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Avaliação da neuroinflamação e da atividade astrocitária em modelo de epilepsia por Li-pilocarpina: S100B possível marcador e alvo farmacológico

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"O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso, existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis."

— Fernando Pessoa

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SUMÁRIO

RESUMO

A epilepsia do lobo temporal (ELT) é a um dos casos mais frequente epilepsia em humanos e de maior refratariedade nos pacientes. A maioria dos fármacos anti-epilépticos são moduladores da atividade neuronal e atuam sobre canais iônicos do receptor $GABA_A$. Estudos vêm demonstrando o papel das células gliais e da neuroinflamação na epileptogênese e a modulação desta resposta pode ser um alvo potencial para drogas adjuvantes aos fármacos anti-epilépticos. Astrócitos são células gliais participantes da sinapse tripartite, moduladores da atividade neuronal. Os astrócitos são capazes de promover a homeostase de íons e de neurotransmissores, são responsáveis pelo metabolismo energético e da produção de fatores neurotróficos, glutationa, glutamina, S100B e citocinas. Neste trabalho, induzimos status epilepticus (SE) em ratos jovens (PN28) através do modelo lítio-pilocarpina que mimetiza alterações neuronais, bioquímicas e morfológicas similares à ELT em humanos. Os animais foram divididos nos tempos 1, 14 e 56 dias após a indução de status epilepticus (SE). Estes períodos são caracterizados respectivamente como a fase aguda, latente e crônica da epilepsia. Inicialmente, analisamos as mudanças neuroquímicas e astrocitárias ao longo do tempo. Foi observada neuroinflamação inicial e transitória que promove morte neuronal e mudanças ao longo do tempo de astrogliose e disfunção astrocitária. Também foi observado que a proteína S100B, proteína ligante de cálcio, predominantemente astrocitária, pode ser considerado um marcador da disfunção neuronal e astrocitária promovida neste modelo de epilepsia. Em seguida, demonstramos que a modulação da secreção de S100B pelo anti-inflamatório dexametasona um dia após indução de *SE* reverte a neuroinflamação, astrogliose e disfunção astrocitária à curto e à longo prazo. Por conseguinte, observamos que a modulação do receptor $GABA_A$ através de agonistas e antagonistas GABAérgicos altera a secreção de S100B em fatias hipocampais agudas e em cultura de astrócitos. Portanto, pode-se sugerir que as alterações astrogliais e a neuroinflamação dependentes do tempo podem estar ligadas à excitabilidade neuronal e/ou à morte neuronal em ratos jovens em modelo de epilepsia; que a proteína S100B pode ser considerada um marcador deste modelo de epilepsia e que a modulação da sua secreção pode ser um possível alvo farmacológico no tratamento da epilepsia.

Palavras chaves: epilepsia, pilocarpina, neuroinflamação, astrócitos, S100B.

ABSTRACT

Temporal lobe epilepsy (TLE) is the most frequent type of epilepsy in humans and is more associated to refractory to anti-epileptic drugs (AED) in patients. The most AEDs are modulators of neuronal activity and act on ion channels, such as $GABA_A$ receptor. Studies have been demonstrating the role of glial cells and neuroinflammation in epileptogenesis. The modulation of this response may be a potential target for adjunctive drugs to anti-epileptic drugs. Astrocytes are glial cells that participated in the tripartite synapse and modulated neuronal activity. Astrocytes are able to promote homeostasis of ions and neurotransmitters, are responsible for energy metabolism and the production of neurotrophic factors, glutathione, glutamine, S100B and cytokines. In this work, we induced status epilepticus (SE) in young rats (PN28) through the lithium-pilocarpine model that mimics neuronal, biochemical and morphological alterations similar to ELT in humans. The animals were divided at times 1, 14 and 56 days after the induction of SE. These periods are characterized respectively as the acute, latent and chronic phase of epilepsy. Initially, we analyzed neurochemical and astrocytic changes over time. Initial and transient neuroinflammation was observed and promoted over time neuronal death, astrogliosis and astrocytic dysfunction. It has also been observed that the protein S100B, a calcium-binding protein, predominantly astrocytic, can be considered a marker of neuronal and astrocytic dysfunction promoted by this model of epilepsy. Next, we demonstrate that the modulation of S100B secretion by the anti-inflammatory dexamethasone one day after *SE* induction reverses neuroinflammation, astrogliosis and astrocytic dysfunction in the acute and chronic time. Therefore, we analyzed that modulation of the $GABA_A$ receptor through GABAergics agonists and antagonists alters the secretion of S100B in acute hippocampal slices and in astrocyte culture. Therefore, it may be suggested that astroglial changes and time dependent neuroinflammation may be related to neuronal excitability and/or neuronal death in young rats in this epilepsy model; that S100B protein can be considered a marker of this epilepsy model and that the modulation of its secretion may be a possible pharmacological target in the treatment of epilepsy.

Key words: epilepsy, pilocarpine, neuroinflammation, astrocytes, S100B.

LISTA DE ABREVIATURAS

AQP4: canal aquaporina 4

βOHB: β-hidroxibutirato

COX1: ciclooxigenase 1

COX2: 2 ciclooxiginase 2

DAMP: proteína associada à dano celular

DEXA: dexametasona

ELT: epilepsia de lobo temporal

FJC: Fluoro-Jade C

GABA: aminoácido γ-aminobutírico

GFAP: proteína glial fibrilar ácida

GS: glutamina sintetase

GSH: glutationa

HMGB1: Proteína de grupo de alta mobilidade 1

IL-1β: interleucina 1 beta

Kir 4.1: canal retificador do íon potássio 4.1

LiCl: cloreto de lítio

LCR: líquido cefalorraquidiano

MG: metilglioxal

PGE2: prostaglandina E2

SE: Status epilepticus

SNC: sistema nervoso central

TNF-α: fator de necrose tumoral alfa

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INTRODUÇÃO

1 Epilepsia

Epilepsias são um conjunto de desordens neurológicas que acometem cerca de 1% da população mundial (OMS, 2017). A epilepsia pode afetar indivíduos de várias idades, entretanto sua maior incidência é em crianças e idosos (Sander, 2003). Por ser crônica, promove alta morbidade e mortalidade na população (Banerjee et al., 2008). As epilepsias são definidas por recorrência de crises epilépticas, isto é, duas ou mais crises epilépticas não induzidas (espontâneas) no período de um ano, resultantes da atividade excessiva, anormal e hipersincrônica de neurônios do cérebro (Banerjee et al., 2008; Dalby & Mody, 2001; Dichter, 1994; Fisher, 2017).

As epilepsias podem ser classificadas em focal e generalizadas. A epilepsia focal é originada numa região específica e afeta a rede neuronal de apenas uma região cerebral. Enquanto que a generalizada é originada em uma área específica e rapidamente altera a rede neuronal dos dois hemisférios (Berg & Scheffer, 2011; Fisher, 2017).

As causas do desenvolvimento das epilepsias ainda não são completamente esclarecidas. Acredita-se que as epilepsias possam ser um sintoma de diversas doenças neurológicas ou de alterações do metabolismo neuronal e do Sistema Nervoso Central (SNC) (Fisher et al., 2005). A sua etiologia atualmente é classificada em (ILAE, 2017): 1) idiopática, 2) sintomática, 3) epilepsia provocada e 4) de causas desconhecidas (Scheffer et al., 2017; Shorvon, 2011).

1) Idiopática: epilepsia de origem genética, sem alterações neuroanatômica ou neuropatológica.

2) Sintomática: epilepsias de causas adquiridas (esclerose hipocampal, problemas de saúde perinatais e infantis, traumatismo cerebral, doenças neurodegenerativas, infecção cerebral, etc) ou genéticas (síndromes de epilepsia infantil, como anomalias do desenvolvimento da estrutura cerebral como doenças congênitas, etc). Causas que promovem alterações neuroanatômicas e neuropatológicas.

3) Epilepsia provocada: epilepsia em que um fator ambiental ou sistêmico promove crises epilépticas e alterações neuroanatômica ou neuropatológica.

4) Causa desconhecida: natureza da causa da epilepsia é desconhecida, podendo ter origem adquirida ou genética. O número de casos desta epilepsia vem reduzindo, provavelmente pela maior quantidade de estudos e melhor diagnóstico.

Cerca de metade dos pacientes epiléticos desenvolvem epilepsias como resposta a danos encefálicos, como traumatismo craniano, acidente vascular encefálico e outros estímulos ambientais deletérios (Englander et al., 2014).

Cabe ressaltar que em outro estudo as causas da epilepsia são classificadas em: genética, estrutural/metabólica e causa desconhecida (Berg & Scheffer, 2011).

1.1 Epilepsia de lobo temporal

A epilepsia do lobo temporal (ELT) é o tipo mais frequente de casos de epilepsia em humanos e compromete a atividade de regiões do sistema límbico como hipocampo, amígdala e córtex entorrinal (Engel, 1996, 2011). Tanto em pacientes quanto em modelos animais, a desordem neuronal é causada por um desequilíbrio na sinalização excitatória e inibitória dos neurotransmissores. Na fisiopatologia desta epilepsia é comum a morte de neurônios e interneurônios no hipocampo como lesão primária, a qual antecede a manifestação dos sintomas neurológicos. Após a lesão inicia-se o processo epileptogênico e de mecanismos de compensação e reparo, como: rearranjo de redes neuronais anormais, brotamento axonal e neurogênese, promovendo a formação de fibras musgosas nesta região, neuroinflamação, astrogliose e microgliose (Curia et al., 2014; Engel, 2011). Todas essas alterações levam ao desenvolvimento da epilepsia.

A ELT é a forma mais comum de epilepsia que desenvolve resistência às drogas antiepilépticas. Cerca de 30% dos pacientes são refratários às drogas disponíveis, mesmo quando se utiliza associações entre dois ou mais antiepilépticos (Engel, 2011; Leite et al., 2002). ELT é uma das epilepsias mais indicadas para tratamento cirúrgico devido ao alto conhecimentos das regiões mais afetadas e por ser considerada refratária (Téllez-Zenteno & Hernández-Ronquillo, 2012).

Desta forma, torna-se de extrema importância o desenvolvimento de tratamentos alternativos que possam amenizar o quadro clínico bem como melhorar a qualidade de vida dos pacientes com ELT.

1.2 Modelo de epilepsia do lobo temporal

Os modelos animais de epilepsia induzidos pelos fármacos pilocarpina e ácido caínico promovem alterações bioquímicas e neurais similares à ELT em humanos (Engel, 2011; Leite et al., 2002).

O modelo de crises epilépticas induzidas por pilocarpina (agonista colinérgico muscarínico) ativa neurônios colinérgicos que promovem um desequilíbrio da sinalização glutamatérgica/GABAérgica induzindo assim um quadro denominado de

status epilepticus (*SE*), o qual é caracterizado por uma crise epiléptica prolongada seguida de crises epilépticas espontâneas e recorrentes. A pilocarpina induz mudanças comportamentais, morfológicas e neuroquímicas, relacionadas com a epileptogênese. As alterações são características e divididas nas fases aguda, latente e crônica (Cavalheiro et al., 1991; Leite et al., 2002; Scorza et al., 2009).

A fase aguda é logo após a administração do fármaco e indução do *SE*, com mudanças bioquímicas e do comportamento animal caracterizado na escala 4-5 de Racine (Lüttjohann et al., 2009; Racine, 1975). A fase latente é uma fase silenciosa em relação ao comportamento epiléptico do animal, entretanto, neste período há mudanças neuroquímicas que possivelmente levem ao desenvolvimento da fase crônica. O período latente é bem variável, podendo ser de 4-44 dias pós indução de *SE* (Scorza et al., 2009) ou 14-15 dias pós *SE* (Cavalheiro et al., 1991). A fase crônica é caracterizada por crises epilépticas espontâneas e recorrentes também variando o período do início desta fase. Estudo anterior caracterizou o comportamento de crises recorrentes e espontâneas em uma média de 3,2 ± 0,9 na escala comportamental de Racine e as primeiras crises recorrentes ocorreram entre 5-7 dias pós indução de *SE* (Goffin et al., 2007). Enquanto que outro trabalho observou a fase crônica cerca de 2 meses após indução de *SE* (Holtman et al., 2013).

Há diferentes modelos de indução de *SE* por pilocarpina intraperitoneal, com injeção prévia ou não de litio (Curia et al., 2008). A administração prévia de lítio (cerca de 2-24horas) aumenta a sensibilidade dos receptores muscarínicos e potencializa o efeito da pilocarpina (Jope et al., 1986; Müller et al., 2009). O lítio reduz a dose de pilocarpina necessária para indução de *SE*, assim como, os efeitos periféricos no organismo, como diarreia e a taxa de mortalidade (Leite et al., 2002). O modelo Li-pilocarpina mimetiza a ELT (Cavalheiro et al., 1991; Goffin et al., 2007; Leite et al., 2002; Turski et al., 1983). Li-pilocapirna promove neurodegeneração (Castro et al., 2010; Wang et al., 2008), neuroinflamação (Auvin et al., 2010; Holtman et al., 2013), ativação glial (de Oliveira et al., 2008; Shapiro et al., 2008), alterações comportamentais e eletrofisiológicas no SNC similares ao modelo com pilocarpina somente (Curia et al., 2008; Goffin et al., 2007; Santos et al., 2000). Entretanto, esses modelos provavelmente ativam mecanismos neuroquímicos distintos o que promove diferenças de sensibilidade e resposta às drogas antiepilépticas (Leite et al., 2002).

A escala de Racine é uma ferramenta utilizada para a identificação do comportamento animal no desenvolvimento das crises epilépticas. Através desta escala

pode se observar a latência e a severidade das crises. O comportamento das crises epilépticas é categorizado em 5 estágios (Lüttjohann et al., 2009; Racine, 1975) conforme respostas motoras.

1- Automatismo facial (movimentos de boca e face);

2- Mioclonias de cabeça e pescoço

3- Clonias de patas anteriores;

4- Clonias de patas posteriores;

5- Elevação e queda.

Neste estudo foi considerado animal epiléptico aquele que atingiu os estágios 4-5 da escala de Racine por 90 minutos (Cavalheiro et al., 1991) e apenas esses animais foram utilizados neste trabalho.

Outro comportamento observado nos ratos epilépticos, principalmente na fase crônica, também foi o comportamento de pulos e de corridas (Furtado et al., 2011; Pinel & Rovner, 1978).

2 Neuroinflamação

A inflamação é uma resposta imune inata no sistema periférico desencadeada por graves perturbações da homeostase do organismo, como infecção, lesão e exposição a contaminantes, estímulos nocivos que promovem a síntese e liberação de mediadores inflamatórios, como citocinas e quimiocinas (Ashley et al., 2012). A inflamação aguda é uma resposta imediata à infecção ou lesão tecidual em que leucócitos se infiltram no tecido danificado a fim de remover o estímulo nocivo e de reparar o tecido. Entretanto, a inflamação crônica é um processo de longa duração de resposta inflamatória, desencadeando prejuízo para o organismo ao destruir tecidos e promover excesso de resposta de reparo tecidual. A persistência da inflamação contribui para o desenvolvimento de doenças em diferentes tecidos (Medzhitov, 2008).

A neuroinflamação é o processo inflamatório no tecido nervoso e permite a comunicação entre o sistema imune e o SNC. É uma complexa resposta celular e molecular a diferentes tipos de lesões promovidas por patógeno, liberação de *debris* celulares ou proteínas intracelulares devido à exposição a um ambiente danoso, como hipóxia, baixo suporte energético, neurotoxicidade, estresse oxidativo, etc (Glass et al., 2010). Inicialmente a neuroinflamação tem como objetivo auxiliar na prevenção do dano e na recuperação do tecido danificado (Glass et al., 2010). Entretanto, esta resposta inflamatória de forma crônica vem sendo cada vez mais relacionada com doenças

neurodegerativas e desordens neurológicas (Ellwardt & Zipp, 2014; Friedman & Dingledine, 2011; Glass et al., 2010; Lucas et al., 2006).

Inflamação e neuroinflamação divergem quanto à sinalização, isto é, diferem quanto a alterações histomorfológicas, de transcriptoma (regulação de genes) (Filiou et al., 2014) e de tipos celulares atuantes. Na inflamação atuam leucócitos, como neutrófilos, monócitos e macrófagos. Enquanto que na neuroinflamação, células gliais como a microglia e astrócitos (Callaghan et al., 2008).

No SNC, microglia e astrócitos são consideradas células de resposta imune inata e promovem resposta de sobrevivência ou morte celular. Estas células produzem e secretam citocinas pró-inflamatórias, como interleucina-1β (IL-1β), interleucina-6 (IL-6), fator de necrose tumoral-α (TNF-α), e anti-inflamatórias, como interleucina-10 (IL-10), e ativam mediadores intracelulares como fatores transcricionais, ativador de proteína-1 (AP1) e fator nuclear-κB (NF-κB) (Carson et al., 2006; Lu et al., 2014).

Neuroinflamação é uma área crescente nos estudos das doenças neurodegenerativas. Entretanto, não é claro se a neuroinflamação é uma causa ou consequência no desenvolvimento de doenças neurológicas (Callaghan et al., 2008; Ellwardt & Zipp, 2014).

Em modelos de epilepsia tem se demonstrado o papel da resposta neuroinflamatória durante a epileptogênese através da análise de níveis de IL-1β, TNF-α, prostaglandina E2 (PGE2), ciclooxigenase 1 e 2 (COX1 e COX2) e enzima óxido nítrico sintase (NOS) (De Simoni et al., 2000; Ravizza et al., 2005; Rizzi et al., 2003; Somera-Molina et al., 2007).

2.1 TNF-α

Fator de necrose tumoral alfa (TNF-α) é uma citocina pró-inflamatória que possui duas formas, uma ligada à membrana (tmTNF) e outra solúvel (solTNF). Inicialmente, é sintetizado na forma de proteína transmembrana (tmTNF) e, quando clivado por metaloproteases responsáveis pela conversão de TNF-α, libera a fração solúvel (solTNF). Ambas formas de TNF-α são biologicamente ativas (Idriss & Naismith, 2000).

TNF-α liga-se em dois receptores: TNFR-1 de 55 KDa e o TNFR-2 de 75 KDa, que além de diferir no peso molecular, varia na expressão, afinidade e estrutura molecular. O receptor TNF-1 (TNFR1) é expresso na maioria dos tipos celulares e é ativado por TNF solúvel (solTNF) ou TNF transmembranar (tmTNF). Enquanto que

TNFR2 é expresso principalmente por microglia e células endoteliais e é preferencialmente ativado por tmTNF (Idriss & Naismith, 2000; McCoy & Tansey, 2008).

A forma de TNF-α solúvel ao ligar-se a TNFR1 ativa resposta de toxicidade e morte celular tanto via apoptose quanto necrose. Esta sinalização ocorre devido à presença do domínio "death" citosólico na estrutura de TNFR1. A ativação de TNFR2 pelo TNF-α transmembrana promove inflamação, sobrevivência e proliferação celular (McCoy & Tansey, 2008; Sedger & Mcdermott, 2014). Entretanto, evidências também sugerem um papel na morte celular através da ativação de TNFR2 (Olmos & Lladó, 2014).

No SNC, ambas formas de TNF-α são biologicamente ativas. TNF-α é predominantemente sintetizado e liberado pela microglia, entretanto astrócitos e neurônios também são capazes de produzir esta citocina pró-inflamatória (Idriss & Naismith, 2000; Sawada et al., 1989; Welser-Alves & Milner, 2013). Após estímulo nocivo pela exposição ao lipossacarídeo (LPS) de bactérias, TNF-α é uma das primeiras citocinas liberadas e de ação muito rápida (Chen et al., 2014). A elevação da forma solúvel de TNF-α é considerado um marcador de lesão aguda e crônica, isto é, sinaliza a neuroinflamação na fisiopatlogia de doenças neurodegenerativas como acidente vascular cerebral isquémico, doença de Alzheimer (AD), doença de Parkinson (PD), esclerose amiotrófica lateral (McCoy & Tansey, 2008).

A ativação de TNFR1 nas células gliais no SNC induz excitotoxicidade no SNC ao promover alterações nas junções comunicantes das células e a liberação de glutamato (Olmos & Lladó, 2014) e inibir a captação glutamatérgica (Pickering et al., 2005; Santello et al., 2011). Além disso, TNF-α desencadeia um desequilíbrio na neurotransmissão excitatória e inibitória, ao alterar a expressão de receptores glutamatérgicos e GABAérgicos. TNF-α aumenta a expressão de receptores AMPA e diminui a expressão de receptores $GABA_A$ na membrana plasmática ao promover a sua endocitose (McCoy & Tansey, 2008). Os aumentos de níveis de TNF-α induz excitotoxicidade glutamatérgica o que ocasiona morte neuronal (Zhu et al., 2010).

