as unit of fluorescence/mg protein and are expressed as percentage of control.

Protein measurement. Protein was measured by Lowry's method [23] using bovine serum albumin as a standard.

Results

Step-down inhibitory avoidance task. Since data were not normally distributed (Kolmogorov–Smirnov goodness-of-fit test, $p < 0.05$), non-parametric statistics were used for this task. Data were expressed as medians (interquartile range). Training and test session at 14 and 15 days after ICV STZ infusion, respectively, were analyzed by Kruskal Wallis followed by Wilcoxon test, assuming $p < 0.05$. Significant differences between STZ group and the other three groups: sham, sham/aminoguanidine and STZ/aminoguanidine were observed in the test session ($p = 0.007$). There was no difference between the training and test sessions in STZ group ($p > 0.05$) what indicates cognitive decline induced by icv-STZ administration, which was prevented by aminoguanidine treatment (Fig. 1A).

Oxidative stress measurement. In addition, we found an increase in DCF oxidation in the STZ group compared to sham group (Fig. 1B). A small, but significant, decrease of ROS production was observed in rats that received aminoguanidine. In fact, aminoguanidine administration in STZ rats prevented ROS production ($F (3, 21) = 5.185$, $p = 0.09$).

Glutamate uptake and glutamine synthetase activities. Glutamate uptake, measured in hippocampal slices, remains unaltered in all groups group (Fig. 2A) ($F (3,39) = 0.792$, $p = 0.505$). However, glutamine synthetase activity decreased in STZ group compared with sham group (Fig 2B). Interestingly, aminoguanidine *per se* increased the glutamine

synthetase activity ($F(3, 22) = 14.400$ $p = 0.028$) and when administered to STZ-treated rats prevented ROS increase ($F(3, 22) = 14.400$, $p = 0.001$).

S100B secretion in hippocampal slices. S100B secretion was measured in basal and high K^+ conditions. In basal conditions, as observed before, no changes were observed among all groups [18]. However, when hippocampal slices were incubated in the high K^+ medium STZ group exhibited a small and no significant decrease of S100B secretion, when compared to other groups ($F(3, 17) = 4.818$, $p = 0.17$). Notice that, again, aminoguanidine was able to prevent the changes induced by STZ.

Discussion

STZ model of dementia has been an extremely useful as a model for the study of many aspects in AD. The behavioral data confirmed a cognitive decline in inhibitory avoidance task in STZ rats, previously evaluated by the water maze task [18]. Both cognitive tasks are dependent on hippocampal integrity. This cognitive deficit was successfully prevented by administration of aminoguanidine. In addition, we observed elevated reactive oxygen species in this brain region, confirming the oxidative stress induced by icv-STZ, which was prevented by aminoguanidine administration as well.

Altered glutamate uptake has been proposed as one pathophysiological mechanism in AD and other neurodegenerative diseases [24]; resultant hippocampal dysfunction could contribute to the cognitive deficit observed in these animals. Moreover, a decrease of hippocampal glutamate uptake could indicate a higher susceptibility of these animals to excitotoxicity. In fact, a decrease of hippocampal glutamate uptake was observed in rats submitted to chronic cerebral hypoperfusion [17]. However, no changes in glutamate uptake activity were observed in the STZ model of dementia. In agreement with our observation, no changes in levels of astrocyte glutamate transporters were observed in STZ-induced diabetic rats [25].

Beyond glutamate uptake, glutamine synthetase activity has been proposed to evaluate astroglial function in AD [10]. A cortical decrease of this protein has also been suggested as a characteristic in AD [26]. In fact, we observed a decrease of glutamine synthetase activity in the hippocampus of STZ rats, differently from rat dementia model by lesion of the nucleus basalis magnocellularis with ibotenic acid [27]. Interestingly, aminoguanidine “per se” increased the activity of this enzyme and prevented the decrease observed in STZ rats.

Other important feature about astroglial dysfunction in this model of dementia refers to S100B protein. Considering that decrease of cerebrospinal fluid S100B in STZ model of dementia in rats [18], as well as chronic cerebral hypoperfusion [17] we hypothesized that S100B secretion in central nervous system would be reduced in these animals. Based on other neurochemical changes found in hippocampus in AD and STZ model of dementia we decide to evaluate S100B secretion in acute hippocampal slices [19, 28]. In this work, as well as in our previous work [18] we did not found changes in basal S100B secretion of hippocampal slices of STZ rats. It is important to emphasize that basal secretion reflects the amount of S100B spontaneously secreted during 1 h after stabilization of acute brain slices. However, from those data, remained a question whether the modulated S100B secretion would be altered in STZ rats, particularly when this secretion involves cross-talk with neurons. A medium containing high K^+ (e.g. 30 mM KCl) causes a neuronal depolarization, but it does not induce an increase of intracellular calcium is astrocytes [29], putatively underlying the S100B secretion [30]. In fact, previously [19] and here we found a decrease of S100B secretion when acute hippocampal slice were exposed to high potassium, possibly due to release of neuronal transmitters (e.g. glutamate) [28, 31] or other factors.

In this work, hippocampal slices from STZ rats in high potassium medium exhibited a smaller decrease in S100B secretion than control slices. In other words, high potassium that depolarizes neurons and secondarily causes a decrease in S100B secretion is altered in STZ rats. This could be due to the lower neuronal response to high potassium, either by lower number of neurons or by lower neuronal sensibility in this condition. Therefore, in spite of the unaltered basal S100B secretion, these data evidence a functional alteration in secretion of this protein in STZ rats. It is possible to conceive that in STZ rats elevated neuronal activity (accompanied by elevation extracellular potassium) could increase extracellular

S100B and this, in turn, would contribute to neuronal death [32]. Interestingly, aminoguanidine also was able to prevent this alteration, suggesting that protein glycation could be underlying this change induced by STZ.

A chronic elevation of extracellular levels of S100B in the hippocampus of STZ rats contribute to understand the hippocampal impairment in this model, but, on the other hand, invalids our hypothesis that reduced S100B content in cerebrospinal fluid is due to the reduced S100B secretion, at least in this brain region.

Regardless of clear decrease of cerebrospinal fluid S100B in dementia models, some features should be taken into consideration to understand the limitations of our results. First, there is no information about which astrocytes and other cells contribute to cerebrospinal fluid. In fact, oligodendrocytes, choroid plexus epithelium and ependymal cells contains S100B [33] and potentially contribute to cerebrospinal fluid S100B content. Second, there is no report about decreases of cerebrospinal fluid S100B in AD. In fact, there is a report about a transient increase in the beginning of disease [15]. Moreover, in spite of the attractive idea of elevated extracellular levels of S100B in the hippocampus of AD [34, 35], supported by these data in STZ rats, there is no clear evidence in human patients. Third, other factors beyond S100B secretion could be involved in its decrease of cerebrospinal fluid, including elevated extracellular degradation, decrease of free S100B (bind to insoluble targets or receptors) or increased of its cerebrospinal fluid clearance. These possibilities deserve further investigation.

In summary, our results indicate that STZ model of dementia, confirmed by cognitive behavior impairment and hippocampal oxidative stress is accompanied by changes in hippocampal glutamine synthetase activity and in the S100B secretion modulated by high K^+. These results emphasize the role of astrocytes in dementias, as well as the importance

of this cell as targets as therapeutic targets in brain diseases. Moreover, the neuroprotective activity of aminoguanidine was confirmed by its ability to prevent those STZ-induced alterations.

Figures

Figure 1. Cognitive alterations in rats submitted to streptozotocin icv infusion and correlated Intracellular ROS production. Step-down inhibitory avoidance task evaluated 21 days after surgery in 10 animals in each group (A). Data expressed as median and interquartile intervals (training session in white; test session in gray). N= 12. [a]Significant difference when compared training and test session ($p < 0.01$, Wilcoxon test). [b]Significant difference when compared to STZ group ($p < 0.001$, Kruskal-Wallis test). Oxidative stress evaluated by DCF fluorescence (B). The data represent the mean±S.E values, five rats *per* group. *Significantly different from control, two-way Anova, $p < 0.05$.

Figure 2. Glutamate uptake and glutamine synthetase activities in the hippocampus of rats submitted to STZ icv. Hippocampi were chopped into 0.3 mm slices for measurement of glutamine synthetase or for glutamate uptake on the 22th day after surgery. Values are mean±standard error of five rats in each group. *Significantly different from controls (two-way Anova, $p < 0.05$).

Figure 3. S100B secretion modulated by K^+. Secretion was measured after 60 min of equilibration in a medium containing 30 mM KCl (High-K^+). The line indicates basal secretion, assumed as 100% in the experiment. Each value is a mean (±standard error) of five independent experiments performed in triplicate. *Significantly different from basal secretion (two-way Anova, $p < 0.05$)

