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AVALIAÇÃO DOS PARÂMETROS ASTROGLIAIS E COMPORTAMENTAL DE RATOS SUBMETIDOS À HIPOPERFUSÃO CEREBRAL CRÔNICA

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O que vale na vida não é o ponto de partida e sim a caminhada. Caminhando e semeando, no fim terás o que colher.

Cora Coralina

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SUMÁRIO

LISTA DE ABREVIATURAS

APP- Proteína precursora amilóide (do inglês, “Amyloid Precursor Protein”)

FSE- Fluxo sangüíneo encefálico

CSF- Líquido cérebro-espinal ou líquor (do Inglês, “Cerebrospinal fluid”)

DA- Doença de Alzheimer

GFAP- Proteína glial fibrilar ácida (do inglês, “Glial Fibrillary Acidic Protein”)

GS- Glutamina Sintetase

LTP- Memória de longa duração (do inglês, “Long-term potentiation”)

SNC- Sistema Nervoso Central

SNP- Sistema nervoso periférico

2VO- Oclusão de dois vasos (do inglês, “2 Vessels Occlusion”)

RESUMO

A oclusão permanente e bilateral das artérias carótidas comuns (2VO), resultando em uma significante redução do fluxo sangüíneo cerebral (hipoperfusão) em ratos, é aceita como um modelo experimental bem estabelecido para investigar dano neuronal e déficit cognitivo que ocorrre com o envelhecimento e na doença de Alzheimer. Neste estudo foram avaliadas duas proteínas gliais: S100B e proteína glial fibrilar ácida (GFAP) no tecido cortical cerebral e hipocampo, bem como a S100B no fluído cerebroespinal, e captação de glutamato e a atividade da glutamina sintetase em hipocampo. A cognição foi avaliada através dos protocolos espaciais de memória de referência e de trabalho. O conteúdo de S100B periférico, particularmente no soro e no fluído cerebroespinal (CSF), tem sido usado como parâmetro de ativação glial em diversas condições patológicas. Preliminarmente, investigamos o efeito de anestésicos (tiopental, cetamina e halotano) no conteúdo de S100B no CSF e soro, isto porque alguns estudos têm sugerido um efeito prejudicial desses compostos. Além disso, foi investigado também o possível efeito dependente do sexo sobre o conteúdo de S100B no CSF, já que vários estudos têm sugerido os astrócitos como um dos alvos do estrogênio. Altos níveis de S100B no CSF foram encontrados em ratos anestesiados com tiopental; estes níveis, independente do anestésico, foram sexo-dependentes. Ratos adultos, machos Wistar foram submetidos a 10 semanas de hipoperfusão cerebral crônica após o método 2VO. Um significante aumento da S100B e GFAP em hipocampo foi observado, como também uma significante redução da captação de glutamato. Entretanto, observamos uma diminuição da S100B no CSF. Com relação à função cognitiva, houve um déficit tanto na memória espacial de referência como na de trabalho no labirinto aquático. Nossos dados reforçam a hipótese de que astrócitos têm um papel crucial nos mecanismos das patologias neurodegenerativas, como a doença de Alzheimer, e que a patologia hipocampal resultante da hipoperfusão crônica contribui para os déficits de memória.

ABSTRACT

The permanent occlusion of common carotid arteries (2VO), resulting in a significant reduction in cerebral blood flow (hypoperfusion) in rats, is accepted as a well established experimental model to investigate neuronal damage and cognitive impairment that occurs in human ageing and Alzheimer's disease. In the present study, we evaluated two astroglial proteins: S100B and glial fibrillary acidic protein (GFAP) in cerebral cortex and hippocampus tissue, as well S100B in cerebrospinal fluid, and glutamate uptake and glutamine synthetase activity in hippocampus tissue. Cognition was assessed by reference and working spatial memory protocols. Peripheral S100B content, particularly in serum and cerebrospinal fluid (CSF), has been used as a parameter of glial activation in several pathological conditions. Preliminary we investigated the effect of anesthetics (thiopental, ketamine and halothane) on CSF S100B levels since some studies have suggested a damage effect of these compounds. Moreover, we investigated a possible gender dependence on CSF S100B, since several studies have suggested astrocytes as putative targets for estrogen. Higher levels of CSF S100B were found when rats were anaesthetized with thiopental; these levels, independently of anesthetic, were gender dependent. Adult male Wistar rats were submitted to 10 weeks of chronic cerebral hypoperfusion after the 2VO method. A significant increase of S100B and GFAP in hippocampus tissue was observed, as well a significant decrease in glutamate uptake. Interestingly, we observed a decrease in S100B in cerebrospinal fluid. As for cognitive function, there was an impairment of both reference and working spatial memory in the water maze. Our data so support the hypothesis that astrocytes play a crucial role in the mechanisms of neurodegenerative disorders, like Alzheimer's disease, and that hippocampal pathology arising after chronic hypoperfusion gives rise to memory deficits.

1. INTRODUÇÃO

1.1 Demência

Demência é um termo genérico que descreve uma disfunção cortical e subcortical crônica ou progressiva que resulta num complexo declínio cognitivo. As mudanças cognitivas são comumente acompanhadas por distúrbios do humor, comportamento e personalidade (Ritchie & Lovestone, 2002).

Os quadros de demência estão associados à idade; enquanto atingem aproximadamente 5% da população idosa acima dos 65 anos, chegam a 25% acima dos 80 anos (Hynd et al, 2004). A prevalência de demência dobra a cada 5 anos a partir dos 65 anos de idade e aproximadamente 40% dos idosos entre 90-95 anos são afetados (Hansson et al, 2006).

Cada vez mais tem se dado atenção à demência não só no que se refere ao quadro clínico mas também seus efeitos na sociedade. Isto porque, a demência é devastadora tanto para o indivíduo como para família e tem um grande impacto socioeconômico (Cerejeira & Mukaetova-Ladinska, 2007). Com o aumento da expectativa de vida, no Brasil e no mundo, tem sido esperado um aumento na sua prevalência, e está rapidamente tornando-se um problema não apenas de saúde mas também social (Hansson et al, 2006).

A doença de Alzheimer (DA) é a maior causa de demência com o avanço da idade (Hynd et al, 2004; Zlokovic, 2005), sendo responsável por mais de 50% dos casos. A DA é um distúrbio neurodegenerativo caracterizada pela deteriorização progressiva das funções cognitivas, perda da memória, mudanças da personalidade e incapacidade no cuidado pessoal (Weinstock & Shoham, 2004; Koistinaho & Koistinaho, 2005; Zarow et al, 2005). A gravidade

do déficit cognitivo pode ser explicada, pela perda sináptica e formação de emaranhados fibrilares intracelulares, particularmente nos neurônios colinérgicos (Weinstock & Shoham, 2004).

A confirmação da DA pode ser feita somente através do exame morfológico e histopatológico *post-mortem* (Hynd et al, 2004). Seu perfil histopatológico caracteriza-se pela presença de numerosas placas corticais, que são depósitos do peptídeo beta-amilóide (Weinstock & Shoham, 2004; Koistinaho & Koistinaho, 2005).

O processo patogênico da DA provavelmente inicia décadas antes de começarem os sintomas clínicos. Durante este período pré-clínico há uma gradual perda neuronal (Hansson et al, 2006). Estudos têm apontado alguns genes envolvidos nessa doença. Entretanto, em mais de 95% dos casos, a DA é esporádica, e a etiopatogenia é desconhecida, heterogênea e provavelmente multifatorial (Sheng et al, 2000; Nixon et al, 2001). Recentemente, foi proposto que a DA esporádica é decorrente de uma disfunção vascular. Esta conclusão é baseada na semelhança entre o quadro clínico da DA e os sintomas cognitivos da demência vascular (Kasparová et al, 2005).

Distúrbios da circulação cerebral têm sido associados com o declínio da função cognitiva em idosos e com o desenvolvimento de demência vascular. A isquemia cerebral, uma disfunção aguda, produz elevados níveis de espécies reativas de oxigênio, iniciando uma cascata de eventos neuropatológicos que podem culminar em doença neurodegenerativa (Kuang et al, 2008).

Há evidências que sugerem uma relação entre a DA e a isquemia cerebral. A isquemia cerebral atinge principalmente a população mais velha.

Dos pacientes com DA, 60-90% apresentam patologia cerebrovascular na autópsia, e a coexistência de isquemia cerebral e DA é maior do que a ocorrência isolada destas patologias (Koistinaho & Koistinaho, 2005).

Além disso, a diminuição do fluxo sangüíneo encefálico (FSE) ocorre no envelhecimento normal e na demência (Farkas et al, 2004; Ritchie et al, 2004). Alguns trabalhos têm mostrado que a diminuição do FSE está relacionado com o aumento das injúrias cognitivas dos pacientes com Alzheimer e, portanto, sugerem que a redução do FSE é um bom indicador para a progressão da DA. Não é conclusivo se a redução do FSE é conseqüência ou causa da disfunção neuronal**.** No entanto, estudos experimentais sugerem que o decréscimo do FSE pode levar a uma disfunção cognitiva e danos neuronais (Farkas et al, 2004).

A hipoperfusão cerebral, que provoca uma redução do FSE, tem sido implicada no mecanismo de aumento da produção da proteína precursora amilóde (APP- do inglês “Amyloid Precursor Protein”) mimetizando a DA (Schmidt-Kastner et al, 2005). Há diversos modelos experimentais em demência com base na expressão de peptídeo beta-amilóide e déficit cognitivo (Cole & Frautschy, 1997). A hipoperfusão cerebral crônica é um modelo experimental relevante para estudar a DA (Weinstock & Shoham, 2004).

O uso da oclusão permanente e bilateral das artérias carótidas comuns (2VO) em ratos mimetiza a hipoperfusão cerebral crônica (Ritchie et al, 2004). Este modelo, 2VO, é bem caracterizado para investigar as conseqüências cognitivas e histopatológicas da hipoperfusão cerebral crônica (Farkas et al, 2004), por gerar uma diminuição do fluxo sangüíneo e por ser um fator de risco

de demência (Wilde et al, 2002), característico durante o envelhecimento normal (Ritchie et al, 2004). Este modelo experimental em ratos reduz o fluxo sangüíneo cortical e hipocampal em 25-50% dos níveis normais em apenas duas horas e meia pós-oclusão, e pode causar déficit de memória e aprendizagem e dano neuronal semelhante aos efeitos observados na demência vascular (Davidson et al, 2000; Kuang et al, 2008).

Diversos testes têm sido desenvolvidos para avaliar a função cognitiva em roedores. Estudos das funções cognitivas em ratos submetidos à hipoperfusão cerebral crônica têm sido investigados pela memória de referência e aprendizado por meio do labirinto aquático de Morris e do labirinto de 8 braços. Os déficits cognitivos encontrados nos ratos submetidos ao modelo 2VO têm sido associados a danos na região CA1 do hipocampo (Sarti et al, 2002).

1.2 Astrócitos

Os astrócitos são as células gliais mais abundantes do sistema nervoso central (SNC), e têm um papel crucial para o funcionamento normal da fisiologia cerebral durante o desenvolvimento e na vida adulta. Estão envolvidos, por exemplo, na produção de fatores neurotróficos, armazenamento de glicogênio como reserva de energia, regulação da composição iônica extracelular, captação e reciclagem de neurotransmissores e na formação da barreira hemato-encefálica (Araque et al, 1999; Takuma et al, 2004). No SNC de vertebrados superiores, após lesão por trauma, distúrbios genéticos ou lesão por substâncias tóxicas, os astrócitos tornam-se reativos e respondem de uma maneira típica, chamado de astrogliose ou gliose reativa

(Eng et al, 2000), a qual é caracterizada pela proliferação e/ou hipertrofia glial (Wishcamper et al, 2003).