Na epilepsia, a expressão de TNF-α é transitória. Logo após a indução de *SE* há elevação dos níveis de mARN, evento necessário para ativar os mecanismos epileptogênicos (De Simoni et al., 2000).

2.2 IL-1β

Interleucina-1 beta (IL-1β) também conhecida por IL-1F2, pertence a família das IL-1. É uma das citocinas pró-inflamatórias mais estudas e está relacionada com resposta autoimune e de diversas doenças de inflamação local e sistêmica (Dinarello, 2009; Dinarellot, 1997). IL-1β liga-se aos receptores IL-1RI e IL-1RII. IL-1R1 é constitutivamente expresso na maioria das células e transduz sinal de inflamação, enquanto o IL-1RII é conchecido como "decoy receptor", isto é, quando a citocina liga-se a este receptor não há tradução de sinal (Dinarello, 2009).

IL-1β é capaz de ativar a transcrição de genes relacionados a resposta inflamatória como ciclooxigenase 2 (COX-2) e fosfolipase A2, enzimas que participam da síntese de prostaglandina E2 (PGE2), óxido nítrico sintase induzível (iNOS), fatores de ativação plaquetários, etc. Também é capaz de aumentar a expressão de moléculas de adesão em células endotelais, promovendo a infiltração de células imunocompetentes no tecido lesionado. A atuação da IL-1β ocorre na maioria das células e de forma sinérgica com outra citocina pró-inflamatória, geralmente o TNF-α (Dinarello, 2009).

A síntese de IL-1 β ocorre na presença de moléculas relacionadas a patógenos (conhecidas pela sigla em inglês PAMPs) e moléculas associadas à dano celular (conhecidas pela sigla em inglês DAMPs). Sua produção é a partir da síntese de um precursor que é processado à molécula ativa através da enzima caspase-1. A molécula madura possui funções biológicas e pode ser secretada pelas células (Eder, 2009).

No SNC, a principal origem de IL-1β em ambiente nocivo é a microglia, entretanto, astrócitos e macrófagos infiltrantes também são capazes de sintetizar e secretar esta citocina (Mabuchi et al., 2000; Pearson et al., 1999). IL-1 β promove excitotoxicidade e contribui para o desenvolvimento de doenças neurodegenerativas agudas e crônicas (Shaftel et al., 2008; Simi et al., 2007). Lesão neuronal causada por hipóxia/isquemia, hemorragia e crises epilépticas, ou acúmulo extracelular de β-amiloide ativam microglia a produzir IL-1β (Simi et al., 2007). Além disso, a ativação astrocitária pode elevar a síntese de IL-1β. Sabe-se que S100B através das vias ERK, JNK e p38MAPK ativam a síntese de IL-1β pela microglia (Kim et al, 2004).

Na epilepsia, observa-se o papel da IL-1β na epileptogênese (De Simoni et al., 2000; Dube et al., 2004), sendo um alvo farmacológico potencial no tratamento da epilepsia. Interessantemente, estudo anterior demonstrou-se que a expressão de IL-1β ocorre inicialmente nos astrócitos (Vezzani et al., 2008).

2.3 PGE2

Prostaglandina E2 (PGE2) é um mediador lipídico pró-inflamatório, pertencente a família dos prostanoides, uma subclasse dos eicosanoides. PGE2 é produzida por uma sequência de reações: 1) metabolismo oxidativo de ácidos graxos de membrana plasmática, principalmente a hidrólise de glicerofosfolipídios através da ação da enzima fosfolipase A2 (PLA2) e a liberação de ácido araquidônico; 2) conversão oxidativa de ácido araquidônico em prostaglandinas através da ciclização catalisada pelas enzimas ciclooxigenases (COX); 3) isomerização para formas ativas através da prostaglandina sintase (Milatovic et al., 2011; Murakami, 2011).

PGE2 é a prostaglandina mais pleiotrópica e mais sintetizada, responsável por diversas respostas celulares basais como controle da reprodução, metabolismo celular e neruronal; e fisiopatológicas como inflamação, febre, dor e câncer. A variedade de respostas de PGE2 é devido à variedade de receptores (Murakami, 2011).

Os receptores EP1 medeiam a ativação da fosfolipase C e a produção de inositol trifosfato, diacilglicerol e elevação do cálcio intracelular. Enquanto EP2 e EP4 promovem resposta celular via adenilil ciclase, produzindo AMPc e ativando a proteína cinase A. O receptor EP3 somente torna-se ativo quando AMPc eleva-se dentro da célula. A PGE2 liga-se a EP3 e EP4 com maior afinidade do que EP1 e EP2 (Andreasson, 2010; Milatovic et al., 2011).

Em resposta inflamatória, IL-1β e TNF-α promovem aumento da síntese de PGE2 ao aumentar a expressão e atividade de PLA2 e COX (Rocha et al., 2003).

No SNC a ação de PGE2 é dual, podendo ser neurotóxica ou neuroprotetora, pró ou anti-inflamatória, depende da concentração, do tipo de receptor ativado e se é resposta aguda ou crônica. Os mecanismos associados à morte neuronal incluem neurotoxicidade direta ou indireta, através de ativação de receptores de glutamato e posterior excitotoxicidade (Minghetti, 2004).

Doenças neurodegerativas, como doença de Alzheimer, doença de Parkinson e esclerose amiotrófica lateral, possuem níveis elevados de PGE2 (Milatovic et al, 2011). Além dessas doenças, PGE2 está relacionada com a diminuição do tempo de latência para o início de crises epilépticas (Salvadori et al., 2012).

2.4 COX

A síntese de prostaglandinas é regulada pela expressão e atividade das ciclooxigenases (COX). Há duas isoformas de COX. A COX-1 possui expressão

constitutiva na maioria das células e é responsável pelas funções fisiológicas. A COX-2 é induzível, isto é, sua expressão é regulada por mediadores inflamatórios, sendo responsável pelos mecanismos fisiopatológicos em resposta à inflamação, ao estresse, distúrbios auto-imunes e tumorogênese, que elevam a síntese de prostanoides.

No SNC, em desordens neuronais, ocorre aumento da expressão de COX-2 em neurônios e células gliais (Andreasson, 2010), promovendo excitotoxicidade glutamatérgica (Oliveira et al., 2008). Evidências sugerem um aumento de COX-2 em doenças neurodegenerativas como doença de Alzheimer (DA), doença de Parkinson (DP) e esclerose amiotrófica lateral (EML) (Milatovic et al., 2011).

A modulação da expressão e atividade de COX, através de aspirina, de anti-inflamatórios relacionados não esteroideos ou de inibidores específicos, pode ser alvo para tratamentos de doenças neurodegenerativas e desordens neurológicas, como epilepsia (Murakami, 2011; Oliveira et al., 2008; Salvadori et al., 2012).

3 Astrócitos

O SNC é constituído basicamente por neurônios e células gliais. As células gliais mantêm o ambiente para a atividade dos neurônios e são agrupadas em 1) microglia, células de defesa, capazes de realizar fagocitose e envolvidas na resposta inflamatória, e 2) macroglia – composta por oligodendrócitos, células que sintetizam mielina e auxiliam na formação da bainha de mielina presente no axônio de neurônios; células ependimais, que revestem os ventrículos cerebrais, e 3) astrócitos (Jessen, 2004; Perea et al., 2009).

Os astrócitos são células que interagem com os neurônios e participam da regulação e organização da transmissão sináptica tornando a sinapse um evento que não apenas inclui os neurônios, mas também as células astrocíticas, o que atualmente caracteriza-se em sinapse tripartite (Araque et al., 1999; Perea et al., 2009).

As populações de astrócitos são heterogêneas possuindo diferentes morfologias e funções. Podem ser classificados em protoplasmáticos ou fibrosos. Os astrócitos protoplasmáticos são localizados na zona cinza do encéfalo e possuem morfologia complexa com diversos processos finos de ramificação. Os astrócitos fibrosos encontram-se na zona branca e de morfologia menos complexa (Oberheim et al., 2012). A área encefálica determina as diferentes funções dos astrócitos.

Os astrócitos são responsáveis por diversas funções no SNC:

- participam da sinaptogênese ao sintetizar moléculas de adesão e fatores tróficos, promovendo o desenvolvimento do SNC (Eriksen & Druse, 2001; Yoo et al., 2003);

- participam da constituição da barreira hemato-encefálica e associado as células endoteliais é responsável pela captação de nutrientes para o SNC. (Abbott et al., 2006);

- modulam a atividade sináptica e regulam a homeostase iônica extracelular ao tamponar água e íons, isto é, promover o fluxo de água e íons como o potássio através dos canais de aquaporina 4 (AQP4) e de potássio 4.1 (Kir 4.1), respectivamente (Butt, 2006; Roberta & Rossella, 2010; Verkhratsky & Steinhäuser, 2000; Yoneda et al., 2001);

- regulam a concentração de neurotransmissores, como o glutamato, na fenda sináptica e também liberam neurotransmissores (ou gliotransmissores como glutamato, GABA, ATP) contribuindo para a atividade sináptica (Araque et al., 1999; Parpura et al., 1994);

- responsáveis pelo metabolismo de aminoácidos e suporte energético dos neurônios (Pellerin et al., 2007; Simpson et al., 2007);

- síntese e liberação de glutationa, peptídeo responsável pela defesa antioxidante não enzimática (Dickinson & Forman, 2002; Dringen, 2000);

- realizam a síntese de glutamina através da enzima glutamina sintetase (GS) (Anlauf, 2013; Suárez et al., 2002);

- sintetizam fatores tróficos (Eriksen & Druse, 2001; Yoo et al., 2003);

- respondem ao ambiente inflamatório (Saha et al., 2006), sendo considerados células imunológicas (Farina et al., 2007) e estimulam a gliose reativa, ativando a microglia e amplificando a resposta inflamatória (Sukumari-Ramesh et al., 2010).

Os astrócitos respondem a ambientes nocivos e algumas proteínas específicas podem ser usadas como marcadores de resposta de astrogliose, especialmente a proteína ácida fibrilar glial (GFAP) e S100B, e de função astrocitária como a expressão de canais AQP4 e Kir 4.1, atividade e expressão de GS, assim como os níveis de GSH.

3.1 GFAP

A proteína glial fibrilar ácida (GFAP) é uma proteína de filamento intermediário tipo III presente no citoesqueleto de todos os vertebrados. GFAP é um marcador de desenvolvimento e diferenciação de astrócitos maduros e de astrogliose (Eng et al., 1971; Eng & Ghirnikarz, 1994). Astrogliose é a resposta rápida dos astrócitos a um

ambiente nocivo agudo ou crônico em virtude da liberação de moléculas que sinalizam a lesão. Durante a astrogliose ocorre hiperplasia e hipertrofia, os astrócitos quiescentes tornam-se ativos, alterando sua morfologia e metabolismo. O aumento da expressão de GFAP é uma importante etapa na astrogliose, permitindo a formação de novas ramificações e hipertrofia celular, promovendo morfologia característica de ativação astrocitária (Buffo et al., 2010; Eng et al., 1992).

O aumento do conteúdo intracelular de GFAP já foi observado em dano tecidual em resposta a lesão criogênica, hipertermia, crises epilépticas induzidas por estímulo elétrico, moléculas tóxicas, etc (Eng & Ghirnikarz, 1994). A expressão de mARN de GFAP é regulada por diversos fatores, como receptores nucleares de hormônios, fatores de crescimento e lipopolissacarídeo (Eng & Ghirnikarz, 1994). Diversos autores propõem que GFAP é um sensível marcador inicial de neurotoxicidade (Sofroniew & Vinters, 2010; Yang & Wang, 2015). Assim como, S100B e glutamina sintetase são considerados marcadores de reatividade astrocitária (Ridet et al., 1997).

Astrogliose está associada com a neurodegeneração (Eng et al., 1992; Hol & Pekny, 2015; Maragakis & Rothstein, 2006). Os astrócitos reativos podem continuar a se multiplicar, a liberar fatores tróficos e citocinas, assim como migrar para o local inicial da lesão tecidual, fomando a cicatriz glial. Neste tecido ocorrerá processos de resposta inflamatória e remodelação tecidual (Buffo et al., 2010; Sofroniew, 2009).

Na epilepsia, a astrogliose e a disfunção astrocitárias estão relacionadas com a epileptogênese (de Oliveira et al., 2008; Shapiro et al., 2008).

3.2 S100B

A proteína S100B, pertence à família de proteínas ligantes de cálcio (Ca^{++}), é uma proteína solúvel produzida e secretada predominantemente pelos astrócitos no SNC (Donato, 2001). Entretanto, sua expressão não é exclusiva de astrócitos, já sendo identificada em oligodendrócitos em maturação e certas populações neuronais (Donato et al., 2009).

A S100B no meio intracelular regula o metabolismo ao induzir a diferenciação, mitose e plasticidade do citoesqueleto (Donato, 2003), e quando secretada para o meio extracelular exerce ação parácrina sobre neurônios e microglia e autócrina sobre os próprios astrócitos (Donato et al., 2009; Ponath et al., 2007). No meio extracelular S100B sinaliza de forma dual dependendo da sua concentração. Em concentração nanomolar atua como um fator neurotrófico, promovendo o crescimento de neuritos, a

modulação sináptica e a sobrevivência neuronal (Van Eldik & Wainwright, 2003). Entretanto, a S100B pode promover efeitos tóxicos quando atinge concentrações mais elevadas, que variam entre micromolar a molar, desencadendo ativação da sinalização de injúria tecidual, resposta inflamatória e apoptose (Donato et al., 2009; Gonçalves et al., 2008). Sabe-se também que os astrócitos respondem a condições inflamatórias e de lesão tecidual elevando a secreção de S100B (Guerra et al., 2011; Souza et al., 2009). Elevada e crônica concentração extracelular podem desencadear processos neurodegenerativos (Donato et al., 2009; Gonçalves et al., 2008). S100B pode se considerado um marcador de dano no tecido nervoso (Gonçalves et al., 2008). Evidências demonstram que S100B também possui papel na epileptogênese e na ativação glial (de Oliveira et al., 2008; Sakatani et al., 2008) e é um possível alvo farmacológico para tratamento de epilepsia refratária (Yamamura et al., 2013).

3.3 GS

Glutamina sintetase (GS) é uma enzima específica de astrócitos e está envolvida no metabolismo glutamatérgico. GS é capaz de converter glutamato à glutamina. No SNC é abundante no hipocampo e co-localiza com células GFAP positivas, isto é, astrócitos próximos de sinapses glutamatérgicas (Anlauf & Derouiche, 2013; Norenberg, 1979).

Na epilepsia há uma redução da atividade e expressão de GS, mesmo com proliferação astroglial em áreas hipocampais (Eid et al., 2004; Van Der Hel, 2005). A redução de GS está relacionada com a excitotoxicidade glutamatérgica e morte neuronal (Eid et al., 2004). Sabe-se que fisiologicamente o glutamato extracelular é captado pelos astrócitos através de transportadores glutamatérgicos, como o EAAT2 (Danbolt, 2001). Nos astrócitos o glutamato será metabolizado, sendo seu primeiro destino a conversão à glutamina através da GS (Eid et al., 2013). A diminuição da atividade/expressão de GS está relacionada com a geração de crises epilépticas, em virtude da diminuição de captação de glutamato e um aumento dos níveis extracelulares deste neurotransmissor, tornando o ambiente despolarizante e excitotóxico (Eid et al., 2008).

3.4 GSH

Glutationa (GSH) é um tripeptídeo com grupamento tiol constituído por y-L-glutamil-L-cisteinil-glicina. É o antioxidante não enzimático mais abundante no SNC, que protege a célula contra danos causados por espécies reativas de oxigênio (EROs) e espécies reativas de nitrogênio (ERNs) e promove detoxificação celular (Dringen, 2000;

Martin & Teismann, 2009; Sukumari-Ramesh et al., 2010). Devido à presença do resíduo de cisteína, GSH é um composto facilmente metabolizado na presença de metais de transição ou é responsável pela manutenção do estado redox da célula ao manter grupamentos sulfidril na forma reduzida nas proteínas (Dale A Dickinson & Forman, 2002; Dringen, 2000).

A glutationa geralmente está em equilíbrio na forma reduzida, GSH, e na forma oxidada, GSSG, podendo alterar os níveis de suas formas conforme o estado redox celular (Griffith, 1999).

GSH é predominantemente sintetizada e secretada pelos astrócitos através da ação consecutiva de duas enzimas. Inicialmente, a enzima y-GluCys sintetase condensa glutamato e cisteína em y-GluCys. Este dipeptídeo é condensado com glicina pela glutationa sintetase, formando glutationa em reações que consomem energia (ATP). GSH age diretamente sobre os radicais livres através de reação não-enzimática ou de forma indireta transferindo elétrons para o NADP através da glutationa redutase, formando NADPH que pode ser usado como cofator da ação da glutationa peroxidase, enzima que converte peróxido de hidrogênio em água e oxigênio (Dickinson & Forman, 2002).

GSH quando secretada é clivada pela y-glutamiltranspeptidase em cisteinilglicina. Este dipeptídeo é novamente clivado em cisteína e glicina pela dipeptidase. Os aminoácidos liberados são captados pelos neurônios e são responsáveis pela ressíntese de glutationa (Dringen, 2000; Griffith, 1999). Os astrócitos possuem papel central na defesa antioxidante do SNC, ao captar aminoácidos da corrente sanguínea, sintetizar e secretar GSH a fim de elevar a defesa antioxidante dos neurônios (Martin & Teismann, 2009).

Doenças neurodegenerativas estão relacionadas com estresse oxidativo e baixa resposta antioxidante. Na epilepsia, o estresse oxidativo também está associado (Cardenas-Rodriguez et al., 2013; Majkowski et al., 2011; Puttachary et al., 2015). A excitotoxicidade glutamatérgica induz estresse oxidativo, assim como a depleção de GSH devido à alteração da captação de cisteína, diminuindo a síntese de GSH (Dringen & Hirrlinger, 2003). E a redução de GSH induz neuroinflamação, ativação de microglia e astrócitos e morte neuronal (Lee et al., 2010).

3.5 Canais AQP4 e Kir 4.1

Aquaporina 4 (AQP4) pertence a família de proteínas de membranas que atuam como canais/permeases de água e controlam o fluxo e tamponamento de água. No SNC, AQP4 é localizada em células gliais como astrócitos, células ependimais e microglia, mas é abundantemente encontrada na terminação das ramificações astrocitárias (Binder & Steinhäuser, 2006; Roberta & Rossella, 2010). A expressão elevada de AQP4 pelos astrócitos é associada à edema em isquemia e traumatismo craniano (Roberta & Rossella, 2010).

Na epilepsia, evidências mostram que há uma redução da expressão e uma mudança da distribuição de canais AQP4 na membrana plasmática (Kim et al., 2010; Lee et al., 2012; Medici et al., 2011). Alterações na homeostase de água pela baixa de AQP4 nos astrócitos pode acarretar um ambiente de excitabilidade e formação de crises epilépticas (Roberta & Rossella, 2010).

Canal de potássio retificador de entrada 4.1 (Kir 4.1) pertence à família de canais de potássio responsáveis pela capatação e reciclagem de potássio extracelular, promovendo o potencial de equilíbrio do potássio. É expresso por diferentes células gliais, mas possui papel importante nos astrócitos de controle iônico do espaço extracelular (Butt & Kalsi, 2006).

O canal Kir 4.1 é importante na captação de K^+ quando este é liberado durante a atividade neuronal (Butt & Kalsi, 2006). Prejuízo na captação de K^+ pode desencadear um ambiente de hiperexcitabilidade. Na epilepsia, a expressão e atividade de canais Kir 4.1 estão alterados (Hinterkeuser et al., 2000; Seifert et al., 2010). Sabe-se que na epilepsia, há uma atividade elevada e anormal de neurônios e isto pode ser decorrência do aumento de K^+ extracelular (Binder & Steinhäuser, 2006; Dinuzzo et al., 2014).