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Figures

Figure 1

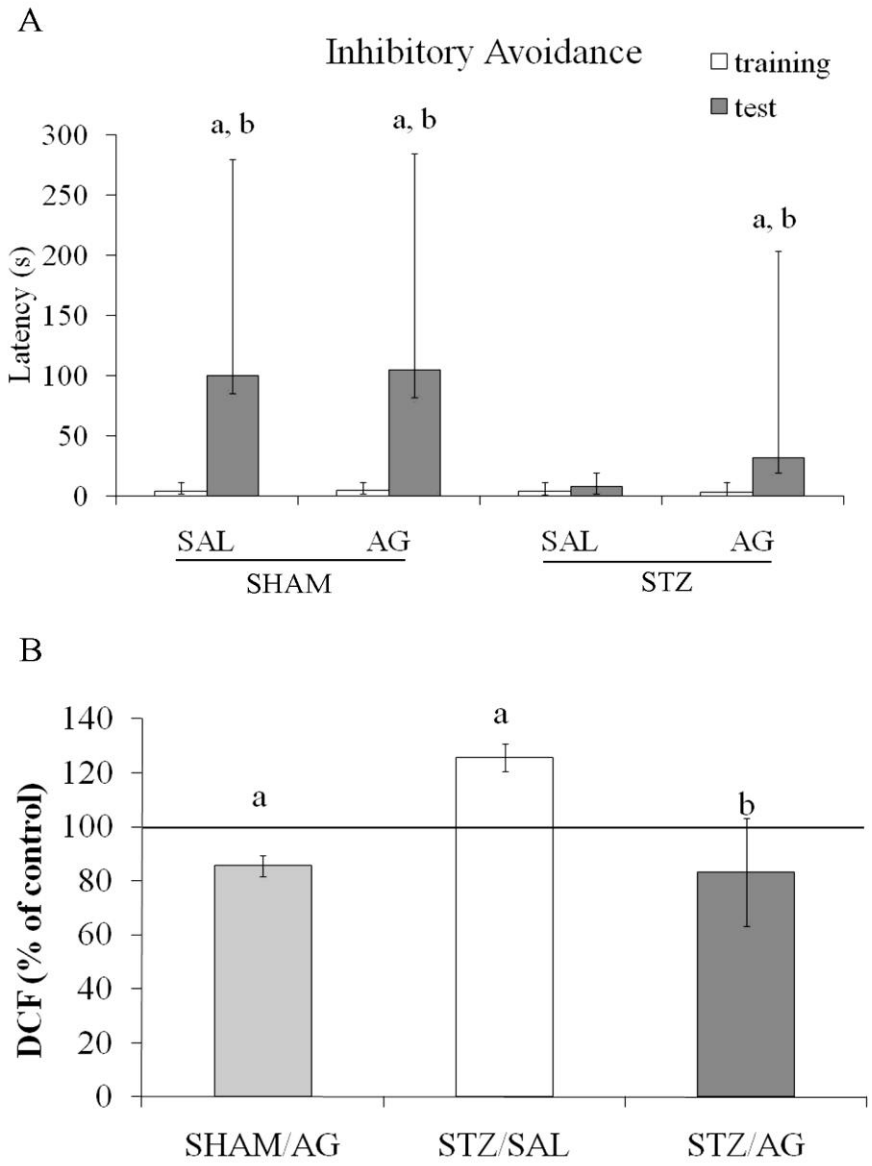

Figure 2

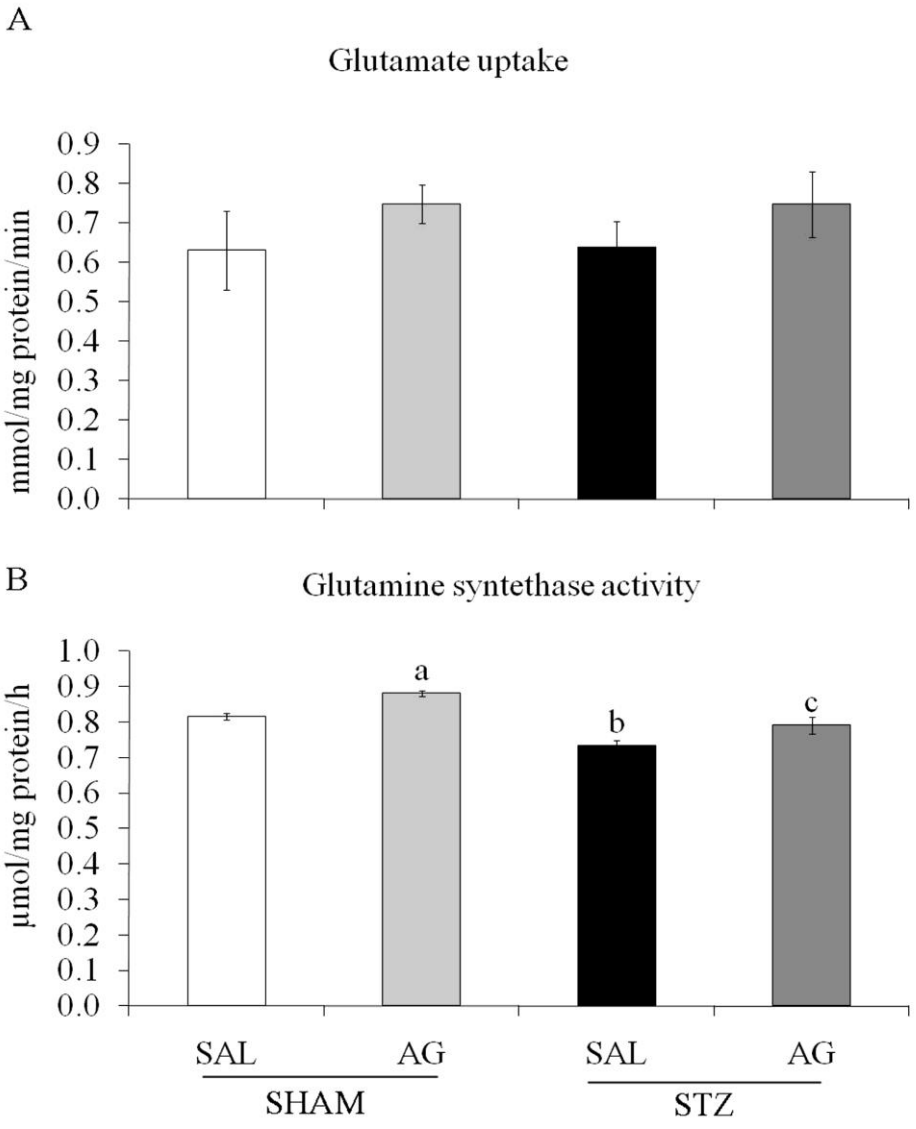
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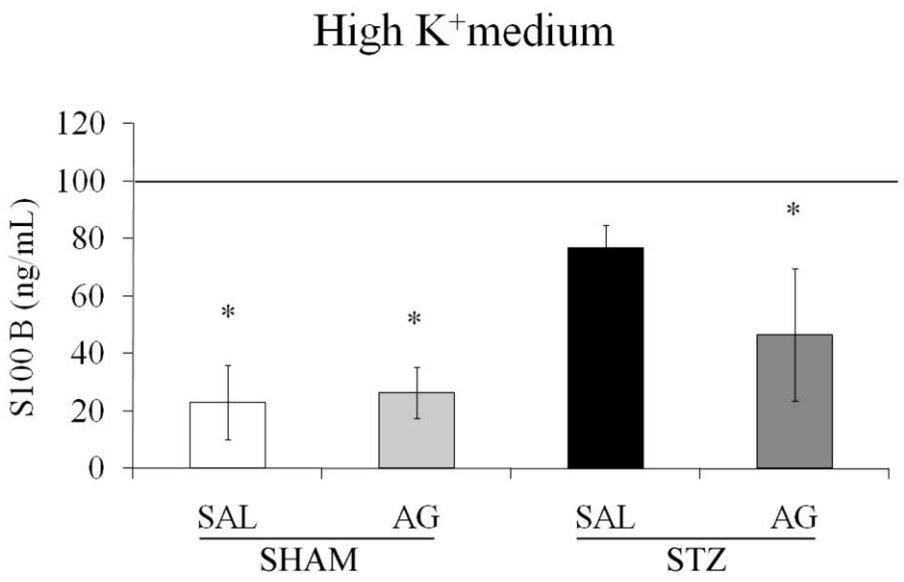
Figure 3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Capítulo III

Tredmill training restores cognitive deficits and hippocampal alterations in rats submitted to streptozotocin-induced dementia model.

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DEMENTIAS - ORIGINAL ARTICLE

Treadmill training restores spatial cognitive deficits and neurochemical alterations in the hippocampus of rats submitted to an intracerebroventricular administration of streptozotocin

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Abstract The intracerebroventricular infusion of streptozotocin (icv-STZ) has been largely used in research to mimic the main characteristics of Alzheimer's disease (AD), including cognitive decline, impairment of cholinergic transmission, oxidative stress and astrogliosis. Moderate physical exercise has a number of beneficial effects on the central nervous system, as demonstrated both in animals and in human studies. This study aimed to evaluate the effect of 5-week treadmill training, in the icv-STZ model of sporadic AD, on cognitive function, oxidative stress (particularly mediated by NO) and on the astrocyte marker proteins, glial fibrillary acidic protein (GFAP) and S100B. Results confirm the spatial cognitive deficit and oxidative stress in this model, as well as astroglial alterations, particularly a decrease in CSF S100B. Physical exercise prevented these alterations, as well as increasing the hippocampal content of glutathione and GFAP per se in the CA1 region. These findings reinforce the potential neuroprotective role of moderate physical exercise. Astroglial changes observed in this dementia model contribute to understanding AD and other diseases that are accompanied by cognitive deficit.

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Keywords Alzheimer's disease · Hippocampus · NO-mediated oxidative stress · S100B · Streptozotocin · Treadmill training

Introduction

As life expectation increases, a greater number of cases of dementia, associated with advanced age, are observed in society, leading to a demand for more research into possible treatments and prevention (Sloane et al. 2002). Dementia, especially the most prevalent of these conditions, Alzheimer's disease (AD), presents a growing incidence and cost for the care of patients all over the world (de la Torre 2010; Middleton and Yaffe 2009). AD can be classified as hereditary or type I, where some genes have been implicated such as presenilin 1 and 2, and as sporadic or type II AD, where there is no specific genetic involvement. The sporadic type of AD comprises the absolute majority of all AD cases (Hoyer 2002). Type II AD is directly related mainly with aging and other risk factors such as diabetes and metabolic disturbance, hypercholesterolemia, atherosclerosis and infection (de la Monte et al. 2009; Erol 2008; Holmes and Cotterell 2009; Martins et al. 2009; Rocchi et al. 2009).

The ethiopathogenesis of sporadic AD is yet unclear and, therefore, adequate animal models are difficult to design. The intracerebroventricular infusion of streptozotocin (icv-STZ) has been largely used in research to mimic the main characteristics of AD, including cognitive decline, impairment of cholinergic transmission, disruption of insulin signal transduction, oxidative stress and astrogliosis (Lannert and Hoyer 1998; Salkovic-Petrisic and Hoyer 2007; Saxena et al. 2008; Sharma and Gupta 2002; Shoham et al. 2007).

The astrocytes play a key role in brain physiology and diverse neurodegenerative diseases, and have become a target for possible therapeutic strategies (Salmina 2009). The activity and protein markers of astrocytes in AD have been widely investigated in the last few years (Damjanac et al. 2007; Edwards and Robinson 2006; Nagele et al. 2004; Nogueira et al. 2009); we have investigated astroglial parameters, particularly glial fibrillary acidic protein (GFAP) and S100B, in icv-STZ (Rodrigues et al. 2009) and other models of dementia, such as lesion of nucleus basalis magnocellularis (Swarowsky et al. 2008) and chronic cerebral hypoperfusion (Vicente et al. 2009). Findings demonstrate that glial activation, in response to injury stimuli, commonly involves changes in GFAP, S100B levels and antioxidant defense. GFAP is a specific astrocyte marker; currently, tissue GFAP increase is taken as a sign of astrogliosis, associated with conditions of brain injury (Eng et al. 2000; Vicente et al. 2004). S100B is a calcium-binding protein found in brain tissue, predominantly in the astrocytes, which secrete this molecule. Extracellular S100B plays a trophic role in neuronal and glial cells, but elevated extracellular levels of this protein could induce apoptosis in neural cells (Van Eldik and Wainwright 2003). Cerebrospinal fluid and serum S100B levels have been used as markers of brain insult (Andreazza et al. 2007; Vicente et al. 2004).

Moderate physical exercise has a number of beneficial effects on the central nervous system, as demonstrated both in animals and in human studies (Cui et al. 2009; Larson et al. 2006). On the other hand, exhaustive exercise could impair, at least in rats, cognitive function (Rosa et al. 2007). Among the beneficial results of physical exercise are the release of neurotrophic factors (Berchtold et al. 2005; Gomez-Pinilla et al. 1998; Kim et al. 2004; Marais et al. 2009; Ploughman et al. 2005), angiogenesis (Ding et al. 2004), neurogenesis (van Praag et al. 1999) and significant cognitive improvements (Cotman and Berchtold 2002; Eggermont et al. 2006; Larson et al. 2006; Nichol et al. 2007; Parachikova et al. 2008). Nevertheless, most of the mechanisms responsible for these effects remain unclear. Various studies have demonstrated the antioxidant effects of moderate exercise protocols on the central nervous system (Aksu et al. 2009; Cechetti et al. 2008; Ogonovszky et al. 2005). A recent report showed that moderate exercise on a treadmill decreased nitrosative stress and improved glutathione levels in a rat anxiety model (Salim et al. 2010). Herein, the aim was to evaluate the effect of 5-week treadmill training, in the icv-SZT model of sporadic AD, on cognitive function, oxidative stress (particularly mediated by NO) and on the astrocyte marker proteins, GFAP and S100B in the hippocampus.