A ativação glial em resposta as lesões do SNC envolve mudanças na proteína glial fibrilar ácida (GFAP) e na proteína S100B, bem como no metabolismo do glutamato.

1.3 GFAP

A GFAP é uma proteína marcadora de astrócitos, presente em filamentos intermediários do grupo III. Exibe uma atividade dinâmica modulada por fosforilação e desfosforilação, efetuando um papel fundamental na plasticidade astrocítica (Rodnight et al, 1997). O seu estado de fosforilação é regulado, dentre outros fatores, pela proteína S100B (Ziegler et al, 1998). O aumento da expressão desta proteína (RNAm e imunoconteúdo), caracterizando a astrogliose, tem sido encontrado em inúmeras patologias agudas e crônicas, como a doença de Alzheimer, e em modelos experimentais de lesão (Eng et al, 2000).

1.4 Proteína S100B

A proteína S100B faz parte de uma família de proteínas ligantes de Ca^{2+} do tipo “EF-hand” (Donato, 1999; Marenholz et al, 2004). Em mamíferos, ela é mais abundante em células gliais do SNC e do sistema nervoso periférico (SNP), sendo considerada uma proteína marcadora de astrócitos. Também é expressa fora do SNC por melanócitos, condrócitos e adipócitos (Donato, 1999).

A proteína S100B possui várias supostas funções e alvos intracelulares que estão sendo estudados. Ela atua regulando a fosforilação de proteínas, a

atividade enzimática, a homeostase do cálcio e a dinâmica do citoesqueleto (Kleindienst & Bullock, 2006). A ação extracelular da proteína S100B, em culturas neurais, depende da sua concentração. Em concentração nanomolar exerce um efeito neurotrófico estimulando a extensão de neuritos, facilitanto a sobrevivência de neurônios durante o desenvolvimento, além de estimular a proliferação de astrócitos e a captação glial de glutamato (Gonçalves et al, 2000; Tramontina et al, 2006). Já em concentrações micromolares pode exercer efeitos neurotóxicos levando a apoptose (Ahlemeyer et al, 2000; Van Eldik & Wainwright, 2003).

Em lesões do SNC, como nas isquemias, e também nas doenças neurodegenerativas, como na DA, há um aumento dos níveis de S100B (Heizmann, 1999), o que leva a hipótese de que a proteína S100B possa estar associado a progressão da doença (Petzold et al, 2003). Há estudos que mostram um significativo aumento da proteína S100B no líquido cérebro-espinhal em pacientes com demência do lobo frontotemporal e nos pacientes com DA (Petzold et al, 2003). Diante disto, a proteína S100B tem sido usada como marcador de dano no sistema nervoso.

1.5 Metabolismo astroglial do glutamato

O glutamato é o neurotransmissor excitatório mais abundante no SNC dos mamíferos (Luján et al, 2005). É crucial que a concentração extracelular do glutamato, assim como de outros neurotransmissores na fenda sináptica, mantenha-se baixa. Além disso, a barreira hemato-encefálica previne a entrada do glutamato no SNC (Nedergaard et al, 2002). O aumento do glutamato na fenda sináptica tem efeito tóxico e pode estar envolvido tanto em

neuropatologias agudas (e.g isquemia), como crônicas (e.g. DA) (Had-Aissounietal et al, 2002; Rajendra et al,2004).

O glutamato liberado na fenda sináptica atua nos receptores pós-sinápticos, mas rapidamente é recaptado pelos astrócitos. A entrada ocorre por transportadores astrogliais específicos dependentes de Na^+, denominados GLAST e GLT-1. Esse glutamato é metabolizado no ciclo de Krebs, e utilizado para a síntese de glutationa ou para a síntese de glutamina pela glutamina sintetase (GS), uma enzima especificamente astroglial no SNC. Portanto, a GS é responsável por manter baixa a concentração de glutamato nos astrócitos e tem um importante papel na reciclagem do glutamato (Nedergaard et al, 2002).

2. OBJETIVOS

2.1 Objetivo Geral

Padronizar a técnica de hipoperfusão cerebral crônica e avaliar parâmetros astrogliais e comportamental possivelmente associados ao prejuízo cognitivo induzido pela hipoperfusão cerebral crônica em ratos Wistar, machos e adultos.

2.2 Objetivos Específicos

1. Avaliar o efeito de anestésicos, em particular do tiopental, cetamina e halotano, sobre o conteúdo de S100B no líquor e no soro de ratos Wistar de ambos os sexos;
2. Padronizar o modelo de indução de *demência* em ratos Wistar machos, adultos, em função do déficit cognitivo (tarefa de Labirinto Aquático);
3. Quantificar o conteúdo de S100B no tecido hipocampal, no córtex frontal e no líquor, após 10 semanas de hipoperfusão cerebral crônica;
4. Medir o conteúdo de GFAP nos tecidos hipocampal e cortical de ratos submetidos à hipoperfusão cerebral crônica;
5. Medir a captação de glutamato e atividade da glutamina sintetase no hipocampo, de ratos submetidos à hipoperfusão cerebral crônica.

3. RESULTADOS – CAPÍTULO I

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S100B LEVELS IN THE CEREBROSPINAL FLUID OF RATS ARE SEX AND ANAESTHETIC DEPENDENT

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SUMMARY

1. S100B is a calcium-binding protein that acts as a neurotrophic cytokine and is expressed in the central nervous system, predominantly by astrocytes. At nanomolar concentrations, S100B stimulates neurite outgrowth and glial glutamate uptake, as well as protecting neurons against glutamate excitoxicity.

2. Peripheral S100B concentrations, particularly in the serum and cerebrospinal fluid (CSF), have been used as a parameter of glial activation or death in several physiological and pathological conditions.

3. In the present study, we investigated the effect of anaesthetics (thiopental, ketamine and halothane) on CSF concentrations of S100B, as well as a possible sex dependence, because several studies have suggested astrocytes as putative targets for oestrogen.

4. Higher levels of CSF S100B were found when rats were anaesthetized with thiopental; these levels, independently of anaesthetic, were sex dependent. Conversely, no effect of anaesthetic or sex was observed on serum concentrations of S100B.

5. The increase in CSF concentrations of S100B induced by thiopental was confirmed in non-anaesthetized neonatal rats and cortical astrocyte cultures.

6. Assuming CSF S100B as a marker of development, glial activation or even brain damage, investigations regarding the sex dependence of its concentration may be useful in gaining an understanding of sex variations in the behaviour and the pathological course of, as well as susceptibility to, many brain disorders. The findings of the present study reinforce the sex effect on synaptic plasticity and suggest a sex dependence of neural communication mediated by extracellular S100B without restricting the influence of astrocytes on the developmental phase.

Key words: anaesthesia, astrocyte, S100B, sex-dependent glial differentiation, thiopental.

INTRODUCTION

Growing evidence indicates the importance of glial cells to overall brain activity. For many decades, astrocytes were considered relatively passive supporters of neuronal function, but over the past few years this concept has altered substantially. Astrocytes are key elements in the understanding of the blood–brain barrier, synaptic plasticity, brain defence and recovery to injuries.[1,2] With regard to glutamate, the main excitatory neurotransmitter, astrocytes are major elements involved in glutamate uptake and exclusively convert glutamate to glutamine using the enzyme glutamine synthetase (GS).[2] In addition to GS, two other proteins are used to characterize astrocytes: glial fibrillary acidic protein (GFAP) and S100B.

S100B is a calcium-binding protein that is predominantly expressed and secreted by astrocytes in vertebrate brain.[3] Intracellular S100B is involved in the regulation of the cytoskeleton and cell cycle. The extracellular effect of the S100B, observed in cell cultures, depends on its concentration; at nanomolar levels it is neurotrophic, whereas at micromolar levels it is apoptotic.[4] Considerable evidence exists to suggest that, at nanomolar concentrations, S100B stimulates neurite outgrowth and glial glutamate uptake, as well as protecting neurons against glutamate excitotoxicity.[4,5] Peripheral S100B concentrations, particularly in the cerebrospinal fluid (CSF) and blood serum, have been used as a parameter of glial activation or death in several situations of brain injury.[6] Moreover, the physiological role of extracellular S100B on behaviour has been investigated by exogenous infusion of this protein[7] or anti-S100B antibodies[8] into brain tissue, as well as in transgenic animals,[9] and, more recently, peripheral levels of S100B have been measured in stress models.[10,11]

Anaesthetics affect many brain activities and some can interfere with the measurement of parameters of brain injury and protection in experimental models and clinical investigations.[12,13] Interestingly, the effect of the anaesthetics commonly used when samples of CSF and serum (by intracardiac puncture) are obtained for measurement of S100B concentrations in experimental models of brain injury has not been investigated.

Moreover, it is well known that levels of circulating adrenal and sex steroids can regulate the expression of GFAP and GS during development. In addition, oestrogen-induced differentiation of astrocytes has been related to the modulation of synaptic plasticity and activity,[14] as well as to inflammatory activity in neurodegenerative diseases.[15] However, little information is available regarding the effect of sex on peripheral levels of S100B. Sex dependence has been reported for S100B concentrations in the CSF of adults and in the serum of children without a history of neurological disorders.[16–18]

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This has not been confirmed in other studies using human samples[19,20] and has not been investigated in other species. Interestingly, a sex-specific hippocampal variation of S100B was found in neonatal rats.[21]

In the present study, we compared S100B concentrations in the CSF and serum of rats anaesthetized by different substances, namely thiopental, ketamine and halothane, with simultaneous evaluation of possible sex-dependent changes in these concentrations.

METHODS

Materials

Sodium thiopental (Tiopentax; Cristália, Porto Alegre, Brazil), ketamine and xylazine (Dopalen; Vetbrands, Jacareí, Brazil) and halothane (Halotano; Cristália) were used in the present study. The anti-S100B (SH-B1) antibody, Dulbecco's modified Eagle's medium (DMEM) and other material for cell culture and ELISA were purchased from Sigma (St Louis, MO, USA). Fetal calf serum (FCS) was purchased from Cultilab (São Paulo, Brazil), rabbit polyclonal anti-S100 was from Dako (São Paulo, Brazil) and peroxidase-conjugated anti-rabbit immunoglobulin was from Amersham (São Paulo, Brazil).

Animals

Adult (90-day-old) and neonatal (2-day-old) Wistar rats (Department of Biochemistry, Universidade Federal do Rio Grande do Sul) were housed with food and water available *ad libitum* under a 12 h light–dark cycle at 25°C. All procedures were in accordance with the international guiding principles for biomedical research involving animals from CIOMS/WHO and were approved by the local authorities at the Federal University of Rio Grande do Sul.

Cerebrospinal fluid and serum samples

Rats were anaesthetized or not (some neonatal rats) by intraperitonial injection of pentobarbital sodium (50 mg/kg)[22] or a mixture of ketamine and xylazine (75 and 10 mg/kg, respectively) or by 2% halothane inhalation. Rats were then positioned in a stereotactic holder and the CSF was obtained by puncture of the cisterna magna using an insulin syringe (27 gauge × 12.7 mm length). A maximum volume of 30 μL was collected over a 3 min period to minimize risk of brain stem damage. Using a similar syringe, intracardiac puncture was performed carefully to obtain 1 mL blood. Blood was transferred to an Eppendorf tube, which was rested for 5 min and then centrifuged (3000 *g*, 5 min) to obtain the serum fraction. Importantly, blood collected by decapitation results in an elevated concentration of S100B, possibly resulting from additional extracerebral sources of S100B (LV Portela, unpubl. obs., 1999). Cerebrospinal fluid and serum samples were frozen (–20°C) until further analysis.