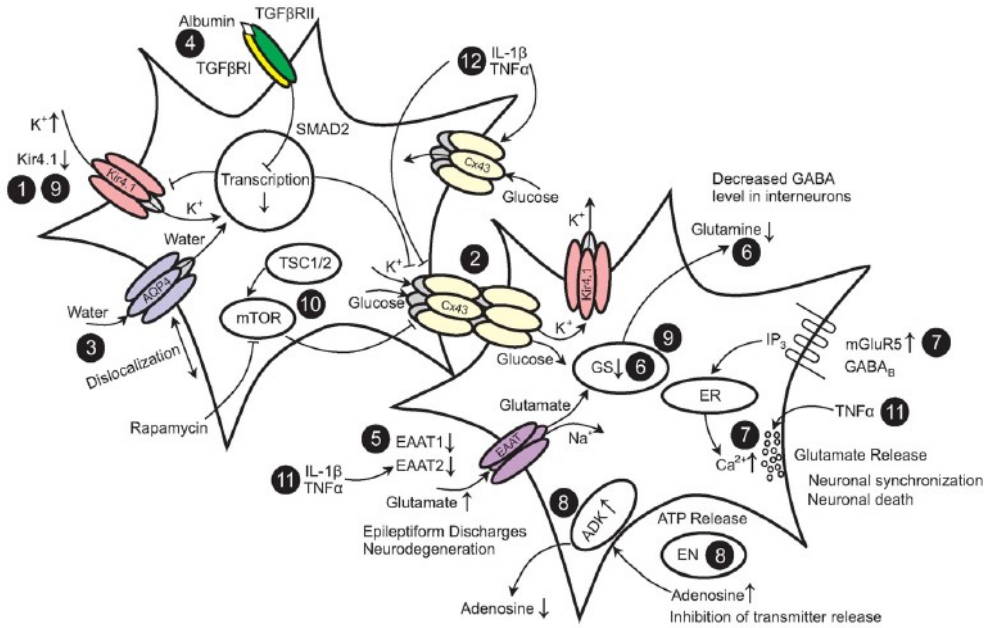

Figura 1: Alterações funcionais dos astrócitos associadas à epilepsia. (1) Aumento dos níveis extracelulares de K^+ devido à baixa expressão dos canais de potássio Kir 4.1. Baixa expressão e atividade de Kir 4.1 compromete a homeostase iônico e desencadeia aumento da despolarização e hiperatividade neuronal (2) Alterações das junções Gap (conexina 43), da redistribuição espacial de K^+ e de nutrientes como a glicose. (3) Alteração na distribuição dos canais de água (AQP4) na membrana astrocitária. (4) Aumento da permeabilidade da barreira hemato-encefálica promove extravazamento de albumina e passagem de grandes moléculas e/ou células pela barreira. (5) Redução da expressão dos transportadores de glutamato EAAT1 e EAAT2 diminui a captação de glutamato pelos astrócitos, desencadeia desequilíbrio na sinalização excitatória/inibitória e desenvolve excitotoxicidade e hiperativação neuronal. (6) Diminuição da expressão e da atividade de glutamina sintetase (GS) reduz a conversão de glutamato à glutamina através da glutamina sintetase (GS). (7) Elevação de Ca^{2+} em astrócitos são mediadas por receptores metabotrópicos, como por exemplo a ativação de mGluR5 aumenta o nível de Ca^{2+} intracelular. (8) Aumento da liberação de ATP. (9) Baixa expressão de Kir 4.1 e GS nos astrócitos. (10) A deleção de TSC1 em astrócitos implica uma regulação negativa de Cx43 e do tamponamento de K^+. (11) Citocinas pró-inflamatórias IL-1β e TNF-α diminuem a captação de glutamato e aumentam a liberação de glutamato glial, o que produz hiperdespolarização. A secreção de TNF-α promove neuroinflamação implicando na excitação e neurodegeneração. (12) IL-1β e TNF-α inibem as junções gap (Cx43), mas aumenta a atividade de hemicanais Cx43, neste hemicanal pode ocorrer passagem de glicose em condições patológicas (figura retirada de Seifert et al., 2010).

Objetivo geral

Avaliar o papel dos astrócitos e da neuroinflamação no processo de epileptogênese em ratos submetidos ao modelo de ELT induzida por Li-pilocarpina.

Objetivos específicos

1. Avaliar mediadores de inflamação (IL-1β, TNF-α, PGE2, COX-1 e COX-2) ao longo do tempo em ratos *Wistar* submetidos ao modelo de indução de *SE*;

2. Avaliar marcadores de astrogliose (S100B, GFAP) ao longo do tempo em ratos *Wistar* submetidos ao modelo de indução de *SE*;

3. Verificar a funcionalidade astrocitária (GS, GSH, canais AQP4 e Kir 4.1) ao longo do tempo em ratos *Wistar* submetidos ao modelo de indução de *SE*;

4. Investigar a proteína S100B como biomarcador e sua regulação como potencial alvo para tratamento em ratos *Wistar* submetidos ao modelo de indução de *SE*;

5. Analisar a sinalização GABAérgica no papel da despolarização na modulação da secreção de S100B *ex vivo* em fatias hipocampais.

CAPÍTULO 1

Artigo publicado

Phase-dependent astroglial alterations in Li-pilocarpine induced *Status Epilepticus* in young rats

Revista: Neurochemical Reasearch

Justificativa: Evidências demonstram a importância da neuroinflamação e da astrogliose no processo epileptogênico. A avaliação destes mecanismos pode auxiliar em uma melhor compreensão das alterações neuroquímicas na epilepsia e no desenvolvimento de novas drogas adjuvantes nos casos de epilepsia refratária.

Objetivo geral: Avaliar o papel dos astrócitos e da neuroinflamação no processo de epileptogênese em ratos jovens submetidos ao modelo de ELT induzida por Li-pilocarpina.

Objetivo específico: Analisar alterações da neuroinflamação, astrogliose e disfunção astrocitária ao longo do tempo em ratos *Wistar* jovens submetidos ao modelo de indução de *SE* por injeção intraperitoneal de Li-pilocarpina

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ORIGINAL PAPER

Phase-Dependent Astroglial Alterations in Li–Pilocarpine-Induced *Status Epilepticus* in Young Rats

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Abstract Epilepsy prevalence is high in infancy and in the elderly population. Lithium–pilocarpine is widely used to induce experimental animal models of epilepsy, leading to similar neurochemical and morphological alterations to those observed in temporal lobe epilepsy. As astrocytes have been implicated in epileptic disorders, we hypothesized that specific astroglial changes accompany and contribute to epileptogenesis. Herein, we evaluated time-dependent astroglial alterations in the hippocampus of young (27-day-old) rats at 1, 14 and 56 days after Li–pilocarpine-induced *status epilepticus* (SE), corresponding to different phases in this model of epilepsy. We determined specific markers of astroglial activation: GFAP, S100B, glutamine synthetase (GS), glutathione (GSH) content, aquaporin-4 (AQP-4) and potassium channel Kir 4.1; as well as epileptic behavioral, inflammatory and neurodegenerative changes. Phase-dependent signs of hippocampal astrogliosis were observed, as demonstrated by increments in GFAP, S100B and GS. Astrocyte dysfunction in the hippocampus was characterized, based on the decrease in GSH content, AQP-4 and Kir 4.1 channels. Degenerating neurons were identified by Fluoro-Jade C staining. We found a clear, early (at SE1) and persistent (at SE56) increase in cerebrospinal fluid (CSF) S100B levels. Additionally, serum S100B was found to decrease soon after SE induction, implicating a rapid-onset increase in the CSF/serum S100B ratio. However, serum S100B increased at SE14, possibly reflecting astroglial activation and/or long-term increase in cerebrovascular permeability. Moreover, we suggest that peripheral S100B levels may represent a useful marker for SE in young rats and for follow up during the chronic phases of this model of epilepsy. Together, results reinforce and extend the idea of astroglial involvement in epileptic disorders.

Keywords Astrocyte dysfunction · Astrogliosis · Epilepsy · Pilocarpine · S100B

Introduction

Epilepsy is a neurological disorder characterized by recurrent and spontaneous seizures, resulting from excessive, abnormal and hypersynchronic neuronal activity [1]. This disorder affects approximately 50 million people worldwide and its prevalence is high during infancy and in the elderly population [2]. Experimentally, pilocarpine and kainic acid are the pharmacological agents most commonly used to induce animal models of epilepsy, leading to similar neurochemical and morphological alterations as those observed in temporal lobe epilepsy, the most common type of refractory epilepsy [3].

Pilocarpine, a muscarinic agonist, promotes activation of cholinergic neurons that, in turn, lead to impaired glutamatergic/GABAergic signaling, resulting in *status epilepticus* (SE) and spontaneous seizures after a latent period [4, 5]. Lithium potentiates the action of pilocarpine [6] and the Li–pilocarpine model mimics the pathophysiolology of human temporal lobe epilepsy [4, 7]. This model exhibits neurodegenerative [8, 9],

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inflammatory [10], glial activation [11, 12], behavioral and electrophsysiological changes in the brain [7].

Several studies have implicated astrocytes in the development of epileptic disorders [13, 14]. Astrocytes are glial cells that interact with neurons and form the tripartite synapse [15]. They are involved in several metabolic functions, such as neuron energy supply [16], water flow mainly by aquaporin-4 (AQP-4) [17], potassium buffering by Kir 4.1 potassium channels [18], and antioxidant defenses, which involves synthesis and secretion of glutathione (GSH) [19] and control/replenishing of neuronal glutamate levels by glutamate transporters and the synthesis of glutamine by glutamine synthetase (GS) [20].

These cells respond to injury and some specific proteins have been used as markers of the astrocytic response, particularly glial fibrillary acidic protein (GFAP) and S100B. Changes in these proteins have been observed in rat models of epilepsy, especially in the hippocampus [11, 12, 21–23]. The activity, expression and distribution of GS are also altered in epilepsy [24, 25]. Alterations in GSH content also are related to astrocyte changes in epileptic disorders [26], in addition to alterations in the expression and distribution of water and potassium channels [13, 27]. Moreover, astrocyte dysfunction in epilepsy is associated with the increased synthesis and secretion of pro-inflammatory cytokines, such as IL-1β and TNF-α [28, 29].

We investigated specific astroglial changes that accompany and contribute to epileptogenesis. For this, we evaluated time-dependent astroglial alterations at 1, 14 and 56 days after SE induction, corresponding to different phases in the Li–pilocarpine model of epilepsy in rats, with specific attention to markers of astroglial activity (GFAP, S100B, GS, AQP-4 and Kir 4.1), as well as epileptic behavioral, inflammatory (TNF-α) and neurodegenerative changes (Fluoro-Jade C staining). We focused this study on young (27-days-old) rats to characterize glial changes from an age corresponding to childhood in rats [30]. It is important to mention that, at this age, rats have developed and matured their blood brain barrier [31], energetic metabolism [32] and GABAergic neurotransmission [33].

Materials and Methods

Animals

Sixty male *Wistar* rats at postnatal day 27 (5 days after weaning) at an age corresponding to childhood in rats [30] were obtained from our breeding colony (Department of Biochemistry, UFRGS) and maintained under controlled light and environmental conditions (12 h light/12 h dark cycle at constant temperature of 22 ± 1 °C). Procedures were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), following the regulations of the local animal house authorities and Committee of Animal Use of UFRGS (project number 24472).

Epilepsy Model

Animals were subjected to the Li–pilocarpine model of TLE accordingly to Cavalheiro [4]. Briefly, rats were treated intraperitoneally with lithium (LiCl, 3 mEq/kg, ip) 12–18 h prior to administration of pilocarpine (45 mg/kg, ip) (Sigma, St. Louis, MO, USA.). Control animals received also LiCl at 12–18 h prior to saline (NaCl 0.9%, ip) administration. Animals were monitored and classified in five stages of epileptic seizure, according to the Racine's scale: (1) mouth and facial movement; (2) head nodding; (3) forelimb clonus; (4) rearing with forelimb clonus; (5) rearing and falling with forelimb clonus [34].

We considered *status epilepticus* (SE) when animals reached stage 4 and keep in this stage for more than 30 min (Table 1). SE induction was stopped after 90 min by administration of diazepam (10 mg/kg, ip) followed by four administrations of HBSS medium (at 1.5, 7, 12 and 24 h after SE onset) containing (in mM): 137 NaCl, 0.63

Table 1 Animals and characterization of status epilepticus (SE), induced by LiCl–pilocarpine

Group	Saline	Pilocarpine	SE development	SE latency (min)	% SE survival	Animal	Videomonitored (days)	Racine scale (phase)	Phase 2 latency (days)
SE1	8	12	10	11:55 ± 3:26	90%				
SE14	8	12	11	13:35 ± 3:19	81%				
SE56	8	12	9	13:49 ± 3:57	90%	1	35	2–3	18
						2	35	2–3	25
						3	35	2–3	18
						4	35	2–3	27

Values of SE are mean ± standard error

Na_2HPO_4, 4.17 $NaHCO_3$ 5.36 KCl, 0.044 KH_2PO_4, 1.26 $CaCl_2.2H_2O$, 0.041 $MgSO_4{\cdot}7H_2O$, 0.049 $MgCl_2{\cdot}6H_2O$, 5.55 glucose, in order to promote a better animal recovery.

It is possible to characterize at least three phases in the Li–pilocarpine model of epilepsy, based on clinical (seizure behavior), neurochemical and EEG parameters: the acute, latent (or silent) and chronic phases [4, 35]. The first phase occurs immediately after pilocarpine administration, during which animals reach SE, based on Racine's scale. The second phase corresponds to a variable period, of between 4 and 44 days after SE [36], in which no epileptic behavior is observed. The last phase corresponds to the reappearance of epileptic behavior, with spontaneous and recurrent seizures. Herein, between 1 and 56 days (7 weeks) after SE onset, rats were video monitored daily 4–5 h for behavioral evaluation of occurrence of spontaneous seizures, which indicated the chronic phase of the epileptic model (Table 1). For further experiments, only animals that had reached stage 4 and presented recurrent seizures were used for analysis at different time points: 1, 14 and 56 days after pilocarpine injection, corresponding to the different phases of epileptic behavior, denominated as SE1, SE14 and SE56, respectively.

Hippocampal Tissue, Blood Serum and CSF Samples

Rats were anaesthetized by injection of ketamine and xylasine (75 mg/kg; 10 mg/kg; ip.) and blood was collected by cardiac puncture; serum was obtained by centrifuging at 1000×*g* for 10 min (Eppendorf 5402, Hamburg, Germany). For ventricular access, the anesthetized rats were placed in a stereotaxic apparatus and cerebrospinal fluid (CSF) was obtained carefully by puncture of the cisterna magna. CSF and serum samples were stored at −70 °C until biochemical and immunological assays. Rats were submitted to intracardiac perfusion with HBSS and the whole brain was removed. The right hemisphere was dissected on ice and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper. The left hemisphere was used for immunofluorescence and fluorojade C (FJC) staining and post-fixed overnight in 4% paraformaldehyde at 4 °C. Tissues were then rinsed in 0.1 M phosphate buffer and transferred to 15 and 30% sucrose solution in NaCl/Pi at 4 °C. After 2–3 days in solution, 50 µm coronal sections of hippocampus were obtained with a vibratome.

Tumor Necrosis Factor-α Measurement

Serum and hippocampal TNF-α were measured by ELISA (eBioscience, Ref. 88-7340, San Diego, USA). Hippocampal slices were homogenized in phosphate buffer saline (mM) (50 NaCl, 18 Na_2HPO_4, 83 $NaH_2PO_4{\cdot}H_2O$, pH 7.4), containing 1 mM EGTA and 1 mM PMSF, followed by centrifugation at 1000×*g* for 5 min at 4 °C. Data are expressed in pg/mg protein or pg/ml.

S100B Measurement

The S100B content in the CSF, serum and hippocampal tissue was measured by ELISA, as described previously [37]. Briefly, 50 µl of sample plus 50 µl of Tris buffer were incubated for 2 h on a microtiter plate that was previously coated with monoclonal anti-S100B SH-B1 (Sigma). Polyclonal anti-S100 (Dako, Carpinteria, CA, USA) was incubated for 30 min and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min. The color reaction with OPD was measured at 492 nm. The standard S100B curve ranged from 0.02 to10 ng/ml. Data are expressed in ng/mg protein or ng/ml.

GFAP Measurement

The GFAP content was measured by ELISA, as described previously [38]. ELISA for GFAP was carried out by coating 100 µl samples containing 70 µg of protein overnight at 4 °C. Incubation with an antibody polyclonal anti-GFAP (Dako) from rabbit for 2 h was followed by incubation with a secondary antibody conjugated with peroxidase for 1 h, at room temperature. The color reaction with OPD was measured at 492 nm. The standard GFAP (Calbiochem, San Diego, CA, USA.) curve ranged from 0.1 to 10 ng/ml. Data are expressed in ng/mg protein.

Glutamine Synthetase Activity

The enzymatic assay for GS was performed, as described previously [39] with modifications. Briefly, hippocampal slices were homogenized in 50 mM imidazole buffer. Homogenates were then incubated with (mM): 50 imidazole, 50 hydroxylamine, 100 L-glutamine, 25 sodium arsenate dibasic hepta-hydrated, 0.2 ADP, 2 manganese chloride, pH 6.2 for 15 min at 37 °C. Reactions were terminated by the addition of 0.2 ml of 0.37 M $FeCl_3$, 50 mM trichloroacetic acid, and 100mM HCl. After centrifugation, supernatant absorbance was measured at 530 nm. The standard γ-glutamylhydroxamate acid (Sigma) curve ranged 0.1–10 mmol/ml. GS activity is expressed as µmol/h/mg protein.

Glutathione (GSH) Content

Total reduced glutathione (GSH) content was determined by a slightly modified assay from Allen et al. (2000). Briefly, slices were homogenized in sodium phosphate buffer (0.1 M, pH 8.0) and protein was precipitated with 1.7% meta-phosphoric acid. O-phthaldialdehyde (1 mg/ml

methanol) (Sigma) was added to the supernatant at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. The standard calibration glutathione solutions curve ranged to 500 µM (Sigma). Glutathione results are expressed as pmol/mg protein.

Protein Measurement

Protein was measured by Lowry's method, modified by Peterson, using bovine serum albumin as a standard [40].

Electrophoresis and Western Blot Analysis

Nitrocellulose membranes were blocked overnight at 4 °C 2% bovine serum albumin (BSA) in Tris-buffered saline (TBS; in mM 10 Tris, 150 NaCl, pH 7.5 and 0.05% Tween 20®) and then incubated overnight at 4 °C in blocking solution containing the following antibodies, anti-Kir 4.1, anti-AQP-4 (diluted 1:1,000), and anti-GS (diluted 1:10,000) (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA). Next, membranes were incubated for 1 h at room temperature in solution containing horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, diluted 1:10,000, HRP anti-mouse IgG diluted 1:10,000 (GE Healthcare, Sao Paulo—Brazil) or HRP anti-goat diluted 1:10,000 (Sigma). Chemiluminescence signal was detected by luminol substrate reaction (ECL Western Blotting System, GE Healthcare®). Immunoblots were quantified by membranes scanning in Image4000, GE Healthcare®, and optical densities of studied proteins were determined by ImageJ software (Packard Instrument Company) and was determined by protein/actin ratio.

Immunofluorescence and FJC Staining

A set of three slices, taken from the same hippocampus in all groups, was used for histochemical analyses. Slices were processed free-floating in wells and incubated with polyclonal rabbit with anti-GFAP antibody (1:3000)(Dako) in NaCl/Pi (0.3% Triton X-100, 2% BSA) for 48 h. Negative controls were carried out with the omission of primary antibodies. After being washed repeatedly in NaCl/Pi, tissue sections were incubated with anti-rabbit Ig conjugated to 647 (Alexa-647) (1:500) in NaCl/Pi,(0.3% Triton X-100, 2% BSA) for 1 h at room temperature. Sections were then washed numerous times in NaCl/Pi, and transferred to gelatinized slides. Neurodegeneration was analyzed by FJC staining according to Wang (2008). Briefly, slides were rinsed for 5 min in distilled water, and then incubated in 0.06% potassium permanganate solution for 15 min. Following a 2-min water rinse, slides were incubated for 10 min in the FJC staining solution (Chemicon-Millipore, Billerica, MA, USA.). Slides were washed, dried, coverslipped in acidic mount media (DPX). Images were obtained with an Olympus IX-81 confocal FV- 1000 microscope and analyzed with ImageJ software.

Statistical Analysis

All results are expressed as means ± standard error of the mean. Results were analyzed by Student's t test, at specific time points of the different phases of the epileptic model (1 or 14 or 56 days after Li–pilocarpine administration). The level of statistical significance was set at $P < 0.05$. In order to evaluate changes over time for astroglial parameters (particularly GFAP and S100B), we performed one-way ANOVA followed by the Tukey's test, in sham or Li–pilocarpine animals.

Results

Li–Pilocarpine-Treated Animals Develop Status Epilepticus (SE) and Spontaneous Recurrent Seizures

All animals used in biochemical analyses reached score 4 on Racine's scale of epileptic seizures and exhibited spontaneous seizures. 30 of the 36 rats treated with Li–pilocarpine reach SE; of these, there were no deaths during 90 min of SE induction and only 13% (4/30) died in the 24 h after diazepam administration. All animals were video monitored and developed scores of 2 or 3 on the Racine scale. The latency of score 2 was between 18 and 27 days after SE induction (Table 1). We also observed jumping and running behavior in SE-induced animals. Note that animals presented spontaneous and recurrent seizures characteristic of the chronic phase (Table 1 and video monitored, Supplementary data S1).