Materials and methods

Chemicals

Sodium carbonate, albumin, Tween-20, streptozotocin, glutamylhydroxamate, sodium nitrate, nitrate reductase, 3,3-diaminobenzidine (DAB), *o*-phenylenediamine (OPD) and anti-S100B monoclonal antibody were purchased from Sigma. Anti-S100 and anti-GFAP antibodies were from Dako. Peroxidase-conjugated secondary antibody was from Amersham.

Animals

A total of 46 male Wistar rats (90 days old, weighing 250–320 g) were obtained from our breeding colony (at the Department of Biochemistry, Universidade Federal do Rio Grande do Sul) and were maintained under controlled light and environmental conditions (12-h light/12-h dark cycle at a constant temperature of 22 ± 1°C) with free access to food and water. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised 1996, and following the regulations of the local animal house authorities.

icv Streptozotocin infusion

Surgery was performed, as described in our previous studies (Rodrigues et al. 2009). Briefly, rats were anesthetized with ketamine/xylazine (75 and 10 mg/kg, respectively, i.p.) and positioned in a stereotaxic apparatus. A midline sagittal incision was made in the scalp and burr holes were drilled in the skull on both sides over the lateral ventricles. The lateral ventricles were accessed using the following coordinates (Paxinos 1997): 0.9 mm posterior to the bregma; 1.5 mm lateral to the sagittal suture; 3.6 mm beneath the surface of the brain. Both STZ groups received a single bilateral infusion of 5 µL STZ (3 mg/kg), while sham groups received vehicle (Hank's balanced salt solution—HBSS—containing in mM: 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$; and 10 glucose, in pH 7.4) using a 10-µL Hamilton syringe. At post-surgery, rats were placed on a heating pad to maintain body temperature at 37.5 ± 0.5°C, and were maintained there until recovery from anesthesia. Only two animals died after surgery, one sham and another STZ.

Animals were initially selected for their ability to walk on a treadmill, as described below. They were grouped as follows: sham sedentary ($N = 11$), sham exercise ($N = 9$), STZ sedentary ($N = 11$) and STZ exercise ($N = 13$). Two

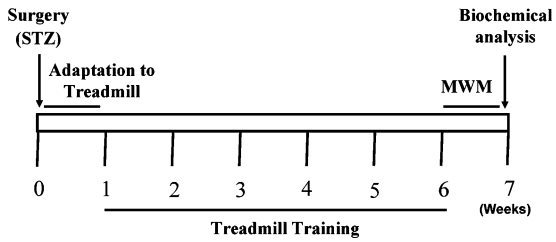

Fig. 1 Schematic representation of the experimental plan**.** Time is represented in weeks. Surgery corresponds to *STZ* or vehicle icv infusion; adaptation to treadmill was based on maximal exercise test (MET, see details in "Materials and methods" section); 5 weeks, treadmill training; cognitive evaluation based on Morris' water maze (*MWM*) task; cerebrospinal fluid, blood and hippocampi were harvested for biochemical evaluation

sets of parameters were evaluated separately. After behavioral tasks, rats were anesthetized, as subsequently described: (i) for CSF puncture, intracardiac blood was collected and intracardiac perfusion performed for immunohistochemistry (first set of measurements); or (ii) for brain tissue preparation, S100B, GFAP, NO and glutathione contents were evaluated (second set of measurements). A schematic representation of the experimental procedure is shown in Fig. 1, indicating times of surgery (STZ or vehicle infusion), adaptation to treadmill, treadmill training, cognitive behavior and biochemical analysis.

Adaptation to treadmill and maximal exercise test

To perform the training, all animals were adapted to walk on a treadmill for three consecutive days (days 1–3) after the surgery (day 0). This adaptation consisted of walking on a treadmill for 10 min at 5 m/min. Four days after the surgery, animals were submitted to the maximal exercise test (MET). The MET was used to determine the maximal exercise capacity (MEC). The test consisted of a graded exercise on the treadmill, with speed increments of 5 m/min every 3 min, starting at 5 m/min and continuing up to the MEC of each rat (Melo et al. 2003). Values attained in the MET were used to plan the treadmill training program. A new MET was repeated at the end of treadmill training to evaluate the efficacy of the training protocol and also the effects of icv infusion of STZ on the rats' MEC.

Treadmill training

The training program was performed on a treadmill designed for human use (Runner, Brazil) and modified for use by rats. Treadmill training was performed between 13:00 and 17:00 h. This training program began 7 days after the surgery and consisted of running on the treadmill for 20 min on the 1st day; this period was then progressively increased every day up to 50 min on the 5th day and 60 min for the next 4 weeks. Each training session included a warm-up period of 5 min of running at 30% of the MEC (maximal velocity), reaching the MET, 10–50 min of running at 40–60% and 5 min recovery at 30% again. This was repeated as five sessions per week, once a day for 5 weeks (Ilha et al. 2008).

Morris water maze task

On the day after the conclusion of treadmill training, rats were submitted to spatial learning evaluation in the Morris water maze using the Reference Memory protocol (Vicente et al. 2009). This procedure was performed between 13:00 and 17:00 h. The apparatus consisted of a circular pool (180 cm diameter, 60 cm high), filled with water (depth 30 cm; 24 ± 1°C) and placed in a room with consistently located spatial clues. An escape platform (10 cm diameter) was placed in the middle of one of the quadrants, 1.5 cm below the water surface, equidistant from the sidewall and the middle of the pool. The platform provided the only escape from water and was located in the same quadrant for every trial. Four different starting positions were equally spaced around the perimeter of the pool. On each training day, all four start positions were used once in a random sequence, i.e., four training trials per day. A trial began by placing the animal in the water facing the wall of the pool at one of the starting points. If the animal failed to escape within 60 s, it was gently conducted to the platform by the experimenter. The rat was allowed to stay there for 20 s. The inter-trial interval was 10 min. After each trial, the rats were dried and returned to their cages at the end of the session. Animals were trained for 5 days. At 24 h after the last training session, the rats were submitted to a test session (7 weeks after surgery). Before this session, the submerged platform was removed. The retention test consisted of placing the animals in the water for 1 min. The latency to arrive at the original platform location, the number of crossings over the previous location and time spent in the target quadrant compared to the opposite quadrant were measured on the day of testing.

Cerebrospinal fluid and serum samples

Animals were anesthetized, as described above, and then positioned in a stereotaxic holder for cerebrospinal fluid collection from the cisterna magna. The puncture was performed using an insulin syringe (27 gauge × 1/2″ length). Rats were then removed from the stereotaxic apparatus and placed in a flat place; whole blood was obtained through an intracardiac puncture using a 0.37-mm diameter needle that was inserted into the intercostal space above the sternum. Serum was separated by centrifugation

at 3000×*g* for 5 min. CSF and serum samples were frozen (−20°C) until further analysis (Netto et al. 2006).

Hippocampal tissue samples

The animals were killed by decapitation, brains were removed, and hippocampi were dissected out and placed in cold saline medium with the following composition (in mM): 120 NaCl; 2 KCl; 1 $CaCl_2$; 1 $MgSO_4$; 25 HEPES; 1 KH_2PO_4 and 10 glucose, adjusted to pH 7.4. Hippocampal samples were then homogenized in specific buffers for biochemical measurements, described as follows.

ELISA for S100B and GFAP

The S100B content in the hippocampus and CSF was measured by ELISA (Leite et al. 2008). Briefly, 50 µL of sample plus 50 µL of Tris buffer were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B (SH-B1). Polyclonal anti-S100B was incubated for 30 min and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min. A colorimetric reaction with *o*-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.025 to 2.5 ng/mL. ELISA for GFAP (Tramontina et al. 2007) was carried out by coating the microtiter plate with 100 µL samples containing 30 µg of protein for 48 h at 4°C. Incubation with a rabbit polyclonal anti-GFAP for 2 h was followed by incubation with a secondary antibody conjugated with peroxidase for 1 h, at room temperature; the standard GFAP curve ranged from 0.1 to 10 ng/mL.

Glutathione content

The glutathione content was determined, as described before (Browne and Armstrong 1998). Briefly, hippocampal samples, homogenized in sodium phosphate buffer (0.1 M, pH 8.0) containing 5 mM EDTA and protein, were precipitated with 1.7% meta-phosphoric acid. The supernatant was assayed with *o*-phthaldialdehyde (1 mg/mL of methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard glutathione solutions (0–500 µM). Glutathione concentrations were expressed as nmol/mg protein.

NO assay

NO metabolites, NO_3- (nitrate) and NO_2- (nitrite), were determined according to (Hevel and Marletta 1994). Briefly, homogenates from hippocampal slices were mixed with 25% trichloroacetic acid and centrifuged at 1800×*g* for 10 min. The supernatant was immediately neutralized with 2 M potassium bicarbonate. NO_3- was reduced to NO_2- by nitrate reductase. The total NO_2- in the incubation was measured by a colorimetric assay at 540 nm, based on the Griess reaction. A standard curve was performed using sodium nitrate (0–80 µM). Results were expressed as µM of nitrite.

Immunohistochemistry for GFAP

Rats were anesthetized using ketamine/xylazine and were perfused through the left cardiac ventricle with 200 ml of saline solution, followed by 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and post-fixed in the same fixative solution at 4°C for 2 h. Subsequently, the material was cryoprotected by immersing the brain in 30% sucrose in phosphate buffer at 4°C (Swarowsky et al. 2008). The brains were sectioned (40 µm) on a cryostat (Leitz) and free-floating sections were pretreated with 3% H_2O_2 for 30 min and then carefully washed and blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.4% Triton X-100 (PBS-Tx) for 30 min. Sections were then incubated with polyclonal GFAP antiserum raised in rabbit (Dako) diluted 1:200 in 2% BSA in PBS-Tx for 48 h at 4°C. After several washes with PBS-Tx, sections were incubated in a PAP-conjugated anti-rabbit IgG (Amersham) diluted 1:50 in PBSTx at room temperature for 2 h. The reaction was developed by incubating the sections in a histochemical medium that contained 0.06% 3,3′-diaminobenzidine (DAB, Sigma Chemical Co., USA) dissolved in PBS for 10 min and in the same solution containing 1 µL of 3% H_2O_2 per mL of DAB medium for an additional 10 min. Finally, the sections were rinsed with PBS, dehydrated in ethanol, cleared with xylene and covered with Permount and coverslips. Control sections were prepared by omitting the primary antibody and replacing it with PBS.

GFAP optical density

The intensity of the GFAP immunoreaction was measured in the stratum radiatum of the CA1 region (identified based on interaural coordinates 5.86–4.16 mm, bregma −3.14 to −4.16 mm) (Paxinos 1997) by semi-quantitative densitometric analysis (Viola et al. 2009), using a Nikon Eclipse E-600 microscope (200×, Japan) coupled to a USB 2.0 Digital Camera Eyepiece (DCE-2, China) and to the Image Pro Plus Software 6.0 (Media Cybernetics, USA). The digitized images obtained from the selected areas were converted to an 8-bit grayscale (0–255 gray levels). All lighting conditions and magnifications were held constant. Picture elements (pixels) employed to measure optical density were obtained from squares with 11484.46 μm^2 (our area of interest, AOI) overlying the grayscale image.