Cell culture

Primary astrocyte cultures from Wistar rats were prepared as described previously.[23] Briefly, the cerebral cortex of newborn Wistar rats (1–2 days old) was removed and dissociated mechanically in Ca^{2+}- and Mg^{2+}-free balanced salt solution (pH 7.4) containing (in mmol/L): NaCl 137; KCl 5.36; Na_2HPO_4 0.27; KH_2PO_4 1.1; glucose 6.1. The cortex were cleaned of the meninges and dissociated mechanically by sequential passage through a Pasteur pipette. After centrifugation at 200 *g* for 5 min, the pellet was resuspended in DMEM (pH 7.6) supplemented with 8.39 mmol/L HEPES, 23.8 mmol/L $NaHCO_3$, 0.1% fungizone, 0.032% garamycin and 10% FCS. Cultures were maintained in DMEM containing 10% FCS in 5% CO_2/95% air at 37°C and allowed to grow to confluence before being used at 14 days *in vitro*. The medium was replaced by DMEM containing thiopental at the given concentrations for 15 min. Extracellular S100B content was referred to as 'secretion', based on cell integrity measurements of lactate dehydrogenase (LDH) activity using a colourimetric commercially available kit (Doles, Goiânia, Brazil).[24]

ELISA for S100B

The ELISA was performed as described previously,[25] with some modification. An S100B standard curve was constructed over the range 0.025–2.5 μg/L. Briefly, 50 μL serum plus 50 μL barbital buffer was incubated for 3 h on a microtitre plate coated previously with monoclonal anti-S100B (SH-B1; Sigma). The plate was then incubated with rabbit polyclonal anti-S100 and then peroxidase-conjugated anti-rabbit immunoglobulin for 1 h. The colour reaction with *o*-phenylenediamine was measured at 492 nm.

Statistical analysis

Parametric data are reported as the mean ± SEM and were analysed by ANOVA followed by Tukey's test or Student's *t*-test, with $P < 0.05$ taken to indicate statistical significance.

RESULTS

The CSF and serum concentrations of S100B in rats are listed in Table 1. Note that the concentration of S100B in the CSF depends on the anaesthetic used. Higher concentrations of S100B were found in the CSF when rats were anaesthetized with thiopental. To determine whether thiopental increases basal values of S100B, samples were collected from anaesthetized (with thiopental) or non-anaesthetized (basal value) 2-day-old rats. Thiopental-anaesthetized rats exhibited higher concentrations of S100B in the CSF compared with non-anaesthetized rats. Serum S100B concentrations were not dependent on the anaesthetic used.

Table 1 Effect of anaesthetics on serum and cerebrospinal fluid concentrations of S100B in rats

	S100B (μg/L)			
	Non-anaesthetized rats	Thiopental-anaesthized rats	Ketamine-anaesthized rats	Halothane-anaesthized rats
Serum (adult)	ND	0.08 ± 0.03	0.11 ± 0.06	0.09 ± 0.06
CSF (adult)	ND	1.15 ± 0.18*	0.60 ± 0.08	0.77 ± 0.10
CSF (neonate)	5.40 ± 0.50	7.10 ± 1.30**	ND	ND

Data are the mean±SEM ($n = 11$–12 per group for adult rats; $n = 6$ for neonatal rats). *$P < 0.05$ compared with the other groups (ANOVA followed by Tukey's test); ** $P < 0.05$ compared with the non-anaesthetized group (Student's *t*-test).

ND, not determined.

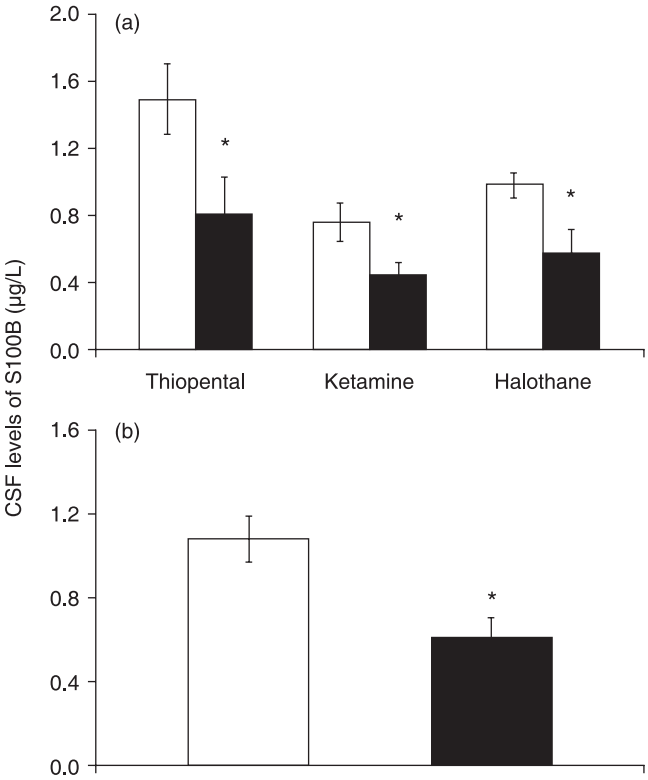

Fig. 1 Sex dependence of cerebrospinal (CSF) concentrations of S100B in rats anaesthetized with different anaesthetic agents. (a) Male (□) and female (■) rats were anaesthetized with thiopental, ketamine or halothane. Data are the mean±SEM ($n = 5–6$ per group). (b) Rats were separated according to sex, independent of the anaesthetic used. (□), male rats; (■), female rats. Data are the mean±SEM ($n = 16–17$ per group). *$P < 0.05$ compared with male rats (Student's *t*-test).

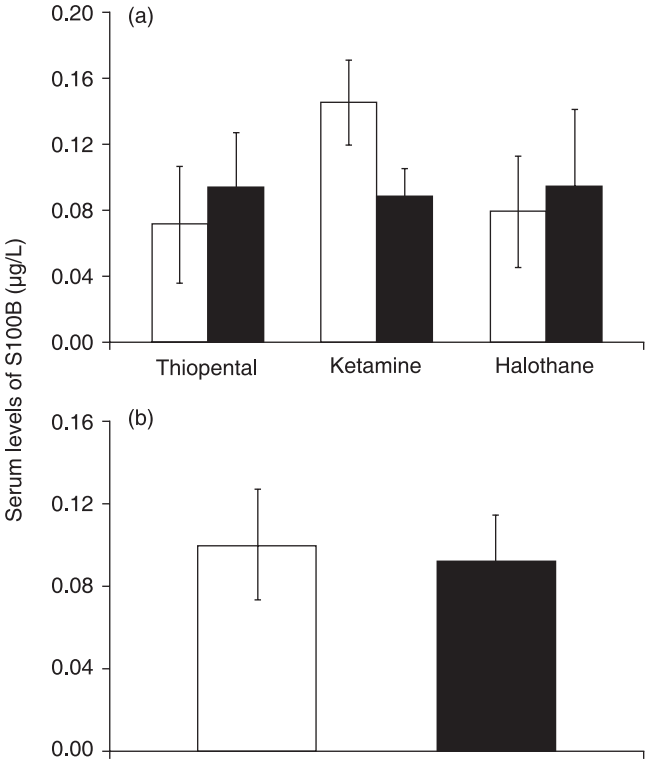

Fig. 2 No effect of sex was found on serum concentrations of S100B in rats anaesthetized with different anaesthetic agents. (a) Male (□) and female (■) rats were anaesthetized with thiopental, ketamine or halothane. Data are the mean±SEM ($n = 5–6$ per group). (b) Rats were separated according to sex, independent of the anaesthetic used. (□), male rats; (■), female rats. Data are the mean±SEM ($n = 16–17$ per group).

When groups exposed to different anaesthetics were separated according to sex, concentrations of S100B in the CSF were clearly observed to depend on sex (Fig. 1a). Male rats had higher concentrations of S100B in the CSF in all three anaesthetic groups and this difference was confirmed when rats were separated according sex (independently of anaesthetic exposure; Fig. 1b).

No sex dependence was found for serum concentrations of S100B in the different groups (Fig. 2a). Furthermore, when rats were separated according to sex (independently of anaesthetic exposure), there were no differences observed in serum concentrations of S100B between male and female rats (Fig. 2b).

In order to investigate any direct effect of thiopental on S100B secretion, cortical astrocyte cultures were acutely exposed to this anaesthetic at concentrations of 0.1 and 1 mmol/L (Fig. 3). A significant thiopental-induced increase in S100B secretion was observed over basal values following exposure of astrocyte cultures to both concentrations of thiopental.

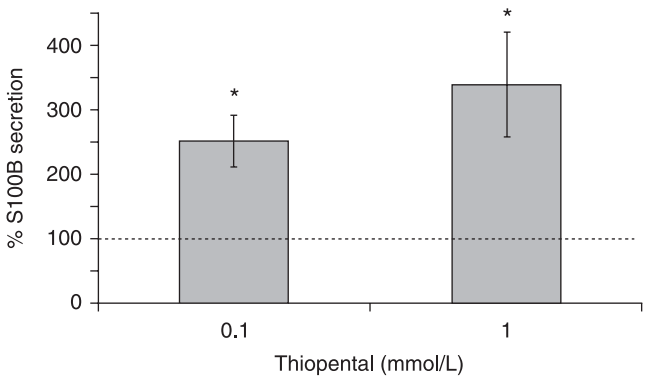

Fig. 3 Effect of thiopental on S100B secretion by cultured astrocytes. Cortical astrocytes were prepared from neonatal rats and cultured for 14 days. Thiopental (0.1 and 1 mmol/L) was added to the cultures and S100B concentrations were determined 15 min later. Data are the mean±SEM from three independent experiments performed in triplicate. Basal secretion (assumed as 100%) is indicated by the dashed line. *$P < 0.05$ compared with basal secretion of S100B.

DISCUSSION

In the central nervous system (CNS), S100B is primarily expressed and secreted by astrocytes. The extracellular effect of S100B, as observed in cell culture, depends on its concentration: it is neurotrophic at nanomolar concentrations and apoptotic at micromolar concentrations.[3,4] There is considerable evidence to suggest that, at nanomolar concentrations, S100B stimulates glial proliferation and neuronal survival, as well as protecting neurons against glutamate excitoxicity.[5] In addition, we recently found that low levels (50 pmol/L) of S100B stimulate glutamate uptake.[26]

The S100B content, particularly extracellular S100B, is used as a parameter of glial activation or death in several situations of brain injury. In fact, increases in serum and CSF concentrations of S100B have been described in many brain disorders, from traumatic brain injury to psychiatric and neurodegenerative diseases.[6] However, there are no standard values for peripheral concentrations of this protein and several interfering factors, such as age, immunoassays and chemical interference, may have an effect. Thus, care should be taken when comparing independent studies.[17–20,27] In addition, extracerebral sources of S100B may contribute to serum concentrations[28] and many authors have reported clinical changes in serum S100B that are not exclusively associated with brain dysfunction.[29]

In the present study, we found that thiopental induced an increase in CSF concentrations of S100B. Induction of S100B secretion was also observed in astrocyte cultures following exposure of cells to thiopental, suggesting that the increase in CSF concentrations of S100B could be due to a direct effect of thiopental on astrocytes. Interestingly, glutamate uptake was reduced in cortical astrocytes exposed to thiopental.[30] It is possible that this reduction in glutamate uptake and the increase in S100B secretion are connected mechanistically;[31] however, the significance of this effect (i.e. whether increased S100B secretion is a response to cell injury or whether the injury itself increases S100B secretion) remains to be elucidated. Importantly, the absence of LDH release suggests that S100B is secreted rather than being released as a result of a loss of cell integrity.