Transitory Increment of Hippocampal TNF-α in the Li–Pilocarpine Model of Epilepsy

Hippocampal increment of TNF-α was only observed on SE1 ($P = 0.01$, Fig. 1a). No changes were observed in the hippocampal tissue later, on SE14 or SE56. However, serum levels of TNF-α were lower than those of the controls at all times analyzed: SE1 ($P = 0.02$), SE14 ($P = 0.02$) and SE56 ($P < 0.01$) (Fig. 1b).

Signs of Astrogliosis and Astroglial Dysfunction in the Li–Pilocarpine Model of Epilepsy

Six biochemical markers of astroglial activity were evaluated in this study; GFAP, S100B, GS, reduced GSH, the

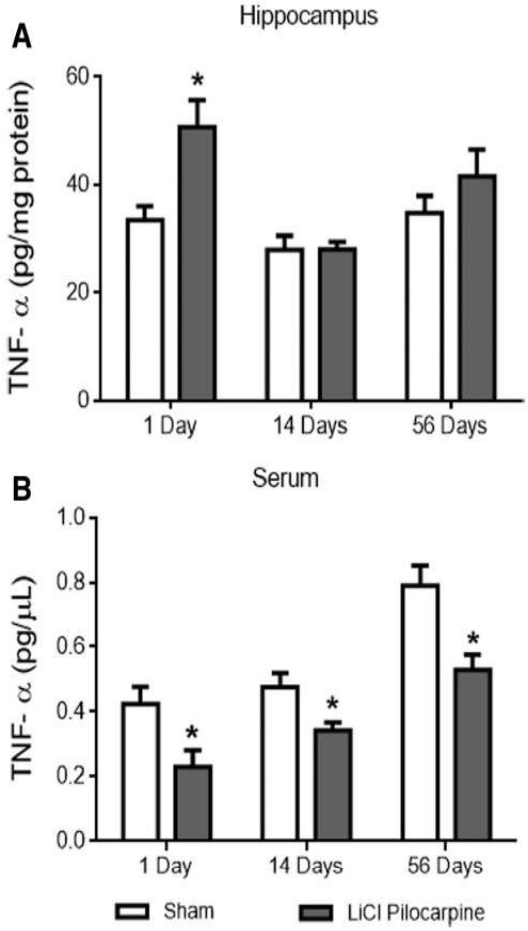

Fig. 1 Li–pilocarpine model alters serum and hippocampal TNFα. Hippocampal (**a**) and serum (**b**) samples were analyzed at 1, 14 and 56 days after SE induction. TNFα was measured by ELISA. Values represent mean ± standard error, of six animals per group. Data were analyzed by Student's t test, assuming $P < 0.05$

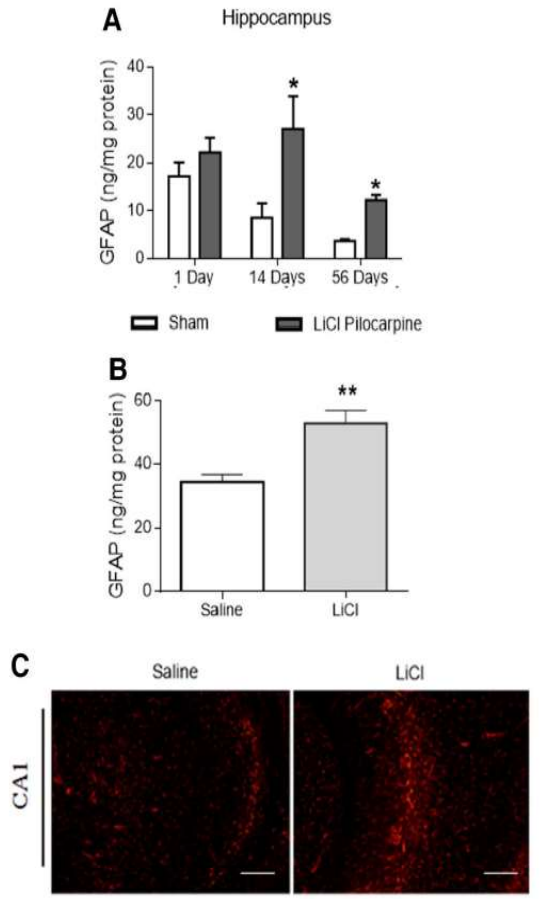

Fig. 2 Li–pilocarpine administration alters hippocampal GFAP. Hippocampal samples were analyzed at 1, 14 and 56 days after SE induction (**a**). Effects of LiCl injection on GFAP hippocampal samples (**b**). GFAP was determined by ELISA. Values represent mean ± standard error, of six animals per group. Data were analyzed by Student's t test, assuming $P < 0.05$. Representative images (from three animals in each group) showing GFAP immunofluorescence in the CA1 region of saline and LiCl animals, respectively, demonstrated induction of astrogliosis by LiCl (**c**). *Scale bar* 30 μm (magnification ×200)

water channel aquaporin-4 (AQP-4) and inwardly rectifying potassium channel 4.1 (Kir 4.1).

Hippocampal GFAP increments were observed in SE14 and SE56, compared to controls ($P = 0.02$ and $P < 0.01$, respectively) (Fig. 2a). Notice a time-dependent decrease in GFAP in sham rats, possibly associated with lithium exposure (One-way ANOVA, $F_{2,18} = 8.23$, $P < 0.01$). Signs of acute astrogliosis, induced by lithium itself, were also observed using ELISA (Fig. 2b, hippocampus) and immunohistochemistry (Fig. 2c, CA1 hippocampal region) at 24 h after administration.

Similarly to GFAP, hippocampal S100B increased on SE14 and SE56 ($P = 0.04$ and $P = 0.02$, respectively) (Fig. 3a), compared to sham rats; a time-dependent alteration in S100B was also observed in the sham rats (One-way ANOVA, $F_{2,19} = 7.75$, $P < 0.01$). Moreover, extracellular S100B is also a marker of astroglial activation that can be measured in CSF and serum.

Elevated levels of S100B were observed in the CSF on SE1 and later on, on SE56 ($P = 0.03$ and $P = 0.02$, respectively) (Fig. 3b). A decrease in CSF S100B depending on time was observed in Li–pilocarpine rats. Interestingly, we also observed a time-dependent decrease in CSF S100B in sham rats exposed to lithium alone (One-way ANOVA, $F_{2,14} = 27.11$, $P < 0.01$).

Serum S100B decreased on SE1 ($P < 0.01$); however, an increase was observed SE1 ($P < 0.01$) (Fig. 3c). No change was observed on SE56. Again, a time-dependent change in serum S100B was observed in control rats (One-way ANOVA, $F_{2,18} = 31.08$, $P < 0.01$). The CSF/serum S100B ratio was greater on SE1 and SE56 ($P < 0.01$ and $P = 0.04$,

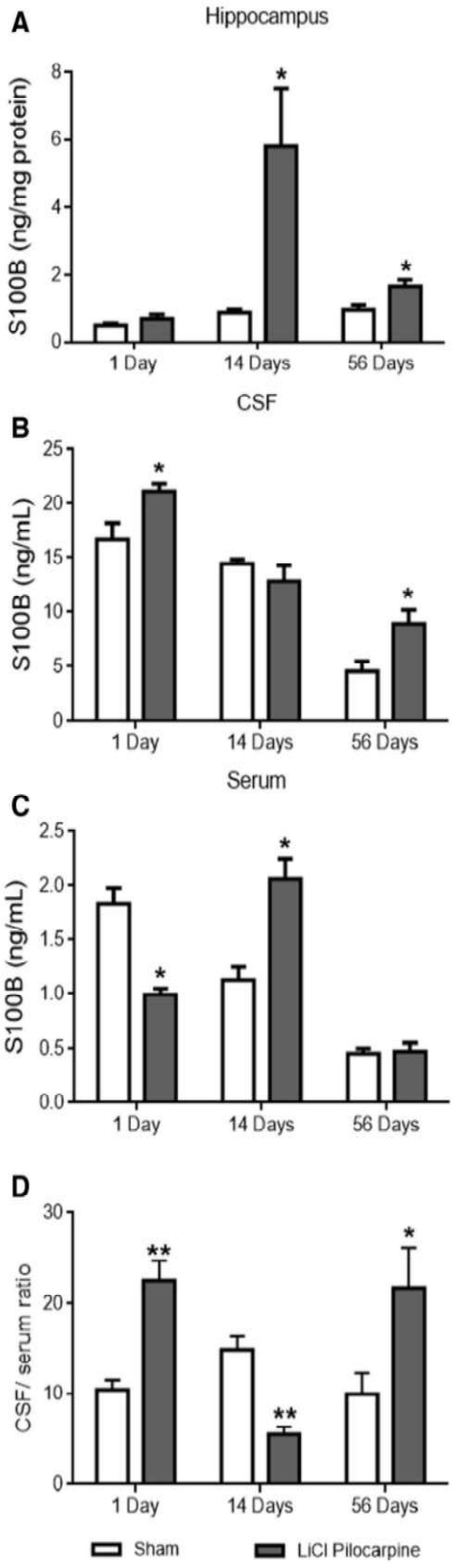

Fig. 3 Li–pilocarpine model alters hippocampal, serum and cerebrospinal fluid levels of S100B. Hippocampal (**a**), cerebrospinal fluid (CSF) (**b**) and serum (**c**) samples were analyzed at 1, 14 and 56 days after SE induction. CSF/serum S100B ratio (**d**). S100B was determined by ELISA. Values represent mean ± standard error of six animals per group. Data were analyzed by Student's t test, assuming $P<0.05$

respectively) (Fig. 3d). This ratio was lower on SE 14 ($P<0.01$). Notice that this ratio remains unchanged in control rats.

Glutamine synthetase activity was increased SE1 ($P=0.04$) (Fig. 4a). Conversely, a decrease was observed later on, on SE14 ($P=0.02$). However, the immunocontent of this enzyme displayed as opposing profile of expression, presenting a decrease on SE1 ($P=0.01$) and an increase later on, on SE14 ($P=0.01$) (Fig. 4b). Moreover, the activity/protein ratio of this enzyme (based on values of a and b) was clearly increased in epileptic rats $P=0.03$ and $P<0.01$, respectively SE 1 and 56 days) (Fig. 4c).

Astrocytes play an important antioxidant role in brain tissue, demonstrating activity that involves the synthesis and recycling of GSH. We observed a significant decrease in hippocampal GSH on SE1 and SE56 ($P=0.02$ and $P=0.04$) (Fig. 5a). Two other astrocyte proteins, Kir 4.1 (Fig. 5b) and AQP-4 (Fig. 5c) were also reduced on SE1 ($P=0.03$ and $P=0.02$, respectively), where the decrease in Kir 4.1 persisted throughout the period analyzed ($P=0.02$ and $P=0.04$ at SE14 and SE56, respectively). AQP-4 was increased at SE14 ($P=0.04$), but unaltered at SE56.

Neurodegenerative Signs are Dependent on Time in Li–Pilocarpine Model of Epilepsy

Neurodegenerative alterations were characterized using Fluoro-Jade C staining and astrogliosis (based on GFAP increment and evaluated by immunohistochemistry) (Fig. 6). Degenerating neurons and astrogliosis was found in the dentate gyrus and CA1 (panel a and b) at SE14. Astrogliosis also occurs in the hippocampal CA3 region at SE14 (data not show). Fluoro-Jace C staining was negative in dentate gyrus, and CA1 and CA3 regions at SE1 and SE56. Astrogliosis was observed on SE56 in all hippocampal regions (data not shown).

Discussion

Neuronal changes have been extensively studied in epilepsy. However, there are few studies regarding specific glial changes during epileptogenesis. We hypothesized that specific and time-dependent glial alterations can occur during the development of the Li–pilocarpine epilepsy model in young rats after weaning. We, herein, showed such glial

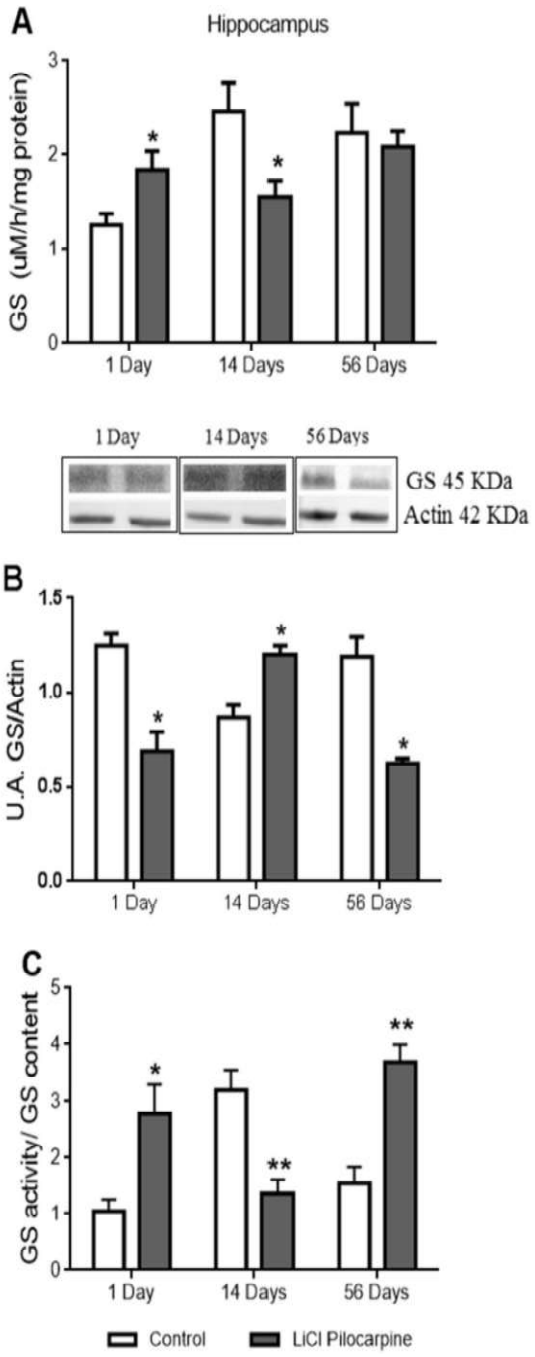

Fig. 4 Li–pilocarpine administration alters glutamine synthetase in the hipocampus. Hippocampal glutamine synthetase (GS) activity (**a**), immunocontent (**b**) and activity/content (**c**) were measured, as described in the Material and Methods section, at 1, 14 and 56 days after SE induction. Values represent mean ± standard error, of 4–6 animals per group. Data were analyzed by Student's t test, assuming $P < 0.05$

changes in parallel to the behavioral changes, inflammation and neuronal death associated with this model.

Clinical signs clearly indicated SE development in young rats and spontaneous recurrent seizures, later on in this model. EEG alterations have been reported in immature (20-day-old) rats in the Li–pilocarpine model and are equivalent to those observed in adult rats [41], reinforcing the usefulness of this model of epilepsy.

The increment of pro-inflammatory cytokines in the brain tissue is considered to be a hallmark of brain inflammation, and such increments, accompanied or not by peripheral changes in cytokine levels, have been observed in epileptic patients and models of seizures [42]. In this work, serum TNF-α levels decreased at different time points after pilocarpine injection. Another study found no significant changes in serum TNF-α levels at SE onset in adult rats submitted to the Li–pilocarpine model [43]. Furthermore, a study that employed systemic administration of different agonists of muscarinic receptors presented a decrease in TNF-α in heart homogenates after pilocarpine injection [44]. Induction of the cholinergic parasympathetic nervous system attenuated systemic inflammation and inhibited TNF-α expression during systemic injection of pilocarpine [45].

In the brain, TNF-α is predominantly synthesized and secreted by microglia, but astrocytes and neurons are also able to produce this cytokine [46]. Hippocampal TNF-α levels were increased at SE1. However, there was no difference between the control and SE14 and SE56 animals, suggesting an acute inflammatory signaling mechanism at the beginning of epileptogenesis in this model. High pro-inflammatory cytokine levels have been observed in the hippocampus region in other animal models of epilepsy and this response appears to be dependent on time and age [28, 29, 47, 48]. A transitory increment in hippocampal TNF-α was also observed after SE, induced by kainic acid administration [49]. However, in TLE patients, TNF-α mRNA production is elevated in the sclerotic hippocampus [50].

Neuroinflammation and astrogliosis are related to neurodegeneration [51, 52]. It is believed that TNF-α inhibits glutamate transport and increases glutamate release from astrocytes [53], leading to glutamatergic excitoxicity and neuronal death [54]. Our study analyzed neuronal death using Fluoro-Jade C (FJC) staining. FJC-positive cells were observed in SE14 (but not in SE1) pilocarpine injection in different hippocampal regions: CA3, CA1 and dentate gyrus. Another study have also detected significant losses at 1 and 3 weeks after SE induction [55]. However, in a study using neonate rats, hippocampal neuronal loss was observed during the first hours after SE [56]. Moreover, using the pilocarpine model in mice, FJC-positive cells were observed from 12 h until 7–14 days in the hippocampal regions CA1 and CA3 [9]. These discrepancies in the

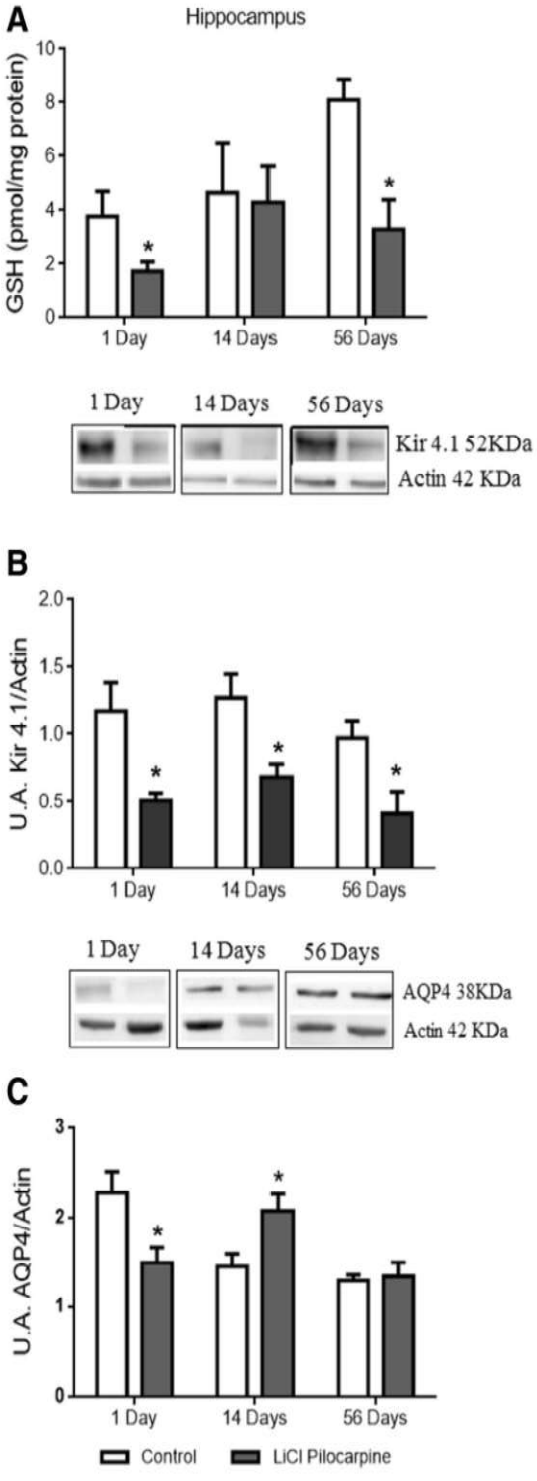

Fig. 5 Li–pilocarpine model alters contents of glutathione and water and potassium channels. Hippocampal content of reduced glutathione (GSH, **a**), Kir 4.1 potassium channel (**b**) and the aquaporin-4 (AQP-4, **c**) were analyzed in samples at 1, 14 and 56 days after SE induction. GSH was measured by a fluorimetric assay with o-phthaldialdehyde. AQP-4 and Kir 4.1 contents were evaluated by Western blotting. Representative blots are presented. Values represent mean ± standard error, of 4–6 animals per group. Data were analyzed by Student's t test, assuming $P < 0.05$

literature could be attributed to methodological differences of SE induction and neuronal loss evaluation.