The obvious blood vessels and other artifacts were avoided. Six sections per animal, from four animals per group, were investigated. At least six readings were performed in each analyzed section and the data average of each section was recorded. Both left and right sides of each brain were used.

Statistical analysis

Parametric data are reported as mean ± SEM and were analyzed by repeated-measures (in Fig. 2a) or two-way analysis of variance (ANOVA), followed by Tukey's test when indicated, with the SPSS-16.0. The Student's *t* test was used only in Fig. 2c. Values of $p < 0.05$ were considered to be significant.

Results

Recovery of spatial learning performance

The four groups were submitted to the Morris water maze 1 week after treadmill training (6 weeks after surgery) for spatial learning evaluation. The results revealed significant differences in the time to find the platform (escape latency) in all groups, compared to STZ/SED, during the water maze training sessions from day 3 onward (Fig. 2a) ($F_{(3,43)} = 10.468$, $p = 0.001$). In the probe trial, the STZ/SED group presented the highest latency to arrive at the original platform location, in relation to all other groups (Fig. 2b) ($F_{(3,43)} = 3.681$, $p = 0.020$). Moreover, the STZ/SED group demonstrated no differences in the time spent in the target quadrant compared with the opposite quadrant (Student's *t* test, $p = 0.746$), in contrast to the other groups (Fig. 2c). In addition, the number of crossings over the previous platform location were significantly lower in the STZ/SED group, compared to all the other groups, showing a clear effect of physical exercise on the cognitive decline observed in this model (Fig. 2d) ($F_{(3,43)} = 4.461$, $p = 0.036$).

Reversal of the decrease in CSF S100B

As demonstrated in our previous study (Rodrigues et al. 2009), a significant decrease in CSF S100B was observed

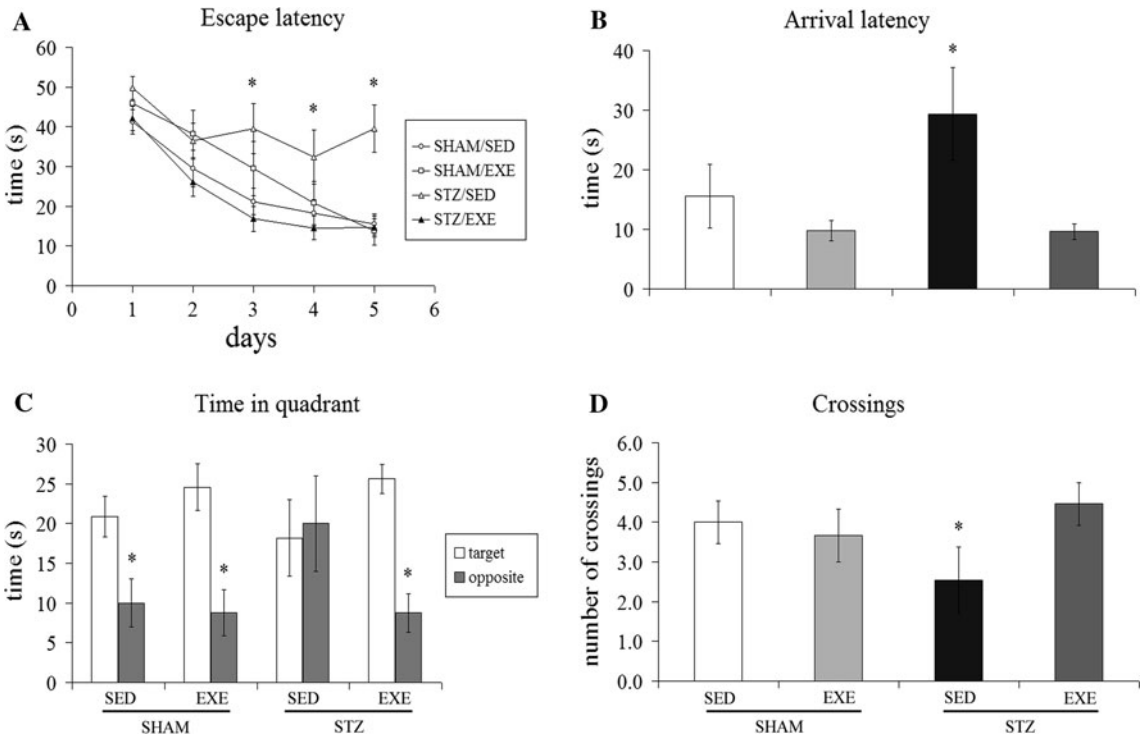

Fig. 2 Cognitive performance of rats submitted to icv-STZ injection, as evaluated by the Morris water maze test. **a** Performance in the reference memory protocol, based on escape latency. *Each point* represents the mean ± standard error. *Significant differences were detected by comparing STZ/SED and other groups from day 3 onward ($N = 9$–13, repeated-measures ANOVA followed by Tukey's test, $p < 0.05$). **b** Memory in the probe trial of reference memory, as measured by latency to arrive at the original place of platform location. Values are mean ± standard error. STZ/SED was different from other groups ($N = 9$–13, two-way ANOVA followed by Tukey's test, $p < 0.05$). **c** Time spent (in s) in the target quadrant compared to the opposite quadrant. Values are mean ± standard error. *Significant difference between the times spent in quadrants in each group ($N = 9$–13, Student's *t* test, $p < 0.05$). **d** Number of crossings over the previous platform position. Values are mean ± standard error. *Significantly different from other groups ($N = 9$–13, two-way ANOVA followed by Tukey's test, $p < 0.05$)

in this model of sporadic AD at 3 weeks after the surgical procedure. The same result was observed 7 weeks later and was remarkably reversed by the aerobic training protocol (Fig. 3a) ($F_{(3,39)} = 3.856$, $p = 0.017$). No significant differences were seen in the serum S100B levels (Fig. 3b) ($F_{(3,21)} = 0.215$, $p = 0.885$), indicating no effect of either the model or the exercise in this parameter.

Hippocampal S100B and GFAP content was not affected by treadmill training

No significant differences in S100B immunocontent, evaluated by ELISA, were observed at 7 weeks after STZ lesion ($F_{(3,28)} = 0.674$, $p = 0.576$), on comparing all four groups (data not shown). In addition, no alterations were observed in the hippocampal GFAP content (Fig. 4a) ($F_{(3,28)} = 1.432$, $p = 0.257$), as also evaluated by ELISA, in agreement with our previous study (Rodrigues et al. 2009).

Immunohistochemistry for GFAP was modified by physical exercise

Interestingly, the immunohistochemistry densitometric analysis revealed significant differences in the *radiatum* of the CA1 region of the hippocampus following treadmill training, despite STZ treatment (Fig. 4b) ($F_{(3,19)} = 6.989$, $p = 0.006$). A representative photomicrograph (Fig. 5) shows a higher GFAP immunoreactivity in both exercised groups, compared to sedentary ones.

Hippocampal nitrosative stress and glutathione content

To evaluate nitrosative stress, NO production was measured through nitrite production. Results show an increase in nitrite content in the STZ/SED group, which was reverted in STZ/EXE (Fig. 6a) ($F_{(3,19)} = 48.146$, $p = 0.001$). The glutathione content was lower in the STZ/SED group and was improved in the exercised animals (Fig. 6b) ($F_{(3,19)} = 95.487$, $p = 0.001$). Moreover, physical exercise

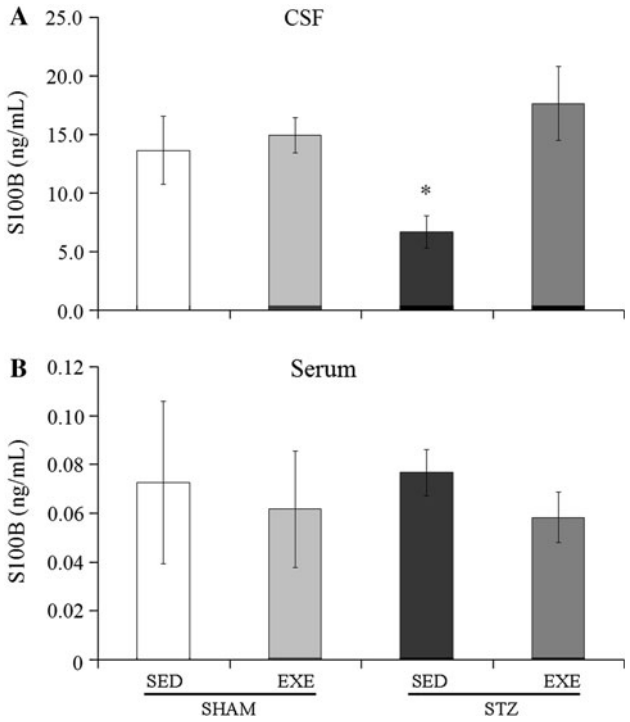

Fig. 3 S100B levels in the cerebrospinal fluid and serum of rats submitted to icv-STZ injection. **a** Seven weeks later, cerebrospinal fluid (CSF) was collected by cisterna magna puncture. S100B content was measured by ELISA. **b** Serum S100B levels (collected by intracardiac puncture) were analyzed 7 weeks after surgery and measured by ELISA. Values are mean ± standard error of six to nine rats in each group. *Significantly different from other groups (two-way ANOVA followed by Tukey's test, $p < 0.05$)

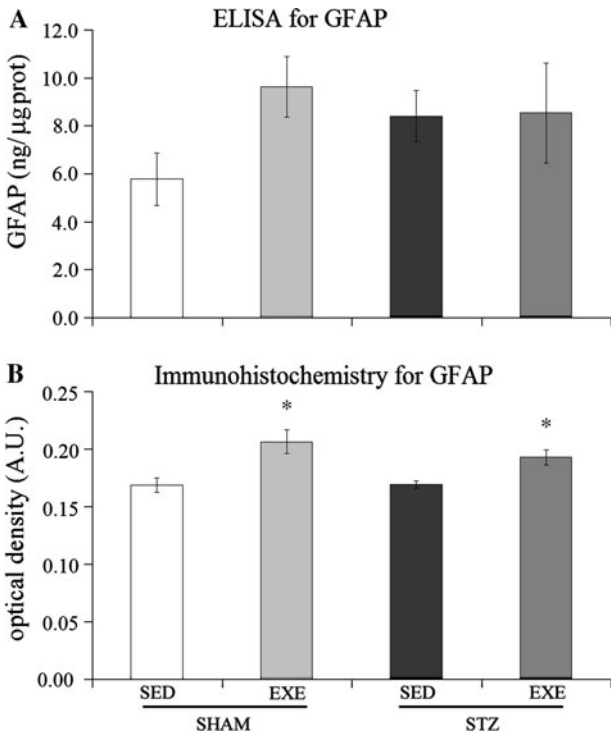

Fig. 4 GFAP content and immunohistochemistry in the hippocampus of rats submitted to icv-STZ injection. Adult rats were submitted to icv injection of STZ. Hippocampi were dissected out and the contents of GFAP (**a**) were measured by ELISA in the 7th week. GFAP optical density measurements (**b**) in immunohistochemical sections of hippocampus from rats submitted to icv injection of STZ (see also Fig 5). Values (in **a** and **b**) are mean ± standard error of six to nine rats in each group. *Significantly different from respective sedentary (SED) group (two-way ANOVA followed by Tukey's test, $p < 0.05$)