At present, it is not possible to establish a mechanistic relationship between the thiopental-induced increment observed in S100B and any neuronal dysfunction or protection; however, many *in vitro* studies have shown a protective affect of S100B against brain insults, such as glutamate toxicity[5] and traumatic injury.[32] In addition, it has been proposed that S100B release in acute brain injury could contribute to functional recovery and even neurogenesis.[33]

A sex effect (particularly mediated by oestrogens) on astrocyte development has been shown for specific proteins, such as GFAP and GS (for a review, see Mong and Blutstein[14]). Therefore, astrocytes are targets and mediators of the effect of sex on brain function. In support of this, prenatal exposure to betamethasone, a synthetic glucocorticoid, induced a decrease in serum and hippocampal S100B content in neonatal male rats.[34] In the present study, we demonstrated that adult rats exhibited sex-dependent differences in S100B concentrations in the CSF, but not in the serum. Therefore, it is possible that the basal (and thiopental-stimulated) rate of glial S100B secretion is dependent upon sex. The present findings reinforce the theory that sex has an effect on synaptic plasticity, as well as suggesting a sex dependence of neural communication, mediated by extracellular S100B, without restricting the influence of astrocytes on the developmental phase. Possible mediation by oestrogen hormones remains to be elucidated, as well as the brain region(s) involved.

In the present study, anaesthetics had no effect on serum S100B concentrations, suggesting that cerebral changes observed in CSF concentrations of S100B are not necessarily accompanied by detectable peripheral alterations. Recently, a discrepancy between brain and serum concentrations of S100B was described in rats subjected to traumatic brain injury.[35] These observations suggest that the passage of S100B from brain tissue to the CSF and from the CSF to the serum is subject to complex modulation involving blood–brain barrier integrity and possibly extracellular degradation beyond S100B synthesis and secretion (for a review, see, Kleindienst and Bullock[36]). Other extracerebral sources of S100B contribute to serum levels of this protein and may quench changes caused by variations in CSF concentrations. We have discussed the contribution of adipocytes as an important source of serum S100B.[28] In contrast with astrocytes, adipocytes did not appear to demonstrate differentiated S100B secretion in response to anaesthetics. Moreover, a sex dependence of serum concentrations of S100B was not observed. This could be due to the indifferent sex hormone sensitivity of adipocytes or result from a more complex arrangement; for example, adipose tissue is more abundant in females, but would have lower S100B secretion than adipose tissue from males. However, at present, there is no evidence to clarify this phenomenon.

Regardless of the cell source and the mechanisms involved in S100B release, the present findings clarify a number of issues. First, the CSF concentration of S100B in rats, in contrast with serum concentrations, is dependent on sex. Second, anaesthesia *per se* is able to affect CSF concentrations of S100B and must be considered when interpreting results. Third, the results of the present study reinforce the concept that changes in CSF concentrations of S100B are not necessarily accompanied changes in serum concentrations of S100B.

Some implications of these findings should be considered. The sex dependence of S100B concentrations in the CSF in rats must be confirmed in other species used as models of brain diseases, in addition to human patients. Moreover, this dependence was originally described in healthy human subjects,[17] but other studies have not confirmed this finding. Despite methodological differences, this issue deserves further clarification; however, most clinical studies in humans performed careful matching of age and sex in healthy subjects and patients. Possibly, in addition to investigating the sex dependence of peripheral levels of S100B in humans of all ages, it may be necessary to investigate putative changes in neuropsychiatric disorders in relation to sex.

Some limitations of the present study should also be commented on, the first being the homogeneity of the population of rats analysed, which makes the extrapolation of the results of the present study to humans difficult. Another uncertainty is the effect of anaesthetics *per se* on astrocyte activity and on the blood–brain barrier.[37] We cannot rule out any brain damage or protector effect of thiopental or other anaesthetics without evaluating other parameters. Moreover, there is evidence indicating that S100B expression in the CNS is not restricted to astrocytes.[38]

In summary, CSF concentrations of S100B are sex dependent and also vary depending on the anaesthetic used. Assuming peripheral S100B as a marker of development, glial activation or even brain damage, its sex dependence may be useful in gaining an understanding of sex variations in the behaviour and the pathological course of, as well as susceptibility to, many brain disorders.

ACKNOWLEDGEMENTS

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4. RESULTADOS – CAPÍTULO II

Manuscrito a ser submetido ao periódico *Brain Research* .

Título: *Astroglial And Cognitive Effects Of Chronic Cerebral Hypoperfusion In The Rat*

ASTROGLIAL AND COGNITIVE EFFECTS OF CHRONIC CEREBRAL HYPOPERFUSION IN THE RAT

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Abstract

The permanent occlusion of common carotid arteries (2VO), resulting in a significant reduction in cerebral blood flow (hypoperfusion) in rats, is accepted as a well established experimental model to investigate neuronal damage and cognitive impairment that occurs in human ageing and Alzheimer's disease. In the present study, we evaluated two astroglial proteins: S100B and glial fibrillary acidic protein (GFAP) in cerebral cortex and hippocampus tissue, as well S100B in cerebrospinal fluid, and glutamate uptake and glutamine synthetase activity in hippocampus tissue. Cognition, as assessed by reference and working spatial memory protocols, was also investigated. Adult male Wistar rats were submitted to 10 weeks of chronic cerebral hypoperfusion after the 2VO method. A significant increase of S100B and GFAP in hippocampus tissue was observed, as well a significant decrease in glutamate uptake. Interestingly, we observed a decrease in S100B in cerebrospinal fluid. As for cognitive function, there was an impairment of both reference and working spatial memory in the water maze. Our data so support the hypothesis that astrocytes play a crucial role in the mechanisms of neurodegenerative disorders, like Alzheimer's disease, and that hippocampal pathology arising after chronic hypoperfusion gives rise to memory deficits.

Section: Disease-Related Neuroscience

Key words: astrocytes; GFAP; hippocampus; hypoperfusion; S100B; 2-VO method

Introduction

A reduction of cerebral blood flow, as consequence of cerebral stroke and advance ageing, is a prominent risk factor of brain dysfunction. In fact, it has been proposed that a state of chronic cerebral hipoperfusion represents one of the physiopathological mechanisms of damage underlying dementia, including Alzheimer's disease (AD) [7, 22, 35]. AD is the major neurodegenerative disorder responsible for dementia in the elderly; it is clinically characterized by a progressive loss of memory and higher cognitive function [13, 18, 43]. The main neuropathological findings of this disease include amyloid plaques, neuronal loss, neurofibrillary tangles, astrogliosis and microgliosis [1]. The exact etiology of AD is not yet established, however there is evidence that neurovascular dysfunction contributes to a cognitive impairment in AD [17].

The permanent occlusion of the common carotid arteries (2VO) results in a significant reduction in cerebral blood flow (hypoperfusion) in rats, and it is accepted as a well established experimental model to investigate neuronal damage and cognitive impairment as it occurs in human ageing and AD [7, 8, 28, 36]. This cognitive impairment is accompanied by progressive loss of hippocampal pyramidal neurons, which is often observed in human ageing and dementia states [28].

Astrocytes are in close morphological and functional association with neurons and the importance of these glial cells in brain disorders has been strongly suggested (e.g. [20]). Glial activation in response to injury stimuli commonly involves changes in glial fibrillary acidic protein (GFAP), S100B levels and glutamate metabolism. GFAP is a specific astrocyte marker;

currently, tissue GFAP increase is taken as a sign of astrogliosis associated with conditions of brain injury [6, 41]. S100B is a calcium-binding protein found in brain tissue, predominantly in astrocytes. This protein has putative intra and extracellular functions. Intracellular functions include regulation of protein phosphorylation, cytoskeleton components and transcriptional factors [4, 33]. Extracellular S100B plays a trophic role on neuronal and glial cells, but elevated extracellular levels of this protein could induce apoptosis in neural cells [40]. Cerebrospinal fluid and serum S100B levels have been used as marker of brain insult [2, 41].

Moreover, astrocytes are key elements in the brain metabolism of glutamate, particularly responsible for removing glutamate from synaptic cleft and for the synthesis of glutamine, which is sent back to the neuron to renew glutamate stocks [12, 24]. Therefore, glutamate uptake and glutamine synthetase activities have also been used to characterize glial response in brain tissue.

The aim of this study was to investigate, in rats submitted to chronic cerebral hyoperfusion by the 2-VO model for 10 weeks: a) astrocyte biochemical parameters, namely GFAP, S100B, glutamate uptake and glutamine synthetase in brain tissue, particularly hippocampus and cerebral cortex. b) S100B content in CSF and astrogliosis as revealed by immunohistochemistry. c) cognitive impairment as evaluated by spatial water maze task. The working hypothesis is that chronic cerebral hypoperfusion will provoke glial alterations and cognitive impairment.

Results

Behavioral Effects

The Morris water maze was used to evaluate reference and working memory. There were no differences between sham and not-operated groups (data not shown), so the cognitive performance and biochemical parameters of hypoperfusion group, were compared to sham group, referred in results and discussion sections as control group. In the reference memory, there was a decline of average time to find the platform from day 3 on in the control group (Fig 1A) ($p < 0.01$). The latency to find the platform position was shorter in the Sham group than in the hypoperfusion group in the probe trial (Fig 1B)($t = -2.011$, $p < 0.05$). The 2VO animals spent less time in the target quadrant, as compared to control groups (Fig 1C) ($t = 2.291$, $p < 0.03$). In the working memory task, from trial 2 on there was a decline in the average time to find the platform in the Sham groups, this effect did not appear in 2-VO rats (Fig 1 D) ($p < 0.01$).

Changes in GFAP and S100B contents

A significant increase ($p < 0.02$) in GFAP immunocontent of the hippocampus was observed in rats submitted to chronic hypoperfusion by the 2VO model (Fig 2A); this effect was not found in cerebral cortex (Fig 2B). Similarly, hippocampal S100B content was higher in rats submitted to hypoperfusion (Fig 2C), however it did not reach statistical significance in cerebral cortex (Fig 2D). Interestingly, a significant decrease in CSF S100B was observed in the hypoperfusion group as compared to controls (Fig 3).

Immunohistochemistry for GFAP

In order to evaluate and confirm the hippocampal GFAP change observed in rats submitted to 2VO model we carried out a GFAP immunohistochemistry study (Fig 4 shows a representative image). The photomicrographs of astrocyte GFAP immunoreactive (ir) clearly show astrogliosis in the 2VO animals.

Marked astrocytes (black arrows) in the radiatum and lacunoso-moleculare layers (white arrows) in the hippocampus of the control animals (4A). Intense markers of astrocytes GFAP-ir (black arrow) in the 2VO group. There are more astrocytes prolongation (white arrows) in the 2VO animals when compared to the control group (4B). We also observed marked neuron nuclei in the pyramidal layer of hippocampus with hematoxylin and eosin staining (4C and 4D). There is more density of nucleus (asterisk) marked in the control group (4C) when compared to the 2VO animals (4D), what indicates neuronal loss provoked by chronic cerebral hypoperfusion.

Glutamate metabolism in the hippocampus

Glutamate uptake and glutamine synthetase activity were measured in hippocampal slices of control and 2VO rats. A significant decrease in the glutamate uptake ($p < 0.03$) was observed in 2VO rats (Fig 5A), however there was no difference in the glutamine synthetase activity between groups (Fig 5B).

Discussion

Several types of animal models have been developed to investigate the consequences of chronic reduction of cerebral blood flow [25, 28, 34]. A progressive cognitive impairment has been associated with the 2VO model, as well as neuronal loss, cholinergic dysfunction and astrogliosis in the hippocampus [3, 34]. In agreement, data presented in Figure 1 shows clear

reference and working spatial memory impairments 10 weeks after 2VO, as evaluated by Morris water maze task.