In parallel to the neuron injury and inflammation induced by SE, we observed clear signals of astrocyte dysfunction in the hippocampus. Our results indicate an astrogliosis during epileptogenesis, based on an increased immunocontent of GFAP and S100B at SE14 and SE56. Immunohistochemical results also contributed to the characterization of astrogliosis. In fact, hyperplasia and hypertrophy of astrocytes have been described as early signals of glial activation that contribute to the mechanism of epileptogenesis [12, 21]. GFAP positive and S100B positive cells were increased in different hippocampal regions at 5 days after SE. Increased S100B positive cells and astrocyte processes were observed in the neocortex of patients with intractable epilepsy increase [57]. It is noteworthy that lithium per se is potentially able to induce astrogliosis [58], which is reduced by gabapentin [59]. Therefore, the time-dependent decrease in GFAP in sham animals may be related to lithium-induced gliosis. Accordingly, we observed an increase in hippocampal GFAP after lithium administration in sham animals.

S100B is a calcium-binding protein that is predominantly synthesized and secreted by astrocytes in the brain tissue, exerting paracrine and autocrine effects on neurons and glia cells [60]. Extracellular changes in S100B have be used as signals of astroglial activation or dysfunction [61]. Herein, we detected an early increase in this protein in the CSF at SE1. This increment was persistent at SE56. A previous study by our group showed a higher CSF S100B in adult rats at 2 weeks after SE [11]. Higher CSF levels of this protein may be due an astrocyte secretion response to neural injury. We have demonstrated S100B secretion in many injury conditions, including inflammation in vivo, acute hippocampal slices and astroglial cultures [62, 63]. More recently, S100B is postulated to represent a damage-associated molecular pattern (DAMP) protein or alarmin that could mediate cell responses [64, 65]. Other studies in epilepsy models and human tissue samples have shown a greater labeling and release of HMGB1, a classical DAMP [64, 66]. Therefore, our study emphasizes the value of CSF S100B as a marker of SE, as has been proposed in other acute brain injury conditions, such as traumatic brain injury and stroke [61, 67, 68]. It is important to emphasize that we have chosen to investigate S100B levels in the hippocampus due to its high astrocyte reactivity [23], but we are aware that it is not possible to directly correlate specific changes in the hippocampus with S100B changes in

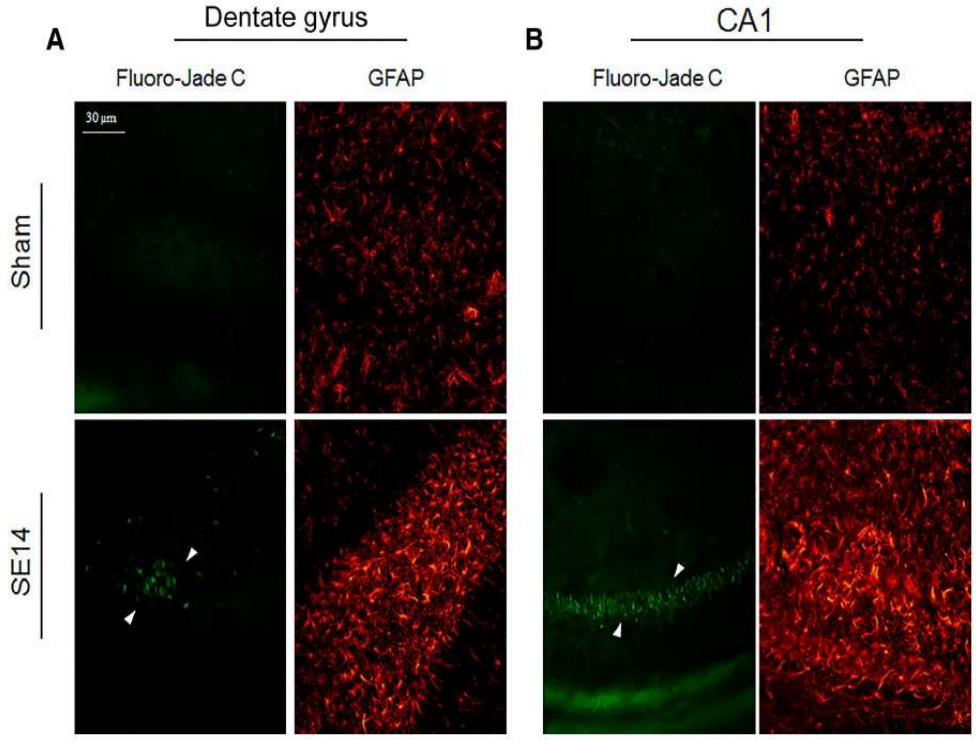

Fig. 6 Li–pilocarpine administration causes astrogliosis and neurodegeneration in the hippocampus. Representative immunofluorescent images (from three animals in each group) for GFAP and Fluoro-Jade C of hippocampal region dentate gyrus (***panel a***) and CA1 (***panel b***) at 14 days from control and SE animals. Fluoroa-Jade C was positive and the astrogliosis marker was observed at 14 days in CA1 and dentate gyrus. *Scale bar* 30 µm (magnification ×200)

the CSF, due to innumerable sources of this protein in the brain tissue [61].

Interestingly, serum S100B decreased at SE1, whereas CSF levels of this protein are elevated. This increase in S100B in the CSF suggests astroglial activation. We know that peripheral levels of S100B do not necessarily reflect cerebrospinal fluid changes and that other cells (e.g. adipocytes) contribute to S100B levels in the blood [61, 68, 69]. The present data confirm this independence. Such independence may be due to the specific changes in the blood brain barrier (BBB) during and after status epilepticus [70–72]. BBB breakdown, induced by brain injury, has been observed under clinical and experimental conditions. However, in spite of this increased cerebrovascular permeability, increments in CSF S100B were not accompanied by S100B leaking from CSF to the serum, during brain injury [67, 68]. At this moment we cannot determine how much of this serum S100B decrease is due to "brain retention" [67, 68] or a reduction in peripheral release, which also is regulated by neural signals. Another unclear aspect, at this moment, is the time-dependent decrease in serum S100B observed in sham animals. It would be necessary to investigate a possible acute lithium effect on peripheral S100B levels. In fact, lithium affects adipose tissue [73], which express and secrete S100B [69]. However, regardless of these uncertainties, we observed a clear early and prominent increase in the CSF/serum S100B ratio after SE, reinforcing the idea that S100B may represent a useful marker for SE.

Moreover, 14 days after SE, we observed a clear increase in serum S100B that, in turn, decreased the CSF/serum S100B ratio. Again, this suggests astroglial activation and/or long-term changes in cerebrovascular permeability. In order to evaluate the usefulness of the CSF/serum S100B ratio, we searched for a correlation with SE severity. In fact, there was a correlation between the latency time of the development of spontaneous seizure (at SE56) and a high CSF/serum ratio (Spearman's correlation, $R = -0.9487$ and $P < 0.001$). However, this is a preliminary result due to the small sample ($n = 4$).

Another classical astrocyte marker is GS. This enzyme catalyzes the conversion of glutamate to glutamine. In the brain, GS is abundant in the hippocampus and co-locates with GFAP positive cells, in association with the glutamatergic synapse [20]. Hippocampal samples of patients with mesial temporal lobe epilepsy and TLE patients with

hippocampal sclerosis showed a decreased content and activity of GS, reinforcing the idea of astrogliosis [25, 74]. Additionally, experimental models of GS activity inhibition by methionine sulfoximine resulted in GS deficiency and recurrent seizures [24].

We found a decrease in hippocampal GS activity in SE1, but an increase at SE14. No difference was found at SE56. Interestingly, GS activity did not correlate to the protein content, as evaluated by Western blotting. The GS content was lower in SE1 and higher in SE14, when compared with the respective controls. Additionally, there was no difference in hippocampal GS activity in SE56; however, the GS content was lower. The GS activity/protein ratio was higher in SE1 and SE56, also suggesting the existence of astrogliosis following SE. The increments observed in this Li–pilocarpine model may demonstrate a compensatory mechanism to remove the excess of glutamate in the synaptic cleft, due to the impaired balance of excitatory and inhibitory neurotransmitters immediately after SE and recurrent seizures in the chronic phase. Another fate for glutamate in astrocytes is the synthesis of GSH [75]. However, glutamatergic excitotoxicity due to oxidative stress consumes GSH and, due to the interference in cysteine uptake, impairs GSH synthesis [19]. We found decreased GSH levels on SE1 and SE56. Other studies have shown reduced levels of GSH 24 h after treatment with pilocarpine [26] and a decrease in GSH was reported in patients with epilepsy [76]. Therefore, the induction of astrogliosis in the Li–pilocarpine model is hampered by the decrease in antioxidant defenses, favoring a neurodegenerative scenario.

The astrogliosis induced by SE is accompanied by changes in expression, localization, distribution and activity of ionic and water channels [27, 77, 78]. AQP-4 and Kir 4.1 are specific astrocyte channels and are functionally very important for the clearance of potassium and water during intense depolarizing activity, as is observed in SE [13, 18, 27, 79]. AQP-4 levels decreased in SE1 and increased in SE14, when compared with sham animals. This pattern was quite similar to that observed for the GS content. On the other hand, Kir 4.1 channels were reduced at all time points analyzed. Together, these data reveal the long-term impairment of the mechanism of potassium and water clearance induced by the Li–pilocarpine model.

Some limitations of this study should be highlighted. Firstly, EEG records at different times would be useful to characterize SE and seizure activities later on, but behavioral changes based on Racine's scale allow us to clearly characterize SE. Therefore, the time-dependent astroglial changes may be linked to the neuronal excitability induced by Li–pilocarpine and/or the neuronal damage identified later by FJC staining. Secondly, the employment of additional time points in future studies will be valuable for understanding some astroglial parameters. Thirdly, some astroglial alterations may be underestimated as these were compared to a sham group that was submitted to a lithium exposure that per se alters some glial parameters. In spite of this, our data reinforce the astrocyte sensitivity in this model, particularly in the hippocampus [11, 12, 20–23].

The Li–pilocarpine model of epilepsy induced SE in 27-day-old rats and the development of chronic phase of epilepsy was confirmed by video monitoring of spontaneous recurrent seizures. Hippocampal inflammation (based on TNF-α content) occurred as an early and transitory signal after SE. Time-dependent signs of hippocampal astrogliosis were observed in this model, based on GFAP, S100B and GS increments. Astrocyte dysfunction in the hippocampus was characterized in this model based on decreases in GSH content, AQP-4 and Kir 4.1 channels. These alterations suggest that glial failure, induced by Li–pilocarpine, triggers and/or exacerbates neuronal damage, possibly to a decrease in antioxidant defense and the ability to regulate the ionic environment (particularly extracellular K^+ levels). This astroglial failure putatively aggravated the glutamatergic excitotoxicity observed in this model and the neuronal damage herein identified by FJC staining. We found a clear, early and persistent increment in CSF S100B during SE, as well as changes in serum S100B after SE. A prominent increase in the CSF/serum S100B ratio was observed soon after SE. This elevated ratio persisted at SE56, possibly correlating with SE severity. The mechanisms involved in these CSF and serum changes in S100B levels, and whether these alterations are associated with SE severity, are unclear at this time. Peripheral S100B may represent a useful marker for SE and for follow up during the chronic phase in this model of epilepsy and the chronic phases of epileptic disorders. Taken together, results reinforce and extend the idea of astroglial involvement in epileptic disorders.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interests.

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CAPÍTULO 2

Artigo submetido

Effects of dexamethasone treatment on epilepsy model*:* revertion of neuroinflammation and astrogliosis in the hippocampus of young rats with induced *Status Epilepticus*

Revista: Journal of Neuroinflammation

Justificativa: No estudo anterior, sugerimos que a proteína S100B pode ser considerada um marcador de alterações e/ou morte neuronal no modelo de epilepsia do lobo tempotal em ratos jovens. Além disso, a regulação da sua secreção poderia ser um potencial alvo para tratamento da epilepsia refratária.

Objetivo geral: Investigar a modulação da secreção da proteína S100B em ratos *Wistar* jovens submetidos ao modelo de indução de *SE*;

Objetivo específico: Analisar a modulação da secreção de S100B *ex vivo* através do anti-inflamatório dexametasona em ratos com *SE*. Assim como, investigar os efeitos *in vivo* da administração da dexametasona sobre parâmetros inflamatórios, disfunção astrocitária e astrogliose em animais com *SE*.

Journal of Neuroinflammation

Effects of dexamethasone treatment on epilepsy model: revertion of neuroinflammation and astrogliosis in the rat hippocampus induced Status Epilepticus.

--Manuscript Draft--

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Abstract:	Background: Temporal lobe epilepsy (TLE) is the most common epilepsy syndrome and is, in one-third of cases, accompanied by resistance to antiepileptic drugs (AED). Most AED target neuronal activity modulated by ionic channels and the steroid sensitivity of these channels has supported the use of corticosteroids as adjunctives to AED. Assuming the importance of astrocytes in neuronal activity, we investigated inflammatory and astroglial markers in the hippocampus, a key structure affected in TLE, using the Li-pilocarpine model of epilepsy. Methods: Initial experiments demonstrated that ex vivo hippocampal slices, incubated with dexamethasone, from sham and status epilepticus (SE)-induced animals exhibited a decrease in S100B secretion. Extracellular S100B is a glial protein that is a widely used marker for brain injury conditions, including epileptic disorders. We investigated the effects of in vivo dexamethasone treatment in the hippocampus of rats submitted to the Li-pilocarpine model of epilepsy, at 1 and 56 days after SE induction. Parameters determined were the COX2 and PGE2 cytokines, and the astroglial markers, glial fibrillary acidic protein (GFAP), S100B, GSH content, glutamine synthetase activity, potassium channel Kir4.1 and the water AQP-4 channel. Cerebrospinal fluid and serum S100B were also investigated. Results: With the exception of AQP-4, all parameters were altered in the Li-pilocarpine model. Dexamethasone administration, at 24h after SE induction, reversed most of these parameters, reinforcing the importance of anti-inflammatory steroid therapy in epilepsy. Conclusions: Data demonstrating specific alterations in astrocytes in this model highlight the importance of these cells in the pathogenesis of epilepsy, where they may represent potential therapeutic targets of AED.
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Effects of dexamethasone treatment on epilepsy model: revertion of neuroinflammation and astrogliosis in the hippocampus of young rats with induced *Status Epilepticus*.

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Abstract

Background: Temporal lobe epilepsy (TLE) is the most common epilepsy syndrome and in one-third of cases of epilpesy is accompanied by resistance to antiepileptic drugs (AED). Most AED target neuronal activity modulated by ionic channels and the steroid sensitivity of these channels has supported the use of corticosteroids as adjunctives to AED. Assuming the importance of astrocytes in neuronal activity, we investigated inflammatory and astroglial markers in the hippocampus, a key structure affected in TLE, using the Li-pilocarpine model of epilepsy.

Methods: Initial experiments demonstrated that *ex vivo* hippocampal slices from young rats, incubated with dexamethasone, from sham and status epilepticus (SE)-induced animals exhibited a decrease in S100B secretion. Extracellular S100B is a glial protein that is a widely used marker for brain injury conditions, including epileptic disorders. We investigated the effects of *in vivo* dexamethasone treatment in the hippocampus of rats submitted to the Li-pilocarpine model of epilepsy, at 1 and 56 days after *SE* induction. Parameters determined were the COX2 and PGE2 cytokines, and the astroglial markers, glial fibrillary acidic protein (GFAP), S100B, GSH content, glutamine synthetase activity, potassium channel Kir4.1 and the water AQP-4 channel. Cerebrospinal fluid and serum S100B were also investigated.

Results: With the exception of AQP-4, all parameters were altered in the Li-pilocarpine model. Dexamethasone administration, at 24h after *SE* induction, reversed most of these parameters, reinforcing the importance of anti-inflammatory steroid therapy in epilepsy.

Conclusions: Data demonstrating specific alterations in astrocytes in this model highlight the importance of these cells in the pathogenesis of temporal lobe epilepsy, where they may represent potential therapeutic targets of AED.

Key words: epilepsy, dexamethasone, neuroinflammation, astrocytes, S100B.

Background

Temporal lobe epilepsy (TLE) is the most common form of epilepsy and is characterized by recurrent and spontaneous seizures, resulting from excessive, abnormal and hypersynchronic neuronal activity in the brain[1–3]. Approximately 50 million people worldwide suffer from this neuronal disorder, which normally affects mostly children and the elderly population [4]. About 30% of epileptic patients develop refractory- and pharmacoresistance to antiepileptic drugs [5,6]. Li-pilocarpine induced model of epilepsy exhibits similar alterations to those observed in TLE patients [7,8] and is accompanied by drug resistance [9].

Brain tissue samples from patients and experimental models of TLE show specific astroglial changes, mainly in the levels of glial fibrillary acidic protein (GFAP) and S100B protein [10–14]. In fact, results from several studies suggest that epileptogenesis involves changes in glial cells beyond neuronal alterations [15,16]. Astrocytes are glial cells that interact with neurons and form tripartite synapses [17]. Some studies have strongly implicated astrocytes in the development of epileptic disorders [18–21]. Astrogliosis and neuroinflammation have been correlated to epileptogenesis, recurrent and spontaneous seizures [22–27]. For this reason, specific astroglial targets (e.g. S100B, glutamine synthetase – GS, potassium channel Kir 4.1 and water channel AQP-4) have been investigated with a view to improving therapeutic approaches and the development of anti-apileptic drugs [28]. The S100B protein is a glial protein that is a widely used marker for brain injury conditions, including epileptic disorders [29,30] and indeed displays an augmented secretion during brain injury condition, working as a neurotrophic cytokine or simply as a damage-associated molecular pattern (DAMP) [31]. Moreover, S100B secretion is modulated by LPS [32] and antiinflamatory drugs [33] (Guerra et al, unpublished data). Chronically elevated extracellular levels of S100B potentially lead to neurodegeneratives prcocesses [34,35].

Therapy with corticosteroids, such as dexamethasone palmitate, has been used to treat refractory epilepsy in children [36]. Dexamethasone has been shown to reduce seizures in epileptic encephalopathy patients [37,38]; while dexamethasone use has been evaluated in the Li-pilocarpine model of epilepsy [39,40], its effect on astroglial targets demands has not been fully investigated. We hypothesized that dexamethasone mediates down-regulation of S100B secretion and that this change could contribute to decrease neuroinflammation and astrogliosis during epileptogenesis. We, herein, evaluated S100B secretion by dexamethasone in acute hippocampal slices 1, 14 and 56

days after the induction of status epilepticus (SE) by Li-pilocarpine administration in young rats. In this experimental model, these times correspond approximately to the acute, silent and chronic phases, respectively [41]. In a second set of experiments, we administered intraperitoneal dexamethasone 1 day after *SE* and analyzed inflammatory and hippocampal astroglial parameters (S100B, GFAP, GS, GSH, AQP-4 and Kir 4.1) at 1 and 56 days afterwards. Cerebrospinal fluid (CSF) and serum S100B were also determined.

Methods

Animals

Male Wistar rats, at postnatal day 27 were used in this study. We focused this study on young (27-days-old) rats to characterize glial changes from an age corresponding to childhood in rats [42]. It is important to mention that, at this age, rats have developed and matured their blood brain barrier [43], energetic metabolism [44] and GABAergic neurotransmission [45]. Animals were obtained from our breeding colony (Department of Biochemistry, UFRGS) and maintained under controlled light and environmental conditions (12 h light/12 h dark cycle at constant temperature of 22 ± 1ºC). Procedures were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) following the regulations of the local animal house authorities and Committee of Animal Use of UFRGS (project number 24472).

This study was divided into two parts. The first was to analyze the S100B secretion in hippocampal acute slice in animals submitted to *SE*. The second part was to observe the effects of dexamethasone, an anti-inflammatory steroid, on *SE* animals.

Epilepsy model

Animals were subjected to the LiCl-pilocarpine model of TLE, according to Cavalheiro [7]. Briefly, rats were treated intraperitoneally with lithium (LiCl, 3 mEq/kg, i.p.) 12-18 hours prior to the administration of pilocarpine (45 mg/kg, i.p.) (Sigma, St. Louis, MO, U.S.A.). Control animals also received LiCl at 12-18 hours prior to saline (0.9% NaCl, i.p.) administration. Animals were monitored and classified into 5 stages of epileptic seizure, according to Racine's scale: 1) mouth and facial movement; 2) head nodding; 3) forelimb clonus; 4) rearing with forelimb clonus; 5) rearing and falling with forelimb clonus.