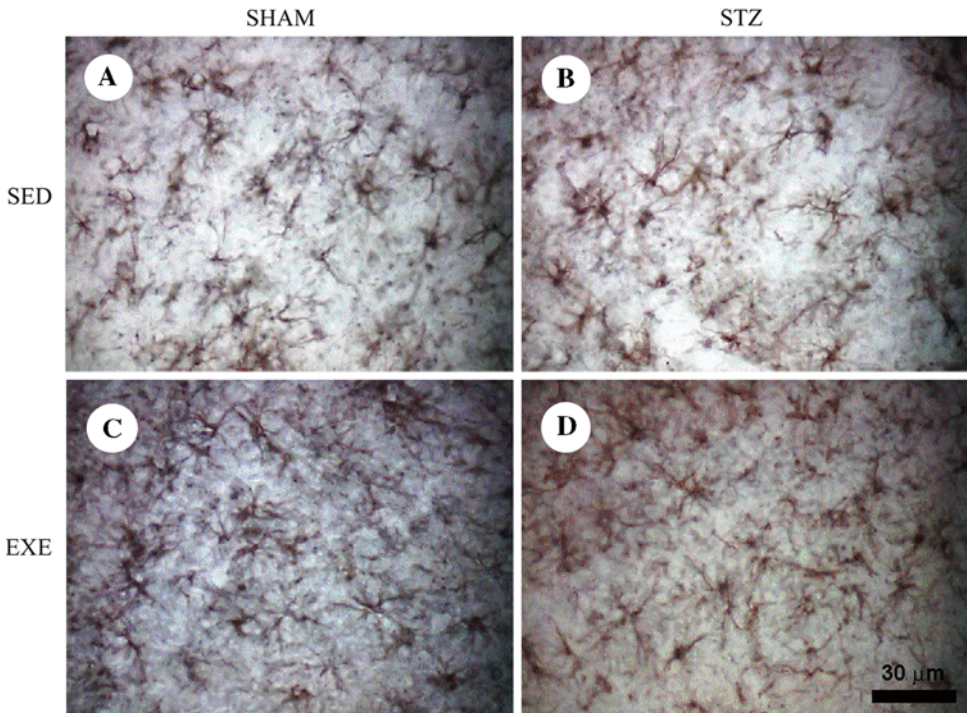

Fig. 5 Immunohistochemistry for GFAP from rats submitted to icv-STZ injection. Representative photomicrographs (from 4 animals in each group) showing GFAP immunoreactive cells in the radiatum layer of the CA1 hippocampal region of sham sedentary (**a**), STZ sedentary (**b**), sham exercised (**c**) and STZ exercised (**d**) groups. 400× Magnification, scale *bar* 30 µm. Optical densitometry (see Fig. 4b) showed an increase in GFAP immunoreactivity in EXE groups, compared to the respective SED ones

itself induced an increase in glutathione content ($p = 0.001$).

Discussion

The oxidative stress generated by STZ itself (e.g., due to NO release) and the induced impairment of glucose uptake and insulin/insulin-like growth factor signaling make this compound very useful in the models for diabetes mellitus and AD (Lannert and Hoyer 1998; Salkovic-Petrisic and Hoyer 2007). The icv-STZ model of sporadic AD has been extremely useful for the study of the histopathological and biochemical features as well as neuroprotective strategies. Our results reinforce previous findings that demonstrate cognitive decline, nitrosative stress and glutathione reduction in this model (de la Monte et al. 2006; Ishrat et al. 2006, 2009; Saxena et al. 2008; Shoham et al. 2003). The behavioral data showed a cognitive decline in the water maze task at 7 weeks after surgery. This decline was successfully prevented by 5-week treadmill running. Many hippocampal gene alterations underlie the memory improvement elicited by running exercise (Stranahan et al. 2008). Among these, the activation of genes associated with synaptic plasticity and mitochondrial function and the downregulation of genes related to oxidative stress have been reported. In another investigation, the memory improvement resulting from physical exercise was shown to be the consequence of histone H3 phospho-acetylation

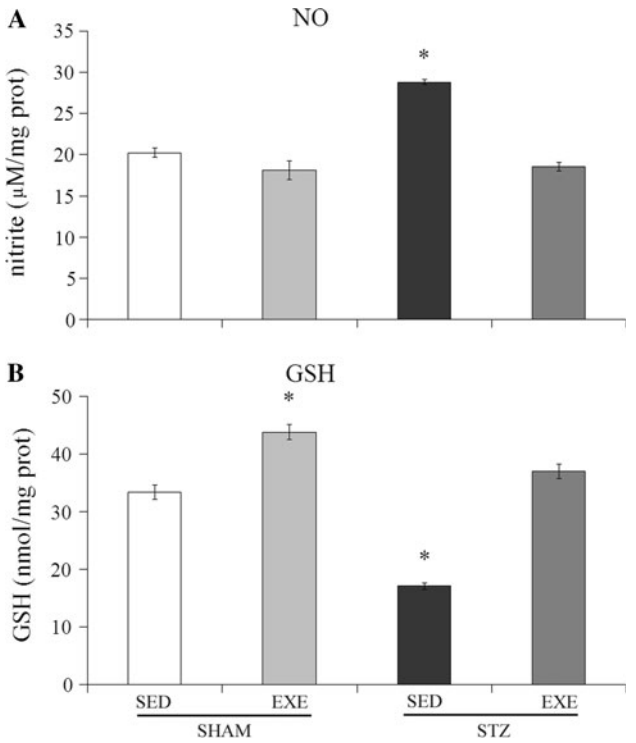

Fig. 6 NO and glutathione levels in the hippocampus of rats submitted to icv-STZ injection. Adult rats were submitted to icv injection of STZ. Seven weeks later, hippocampi were dissected out and homogenized for measurement of NO (**a**) or glutathione (**b**). Values are mean ± standard error of six to nine rats in each group. *Significantly different from other groups (two-way ANOVA, $p < 0.05$)

and c-Fos in the dentate gyrus neurons, in response to novelty stress (Collins et al. 2009). A 2-week treadmill running program promoted a cognitive improvement in memory, as assessed by an inhibitory avoidance task in rats submitted to icv-STZ, and also demonstrated increased c-Fos expression in the hippocampus (Jee et al. 2008).

As observed in our previous study, STZ (infused via intracerebroventricular) causes a decrease in the S100B immunocontent in the CSF (Rodrigues et al. 2009). The same was observed in the present investigation, and physical exercise was found to prevent this effect. A decrease in CSF S100B was also found in another dementia model induced by chronic cerebral hypoperfusion (Vicente et al. 2009). Interestingly, a decrease in serum S100B was recently observed in AD patients (Chaves et al. 2010). However, all these data apparently contrast with the increase in S100B reported in the brain tissue of these patients (Griffin et al. 1998). In addition, during development, S100B accumulation in the brain tissue is accompanied by a decrease in extracellular levels of this protein (Netto et al. 2005; Portela et al. 2002; Tramontina et al. 2002). Interestingly, a transitory increase in CSF S100B was observed during the initial stage of AD, but not during further stages (Peskind et al. 2001). In our rats submitted to the icv-STZ model of AD, no changes in serum S100B were observed. Assuming that S100B has a neurotrophic extracellular effect (Donato et al. 2009; Van Eldik and Wainwright 2003), the decrease observed in the CSF S100B of icv-STZ rats could represent a failure to respond to injury. Five-week treadmill exercise seems to normalize extracellular levels of S100B, as observed before using aminoguanidine, a compound used to prevent glycation, which also prevented cognitive deficit (Rodrigues et al. 2009).

No changes were seen in the S100B immunocontent in the hippocampal tissue; as also shown in our previous study, the expression and secretion of S100B are independent events (Goncalves et al. 2008). On the other hand, immunohistochemistry demonstrated a significant increase in GFAP immunoreactivity in the *radiatum* layer of the CA1 region only in the exercised group, independently of the STZ administration. In agreement, an augment in GFAP immunoreactivity was observed, as demonstrated by immunohistochemistry in the cortex and striatum of rats after 3 or 6 weeks of treadmill running exercise (Li et al. 2005). It is important to note that the mean value of GFAP content in the hippocampal slices did not change, as measured by ELISA. Therefore, we assume that a specific and localized increment in the radiatum layer of CA1 occurred. It is important to mention that changes in the hippocampal CA1 field are the best correlates of cognitive deterioration in human brain aging (Giannakopoulos et al. 2009). Astrogliosis in rats has been previously attributed to the reinforcement of the blood brain barrier and angiogenesis caused by exercise (Ding et al. 2004; Li et al. 2005). It is important to emphasize the fact that increased GFAP does not signify only a harmful response to an injury, but also represents a protective reaction of the CNS (Liberto et al. 2004).

Astrocytes are responsible for the main antioxidant defenses in brain tissue and oxidative stress is a common feature in several neurodegenerative diseases (Jellinger 2009; Zhu et al. 2005). In fact, the decrease in glutathione content suggests a failure of astroglial antioxidant defense in this model of sporadic AD. In addition, hippocampal NO production, as measured by nitrate and nitrite, indicates nitrosative stress in this model. This could be a consequence of the decrease in glutathione. Moderate physical exercise plays a remarkable role in the maintenance of cognition in the elderly (Laurin et al. 2001; Pope et al. 2003; Shen et al. 2001). This is attributed mostly to the improvement of cardiovascular and neuronal function, associated with antioxidant effects. Furthermore, studies with transgenic animal models of AD have shown that physical exercise, besides improving cognition, causes a decrease in beta amyloid deposition (Adlard et al. 2005; Wolf et al. 2006).

Our results have shown a positive effect of 5-week treadmill running on glutathione levels, which not only restored the decrease in glutathione caused by the STZ injection, but also caused an elevation of this parameter in the sham exercised group. Conversely, a decrease in glutathione levels was demonstrated as a result of physical exercise per se, although a beneficial effect of moderate exercise by the reversal of the glutathione decrease caused by reserpine has also been demonstrated (Teixeira et al. 2008). In that study, the brain region analyzed was *striatum* and forced swimming was employed as physical exercise. Moreover, moderate treadmill running physical exercise showed a positive effect on glutathione levels in the liver of rats submitted to ethanol insult (Mallikarjuna et al. 2009). In our study, we also observed that NO levels also return to normal levels in the physically exercised group, possibly also as a consequence of the restoration of glutathione levels.

Conclusions

Results from icv-injected STZ rats confirm the spatial cognitive deficit and oxidative stress in this model (hippocampal decrease of glutathione and increase of NO), and also show astroglial alterations, particularly a decrease in CSF S100B. Five-week treadmill training prevented these alterations and also increased per se the hippocampal content of glutathione and GFAP content in the CA1 region. These findings reinforce the potential neuroprotective role

of moderate physical exercise for rodents, as well as the clinical application of this therapeutic strategy as being neuroprotective. Astroglial changes observed in this sporadic AD model contribute to the understanding of AD and other diseases that are accompanied by cognitive deficits.