It was also shown that chronic cerebral hypoperfusion in rats by 2VO cause a significant increase in two glial proteins GFAP and S100B, and a decrease in glutamate uptake in hippocampal tissue. Interestingly, a decrease of S100B in the CSF was also demonstrated.

GFAP is commonly used as a marker of changes in astroglial cells during brain development or injury [6]. In fact, injury of the central nervous system, either as a result of trauma, disease, genetic disorders, or chemical insult, cause astrocytes to become reactive, a condition characterized by an increase in GFAP [27]. It is important to mention that astrocyte reaction is not necessarily accompanied by neuronal death and that astrocytes themselves react to reduction of cerebral blood flow through mechanisms mediated by changes in energetic metabolism, oxygen radical formation and/or cytokine production [39, 42].

Specific glial changes observed in 2VO model indicate GFAP increase, as evaluated by immunohistochemistry already in the first week after reduction of cerebral blood flow [34]. The increment was diffuse and transient in neocortex, but was not observed in hippocampus. However, GFAP increment was observed later (6 months) in the hippocampus, apparently without signals of neuronal damage. Variable hippocampal damage has been reported in 2VO model, possibly involving differences in the age, rat strains, anaesthesia and time of occlusion [7, 29, 34]. In our study, 10 weeks of chronic hypoperfusion

caused a significant GFAP increment in hippocampus measured by ELISA. Immunohistochemistry of hippocampus confirmed the astrogliosis.

S100B is a calcium binding protein predominantly expressed and secreted by astrocytes in vertebrate brain [21]. Intracellularly, S100B binds to many protein targets, possibly modulating cytoskeleton plasticity, cell proliferation and astrocyte energy metabolism [5, 40]; however high levels of brain tissue S100B have been observed in neurodegenerative disorders, including Alzheimer's disease [11]. Interestingly, S100B is able to stimulate the protein phosphatase calcineurin [15], which appears to be involved in the inflammatory activation of astrocytes in transgenic Alzheimer's models [26].

Brain trauma and acute ischemia is associated with peripheral (CSF and/or serum) S100B elevation, probably due to astrocyte S100B secretion or released from damaged cells [32]. It has been proposed that acute increments of S100B may improve neurogenesis, particularly in the hippocampus. Moreover, several studies in cell culture suggest that, from pico to nanomolar concentrations, S100B stimulates glial proliferation, neuronal survival and protects neurons against glutamate excitotoxicity [37, 40]. However, there is no information about the content of this protein in cerebrospinal fluid or brain tissue in rats submitted to chronic reduction of cerebral blood flow.

The present study showed that S100B, like GFAP, is increased in brain tissue exposed to hypoperfusion, particularly hippocampus, as observed in neurodegenerative disorders. Moreover, we found low levels of CSF S100B, which could somehow indicate a lower trophic activity of this protein in rats submitted to chronic reduction of cerebral blood flow.

Other astroglial parameters, namely glutamate uptake and glutamine synthetase activities, were investigated in the hippocampus of rats submitted to 2VO. Interestingly, we observed a decrease of glutamate uptake, indicating a higher susceptibility of these animals to excitotoxicity. In fact, an altered glutamate uptake has been proposed in Alzheimer's disease and other neurodegenerative diseases [19]; this hippocampal dysfunction could contribute to the cognitive deficit observed in these animals. However, no change was observed in GS activity. Recently, we proposed a correlation between glutamate uptake activity and S100B secretion [37] and data presented here reinforce this idea; the reduced influx of glutamate would then result in a decrease of S100B secretion.

In summary, the present study showed specific astroglial alterations in rats after 10 weeks of 2VO: hippocampal gliosis (based on GFAP and S100B contents), reduced CSF S100B and reduced hippocampal glutamate uptake. These alterations (except the decrease in CSF S100B) have been described in patients with neurodegenerative disorders, including Alzheimer's disease, and so reinforce the importance of this model to the investigation of astrocytes as targets of future therapeutic strategies. Such alterations could also be possibly involved in the cognitive effects observed in rats submitted to chronic hypoperfusion.

Experimental Procedure

Chemicals. Antibody anti-S100B (clone SH-B1), glutamate, γ-glutamylhydroxamate and fast OPD were purchased from Sigma-Aldrich. L-[2,3-^{3}H] glutamic acid and conjugated-peroxidase anti-rabbit IgG were purchased

from Amersham; Rabbit policlonal anti-S100 and anti-GFAP were obtained from DAKO.

Animals. Adult (120-days old) male Wistar rats, weighing 280 - 300 g, obtained from the Department of Biochemistry colony of the Federal University of Rio Grande do Sul, were housed with food and water *ad libitum* under a 12 h light/dark cycle at a temperature of 25 °C. Rats were divided into three groups: not-operated, sham, and 2-VO. All procedures were in accordance with the international guiding principles for biomedical research involving animals from CIOMS/WHO and were approved by the local authorities at the Federal University of Rio Grande do Sul.

*Surgery procedure***.** Rats were anesthetized with Ketamine and Xylazine (75 and 10 mg/Kg, respectively), common carotid arteries of the animals were exposed and carefully separated from carotid sheath, cervical sympathetic and vagal nerves. Both common carotid arteries were ligated with surgical silk in 2VO rats; sham-operated animals suffered no artery ligature. After the surgical procedure, rats were put on a heating pad to maintain body temperature at 37.5 ± 0.5 °C, and were kept on it until recovery from anesthesia [14]. The animals were submitted to behavioral tasks and biochemical analysis after 10 weeks of chronic hypoperfusion.

*Behavioral Procedure***.** After 10 weeks of chronic cerebral hypoperfusion, rats were submitted to behavioral testing to study reference and work memory in the Morris water maze. The maze consisted of a black circular pool with 180 cm diameter and 60 cm high filled with water (temperature around 24 ± 1 °C, depth 30 cm), situated in a room that was rich in consistently located spatial

cues. The pool was conceptually divided in four quadrants and had four points designed as starting positions (N, S, W or E). An escape platform (10 cm diameter) was placed in the middle of one of the quadrants, 1.5 cm below the water surface [23]. Two behavioral protocols, for reference and working memory, were utilized.

*Reference memory protocol***:** In this task the rats received five training days (sessions) and a probe trial in the 6th day. Each session consisted of four trials with a 15 min intertrial interval. A trial began when the rat was placed in the water at one of the four starting positions, chosen at random, facing the wall. The order of starting position varied in every trial and any given sequence was not repeated on acquisition phase days. The rats were given 60 s to located the platform; if the animal did not succeed it was gently guided to the platform and left on it for 10 s. Rats were dried and returned to their home cages after each trial. The latency to find the platform was measured in each trial and the mean latency for every training day was calculated. The probe trial consisted of a single trial, as described before, with the platform removed. Here, the latency to reach the original platform position, as well as the time spent in the target quadrant, were measured [30, 41]. Sessions were recorded by a video system to allow for post-hoc analysis.

Working memory protocol: This protocol consisted of four trial/day, during four consecutive days, with the platform changed daily. Each trial was conducted as described in the reference memory protocol, with intertrial interval of 5 min. Latency to find the platform was measured in each trial and the mean latency for each trial was calculated, allowing to observe the ability of the

animals in locating the novel position of the platform in the day [30]. After behavioral tasks, rats were anaesthetized as describe previously and decapited for biochemical analysis and Immunohistochemistry.

Quantification of S100B and GFAP. S100B content in the hippocampus and cerebrospinal fluid (CSF) was measured by ELISA [16]. 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 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 polyclonal anti-GFAP from rabbit 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 [38].

*Immunohistochemistry for GFAP***.** Rats were anesthetized using ketamine/xylazine and were perfused through the left cardiac ventricle with 20 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. The brains were sectioned (60 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 4^{o}C. After washing several times, tissue sections were incubated in a rabbit PAP-conjugated anti-rabbit IgG diluted 1:50 in PBS at room temperature for 2 h. The immunohistochemical reaction was revealed by incubating the sections in a histochemical medium that contained 0.06% 3,3-diaminobenzidine (DAB) dissolved in PBS for 10 min and then, in the same solution containing 1μM of 3% H_2O_2 per ml of DAB medium for approximately 10 min. Afterwards, the sections were rinsed in PBS, dehydrated in ethanol, cleared with xylene and covered with Entellan and coverslips. Images were viewed with a Nikon inverted microscope and images transferred to computer with a digital camera (Sound Vision Inc. Wayland, MA) [10].

Glutamate uptake. The animals were sacrificed 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 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 [9]. 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.

Glutamine synthetase (GS) activity. The enzymatic assay was performed, as described previously [9]. Briefly, homogenized tissue samples were added to a reaction mixture containing (in mM): 10 $MgCl_2$; 50 L-glutamate; 100 imidazole-HCl buffer (pH 7.4); 10 2-mercaptoethanol; 50 hydroxylamine-HCl; 10 ATP and incubated for 15 min at 37ºC. The reaction was stopped by the addition of 0.4 mL of a solution containing: 370 mM ferric chloride; 670 mM HCl; 200 mM trichloroacetic acid. After centrifugation, the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate treated with ferric chloride reagent.

Protein measurement. Protein was measured by Lowry's method [31] using bovine serum albumin as a standard.

Statistical Analysis. Parametric data are reported as mean ± SEM and were analyzed by Student's *t* test (when two groups were considered) or by repeated measures ANOVA. Values of $p< 0.05$ were considered significant.

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Figure legends

Fig 1. ***Cognitive alterations in of rats submitted to chronic cerebral hypoperfusion evaluated by water maze task*****. (A)** Performance in the reference memory protocol. Each line represents the mean ± standard error. * Significant difference were detected from day 3 on when compared to control group (N= 8, repeated-measures ANOVA, $p < 0.05$); **(B)** Probe trial of the reference memory protocol measured by latency (in s) to find the platform position. Values are mean ± standard error. * Significant different from control group (N= 8, Student's *t* test, $p < 0.05$); **(C)** Probe trial of the reference memory protocol measured by the time spent in the target quadrant (in s). Values are mean ± standard error. * Significant different from control group (N= 8, Student's *t* test, $p < 0.05$); **(D)** Performance in the working memory protocol. Each line represents the mean ± standard error. * Significant difference were detected from trial 2 on when compared to control group (N= 8, repeated-measures ANOVA, $p < 0.05$).

Fig 2. ***GFAP and S100B content in hippocampus and cerebrax cortex of rats submitted to chronic cerebral hypoperfusion*****.** Adults rats were submitted to hypoperfusion for 10 weeks. Hippocampi and cerebral cortex were dissected out and the contents of GFAP (panels A and B, respectively) and S100B (panels C and D, respectively) were measured by ELISA. Values are mean ± standard error of 6-8 rats in each group. * Significantly different from control (Student´s *t* test, $p < 0.05$).

Fig 3*. S100B levels in the cerebrospinal fluid of rats submitted to chronic cerebral hypoperfusion*. Adults rats were submitted to hypoperfusion for 10 weeks. Cerebrospinal fluid (CSF) was collected by cisterna magna puncture and the content of S100B was measured by ELISA. Values are mean ± standard error of 6-8 rats in each group. * Significantly different from control (Student´s *t* test, $p < 0.05$).

Fig 4. *Immunohistochemistry for GFAP and histological analysis of the hippocampi from rats submitted to chronic cerebral hypoperfusion*. Photomicrographs showing GFAP immunoreactive cells (ir) in the hippocampus of control (panel A) and hypoperfusion (panel B) groups. Notice the difference in GFAP-ir in the lacunosum moleculare (white arrows) and radiatum layers of CA1 regions (black arrow-heads) between groups. Hippocampal sections show a higher density of hematoxylin-eosin stained nuclei of pyramidal layers (indicated by an asterisk) in control rats (panel C) than rats submitted to chronic hypoperfusion (panel D). Magnification 100x, scale bar = 150 μm.