We considered *SE* when animals reached stage 4 and stayed at this stage for more than 30 min (Table 1). *SE* induction was stopped after 90 minutes by administration of diazepam (10 mg/kg, i.p.) followed by 4 administrations of HBSS medium (at 1.5, 7, 12 and 24 hours after *SE* onset) containing (in mM): 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$ 5.36 KCl, 0.044 KH_2PO_4, 1.26 $CaCl_2.2H_2O$, 0.041 $MgSO_4.7H_2O$, 0.049 $MgCl_2.6H_2O$, 5.55 glucose, in order to promote a better animal recovery.

For further experiments, only animals that reached stage 4 and presented recurrent seizures were used. These animals were analyzed at different times: 1, 14 and 56 days after pilocarpine injection, denominated as SE1, SE14 and SE56, respectively. These time points was used conformed previous study [46] and was characterized in acute, latent and chronic phase of epilepsy induced by Li-pilocarpine model.

First study – S100B secretion in acute hippocampal slices of *SE* rats

Sixty male rats were divided into 1) control and 2) *SE* animals at different times (1, 14 and 56 days) after pilocarpine injection. These animals were killed by decapitation, and their brains were removed and placed in cold saline medium of 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. 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 saline medium and only one slice. The medium was replaced every 15 min with fresh saline medium at room temperature. Following a 120-min equilibration period, the medium was removed and replaced with basal or specific treatments (high potassium-30 mM KCl; 0.1µM dexamethasone; vehicle - 0.01% DMSO) for 60 min at 30°C on a warm plate [53].

S100B secretion

S100B content in the supernatant was measured by ELISA, as described previously [47]. Briefly, 50 µl of sample plus 50 µl of Tris buffer were incubated for 2 h on a microtiter plate that was previously coated with monoclonal anti-S100B SH-B1 (Sigma-Aldrich, St. Louis, MO. USA). Polyclonal anti-S100 (Dako, Carpinteria, CA, U.S.A.) was incubated for 30 min and peroxidase-conjugated anti-rabbit antibody was then added for a further 30 min. The color reaction with OPD was measured at 492 nm.

The standard S100B curve ranged from 0.02 to 10 ng/ml and data expressed as ng/mg or ng/ml. Results are shown as percentages of the control.

Lactate dehydrogenase assay

Slice integrity was evaluated by Lactate dehydrogenase (LDH) kinetic activity using a commercial kit (BioClin, Brazil). The assay was performed by according to the manufacturer's instructions.

Second study – Effect of dexamethasone on the *SE* model

Sixty male rats received an administration of vehicle (DMSO, 0.1%, i.p.) or dexamethasone (10 mg/kg, i.p., 12h/12h) after 1 day of the *SE* induction by LiCl-pilocarpine administration. Group animals were divided into 1) control + vehicle, 2) *SE* + vehicle and 3) *SE* + dexamethasone after 1 and 56 days from pilocarpine injection.

Brain tissue, serum and CSF samples

Rats were anaesthetized by intraperitoneal injection of ketamine (75mg/kg) and xylazine (10mg/kg) and blood was collected by cardiac puncture; serum was obtained by centrifuging at 1 000 X g for 10 min (Eppendorf 5402, Hamburg, Germany) before storing at −80°C. For ventricular access, the anesthetized rats were placed in a stereotaxic apparatus and cerebrospinal fluid (CSF) was obtained carefully by puncturing the cisterna magna with an insulin syringe. A maximum volume of 30 µl was collected over a 3-min period to minimize the risk of brain stem damage. Hippocampi were dissected and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper as described above. Samples were stored at −80°C until biochemical and immunological assays.

Cytokine measurement

Hippocampal slices were homogenized in phosphate buffer saline (PBS) containing (in mM) 50 NaCl, 18 Na_2HPO_4, 83 $NaH_2PO_4.H_2O$, pH 7.4, with 1 mM EGTA and 1 mM phenylmethyl-sulphonyl fluoride (PMSF), followed by centrifugation at 1 000 X g for 5 min at 4ºC. Cytokines were measured in supernatants using rat TNF-α, IL-1β, IL-10 (eBioscience, San Diego, USA) and PGE2 (Enzo Life Science, Farmingdale, NY , USA) ELISA Kits. Serum TNF-α content was also evaluated. Data are expressed in pg/mg protein (tissue samples) or pg/ml (serum).

S100B measurement

Slices were homogenized in PBS with 1 mM EGTA and 1 mM PMSF. The S100B content in the CSF, serum and brain tissue was measured by ELISA, as described above. Data are expressed in ng/mg protein (tissue samples) or ng/ml (CSF and serum).

GFAP measurement

GFAP content was measured by ELISA, as described previously [48]. The ELISA for GFAP was carried out by coating wells of 96-well plates with 100 µl samples containing 70 µg of protein overnight at 4°C. Wells were incubated with a polyclonal anti-GFAP antibody (Dako, Carpinteria, CA, U.S.A.) from rabbit for 2 h, followed by incubation with a secondary antibody conjugated with peroxidase for 1 h, at room temperature. The color reaction with OPD was measured at 492 nm. The standard GFAP (Calbiochem, San Diego, CA, U.S.A.) curve ranged from 0.1 to 10 ng/ml. Data are expressed in ng/mg protein.

Glutamine Synthetase (GS) activity

The enzymatic assay for glutamine synthetase (GS) was performed, as described previously [49] with modifications. Briefly, hippocampal slices were homogenized in 50 mM imidazole buffer. Homogenates were then incubated with (mM): 50 imidazole, 50 hydroxylamine, 100 L-glutamine, 25 sodium arsenate dibasic hepta-hydrated, 0.2 ADP, 2 manganese chloride, pH 6.2 for 15 min at 37°C. The reactions were terminated by the addition of 0.2 ml of 0.37 M $FeCl_3$, 200 mM trichloroacetic acid, and 670 mM HCl. After centrifugation, supernatant absorbance was measured at 530 nm. The standard γ-glutamylhydroxamate acid (Sigma-Aldrich, St. Louis, MO. USA) curve ranged from 0.1 to 10 mmol/ml. GS activity is expressed as µmol/h/mg protein.

Glutathione (GSH) content

Reduced glutathione (GSH) content was determined based on [50]. Briefly, slices were homogenized in sodium phosphate buffer (0.1 M, pH 8.0) and protein was precipitated with 1.7% meta-phosphoric acid. O-phthaldialdehyde (1 mg/ml methanol) (Sigma) was added to the supernatant at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively.

The standard calibration glutathione (Sigma-Aldrich, St. Louis, MO. USA) solutions curve ranged from 0 to 500 μM. Glutathione results are expressed as nmol/mg protein.

Western Blotting

Nitrocellulose membranes were blocked overnight at 4ºC with 2% bovine serum albumin (BSA) in Tris-buffered saline (TBS; in mM 10 Tris, 150 NaCl, pH 7.5 and 0.05% Tween 20®) and then incubated overnight at 4°C in blocking solution containing the following antibodies, anti-Kir 4.1, anti-AQP-4, anti-COX1, anti-COX2 (diluted 1:1,000), and anti-GS (diluted 1:10,000) (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) and anti-actin (1:2000) (Sigma). Subsequently, membranes were incubated for 1 h at room temperature in solution containing horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (diluted 1:10,000), HRP anti-mouse IgG (diluted 1:10,000) (GE Healthcare, Sao Paulo—Brazil) or HRP anti-goat diluted 1:10000 (Sigma). Chemiluminescence signal was detected by luminol substrate reaction (ECL Western Blotting System, GE Healthcare®). Immunoblots were quantified by membranes scanning in Image4000, GE Healthcare®, and optical densities of studied proteins were determined by ImageJ software (Packard Instrument Company) and was determined by protein/actin ratio.

Protein measurement

Protein was measured by Lowry's method, modified by Peterson, using bovine serum albumin as a standard [51].

Statistical Analysis

All results were expressed as mean ± standard error mean (SEM) and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's test. The level of statistical significance was set at $P<0.05$. All analyses were performed using the Prism 5.0 software (GraphPad).

Results

S100B secretion in acute hippocampal slices of epileptic rats

All animals from the Li-pilocarpine group, used in neurochemical assays, reached at least phase 4 of the epileptic seizure Racine's scale within 13 min after pilocarpine administration (data not shown). At 1, 14 and 56 days after Li-pilocarpine

treatment, we analyzed basal S100B secretion or S100B secretion in the presence of dexamethasone or in high-potassium medium, in acute hippocampal slices from control and Li-pilocarpine treated animals (Figure 1).

S100B secretion in hippocampal slices from sham animals is presented in panels 1A, 1C and 1E, which correspond to 1, 14 and 56 days after saline administration. Li-pilocarpine-treated animals are shown in panels B, D and F. Basal secretion did not differ between the sham and Li-pilocarpine-treated rats at all times (data not shown) and was assumed as 100%. Dexamethasone down-regulated S100B secretion (P=0.0073 in A, P=0.0184 in C and P=0.0171 in E) in high-potassium medium at all times analyzed. Ex-vivo S100B secretion of hippocampi after *SE* induction of rats by Li-pilocarpine was not different in normal or high-potassium medium for all times analyzed. However, dexamethasone down regulated S100B secretion at 1 (panel 1B) and 56 days (panel 1F) following *SE* induction (P=0.0184 and P=0.0171, respectively). Dexamethasone did not affect S100B secretion at 14 days (panel 1C) after *SE* induction (P=0.9242).

Inflammatory signals and astroglial parameters in the hippocampus of epileptic rats treated with dexamethasone

Based on previous *ex vivo* results, in this set of experiments we administrated intraperitoneal dexamethasone 1 day after *SE* induction and analyzed inflammatory signals and astroglial parameters at two times: 1 and 56 days afterwards.

Dexamethasone reverses the increment in inflammatory cytokines, PGE2 and COX2

Dexamethasone, administered at 24h after *SE* induction, was able to reverse inflammatory signals of hippocampal inflammation caused by *SE* induction (Figure 2). Dexamethasone reversed the augmentation in IL-1β (Panel 2A) and PGE2 (Panel 2C) levels in the hippocampus of *SE* animals at 1 day after pilocarpine injection (P=0.0001 and P=0.0002, respectively). The treatment with dexamethasone reduced TNF-α levels (Panel 2B), when compared with *SE* and sham animals (P=0.0286). No change was observed in IL-10 at 1 day after *SE* and dexamethasone did not affect the levels of this anti-inflammatory cytokine at this time (P = 0.9221; Panel 2D). However, interestingly, we observed an increase in hippocampal IL-10 in dexamethasone-treated animals at day 56 after *SE* (P= 0.0165, Panel 1E). At 56 days after SE, the increment in PGE2 was not significant (P=0.572; Panel F) and dexamethasone did not affect this parameter.

In order to further investigate the effect on inflammatory parameters in this model we measured the contents of COX1 and 2 (Figure 3). The immunocontent of COX1 was the same in all groups at 1 (Panel C) and 56 (panel D) days ($p = 0.8938$ and $p = 0.4244$, respectively). However, dexamethasone reversed the increase in COX2 at 1 (Panel E) and 56 (Panel F) days (*P*=0.0109 and *P*=0.00913, respectively).

Dexamethasone reverses astrogliosis markers in the SE model: GFAP and S100B

The high GFAP content induced by *SE* was reversed by dexamethasone in animals at 1 (Panel 4A) and 56 (Panel 4B) days after pilocarpine injection (*P*=0.0035 and *P*=0.0245, respectively). Hippocampal S100B content also increased following *SE* induction (Panels 5A and 5B), and this was reversed by dexamethasone at 1 day (Panel 5C, *P*=0.0062). However, the elevation in hippocampal S100B at 56 days in *SE* animals was partially reversed by dexamethasone administration (Panel 5B, *P*=0.0445).

Dexamethasone altered levels of cerebrospinal fluid S100B

CSF S100B content was increased after *SE* induction at 1 (Panel 5C) and 56 (Panel 5D) days (*P*=0.0125 and *P*=0.0096, respectively). Dexamethasone was not able to reverse this increase at 1 day after pilocarpine administration (Panel 5C). However, it completely reversed the elevation in CSF S100B at 56 days (Panel 5C). Serum S100B content diminished in *SE* animals at 1 day (*P*= 0.0005; Panel 5E) and dexamethasone did not affect this change. At 56 days, serum S100B was not different in *SE* animals or affected by dexamethasone (*P*=0.3719; Panel 5E)

Glutathione content is diminished by SE induction and reversed by dexamethasone

Based on the astrogliosis signals found in this model, we investigated other astroglial parameters related to astrocyte functionality, namely glutamine synthetase (GS) activity, GSH content, potassium channel Kir 4.1 and aquaporin-4 (AQP-4). GS activity decreased in *SE* animals (Figure 6) at 1 (Panel A) and 56 (Panel B) days (*P*=0.0003 and *P*=0.0030, respectively) and dexamethasone did not reverse this specific parameter at either time. GSH content is not a suitable marker for astrocytes, but its synthesis and recycling is totally dependent on astrocyte activity. As such, we found a decrease in GSH content at 1 (Panel 6C) and 56 (Panel 6D) days (*P*=0.0174 and *P*=0.0500, respectively), reflecting astroglial dysfunction, and dexamethasone administration completely reversed this alteration.

Two other astroglial parameters, Kir 4.1 and AQP-4, are affected by dexamethasone administration

The content of the Kir 4.1 potassium channel was lower in *SE* animals at 1 (Figure 7C) and 56 (Figure 7D) days (P=0.0024 and P=0.0418, respectively), and dexamethasone was able to reverse this effect at 56 days after *SE* induction, but not at 1 day after. There were no differences in AQP-4 levels in *SE* animals at 1 (Figure 7E and 56 (Figure 7F) days (P=0.6905 and P=0.1419, respectively), and dexamethasone did not affect AQP-4 content at 1 and 56 days after *SE* induction.

Discussion

Most AED target neuronal activity modulated by ionic channels. The steroid sensitivity of GABA receptors has led to the use of steroids as adjunctive drugs for epilepsy [52–54]. Moreover, 30% of epileptic patients develop resistance to AED [55] and additional strategies for therapeutic approaches are welcome. Mounting evidence suggests that astrocytes and neuroinflammation (which is modulated by astrocytes and microglia in brain tissue) contribute to epileptogenesis, and are potential targets for therapies being developed against epileptic disorders [56].

Dexamethasone effects on S100B secretion

As previously mentioned, S100B is widely used as a marker for epileptic disorders [29,30] and its secretion is modulated by LPS [32] and antiinflamatory drugs [33] (Guerra et al., unpublished data). Our studies have suggested that hippocampal and CSF S100B are altered after *SE* induction in the Li-pilocarpine model [12]. We, herein, confirm that hippocampal slices incubated in high-potassim medium secrete less S100B [57,58]; this effect was observed in slices from young (14 days old) and adult (70 days old) rats. However, in rats submited to the Li-pilocarpine model of epilepsy, this S100B secretion did not respond to high-potassium stimulation as did corresponding controls. The mechanism involved in this difference is unclear at the moment [57], as well as the mechanism of S100B secretion [34]. However, as we found a decrease in potassium channels 4.1 in *SE* animals this may be related to a lower potassium influx in astrocytes in these animals [46,59,60].

Many modulators of S100B secretions have been described [35]. Dexamethasone *per se* decreased S100B secretion in acute hippocampal slices from

control animals (in all analyzed times) and in *SE* animals (at 1 and 56 days after pilocarpine administration). No in vitro effect of dexamethasone occurred at 14 days after SE, which corresponds to the "silent period" of this model [41]. The acute effect of dexamethasone on epileptiform activity of hippocampal slices has been previously reported [61], but the mechanism underlying this activity remains unclear. Based on the dexamethasone efffect on S100B secretion at 1 day after *SE* induction. We decided to investigate if dexamethasone administration only 1 day after pilocarpine injection cause a reversion effect to inflammatory and astroglial markers at 1 and 56 days after dexamethasone injection. We did not use 14 days group to reduce the number of experimental animals.

Dexamethasone reverts neuroinflammation

The long-term effect of dexamethasone that we observed on astroglial and inflammatory parameters involves changes in gene expression. In fact, dexamethasone reversed the increases in IL-1β, TNF-α and COX2 (and consequently PGE2 levels) in the hippocampus of *SE* animals at 1 day after dexamethasone administration. Of note, in *SE* animals treated with dexamethasone TNF-α was decreased to levels lower than those of sham animals, but unfortunately we did not carry out measurements of cytokines in a sham group treated with dexamethasone. Furthermore, dexamethasone increased IL-10, an anti-inflammatory cytokine, in *SE* animals at 56 days after pilocarpine administration. Taken together, these data suggest a non-inflammatory scenario induced by dexamethasone in the hippocampus of Li-pilocarpine treated animals over the short and long-term.

Dexamethasone reverts astrogliosis

Based on two classical glial markers, GFAP and S100B, we found that dexamethasone administration at 24 h after pilocarpine-induced *SE* administration was able to reverse astrogliosis. It is well known that GFAP and S100B expressions are down regulated in glial cultures by dexamethasone [62,63] and that the *in vivo* administration of this corticoid has been used to reduce inflammatory response and gliosis [64,65]. Moreover, although dexamethasone was not able to reduce CSF S100B during a short time (2 days after SE), it was effective later on (at 56 day after SE); an effect that may be of relevance because elevated chronic levels of this protein contribute to neurodegenerative diseases [34,35]. Note that we found a decrease in serum S100B

after SE. This could be due to brain "retention" of this protein, as proposed in some cases of acute brain injury [66] or could be due to its peripheral alteration (independent of brain source) [67]. It is also important to mention that in another model of *SE* induction, using scopolamine/pilocarpine, an increase in serum S100B was reported and that the previous administration of dexamethasone prevented this increment [68].

Dexamethasone effects on astrocyte functions

Glutamine synthetase (GS) is a specific astrocyte enzyme that is critical to glutamate metabolism in the brain, and closely related to glutamatergic and GABAergic neurotransmission and reduced in the human hippocampus in TLE [69]. Reduced expression of GS was reported at 2 weeks after *SE* in the Li-pilocarpine model [70]. In this study, we detected an earlier decrease in GS that persisted until the chronic phase. It is well-known that dexamethasone induces the expression of this enzyme [71]; however, inflammatory cytokines are able to block this induction in astrocyte cultures [72]. We assumed that the hippocampal GS decrease in the Li-pilocarpine model is due to inflammation, but it is unclear at the moment why dexamethasone did not reverse this effect. This effect may depend on the dose and time of corticoid administration. On the other hand, the hippocampal oxidative stress observed in this model [73,74], characterized here by the decrease in GSH, was completely reversed by dexamethasone administration. Rosiglitazone, an agonist of peroxisome proliferator-activated receptor gamma that has anti-inflammatory activity, also prevented GSH imbalance in the hippocampus after *SE* induced by pilocarpine [75].

Neuronal excitability is highly dependent on extracellular levels of K+, which are regulated mainly by the astrocytic Kir4.1 potassium channels that are in turn functionally coupled to the AQP-4 water channels [28]. In a previous study reporting on the induction of *SE* in rats with pilocarpine (without lithium), an increase in Kir4.1 in the cortical regions, but not in the hippocampus, was observed at 8 weeks after *SE* induction [76]. We found an early (1 day after SE) and persistent (56 days after SE) decrease in Kir4.1 content in the hippocampus. This apparently contradictory result is possibly due to methodological differences. Dexamethasone reversed the decrease in Kir4.1 at 56 days after *SE* induction, but not at 1 day afterwards. Accordingly, in the eye retina, dexamethasone (used to treat macular edema) selectively upregulated Kir4.1 (but not AQP-4) channels [77]. Regardless of the changes in Kir4.1 observed in *SE* animals, no changes in AQP-4 were observed in this model.

Corticosteroid therapy in epilepsy disorders is a matter of debate, due to the pro- and anti-epileptic effects observed. Experimental studies have administered dexamethasone before *SE* induction and observed behavioral alterations, changes in the latency period of *SE* and mortality, biochemical, neurological and inflammatory modifications [39,78,79]. However, if dexamethasone administration occurred during SE, there were no changes in latency period, increased mortality ratio and exacerbated cerebral edema [40]. Data relating to COX2 expression in experimental models suggests that the corticoid effect depends on the dose, the time point of administration, the type of inhibitor (selective or non-selective) and differences among models of *SE* induction [80]. Some limitations of this study should be highlighted. Firstly, EEG records at different times would be useful to characterize *SE* and seizures later on. The abcense of this data was due to avoid surgery and the activation of neuroinflammation and astrogliosis by the lesion followed the introduction of electrodes. The different time points 1, 14 and 56 days correspond to acute, latent and chronic epileptic phases and was determined in previous study [46]. This study demonstrated that dexamethasone administration (24h after *SE*) promotes effects on acute and chronic phases of epilepsy. Our results show a decrease in neuroinflammation, astrogliosis and astroglial dysfunction, as well as improvements in epileptic behavior (data not shown). Taken together, our data reinforce the neuroprotective role of dexamethasone in epilepsy disorders and resulting neurodegenerative diseases.