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Parte III

Discussão

1. Resumo dos resultados.

1.1 Achados decorrentes da infusão icv de STZ em ratos três e sete semanas após a cirurgia:

Parâmetros Investigados	Três semanas após a cirurgia	Sete semanas após a cirurgia
Cognição	déficit cognitivo	déficit cognitivo
Conteúdo de GSH hipocampal	decréscimo	decréscimo
Estresse nitrosativo	aumento de nitritos e nitratos hipocampais	aumento de nitritos e nitratos hipocampais
Espécies reativas de oxigênio	aumento determinado por DCF	não investigado
S100B hipocampal	aumento	-
S100B no LCR	diminuição	diminuição
Secreção de S100B em fatias hipocampais	alterada	não investigado
S100B no soro	não investigado	inalterada
GFAP hipocampal	inalterada	inalterada
Captação de glutamato hipocampal	inalterada	não investigado
Atividade da glutamina sintetase hipocampal	diminuição	não investigado
Neurônios hipocampais	morte de neurônios corados com HE.	não investigado

1.2 Efeito da aminoguanidina sobre o modelo doença de Alzheimer esporádica por infusão icv de STZ três semanas após a cirurgia:

Déficit cognitivo avaliado em labirinto aquático de Morris	prevenção
Déficit cognitivo avaliado em esquiva inibitória	prevenção
Diminuição de GSH hipocampal	prevenção
Aumento de nitritos e nitratos hipocampais	prevenção
Aumento de ERO hipocampais	prevenção
Aumento de S100B hipocampal	não previne
Diminuição da S100B em LCR	prevenção
Secreção de S100B	não altera
Diminuição da atividade da enzima GS	prevenção
Morte de neurônio hipocampais	prevenção

1.3 Efeito da corrida em esteira sobre o modelo doença de Alzheimer esporádica por infusão icv de STZ sete semanas após a cirurgia:

Déficit cognitivo avaliado em labirinto aquático de Morris	prevenção
Diminuição da GSH hipocampal	prevenção
Aumento de nitritos e nitratos hipocampais	prevenção
Diminuição de S100B em LCR	prevenção

1.4 Efeitos terapêuticos sobre os animais controle:

Parâmetros Investigados	Aminoguanidina	Corrida em Esteira
GSH hipocampal	aumento	aumento
Estresse nitrosativo	diminuição	diminuição
Atividade da enzima GS	aumento	-
Imunorreatividade da GFAP hipocamapal	não investigado	aumento

2. Prevenção do declínio cognitivo causado pelo modelo de doença de Alzheimer esporádica por infusão icv de STZ.

Como já demonstrado por vários autores, a infusão icv única e bilateral de STZ na dose de 1,5 mg/Kg foi efetiva na causa do déficit cognitivo avaliado no teste de memória espacial no Labirinto Aquático de Morris (LAM), de duas a oito semanas depois da cirurgia (Prickaerts *et al.*, 1995; Pathan *et al.*, 2006; Grunblatt *et al.*, 2007; Salkovic-Petrisic *et al.*, 2006). Da mesma maneira, tem-se encontrado prejuízo de memória observada através da tarefa de esquiva inibitória (EI), também chamada de esquiva passiva (Sharma e Gupta, 2001; Sharma e Gupta, 2002; Jee *et al.*, 2008). A navegação espacial é uma tarefa essencialmente dependente da integridade hipocampal; já a esquiva inibitória recruta estruturas associadas ao hipocampo, tais como amígdala, córtex entorrinal e perirrinal (Nader *et al.*, 2000; Foster *et al.*, 2000).

O principal achado neuroquímico justificando o déficit cognitivo encontrado no modelo é a deficiência na transmissão colinérgica e no metabolismo da acetilcolina encontrada em hipocampo por Prickaerts e colaboradores (Prickaerts *et al.*, 1995) e diversos outros desde então (Lannert e Hoyer, 1998; Ishrat *et al.*, 2006; Sonkusare *et al.*, 2005; Pinton *et al.*), tanto em hipocampo quanto em córtex pré-frontal.

2.1 Ação da aminoguanidina.

Em nosso estudo, aminoguanidina mostrou-se efetiva em prevenir o dano cognitivo vinte e um dias após a cirurgia, avaliado tanto em LAM quanto em EI (capítulos I e III). Existe uma série trabalhos mostrando o efeito de agentes antioxidantes sobre o déficit cognitivo neste modelo (Sharma e Gupta, 2002; Sharma e Gupta, 2003; Tiwari *et al.*, 2009; Ishrat *et al.*, 2009a). Em nosso trabalho, a

aminoguanidina teve ação antioxidante significativa, o que pode explicar, em parte, sua capacidade em prevenir o prejuízo de memória tanto em LAM quanto em EI.

Além disso, demonstramos de maneira qualitativa a visível preservação dos neurônios piramidais da região CA1 hipocampal com a administração da aminoguanidina nos animais submetidos à STZ icv (capítulo I), justificando a preservação da memória após a administração de AG.

2.2 Efeito da corrida em esteira.

O exercício físico é reconhecido em inúmeros estudos como sendo benéfico para as funções cerebrais, tendo como principal efeito a capacidade de prevenir o déficit cognitivo decorrente do envelhecimento e de doenças neurodegenerativas (Cotman e Berchtold, 2002; Berchtold *et al.*). Um dos mecanismos que justifica tal benefício é o aumento das defesas antioxidantes (Foster, 2006). Em nosso trabalho (capíltulo III), o exercício físico reforçou as defesas antioxidantes nos animais submetidos à corrida em esteira, o que foi demonstrado pelo aumento da GSH e pela diminuição do estresse nitrosativo.

Em trabalho realizado por Jee e colaboradores (Jee *et al.*, 2008) com o mesmo modelo de demência utilizado neste trabalho, a corrida em esteira durante quatorze dias consecutivos, trinta minutos diários, já teve efeito sobre o prejuízo à memória causado pelo modelo, avaliado pela tarefa de esquiva passiva. Nesse trabalho, a melhora cognitiva em decorrência do exercício foi acompanhada pela expressão aumentada da proteína *c-fos*. No nosso caso, o exercício teve duração de cinco semanas, onde nas últimas quatro os animais correram durante sessenta minutos diários, em um protocolo adaptado de Ilha e colaboradores (Ilha *et al.*, 2008). Tal protocolo mostrou-se efetivo na

reversão do déficit na memória espacial causada pela infusão icv de STZ avaliado sete semanas após a cirurgia.

3. Prevenção do estresse oxidativo e nitrosativo

3.1 Ação da aminoguanidina.

Tanto na DA quanto no modelo de doença de Alzheimer esporádica por infusão icv de STZ, incluindo os achados do nosso trabalho, temos o desequilíbrio entre as defesas antioxidantes e a produção de substâncias redox ativas como base dos processos geradores de disfunção e dano celular (Ishrat *et al.*, 2009b; Zhu *et al.*, 2007). Estudos datados na metade do século passado já atribuem ao estresse oxidativo a base da deterioração das funções cognitivas decorrente do envelhecimento e, consequentemente, da demência (Harman, 1956).

Pela própria DA e o modelo de demência aqui estudado apresentarem características metabólicas semelhantes ao diabetes tipo II, tais como a diminuição da transdução de sinal dos receptores de insulina, diminuição da densidade de receptores de IGF-I, estresse oxidativo e aumento de AGE (Steen *et al.*, 2005; Rivera *et al.*, 2005), guardadas as diferenças de compartimentalização, foi interessante observar os efeitos antioxidantes de uma droga inicialmente utilizada no tratamento do diabetes. Além disso, a aminoguanidina em única administração i.p. na mesma dose utilizada em nosso trabalho (100 mg/Kg) possui efeitos importantes sobre o SNC como a prevenção de edema após lesão por traumatismo encefálico em ratos (Louin *et al.*, 2006).

Primeiramente, além de prevenir o aumento dos níveis de nitritos e nitratos hipocampais, a amioguanidina causou nos animais *sham* operados uma diminuilção *per se* dos metabólitos do NO. Alguns trabalhos na literatura apontam a aminoguanidina como sendo um inibidor da enzima óxido nítrico sintase induzível (iNOS), além de seu

potencial anti glicação (Tilton *et al.*, 1993), e como agente antioxidante (Giardino et al., 1998). Em estudo utilizando camundongos *knokout* para a enzima iNOS, a aminoguanidina foi capaz diminuir o volume da lesão isquêmica em camundongos selvagens a níveis semelhantes aos *knokout,* mostrando que o NO participa deste tipo de lesão e a aminoguanidina age inibindo a iNOS encefálica (Sugimoto e Iadecola, 2002). Outro trabalho mostra o efeito da aminoguanidina sobre a expressão da iNOS em medula espinal de ratos após lesão compressiva, onde tem-se uma importante diminuição da imunodetecção da enzima após a administração intraperitoneal única de aminoguanidina, vinte e quatro horas após a lesão, na dose de 150 mg/Kg (Fan *et al.*).

Ao mesmo tempo, a aminoguanidina também preveniu a diminuição no conteúdo de GSH causada pela infusão icv de STZ e, da mesma maneira, foi capaz de, por si só, aumentar as reservas de GSH nos animais controle, como demonstrado no capítulo I. Os poucos estudos investigando o efeito da aminoguanidina sobre a GSH demonstram aumento da mesma em estômago de ratos após tratamento agudo para isquemia (Hung, 2006), aumento no coração de ratos lesados com doxorubicina (Cigremis *et al.*, 2006), a não alteração em retina de ratos diabéticos (Giardino *et al.*, 1998) e efeitos positivos indiretos sobre a GSH, observados em cultura de astrócitos, prevenindo a morte celular (McNaught e Jenner, 1999). Em recente trabalho (Fan et al.), tem-se o relato do efeito da aminoguanidina na dose de 150 mg/kg, administrada i.p. sobre o conteúdo de GSH na medula espinal de ratos após lesão por compressão mostrando um aumento bastante significativo da mesma. Porém, o referido trabalho não relata a ação da aminoguanidina por *per se*.

No capítulo II, demonstramos o estresse oxidativo aumentado nos animais submetidos ao modelo através da medida das espécies reativas de oxigênio totais com diacetato de 2,7-diclofluoresceína (DCF), o que corrobora o desequilíbrio do estado

redox como característica central neste modelo de demência. Mais uma vez, a aminoguanidina foi efetiva na recuperação de tal dano.

3.2 Efeito da corrida em esteira.

Da mesma maneira, a corrida em esteira foi capaz de prevenir o decaimento de GSH resultante do modelo de demência e, mais do que isso,foi capaz de, por si só, aumentar os níveis de GSH hipocampais. Alguns trabalhos mostram o efeito positivo da corrida em esteira sobre os níveis de GSH no fígado (Mallikarjuna *et al.*, 2009) e coração (Liu *et al.*, 2000). No SNC, um trabalho mostra o efeito também positivo do exercício físico moderado, no caso a natação forçada, sobre a GSH encefálica após insulto com reserpina em ratos (Teixeira et al., 2008).