Fig 5. *Glutamate uptake and glutamine synthetase activity in the hippocampus of rats submitted to chronic cerebral hypoperfusion.* Adult rats were submitted to hypoperfusion for 10 weeks. Hippocampi were dissected out and chopped into 0.3 mm slices for measurement of glutamate uptake (panel A) or homogenized for measurement of glutamine synthetase activity (in panel B). Values are mean ± standard error of 6-8 rats in each group. * Significantly different from controls (Student´s *t* test, $p < 0.05$).

Figure 1

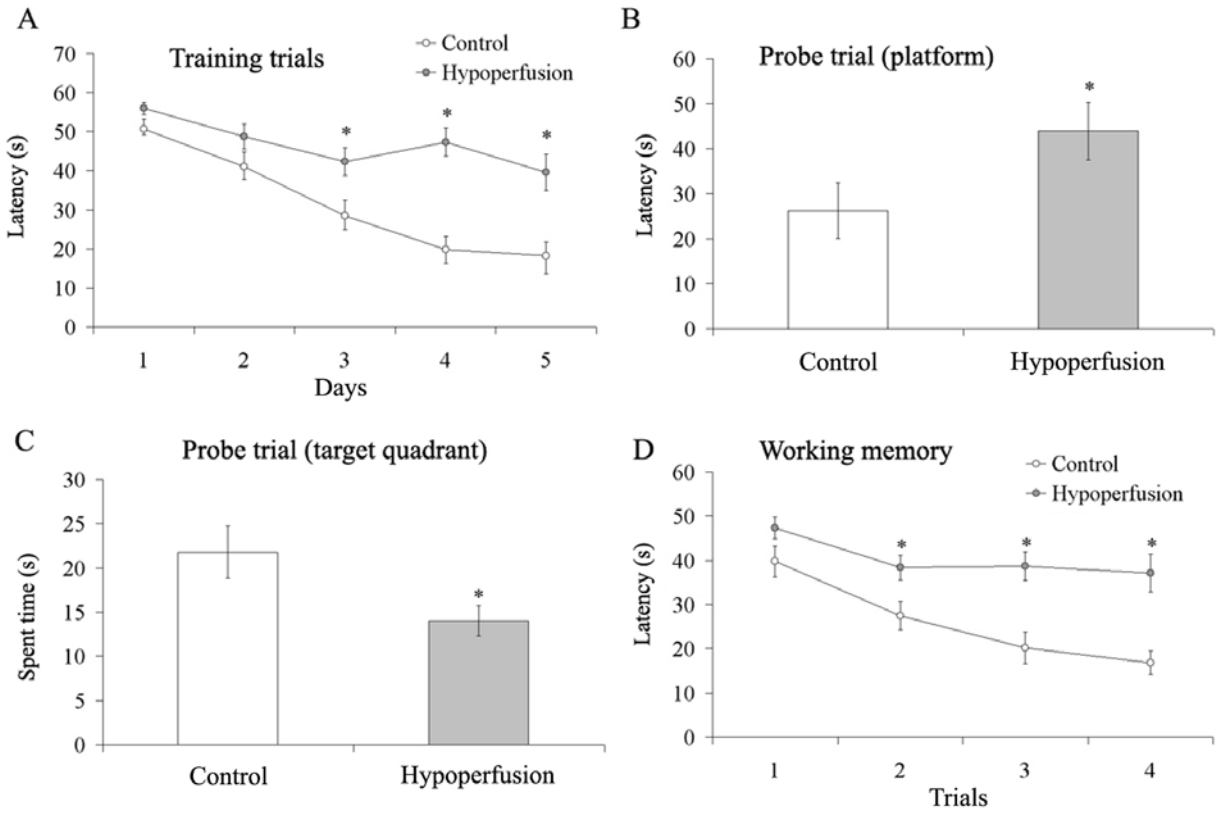

A
Training trials
Control
Hypoperfusion
Latency (s)
Days
B
Probe trial (platform)
Latency (s)
Control
Hypoperfusion
C
Probe trial (target quadrant)
Spent time (s)
Control
Hypoperfusion
D
Working memory
Control
Hypoperfusion
Latency (s)
Trials

Figure 2

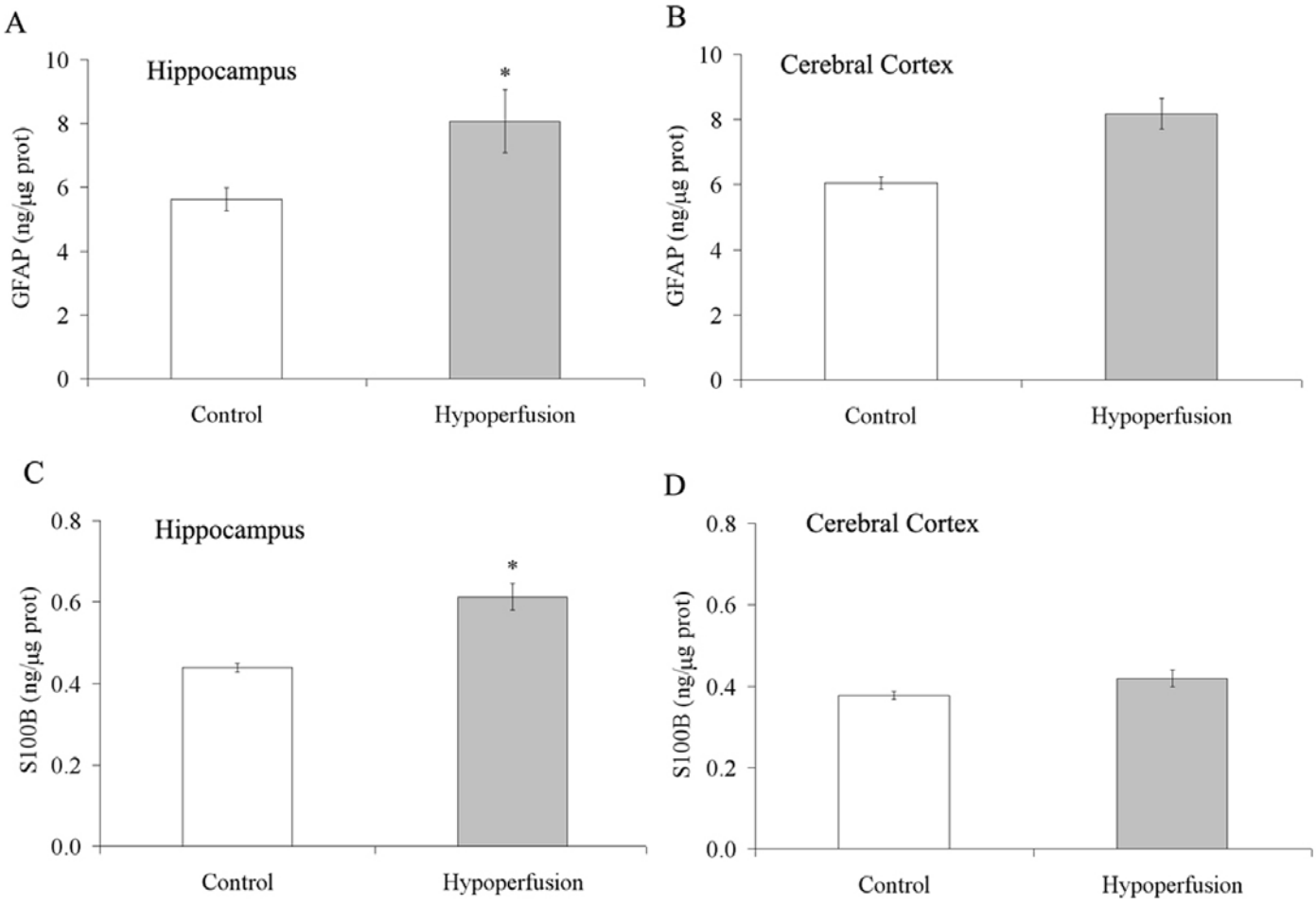

A
Hippocampus
GFAP (ng/µg prot)
10
8
6
4
2
0
*
Control
Hypoperfusion
B
Cerebral Cortex
GFAP (ng/µg prot)
10
8
6
4
2
0
Control
Hypoperfusion
C
Hippocampus
S100B (ng/µg prot)
0.8
0.6
0.4
0.2
0.0
*
Control
Hypoperfusion
D
Cerebral Cortex
S100B (ng/µg prot)
0.8
0.6
0.4
0.2
0.0
Control
Hypoperfusion