Conclusions

In summary, we initially demonstrated in *ex vivo* hippocampal slices from sham and *SE* animals that dexamethasone was able to acutely reduce S100B secretion, whereas a high-potassium medium did not reduce S100B secretion in slices from *SE* animals as it did in control animals. In another set of experiments, we found alterations in: inflammatory parameters (cytokines, COX2 and PGE2), as wells as clear alterations in astroglial markers (GFAP and S100B) and indicators of astrocyte dysfunction (GS activity, GSH content, Kir4.1 potassium channel and AQP-4 water channel) in the hippocampus of rats submitted to the Li-pilocarpine model of epilepsy, at 1 and 56 days after SE. CSF and serum S100B were also investigated. All these parameters, with the exception of AQP-4, were altered, emphasizing the importance of this model for understanding alterations and mechanisms of epileptic disorders. *In vivo* dexamethasone administration, 24h after *SE* induction, reversed most the parameters analyzed,

reinforcing the importance of anti-inflammatory steroid therapy in epilepsy. Our data on specific alterations in astrocytes in this model clearly contribute to our understanding of the importance of these cells in the pathogenesis of epilepsy as well as suggesting potential therapeutic targets for AED.

List of abbreviations:

SE: status epilepticus, GFAP: glial fibrillary acid protein, LDH: lactate dehydrogenase, CSF: cerebrospiinal fluid, TNF-α: tumor necrosis factor alpha, IL-1β: interleukin-1 beta, IL-10: interleukin-10, PGE2: prostaglandin E2, GS: glutamine synthetase, GSH: glutathione, Kir 4.1: inward-rectifier potassium ion channel4.1, AQP4: aquaporin channel 4, COX1: cyclooxygenase 1, COX2: cyclooxygenase 2.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

AFKV organized and conducted all study. FH, EN and MCL helped to animal experimentation. DLO and CAG aided in discussion of data and writing the manuscript. All authors have read and approved of the final manuscript.

Competing interests

The authors declare that they have no conflicts of interests.

Consent for publication

Not applicable.

Consent

The study was approved by the Committee of Animal Use of the Federal University of Rio Grande do Sul (project number 24472) and the animal procedures were in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23).

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Figure legends

Figure 1: *Dexamethasone and high potassium levels modulate S100B secretion in acute hippocampal slice*. S100B secretion from the hippocampus was measured by ELISA. Dexamethasone and high potassium levels decreased S100B secretion in control (saline) animals at 1, 14 and 56 days (A, C and E). Dexamethasone reduced S100B secretion at 1 and 56 days after pilocarpine injection. *SE* animals were not affected by high potassium at 1, 14 and 56 days (B, D and F). Data are expressed as percentages compared to the basal condition and values represent mean ± standard error, of 6-8 animals per group. Data were analyzed by ANOVA, following by the Dunnet test. Bars without a common letter differ significantly, assuming $p < 0.05$.

Figure 2: *Dexamethasone reverses neuroinflammation in the hippocampus*. Pro-inflammatory and anti-inflammatory cytokines were measured by ELISA. Dexamethasone decreased TNFα (A) and reversed IL-1β (B) and PGE-2 (C) levels at 1 day after treatment. Dexamethasone did not affect PGE2 (D) content at 56 days nor IL-10 levels at 1 day after treatment. At 56 days, dexamethasone increased IL-10 content. Values were expressed by mean ± standard error, of 4-6 animals per group. Data were analyzed by ANOVA, followed by the Tukey test. Bars without a common letter differ significantly, assuming $p < 0.05$.

Figure 3: *Dexamethasone reverses COX2 content in the hippocampus*. COX1 and COX2 content was measured by Western Blot. Representative images of COX1 and COX2 in the hippocampus at 1 and 56 days after treatment (A-B). Chemiluminescent quantification of protein/actin of COX 1 (C-D) and COX 2 (E-F). Dexamethasone reversed the increase in COX2 content in the hippocampus of *SE* animals at 1 and 56 days after treatment (E and F respectively). Values represent mean ± standard error, of 4-6 animals per group. Data were analyzed by ANOVA, following by the Tukey test. Bars without a common letter differ significantly, assuming $p < 0.05$.

Figure 4: *Dexamethasone reverses astrogliosis in the hippocampus*. GFAP was measured by ELISA. Dexamethasone reversed the increase in GFAP content in the hippocampus of *SE* animals at 1 and 56 days after treatment (A and B, respectively). Values represent mean ± standard error, of 4-6 animals per group. Data were analyzed

by ANOVA, followed by the Tukey test. Bars without a common letter differ significantly, assuming $p < 0.05$.

Figure 5: *Dexamethasone modulates S100B levels in the hippocampus and in the cerebrospinal fluid*. S100B content was measured by ELISA. Dexamethasone reversed the augmentation in S100B content in the hippocampus of *SE* animals at 1 day after treatment (A) and did not affect S100B content at 56 days after *SE* induction (B). The augmentation in S100B levels in the CSF were reversed by dexamethasone at 56 days after *SE* induction (D). Serum S100B levels were not altered by dexamethasone at any of the times (E-F) Values represent mean ± standard error, of 4-6 animals per group. Data were analyzed by ANOVA, followed by the Tukey test. Bars without a common letter differ significantly, assuming $p < 0.05$.

Figure 6: *Dexamethasone does not modulate decreased GS activity, but reverses GSH content in the hippocampus*. The decrease in GS activity was not modulated by dexamethasone in the *SE* animals (A-B). Dexamethasone reversed the reduction in GSH reduced at 1 and 56 days after treatment (C-D). Values represent mean ± standard error, of 4-6 animals per group. Data were analyzed by ANOVA, followed by Tukey test. Bars without a common letter differ significantly, assuming $p < 0.05$.

Figure 7: *Dexamethasone does not alter Kir 4.1 and AQP-4 astrocyte channel content in the hippocampus*. Kir 4.1 and AQP-4 contents were determined by Western Blot. Representative image of Kir 4.1 and AQP-4 in the hippocampus at 1 and 56 days after treatment (A-B). Chemiluminescent quantification of Kir 4.1 (C-D) and AQP-4 (E-F) protein/actin. Dexamethasone did not reverse the reduction in Kir 4.1 content in the hippocampus of *SE* animals at 1 day after treatment (C), however, dexamethasone reverses Kir4.1 content of *SE* animals at 56 days after treatment (D). No differences of AQP-4 channel was observed between groups (E-F). Values represent mean ± standard error, of 4-6 animals per group. Data were analyzed by ANOVA, following Tukey test. Bars without a common letter differ significantly, assuming $p < 0.05$.

Additional file 1

File name: Figure studies

File format: tiff

Title data: Schematic experimental design.

Description data: Schematic experimental design of the two studies. The first study was the S100B secretion analysis in acute hippocampal slices of *SE* rats. The second study was the analysis of dexamethasone effects on neuroinflammation, astrogliosis and astrocyte functions on *SE* model.

Figure 1

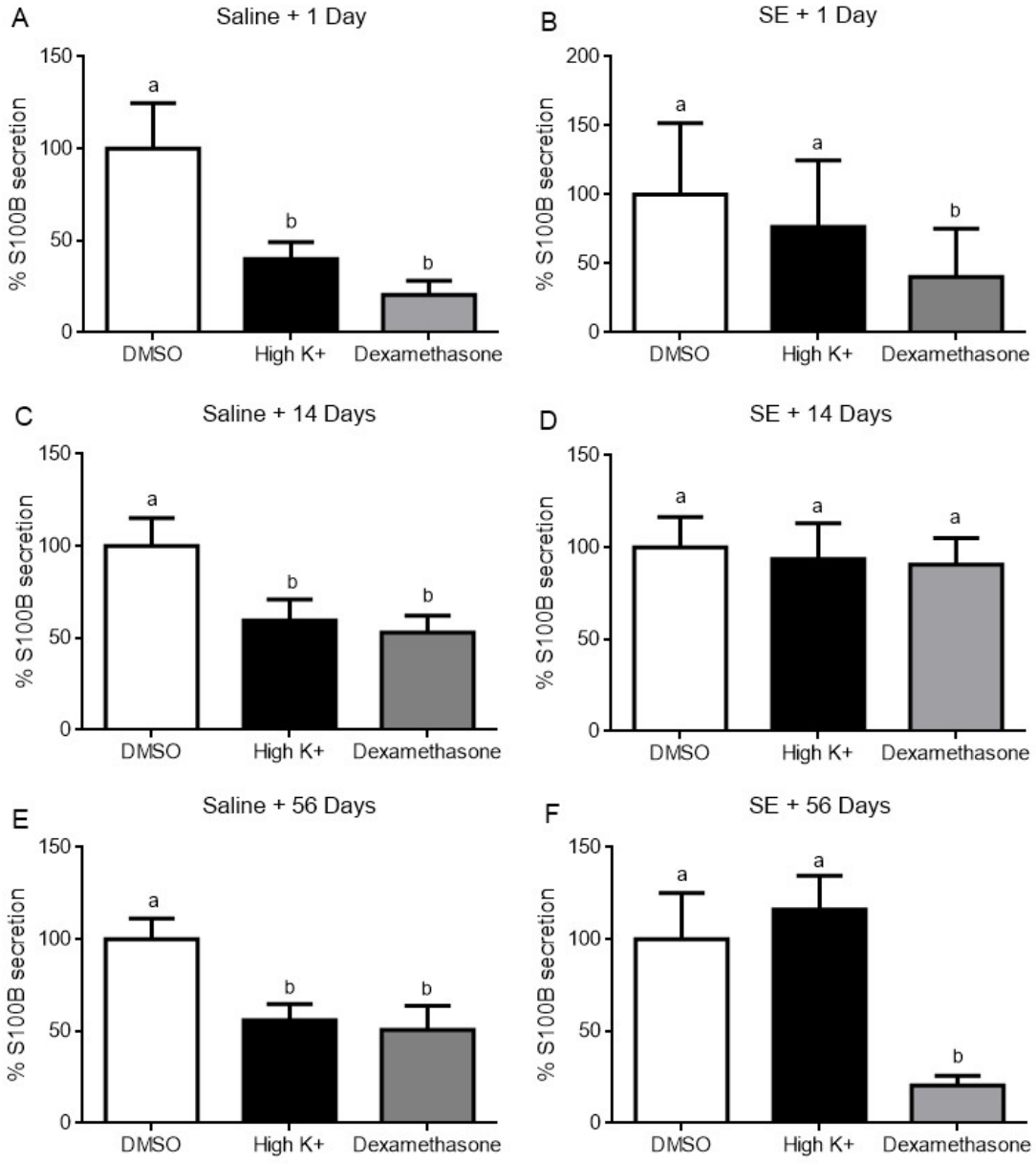

A
Saline + 1 Day
% S100B secretion
150
100
50
0
a
b
b
DMSO
High K+
Dexamethasone
B
SE + 1 Day
% S100B secretion
200
150
100
50
0
a
a
b
DMSO
High K+
Dexamethasone
C
Saline + 14 Days
% S100B secretion
150
100
50
0
a
b
b
DMSO
High K+
Dexamethasone
D
SE + 14 Days
% S100B secretion
150
100
50
0
a
a
a
DMSO
High K+
Dexamethasone
E
Saline + 56 Days
% S100B secretion
150
100
50
0
a
b
b
DMSO
High K+
Dexamethasone
F
SE + 56 Days
% S100B secretion
150
100
50
0
a
a
b
DMSO
High K+
Dexamethasone

Figure 2

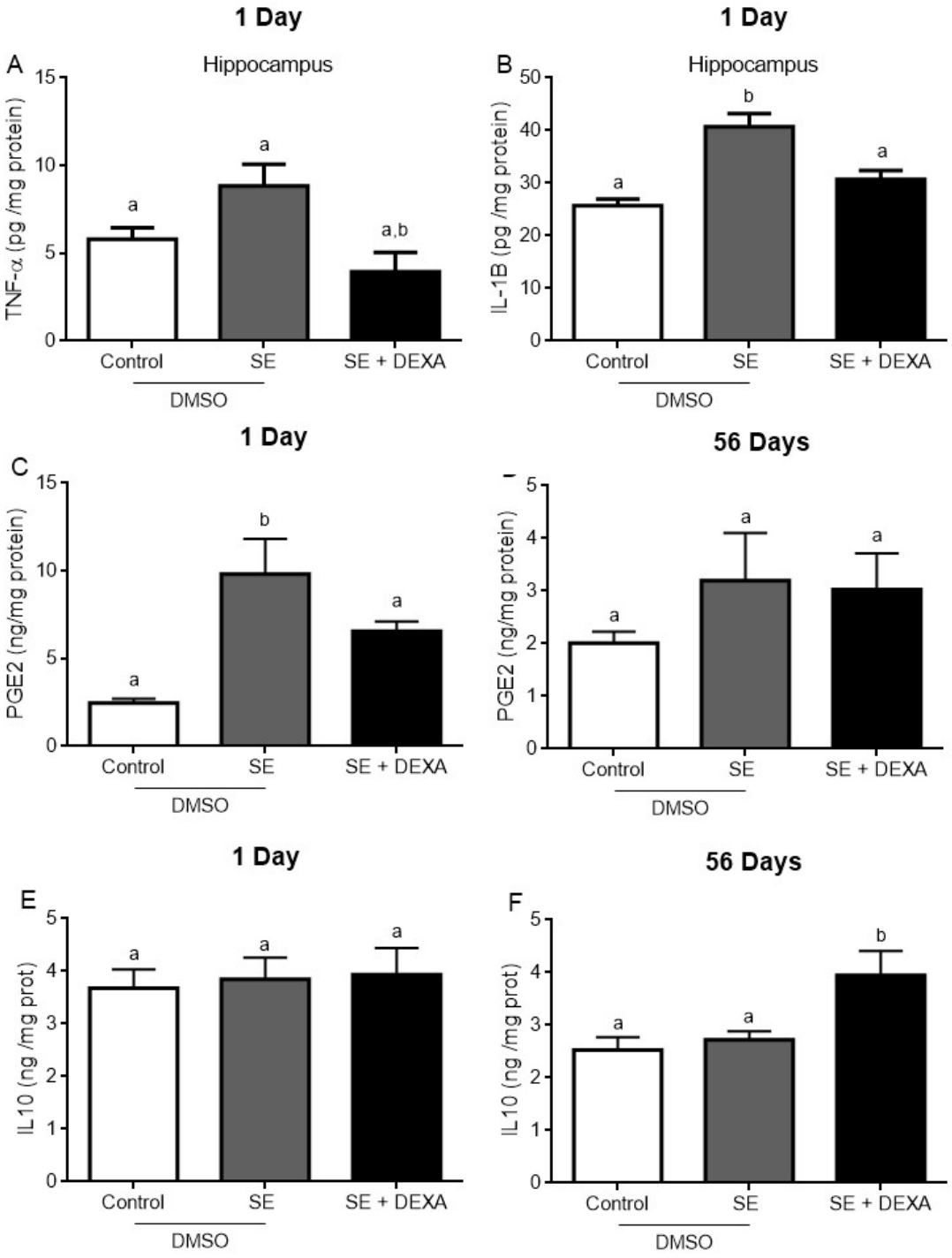

1 Day
A
Hippocampus
TNF-α (pg /mg protein)
15
10
5
0
a
a
a,b
Control
SE
SE + DEXA
DMSO
1 Day
B
Hippocampus
IL-1B (pg /mg protein)
50
40
30
20
10
0
a
b
a
Control
SE
SE + DEXA
DMSO
1 Day
C
PGE2 (ng/mg protein)
15
10
5
0
a
b
a
Control
SE
SE + DEXA
DMSO
56 Days
PGE2 (ng/mg protein)
5
4
3
2
1
0
a
a
a
Control
SE
SE + DEXA
DMSO
1 Day
E
IL10 (ng /mg prot)
5
4
3
2
1
0
a
a
a
Control
SE
SE + DEXA
DMSO
56 Days
F
IL10 (ng /mg prot)
5
4
3
2
1
0
a
a
b
Control
SE
SE + DEXA
DMSO

Figure 3

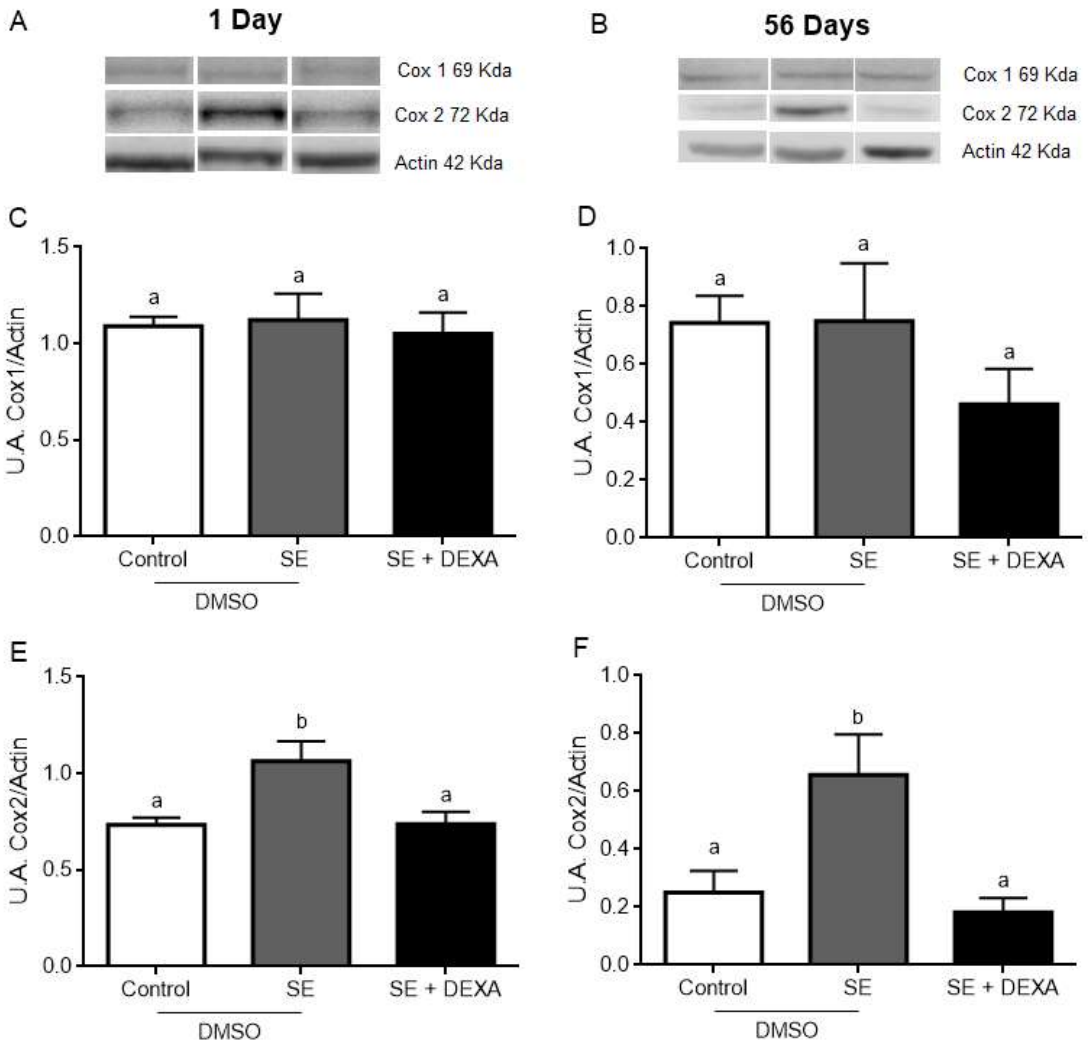

A
1 Day
Cox 1 69 Kda
Cox 2 72 Kda
Actin 42 Kda
B
56 Days
Cox 1 69 Kda
Cox 2 72 Kda
Actin 42 Kda
C
U.A. Cox1/Actin
1.5
1.0
0.5
0.0
a
a
a
Control
SE
SE + DEXA
DMSO
D
U.A. Cox1/Actin
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
Control
SE
SE + DEXA
DMSO
E
U.A. Cox2/Actin
1.5
1.0
0.5
0.0
a
b
a
Control
SE
SE + DEXA
DMSO
F
U.A. Cox2/Actin
1.0
0.8
0.6
0.4
0.2
0.0
a
b
a
Control
SE
SE + DEXA
DMSO