Ainda, a corrida em esteira mostrou-se efetiva em impedir o aumento dos metabólitos do óxido nítrico hipocampais causados pela STZ. Não existem muitos estudos correlacionando a corrida em esteira com estresse nitrosativo avaliado pela medida de nitritos/nitratos. Um estudo apenas com exercício crônico realizado em esteira (uma hora por dia, cinco dias por semana durante oito semanas) não demonstrou alterações nos metabólitos do NO em encéfalo dos ratos exercitados, porém demonstraram aumento na atividade da enzima superóxido dismutase (SOD), possivelmente causando uma diminuição na produção do radical superóxido (Aksu *et al.*, 2009).

4. Metabolismo do glutamatérgico no modelo de doença de Alzheimer esporádica por infusão icv de STZ

A fim de compreendermos um pouco melhor o funcionamento astrocítico neste modelo de demência, no capítulo II foi realizada a medida da captação de glutamato em

fatias hipocamapais, a qual se mostrou inalterada nas condições avaliadas. Em outros modelos de demência estudados em nosso laboratório, foi encontrada diminuição da capatação de glutamato, tanto no modelo de destruição do núcleo basal magnocelular (Swarowsky *et al.*, 2008) quanto no modelo de hipoperfusão cerebral crônica (Vicente *et al.*, 2009). Curiosamente, este parâmetro não se alterou no modelo da STZ. Apesar da perda de neurônios piramidais hipocampais, demonstrada no capítulo I e também por outros autores (Lester-Coll *et al.*, 2006; de la Monte *et al.*, 2006), não podemos afirmar que a excitotoxicidade glutamatérgica não está envolvida neste modelo de doença de Alzheimer.

Por outro lado, a atividade da GS, a enzima que converte o glutamato captado pelo astrócito em glutamina, mostrou-se diminuída após a lesão por STZ, como também ocorre no modelo de demência por lesão com ibotenato (Swarowsky *et al.*, 2008). Este achado poderia representar um "desvio" do glutamato astrocítico para compensar a diminuição de GSH consequente ao modelo, já que a síntese da mesma ocorre a partir de glutamato, glicina e cisteína. Ao mesmo tempo, como temos também uma redução no metabolismo energético neste modelo, pode-se inferir que a atividade da GS diminuída pode significar uma quantidade menor de glutamina pra o neurônio converter em glutamato, o qual pode entrar no ciclo de Krebs para a produção de energia.

Mais uma vez, a AG teve não só um efeito preventivo sobre a diminuição da atividade da GS nos animais submetidos à infusão icv de STZ, como também, por si só, foi capaz de aumentar a atividade da mesma nos animais controle *Sham* operados. A atividade da enzima GS é diretamente influenciada pelos níveis de óxido nítrico, como demonstrado por Kosenko e colaboradores (Kosenko *et al.*, 2003). Os autores demonstram que o NO diminui a atividade da GS através da nitração ou nitrosilação da mesma, o que explica o aumento da atividade causada pela AG, um inibidor da iNOS e

a prevenção da diminuição da atividade nos animais do grupo STZ. E ainda, podemos inferir que no modelo de demência por infusão icv de STZ o decréscimo na atividade da GS pode estar ocorrendo devido ao estresse nitrosativo.

5. Alterações no imunoconteúdo de GFAP e S100B hipocampais e de S100B no LCR

5.1 Ação da aminoguanidina.

A proteína S100B possui ações tanto intra quanto extracelulares. Dentro da célula, está envolvida com a regulação da plasticidade do citoesqueleto, a inibição da fosforilação de proteínas, a regulação de enzimas e o controle do crescimento e diferenciação celular (Donato, 2001; Donato, 2003). Sabe-se que enzimas da via glicolítica são alvos potenciais da ação da S100B intracelular (Donato, 2001). Considerando que o modelo de demência estudado apresenta uma importante redução do metabolismo oxidativo, juntamente com déficit energético encefálico (Lannert e Hoyer, 1998), isto pode estar relacionado à alteração no imunoconteúdo de S100B hipocampal encontrada nos animais que receberam STZ icv (capítulo I). Além disso, o aumento da S100B intracelular tem sido encontrado em análise *post mortem* de tecido cerebral de paciente com DA (Van Eldik e Griffin, 1994). Mais uma vez, a aminoguanidina atenuou este efeito causado pela STZ.

Ao mesmo tempo, foi observada uma diminuição no imunoconteúdo de S100B no LCR dos animais tratados com STZ, representando uma porção da proteína secretada. Levando-se em conta que a S100B extracelular funciona como um fator neurotrófico, promovendo aumento da sobrevivência neuronal, neuritogênese e até mesmo o aumento da função sináptica (Van Eldik e Wainwright, 2003) e os achados

deletérios encontrados neste modelo de doença de Alzheimer esporádica, podemos inferir que possivelmente a atividade neuronal está comprometida.

Quanto à GFAP, não encontramos alteração no imunoconteúdo desta proteína no hipocampo dos animais submetidos à infusão icv de STZ vinte e um dias após a cirurgia. Aparentemente houve um aumento, porém não foi estatisticamente significativo (capítulo I). Com a realização de uma imunoistoquímica por fluorescência para GFAP hipocampal, confirmamos não haver diferenças regionais entre os grupos e tampouco alterações na morfologia astrocítica.

É interessante enfatizar o fato de houve alteração numa importante proteína astrocítica, a S100B, sem ter havido alteração no tradicional marcador de astrogliose, a proteína de citoesqueleto GFAP. Tal achado também salienta o fato de que a resposta astrocítica em eventos de insulto, ou astrogliose, pode ser complexa e ampla, devendo ser analisada por diferentes parâmetros.

5.2 Efeito da corrida em esteira.

Como observado no capítulo I, o modelo de doença de Alzheimer esporádica por infusão icv de STZ também provocou alterações no imunoconteúdo de S100B no LCR, que perduraram, como vimos neste estudo, até pelo menos sete semanas (capítulo III). Por outro lado, não foram observadas alterações no conteúdo intracelular hipocampal neste estudo. O que podemos inferir, mais uma vez, é que a produção e a secreção da S100B aparentemente não têm correlação direta, sendo eventos totalmente independentes. Talvez o aumento de S100B hipocampal seja característico nas primeiras semanas para o estabelecimento deste modelo de lesão, já tendo sido restabelecido após sete semanas da cirurgia.

Quanto à diminuição da S100B liquórica, a corrida em esteira foi capaz de prevenir esta alteração, da mesma forma que preveniu o declínio cognitivo e o desequilíbrio das defesas antioxidantes e a produção de NO. Os mecanismos de tais benefícios não são totalmente compreendidos, especialmente sua relação direta com estes resultados, da mesma forma que ainda não se conhecem totalmente as razões pelas quais o exercício físico diminui os riscos para o desenvolvimento da DA e outras demências (Cotman e Berchtold, 2007).

Também foi feita a medida do imunoconteúdo de S100B no soro, a qual não se mostrou alterada nem em consequência do modelo, nem do exercício físico isoladamente. Em trabalho prévio com humanos, foi demonstrado o efeito do exercício físico *per se* sobre este parâmetro, apontando aumento da S100B agudamente no soro de atletas após natação (Dietrich, 2003). Também, alguns trabalhos relatam alterações na proteínas S100B, como em Chaves e colaboradores (Chaves *et. al.*, 2010).

5.2.1 Efeito da corrida em esteira sobre a GFAP hipocampal.

Um interessante resultado, embora independente do estudo do modelo de demência, é o aumento de GFAP hipocampal em conseqüência do exercício. Por si só, esta alteração demonstra um importante efeito da corrida em esteira sobre o encéfalo dos animais exercitados.

Apenas um estudo correlaciona o exercício físico em esteira e a análise densitométrica da GFAP. Nesse estudo (Li *et al.*, 2005), os autores observaram um aumento de células imunomarcadas para a proteína após três e seis semanas de corrida, em córtex frontoparietal e estriado Em trabalho prévio com os mesmo parâmetros de exercício e mesmas estruturas analisadas (Ding *et al.*, 2004), o mesmo grupo de autores demonstrou um aumento de angiogênese de micro vasos, atribuindo ao exercício físico

em esteira a capacidade de interferir com a barreira hematoencefálica, especificamente fortalecendo a unidade formada entre os vasos sanguineos e os astrócitos.

Em nosso trabalho (capítulo III), a análise por densitometria óptica da camada *radiatum* da região CA1 hipocampal mostra aumento significativo da imunomarcação de GFAP em ambos os grupos exercitados, em relação aos sedentários. Podemos inferir que, através dessa interferência com a BHE discutida no capítulo anterior, o exercício físico possa proteger os animais exercitados de eventos isquêmicos, como demonstrado pelos autores citados acima (Ding *et al.*, 2006). Como é uma questão ainda pouco explorada, deve haver outros significados que merecem ser melhor investigados futuramente. Por exemplo, o aumento de GFAP hipocampal decorrente do exercício físico poderia pode estar relacionado a um aumento do fluxo sanguíneo nesta região, pois se sabe que os astrócitos também estão envolvidos no controle do fluxo sanguíneo (Wilhelmsson *et al.*, 2006; Gordon *et al.*, 2007).

Além disso, também vem à luz o questionamento sobre o significado da palavra astrogliose em seu conceito clássico definida como aumento da marcação imunoistoquímica da GFAP devido à hipertrofia do astrócito em resposta a um insulto. Uma definição mais abrangente e atual define astrogliose reativa como um espectro de alterações moleculares, celulares e funcionais em potencial, de astrócitos em resposta a todas às formas e graus de severidade de injúria ou distúrbios do SNC, incluindo as perturbações mais sutis (Sofroniew, 2009). Assim, surge a idéia de que a gliose reativa possa também fazer parte de um mecanismo protetor e assim ser considerado um evento positivo como interpretamos ocorrer em consequência do exercício físico.

5.3 Secreção de S100B em fatias hipocampais

Considerando o decréscimo de S100B observada no líquor dos animais submetidos ao modelo de demência, avaliamos se esta redução seria decorrente da redução na secreção da proteína. No entanto, não há informação sobre que região ou tipos celulares que mais contribuem para a S100B no líquor. Com base nas mudanças observadas no hipocampo, avaliamos se fatias desta região apresentavam uma menor secreção. O primeiro trabalho (Capítulo I) mostrou que a secreção basal de S100B observada em fatias frescas hipocampais não era alterada nos animais tratados com STZ. A secreção basal reflete a quantidade espontaneamente secretada por fatias hipocampais durante 1 hora após a estabilização metabólica (Nardin *et al.*, 2009).