Figure 3

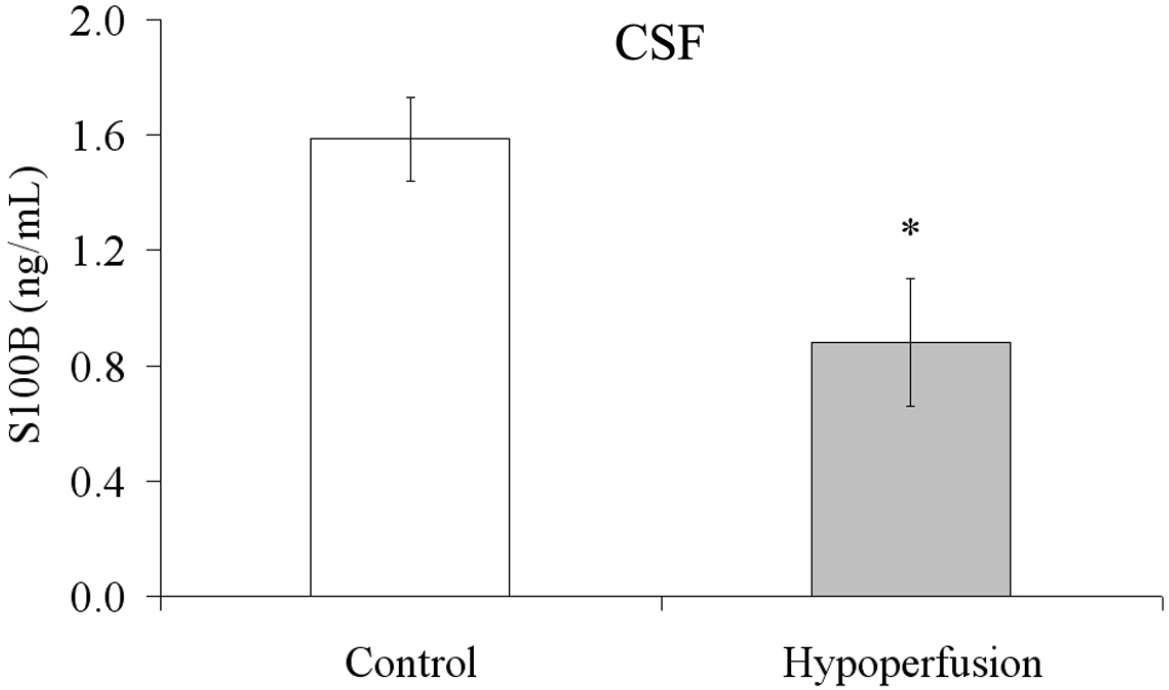


Figure 4

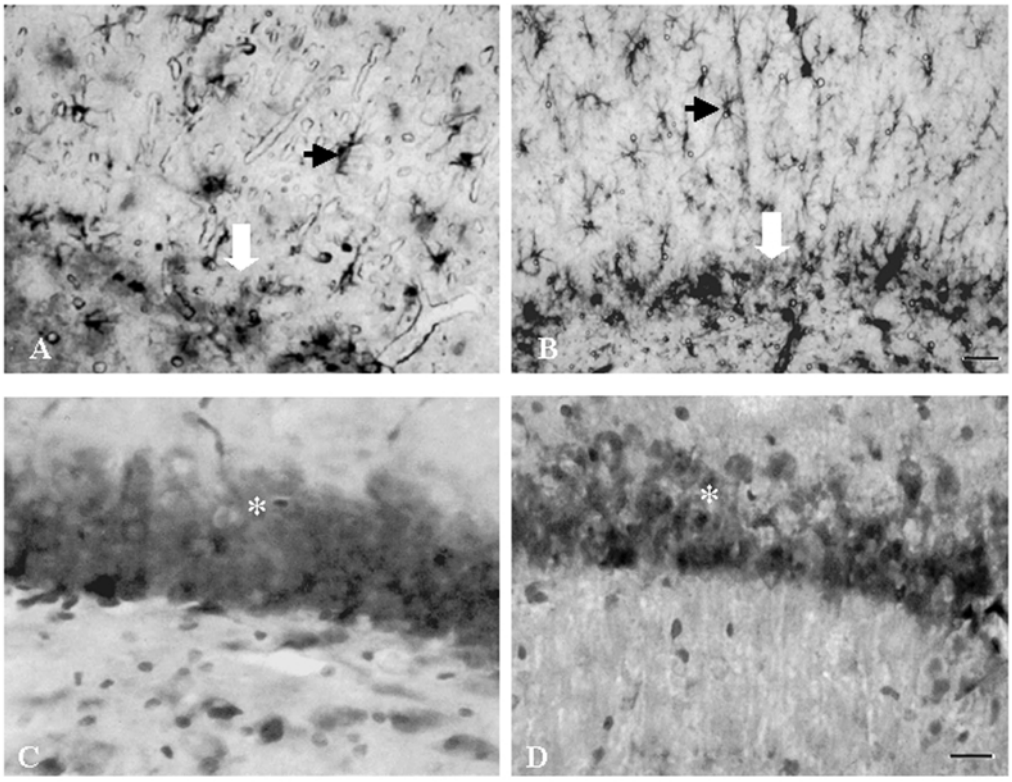
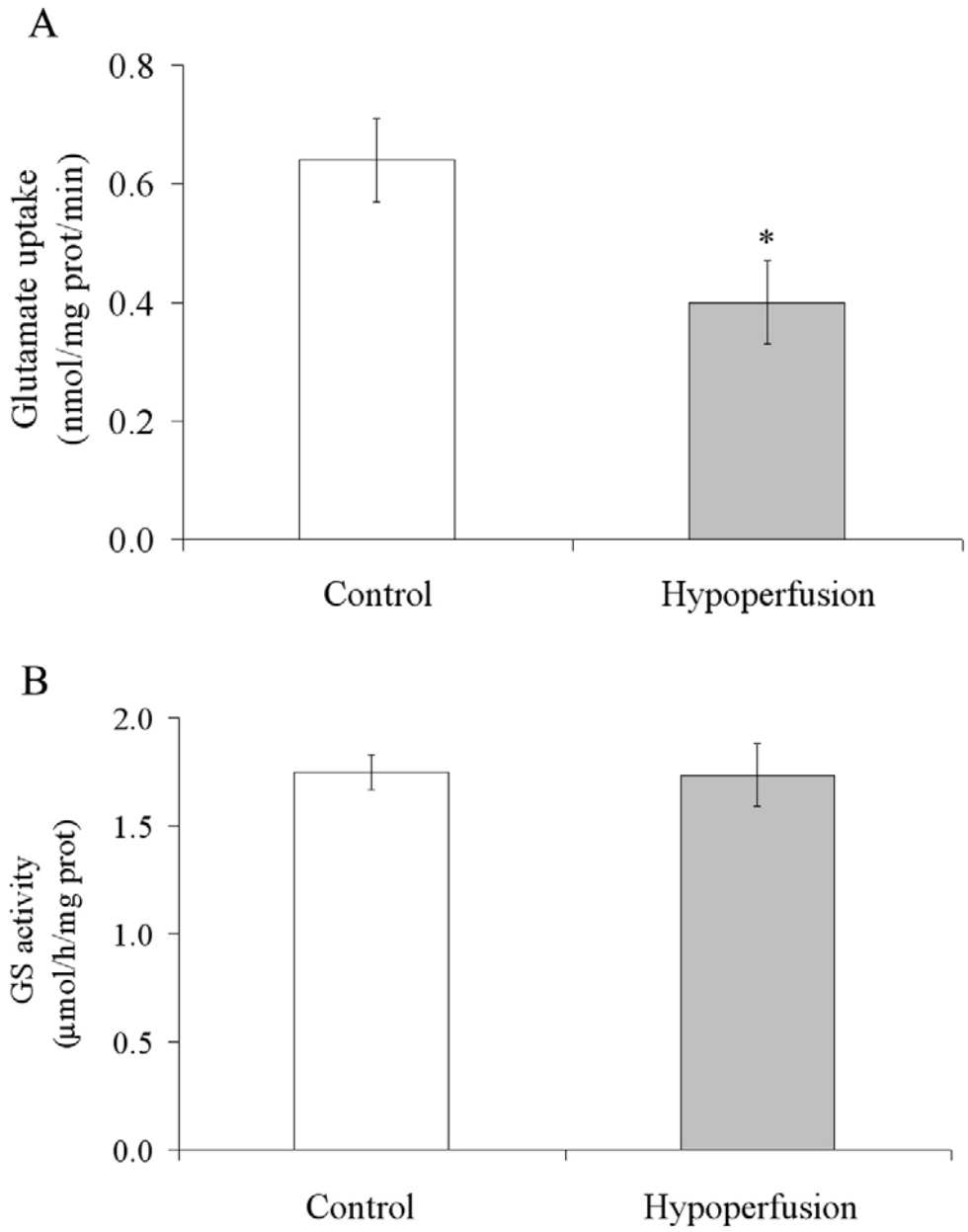


Figure 5


A
Glutamate uptake (nmol/mg prot/min)
0.8
0.6
0.4
0.2
0.0
*
Control
Hypoperfusion
B
GS activity (µmol/h/mg prot)
2.0
1.5
1.0
0.5
0.0
Control
Hypoperfusion

5. DISCUSSÃO

5.1 Sumário dos resultados

Os resultados, neuroquímicos e comportamental, encontrados nos ratos submetidos à hipoperfusão cerebral crônica por 10 semanas podem ser resumidos na tabela abaixo.

Hipoperfusão Crônica	**Efeito**
Comportamento (Labirinto Aquático de Morris)	Déficit cognitivo
Conteúdo de S100B no córtex frontal e hipocampo	Aumento em hipocampo
Conteúdo de S100B no líquor	Redução
Conteúdo de GFAP no córtex frontal e hipocampo	Aumento em hipocampo
Imunoistoquímica para GFAP no hipocampo	Astrogliose
Captação de glutamato hipocampal	Redução
Atividade da glutamina sintetase no hipocampo	Inalterada

Os efeitos da hipoperfusão cerebral crônica, que aumentam com a idade, induzem a mudanças metabólicas em várias regiões do encéfalo (Farkas et al, 2004; Yanpallewar et al, 2004). Embora descrito originalmente como um modelo de demência vascular, a hipoperfusão cerebral crônica induzida pela ligação das duas carótidas comuns (2VO) é relevante para a DA, pois

desenvolve uma lenta e progressiva redução do CBF acompanhadas de mudanças metabólicas semelhante ao que ocorre nesta patologia (Weinstock & Shoam, 2004). O modelo 2VO causa uma redução do CBF entre 70 a 80% do nível normal, podendo desencadear fatores que acelerem a morte de neurônios e levar a progressão da demência (Ritchie et al, 2004). Com 9 semanas de hipoperfusão cerebral crônica observa-se danos em células piramidais do hipocampo, mais especificamente em CA1 (Weinstock & Shoham, 2004), o que justifica o déficit cognitivo apresentado nos ratos submetidos ao modelo 2VO. O mecanismo exato dos efeitos da redução do fluxo sangüíneo encefálico ainda é desconhecido, porém aminoácidos excitatórios, o cálcio, radicais livres, óxido nítrico e o sistema inflamatório parecem ser importantes para o entendimento desse mecanismo (Takita et al, 2004).

Em nosso estudo encontramos um aumento significativo do imunoconteúdo de S100B em hipocampo. No córtex cerebral o aumento não foi significativo. Além disso, encontramos uma redução dos níveis de S100B no líquido cérebro-espinhal. Uma importante redução da captação de glutamato foi observada no hipocampo. Com relação ao comportamento cognitivo, observamos uma diminuição significativa tanto na memória de referência como na de trabalho nos animais submetidos à hipoperfusão, confirmando o modelo de demência. Os significados deste achados serão discutidos a seguir. Entretanto, discutiremos inicialmente os resultados do primeiro artigo.

5.2 Anestésicos e S100B extracelular

A função da proteína S100B no meio extracelular, em culturas neurais, é dependente da sua concentração, sendo neurotrófica em concentrações nanomolares e neurotóxica em concentrações micromolares (Gonçalves et al, 2000; Tramontina et al, 2006; Ahlemeyer et al, 2000; Van Eldik & Wainwright, 2003). Além disso, tem sido considerada uma proteína marcadora de danos no SNC. De fato, o aumento da proteína S100B no soro e no líquido cérebro-espinhal tem sido observado em muitas patologias psiquiátricas e neurodegenerativas (Heizmann, 1999; Steiner et al, 2007)

Um dos aspectos vistos nesse estudo foi que a concentração de S100B no líquor é sensível ao tipo de anestésico utilizado em ratos, sendo que com a cetamina temos menores níveis de S100B no líquido cérebro-espinhal quando comparado, principalmente, com o tiopental. Os ratos anestesiados com tiopental apresentaram um aumento do imunoconteúdo de S100B no líquor e também em culturas expostas a este anestésico. Cabe salientar que em um outro estudo foi observado também uma redução da captação de glutamato em células astrocíticas corticais tratadas com tiopental (Qu et al, 1999). Esse aumento da S100B e redução da captação de glutamato podem estar inter-relacionados e associados à função de proteção dos astrócitos às injúrias do SNC, numa tentativa de evitar a morte neuronal.

Um outro resultado observado foi que a proteína S100B no líquor é sexo-dependente. Ou seja, ratos adultos apresentaram diferença dependente do sexo no conteúdo de S100B no líquor. Essa diferença não foi encontrada no

soro. Esse achado sugere a possibilidade de existir uma diferença de genero com relação ao conteúdo da S100B no líquor.

Com relação ao soro, não houve diferença no conteúdo de S100B. Uma hipótese é que a origem da S100B no soro não é apenas do SNC, mas de outros tecidos, como por exemplo, tecido adiposo, portanto nem sempre um dano no SNC é acompanhado de variação no conteúdo da S100B no soro e vice-versa.

Outro fato importante, é que a anestesia por si só causa uma alteração do conteúdo de S100B no líquor, o que sugere uma atenção maior ao tipo de anestésico a ser usado nos experimentos.

5.3 S100B extracelular e comportamento

A oclusão bilateral e permanente das artérias carótidas comuns em ratos tem mostrado um progressivo déficit cognitivo devido à dificuldade na retenção da memória nas tarefas, por exemplo, do braço radial e labirinto de Morris (Ni et al, 1994; Yamada et al, 2000; Sarti et al, 2002). O hipocampo, uma estrutura particularmente associada com a memória e aprendizado, é altamente vulnerável durante eventos isquêmicos encefálicos (Zhou et al, 2007).