No entanto, permaneceu a dúvida se a secreção modulada de S100B poderia estar afetada nos animais tratados com STZ, particularmente na modulação que envolve a atividade neuronal. Um estímulo com alta concentração de potássio (e.g. 30 mM KCl) leva a despolarização neuronal, mas não provoca um alteração da concentração intracelular de cálcio nos astrócitos (Dallwig e Deitmer, 2002), supostamente envolvida na secreção de S100B (Naraynsingh *et al.*, 2009). De fato, uma despolarização por elevadas concentrações potássio em fatias, leva a uma redução da secreção de S100B, possivelmente secundária à liberação de neurotransmissores (e.g. glutamato) (Nardin *et al.*, 2009; Goncalves *et al.*, 2002; Buyukuysal, 2005). Neste trabalho (Capítulo II), as fatias hipocampais de animais tratados com STZ, expostas ao KCl 30 mM, apresentaram uma menor queda na secreção de S100B se comparada aos controles. Ou seja, a alta concentração de potássio, responsável pela despolarização neuronal e secundariamente causa uma menor secreção astroglial de S100B, está alterada nos animais tratados com STZ. Isto poderia ocorrer devido a uma menor resposta neuronal ao potássio, seja pelo menor número de neurônios responsivos ao estímulo, seja pela

menor responsividade neuronal nesta condição. Portanto este dado reforça a ausência da mudança na secreção basal de S100B, mas evidencia uma alteração funcional da secreção de S100B no hipocampo de animais tratados com STZ. A alteração induzida pela administração icv de STZ poderia de alguma forma e em outras regiões contribuir para a redução de S100B observada no líquor destes animais.

Obviamente outros fatores podem estar envolvidos com a redução de S100B no líquor, como uma maior degradação extracelular, uma redução da S100B livre ou uma redução da depuração liquórica, i.e., uma saída mais intensa do compartimento liquórico. Essas possibilidades precisam ser investigadas futuramente.

6. Conclusões

Em primeiro lugar, neste trabalho foi padronizado o modelo de doença de Alzheimer esporádica do tipo Alzheimer através da infusão intracerebroventricular de estreptozocina, o qual vem sendo investigado sob diferentes aspectos em nosso laboratório desde então.

Foi confirmado o déficit cognitivo, requisito essencial para legitimar um modelo de doença de Alzheimer esporádica. O mesmo foi demonstrado em duas tarefas comportamentais, o labirinto aquático de Morris e a esquiva inibitória.

Ao mesmo tempo, foi confirmado o estresse oxidativo e nitrosativo, detectado através da medida das espécies reativas de oxigênio, dos metabólitos do óxido nítrico e do conteúdo de glutationa hipocampais, corroborando outros dados da literatura.

Pela primeira vez, foram investigadas características específicas de atividade astrocítica, como a captação de glutamato e a atividade da enzima glutamina sintesase (GS), o imunoconteúdo da proteína S100B e GFAP e a secreção de S100B em líquor e

fatias hipocampais. Os resultados obtidos mostram diminuição da atividade da GS, aumento da S100B no tecido hipocampal, diminuição no líquor e o comprometimento da secreção de S100B *ex vivo* em fatias hipocampais decorrentes do modelo.

Interessantemente, as intervenções terapêuticas e ambientais utilizadas neste trabalho, a droga anti-glicação aminoguanidina e a corrida em esteira, foram efetivos da prevenção do comprometimento descrito acima.

Por fim, estes resultados reforçam participação do estresse oxidativo/nitrosativo no modelo de infusão icv de estreptozotocina e na Doença de Alzheimer, bem como de alterações astrogliais hipocampais.

Considerações Finais

Os resultados deste trabalho demonstram o envolvimento dos astrócitos no modelo de demência por infusão intracerebroventricular de estreptozotocina, mostradas principalmente pelas alterações na secreção da proteína S100B e atividade da glutamina sintetase. Ao mesmo tempo, confirmam o dano cognitivo e o estresse oxidativo e nitrosativo, especificamente em hipocampo.

Com estes achados, enfatizou-se o papel dos astrócitos nas demências, bem como a importância destas células como possíveis alvos terapêuticos nos acometimentos do SNC. Além disso, as estratégias terapêuticas utilizadas foram capazes de prevenir as alterações induzidas pelo modelo, confirmando e reforçando o potencial neuroprotetor da aminoguanidina e do exercício físico, especificamente a corrida em esteira.

Por fim, as alterações astrogliais observadas neste modelo de demência contribuem com a compreensão da fisiopatologia da doença de Alzheimer e de outras doenças acompanhadas de déficit cognitivo, bem como com a busca por estratégias de terapia e prevenção.

Perpectivas

1. Análise munoistoquímica do envolvimento da microglia no modelo.
2. Medida de glicação e nitrosilação protéica por *immunoblotting* e imunoistoquimica.
3. Medida do RAGE (solúvel e insolúvel) por *immunoblotting* e imunoistoquimica.
4. Investigação da presença de depósito beta amilóide pela técnica do Vermelho Congo e imunoistoquímica.
5. Estudo da sinalização pela insulina hipocampal.

ANEXOS

Anexo I

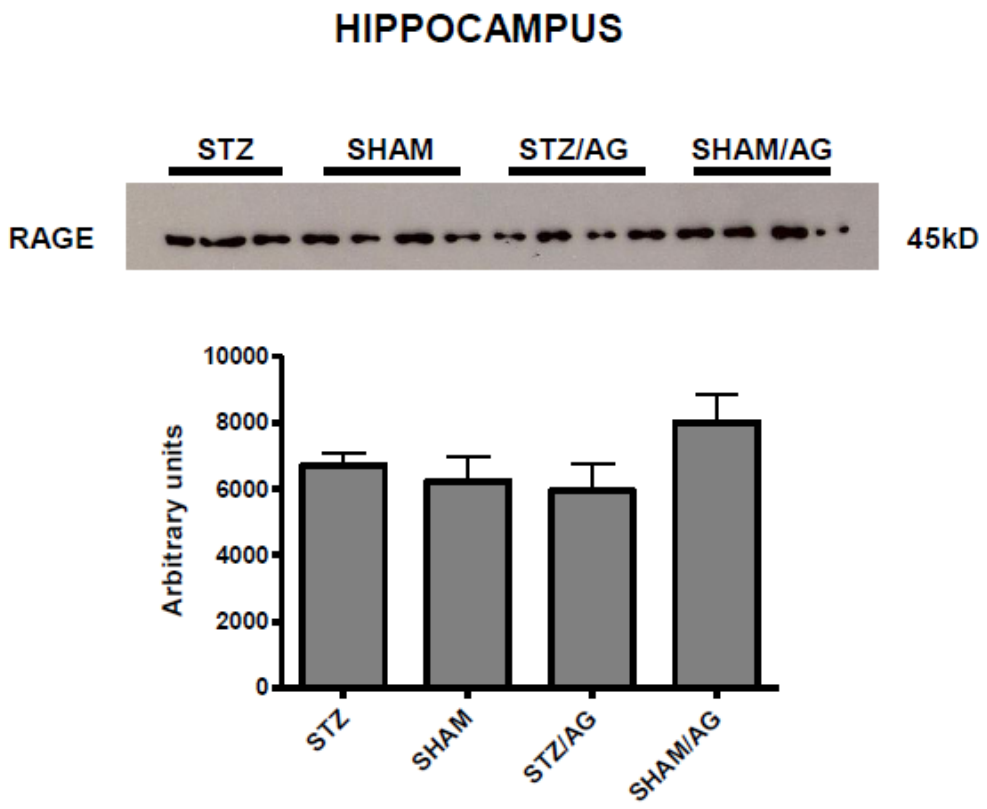
O artigo correspondente ao capítulo I foi selecionado para ser publicado junto ao *Handbook of Journal of Alzheimer's Disease*:

Hippocampal alterations in rats submitted to streptozotocin-induced dementia model: Neuroprotection with aminoguanidine. Handbook of Journal of Alzheimer's Disease Letícia Rodrigues, Regina Biasibetti, Alessandra Swarowsky, Marina Concli Leite, André Quincozes-Santos, Matilde Achaval, Carlos-Alberto Gonçalves (in press)

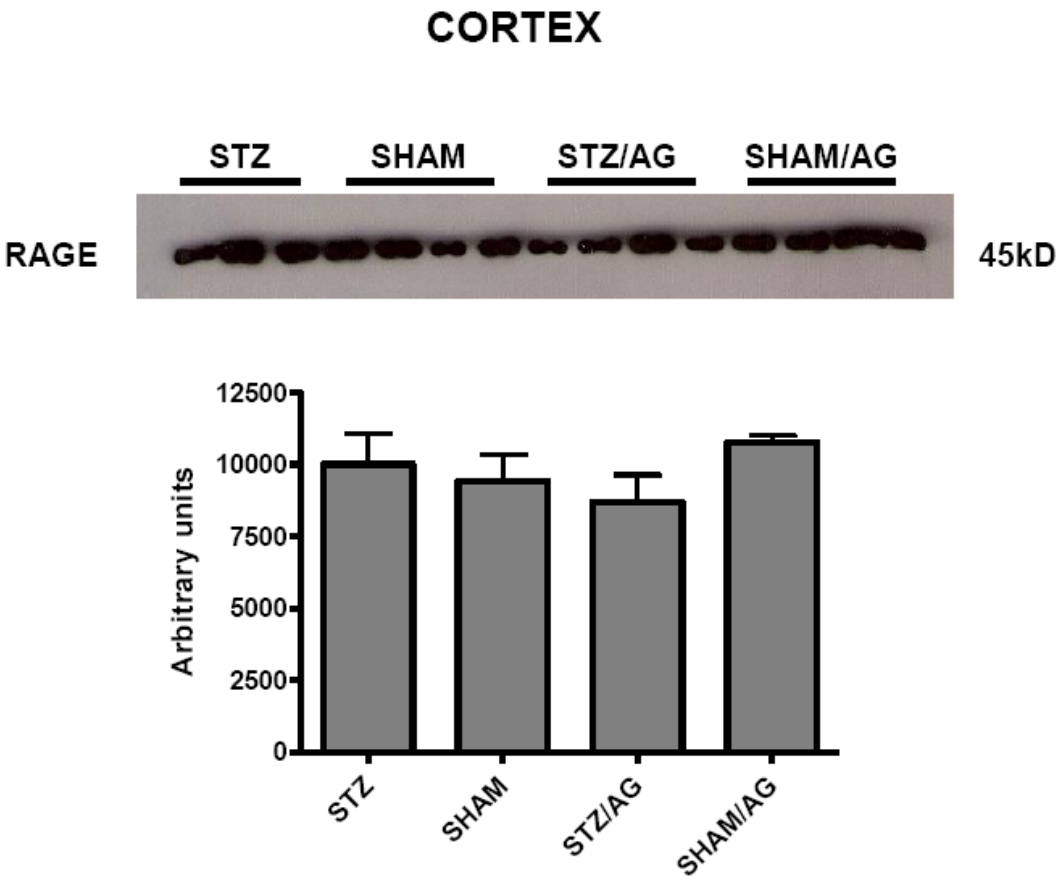
Anexo II

Resultados Preliminares

- Medida do receptor para produtos avançados de glicação em hipocampo e córtex frontal de ratos submetidos ao modelo de doença de Alzheimer esporádica por infusão icv de STZ



Análise quantitativa do RAGE hipocampal. O hipocampo de três animais por grupo foi dissecado e homogeneizado para imuno detecção por *Western blotting*. A figura representa a análise densitométrica do *immunoblot*. Dados expressos como média±SEM (ANOVA de duas vias)



Análise quantativa do RAGE em córtex frontal. O córtex de três animais por grupo foi dissecado e homogeneizado para imuno detecção por *Western blotting*. A figura representa a análise densitométrica do *immunoblot*. Dados expressos como média±SEM (ANOVA de duas vias).

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