A S100B é uma proteína marcadora que reflete as injúrias do SNC (Rothermundt et al, 2003; Bloomfield et al, 2007). Uma específica função desta proteína tem sido proposta no desenvolvimento da plasticidade e processos celulares envolvidos na memória e aprendizagem. Camundongos transgênicos com um aumento da produção de S100B apresentaram déficit cognitivo. Os autores sugerem que a S100B possa acelerar o desenvolvimento hipocampal,

devido ao aumento da densidade dos dendritos hipocampais nos estágios iniciais do desenvolvimento. Por outro lado, elevações crônicas desta proteína têm sido relacionadas ao envelhecimento e perda de dendritos (Kleindienst & Bullock, 2006).

Outros estudos envolvendo animais transgênicos, sugerem a correlação entre a expressão de proteínas ligantes de cálcio (calmodulina, S100) e o aprendizado e a memória. Camundongos super-expressando a proteína S100B tiveram aprendizagem reduzida em tarefas comportamentais, como o Labirinto Aquático de Morris. Além disso, apresentaram uma redução da potenciação de longa duração (LTP – do inglês "Long-term potentiation) (Gerlai et al, 1995). Nishiyama e colaboradores (2002), utilizando camundongos nocaute para S100B, observaram que estes tinham a LTP e aprendizado aumentados. Diversos outros trabalhos, em animais não transgênicos, têm relacionado o conteúdo extracelular de S100B e comportamento, utilizando anticorpos para bloquear a ação da S100B ou injetando a proteína no tecido cerebral (O'Dowd et al, 1997; Mello e Souza et al, 2000).

O resultado encontrado no líquido cérebro-espinhal indica uma alteração na concentração extracelular da proteína S100B nos animais submetidos a hipoperfusão. Essa mudança poderia interferir na sua função extracelular e contribuir, de alguma forma, para o déficit cognitivo encontrado nesses animais. Curiosamente, nos pacientes com DA tem se observado um aumento da proteína S100B no líquor no início do quadro demencial, mas não nas fases mais avançadas (Petzold et al, 2003).

5.4 Aumento de S100B, astrogliose e alterações intracelulares

A hipoperfusão por 10 semanas causou um aumento significativo de GFAP no hipocampo, caracterizando nesta região uma astrogliose (Eng et al, 2000), confirmada pela análise imunoistoquímica. A astrogliose associada à hipoperfusão tem sido observada em outros trabalhos (Farkas et al, 2004; Weinstock & Shoham, 2004).

A ativação glial, estimulada pela redução do fluxo sanguíneo cerebral, é apontada como um fator para o progressivo dano neuronal na demência vascular e DA (Schubert et al, 2000). A hipótese é de que os astrócitos, nas injúrias do SNC, podem perder a capacidade de manter a homeostase iônica extracelular favorecendo danos excitotóxicos (Schubert et al, 2001). Além disso, aumento da resposta inflamatória, também por diminuição do CBF, deixa o tecido neural mais suscetível à subsequentes injúrias (Koistinaho & Koistinaho, 2005).

Embora a S100B seja menos astrócito-específica que a GFAP a variação em seu conteúdo (aumento ou redução) tem sido observada em situações de astrogliose (Vicente et al, 2004; Feoli et al, 2008). Neste caso específico observamos um aumento da S100B e algumas possíveis conseqüências poderiam ser discutidas em função da diversidade de supostos alvos intracelulares, tais como a própria GFAP, a proteína fosfatase calcineurina e a proteína p53 envolvida na modulação do ciclo celular.

A GFAP tem sua polimerização e fosforilação modulada pela proteína S100B (Ziegler et al, 1998; Donato, 2001; Frizzo et al, 2004). Comumente a fosforilação da GFAP é associada à despolimerização dos filamentos

intermediários gliais e pode ser induzida por diversas cinases, incluindo PKA (cinase dependente de AMPc), PKC (cinase dependente de Ca^{2+} e fosfolipídeo) e CaMK II (cinase dependente de Ca^{2+}-calmodulina, tipo II). A proteína S100B pode tanto se ligar à GFAP e impedir a sua polimerização (Donato, 2001), quanto pode se ligar à GFAP e impedir sua fosforilação (i.e. sua despolimerização) (Ziegler et al, 1998). Embora isso pareça contraditório, na verdade, pode indicar que a S100B atue em dois momentos distintos do ciclo de polimerização/despolimerização dos filamentos intermediários gliais (Karl et al, 2004). Embora haja um aumento de ambas as proteínas (GFAP e S100B) é possível que haja um deslocamento no sentido de uma maior despolimerização, favorável às mudanças plásticas associadas a proliferação glial.

É importante salientar que num trabalho com culturas de astrócitos expostas a um meio com alta glicose (12 mM) foram observadas redução da proliferação e redução do conteúdo intracelular de S100B (Nardin et al, 2007). Além disso, tem sido sugerido que o decréscimo de S100B intracelular poderia desfavorecer a proliferação, decorrente da interação da proteína S100B com a proteína p53, a proteína supressora de tumores (Baudier et al, 1992; Scotto et al, 1999). Por outro lado, um acúmulo de S100B nuclear poderia estimular a proliferação glial observada na astrogliose ou em melanomas, que tipicamente expressam altos níveis da proteína S100B (Harpio & Einarsson, 2004). Entretanto, outros dados na literatura contestam uma relação direta entre S100B e p53 e deixa esta possibilidade como uma especulação a ser investigada.

Outra possibilidade de mudança astroglial em função do aumento de S100B poderia ser mediada pela calcineurina. Os astrócitos reativos observados em modelos transgênicos e senis de doenças neurodegenerativas apresentam um aumento da atividade de calcineurina (Norris et al, 2005). Curiosamente isso não se deve a um aumento da expressão desta proteína, mas talvez do cálcio intracelular. Há 4 anos foi demonstrado que a atividade da calcineurina pode ser estimulada pela S100B, de uma maneira similar como faz a calmodulina (Leal et al, 2004). Isso permite supor que a ativação astroglial da calcineurina, que favoreceria ao fenótipo observado em doenças neurodegenerativas, poderia estar associada a uma mudança envolvendo inicialmente um aumento intracelular de S100B.

É interessante também notar que os níveis intracelulares de S100B aumentam nos astrócitos nas placas neuríticas, nas fases iniciais e intermediárias. Por outro lado, o aumento não é observado nas fases mais avançadas (Sheng et al, 1996). Isso talvez possa ser relacionado com as mudanças observadas no líquor de pacientes que tem um aumento apenas nas fases iniciais da DA (Peskind et al, 2001). Entretanto, neste momento não podemos estabelecer uma relação entre o aumento intracelular (observado no hipocampo) e o decréscimo no líquor observado no modelo de hipoperfusão. Além disso, é importante assinalar que o conteúdo intracelular aumentado não necessariamente reflete um aumento na secreção da proteína e conseqüentemente um aumento no conteúdo extracelular (Nardin et al, 2007).

5.5 Astrócitos, metabolismo do glutamato e demência

Os astrócitos no SNC, como já foi dito, desempenham diversas funções, incluindo o suporte metabólico neuronal, a remoção de neurotransmissores da fenda sináptica (particularmente glutamato), defesa antioxidante e secreção de fatores neurotróficos. Nesse contexto, muitas destas atividades podem ser, didaticamente, vistas a partir da captação de glutamato. Além disso, há vários indicadores para o envolvimento do mecanismo neuroexcitatório nos danos neuronais nas injúrias do SNC (Diemer et al, 1993).

A captação de glutamato astroglial é responsável por mais de 90% da remoção desse transmissor da fenda, envolvendo basicamente dois transportadores de glutamato GLAST e GLT-1, dependentes de um co-transporte de Na+ (Had-Aissouni et al, 2002). Os transportadores gliais de glutamato têm papel crucial em manter a concentração extracelular de glutamato abaixo dos valores neurotóxicos (Phillis et al, 2000). Portanto, de acordo com Magistretti (2006), a atividade de captação do glutamato na fenda, carrega para dentro muito Na+, que por sua vez exige uma exportação dependente da bomba da Na+/K+-ATPase. Ou seja, o astrócito (particularmente em sinapses glutamatérgicas) usa muito da sua energia para remover glutamato da fenda. Alguns dados na literatura têm permitido supor que a captação de glutamato seja deficitária em muitas doenças neurodegenerativas, tanto por redução do número de transportadores ou pela redução da atividade desses transportadores (Maragakis & Rothstein, 2004;

Dabir et al, 2006). Essa redução tornaria determinados neurônios, mais suscetíveis aos danos excitotóxicos.

No modelo de hipoperfusão, encontramos uma redução na atividade de captação de glutamato em fatias hipocampais, favorecendo essa idéia de uma maior suscetibilidade, mas os mecanismos envolvidos (redução do número de transportadores ou sua atividade) demandam uma investigação posterior. Esse resultado assemelha-se com o estudo de Moretto e colaboradores (2005), onde ratos jovens de 3 a 5 dias de hipóxia-isquemia apresentaram diminuição da captação de glutamato em hipocampo. Entretanto, sabe-se que os transportadores de glutamato são bastante suscetíveis ao dano oxidativo e poderíamos especular que a redução poderia ser devida ao aumento de radicais livres observados na hipoperfusão. Cabe também salientar, que o glutamato captado contribui para manutenção da defesa antioxidante no SNC.

O glutamato captado tem muitos destinos: síntese de glutamina, degradação no ciclo de Krebs, síntese de glutationa e troca por cistina, através de um trocador específico (Had-Aissouni et al, 2002). A síntese de glutationa, a molécula antioxidante mais abundante nos astrócitos, depende do glutamato diretamente e indiretamente para captação da cistina. Portanto, a redução da atividade de captação observada no modelo de hipoperfusão poderia ser causada por e causar estresse oxidativo.

Repare que o metabolismo energético, a captação de glutamato e a defesa antioxidante estão intimamente relacionadas e dão suporte às idéias de que uma disfunção energética pode estar na base de muitas doenças neurodegenerativas, incluindo a DA (Had-Aissouni et al, 2002).

Mais recentemente, o nosso grupo propôs que a secreção de S100B pode estar relacionada ao transporte de glutamato (Tramontina et al, 2006). Observamos que elevados níveis de glutamato, em culturas primárias de astrócitos (com captação normal de glutamato), reduzem a secreção de S100B. Esse mecanismo envolve uma redução de AMPc (Gonçalves et al, 2002). Podemos pensar que o influxo de glutamato cause uma redução da secreção de S100B. Entretanto, em situações em que o influxo de glutamato está diminuído (e.g. na hipoperfusão encefálica), a secreção poderia estar aumentada. Não sabemos se, de fato, no hipocampo, onde há uma redução da captação de glutamato, há também um aumento da secreção basal de S100B. Este assunto também merece uma investigação posterior, usando fatias hipocampais (P Nardin, dados não publicados). Por outro lado, essa possibilidade contraria os níveis diminuídos de S100B encontrados no líquor. Obviamante, níveis de S100B no líquor são resultantes de uma média ponderada dos diversos sítios de secreção astroglial, de possíveis outras fontes não-astrogliais e da degradação extracelular, que poderia estar aumentada em doenças neurodegenerativas (Castillo et al, 2004).

5.6 Considerações Finais

As alterações astrogliais apresentadas nesse estudo, com exceção da redução da S100B no líquor, têm sido descritas em pacientes com doenças neurodegenerativas, incluindo a DA. Estas alterações reforçam a importância desse modelo experimental para investigar os astrócitos como alvos de futuras estratégias terapêuticas. As alterações observadas (incluindo a redução de

S100B no líquor) poderiam também estar, de alguma maneira, contribuindo para o déficit cognitivo observado nesses animais submetidos a hipoperfusão crônica.

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