Figure 4

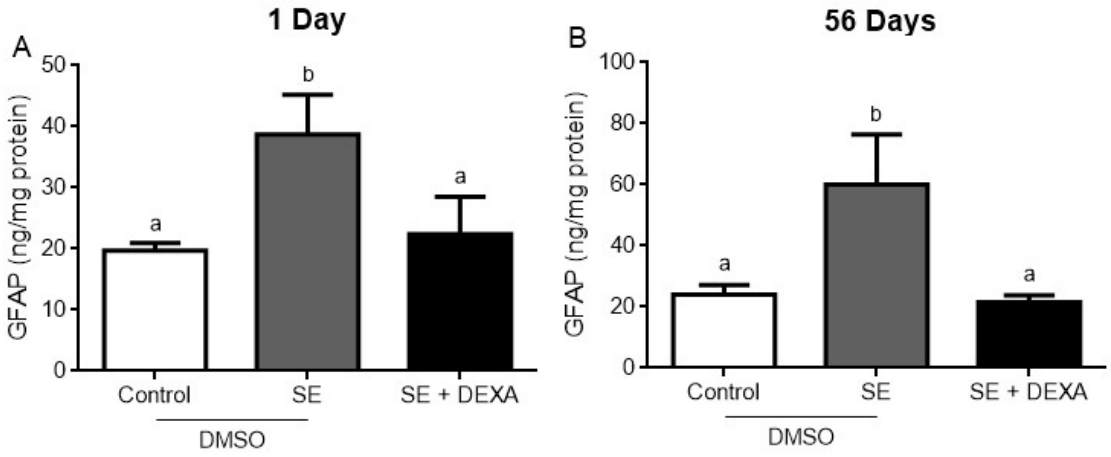

A
1 Day
GFAP (ng/mg protein)
50
40
30
20
10
0
a
b
a
Control
SE
SE + DEXA
DMSO
B
56 Days
GFAP (ng/mg protein)
100
80
60
40
20
0
a
b
a
Control
SE
SE + DEXA
DMSO

Figure 5

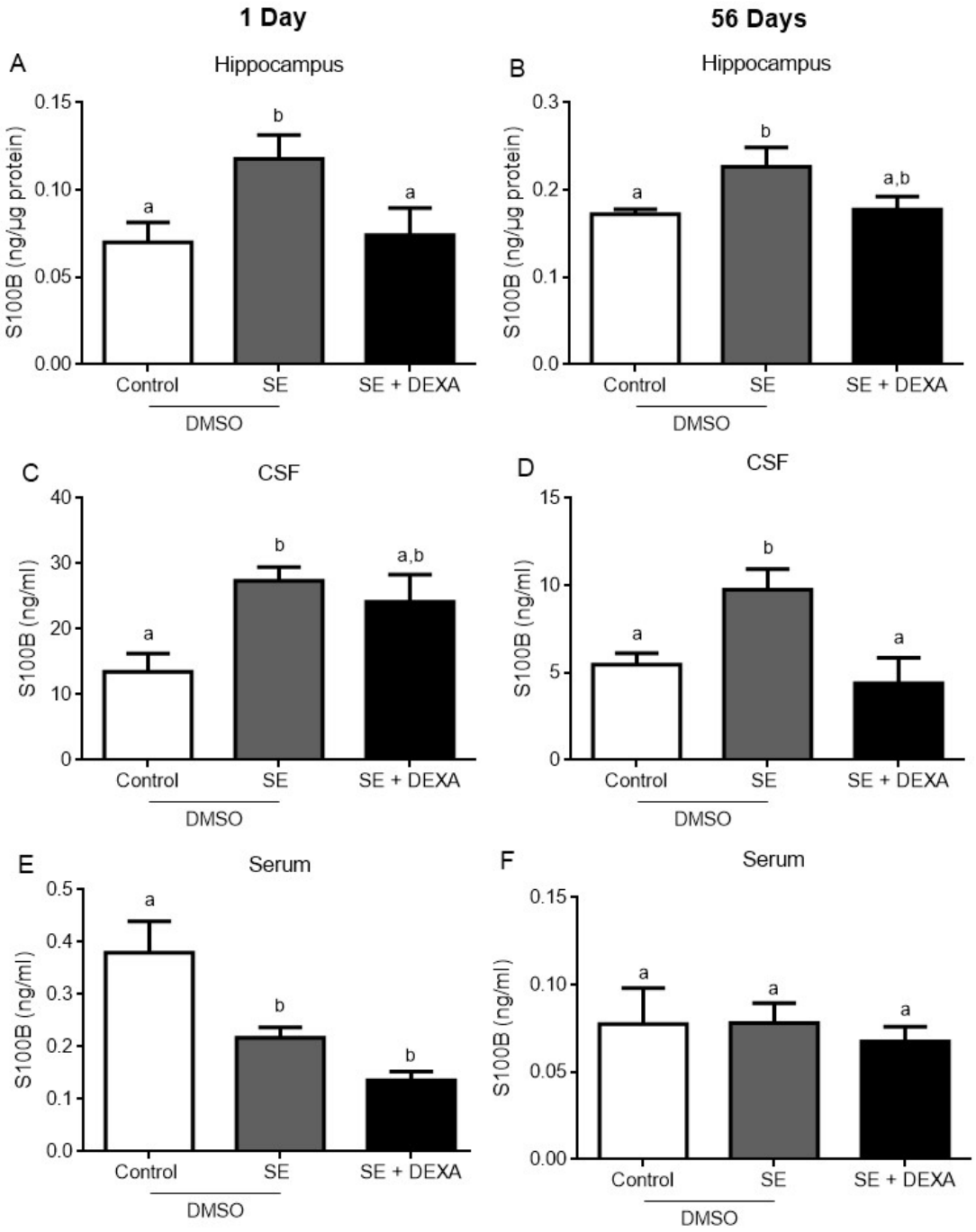

1 Day
56 Days
A
Hippocampus
B
Hippocampus
S100B (ng/µg protein)
0.15
0.10
0.05
0.00
0.3
0.2
0.1
0.0
a
b
a
a,b
Control
SE
SE + DEXA
DMSO
C
CSF
D
CSF
S100B (ng/ml)
40
30
20
10
0
15
10
5
E
Serum
F
Serum
0.5
0.4
0.3
0.2
0.1
0.0

Figure 6

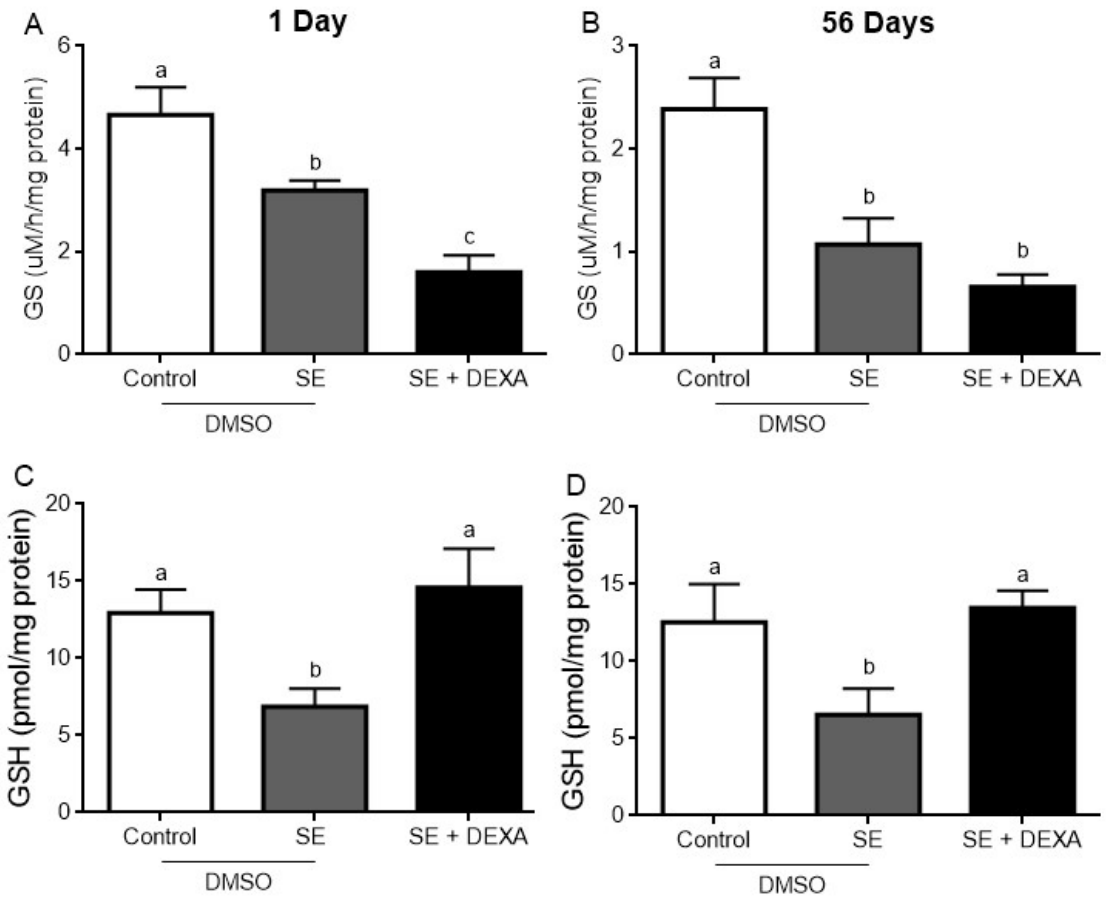
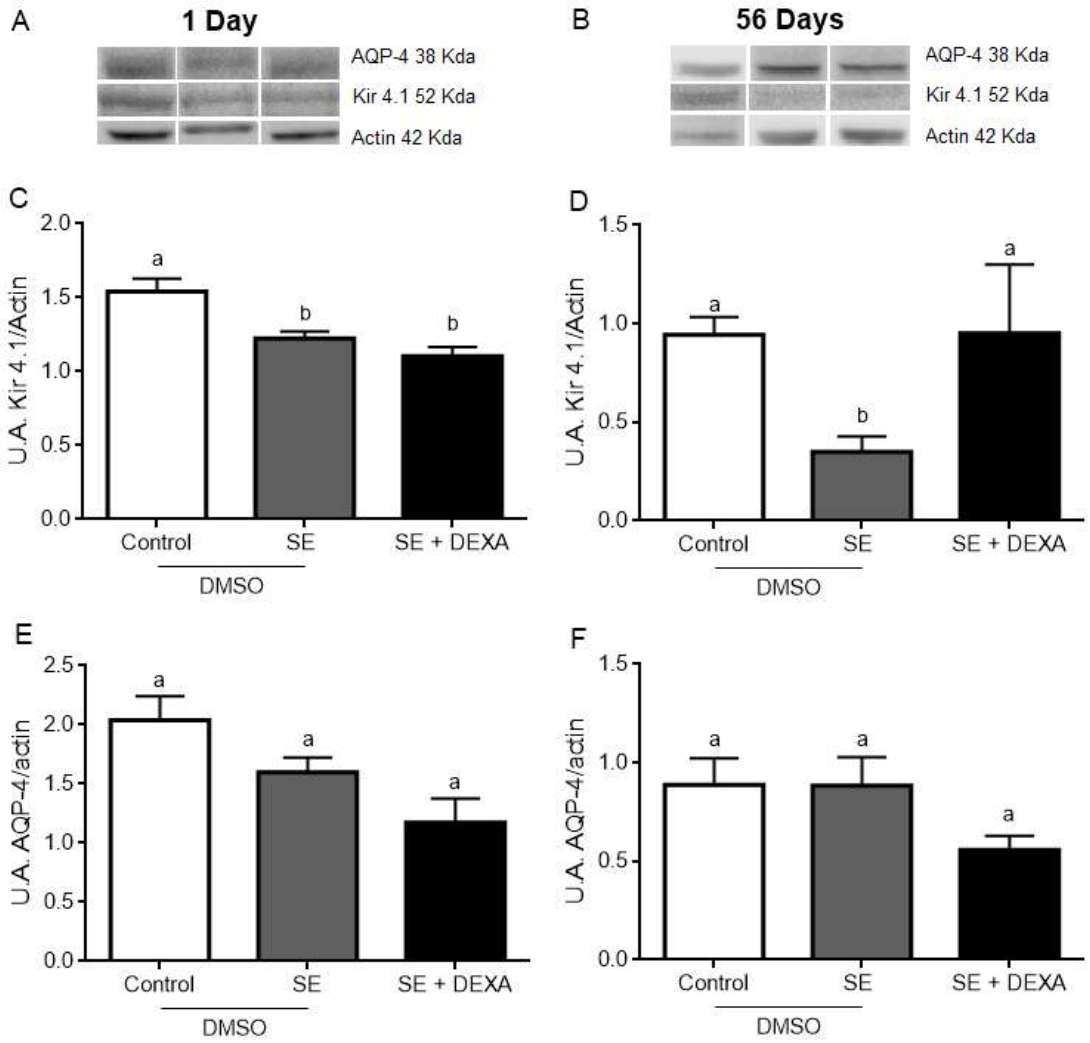
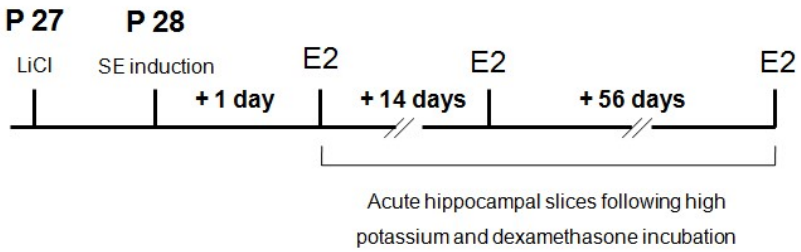

A
1 Day
GS (uM/h/mg protein)
a
b
c
Control
SE
SE + DEXA
DMSO
B
56 Days
GS (uM/h/mg protein)
a
b
b
Control
SE
SE + DEXA
DMSO
C
GSH (pmol/mg protein)
a
b
a
Control
SE
SE + DEXA
DMSO
D
GSH (pmol/mg protein)
a
b
a
Control
SE
SE + DEXA
DMSO

Figure 7


A
1 Day
AQP-4 38 Kda
Kir 4.1 52 Kda
Actin 42 Kda
B
56 Days
AQP-4 38 Kda
Kir 4.1 52 Kda
Actin 42 Kda
C
U.A. Kir 4.1/Actin
2.0
1.5
1.0
0.5
0.0
a
b
b
Control
SE
SE + DEXA
DMSO
D
U.A. Kir 4.1/Actin
1.5
1.0
0.5
0.0
a
b
a
Control
SE
SE + DEXA
DMSO
E
U.A. AQP-4/actin
2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
Control
SE
SE + DEXA
DMSO
F
U.A. AQP-4/actin
1.5
1.0
0.5
0.0
a
a
a
Control
SE
SE + DEXA
DMSO

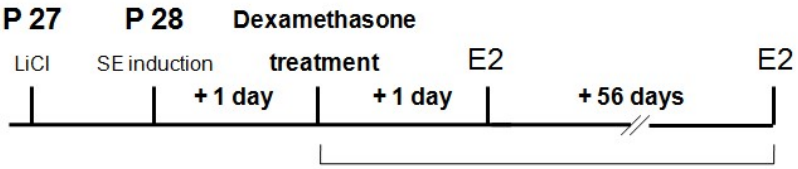
Additional file 1

1) First study - Preparation and incubation of hippocampal slices



Acute hippocampal slices following high potassium and dexamethasone incubation

2) Second study - Protection of dexamethasone in animals submitted to SE model



Inflammatory, astrogliosis and astroglial dysfunction parameters

CAPÍTULO 3

Artigo em preparação

$GABA_A$ receptor modulation affects S100B secretion

Justificativa: Os astrócitos são células gliais participantes da sinapse tripartite e regulam a atividade neuronal. Astrócitos comunicam-se com neurônios e podem sinalizar condições fisiológicas e patológicas do SNC através de diversos fatores, como a proteínaS100B.

Objetivo geral: Investigar a sinalização do receptor $GABA_A$ na modulação da secreção de S100B *ex vivo* em fatias hipocampais em ratos *Wistar* jovens.

Objetivo específico: Analisar a modulação da secreção de S100B através de antagonistas e agonistas GABAérgicos.

$GABA_A$ receptor modulation affects S100B secretion

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Introduction

Astrocytes are the major cell of glial population and participates of many brain mechanisms. Currently it is known that there is a cross talk between astrocytes and neurons in a cleft forming the tripartite synapse (Perea et al., 2009). Astrocytes are cells responsible for energy metabolism (Pellerin et al., 2007; Simpson et al., 2007) and uptake of metabolites through the blood-brain barrier (BBB) (Abbott et al., 2006). In the central nervous system (CNS), the astroglia participates in the development of brain (Eriksen and Druse, 2001). Astrocytes modulates synaptic activity by controlling ion homeostasis (Butt and Kalsi, 2006; Roberta and Rossella, 2010) and uptake of neurotransmitters, as glutamate (Danbolt, 2001; Parpura et al., 1994). Glutamine and glutathione (GSH) are possible pathways of cytosolic glutamate in astrocytes (Anlauf and Derouiche, 2013; Dickinson and Forman, 2002). Moreover, in astrocytes, glutamate may be converted in γ-aminobutiric acid (GABA) (Martin and Rimvall, 1993).

Excessive neuron depolarization affects astrocytic function and promotes neuroinflammation (Xanthos and Sandkühler, 2014). In brain, astrocytes are the cells that predominantly synthesize and secrete S100B, a calcium-binding protein (Donato et al., 2009). Extracellular S100B acts in an autocrine and paracrine form on neurons and glial cells (Donato, 2003). Alteration of extracellular concentration promotes dual effects, nanomolar has a neurotrophic effect. However, micromolar concentration can lead to neurotoxic responses (Van Eldik and Wainwright, 2003). An increase of S100B extracellular concentration have been related to astrogliosis or astrocyte dysfunction. In fact, many modulator of its secretion have been described (Gonçalves et al., 2008). Neuroinflammation induced by LPS or pro-inflammatory cytokines (de Souza et al., 2013; Guerra et al., 2011), depolarization conditions by high potassium medium (Nardin et al., 2009), inhibition of Kir 4.1 channel (Zanotto et al., 2013) or kainate acid (Sakatani et al., 2008) affects astrocytes S100B secretion. The amino acid GABA is a major inhibitory neurotransmitter in the adult CNS and is released by neurons and glial cells (Angulo et al., 2008).

Astrocytes are involved in GABAergic signalling and consequently modulate the conductance in brain. Recent studies observed GABA colocalized with GFAP, a cytoskeleton marker of astrocytes (Lake, 1992; Yoon and Lee, 2014), glutamic acid decarboxylase (isoform GAD67) and monoamine oxidase, enzymes responsible to synthesize GABA from glutamate and an alternative pathway from putrescine, respectively (Angulo et al., 2008). Astrocytes express $GABA_A$ and $GABA_B$ receptors

(Lee et al., 2011) and have an important role in its uptake through GABA transporter 3 (GAT-3) localized in CA3 and dentate gyrus (Kersanté et al., 2013). Nevertheless, there is no information about the effect of GABA on S100B secretion. Therefore, we investigated specific effects of GABAergic signaling on astrocyte S100B secretion in acute hippocampus slice from young rats (30-days-old). It was also studied the effect of astrocyte activity inhibition after agonist and antagonist $GABA_A$ treatment and the interaction between neurons and astrocytes in GABAergic signaling and S100B secretion.

Materials and Methods

Materials

Poly-L-lysine and methylthiazolyldiphenyltetrazolium bromide (MTT), methylglyoxal (MG), anti-S100B (SH-B1), 4-(2-hydroxyethyl) piperazine-l-ethanesulfonic acid (HEPES), o-phenylenediamine (OPD), [3(4,5-dimethylthi-azol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT), γ-glutamylhydroxamate acid, muscimol, β-hydroxybutyrate and fluocitrate were purchased from Sigma (Saint Louis, MO, USA). Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM) and other materials for cell culture were purchased from Gibco. Pentylenetetrazol, bicuculline and kainic acid were purchased from TOCRIS (Bristol, United Kingdom. Polyclonal anti-S100B and anti-rabbit peroxidase-linked antibodies were purchased from DAKO (São Paulo, Brazil) and GE, respectively (Little Chalfont, United Kingdom). Tetrodotoxin from Abcam (Cambridge, MA, USA). LDK kit assay was purchased from BioClin, Brazil.

Animals

Forty-five male Wistar rats at postnatal day 30 were obtained from our breeding colony (Department of Biochemistry, UFRGS) and maintained under controlled light and environmental conditions (12 h light/12 h dark cycle at constant temperature of 22 ± 1ºC). We focused at this animal age due to developed and matured GABAergic neurotransmission in rats (Ben-Ari, 2002).

Procedures were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) following the

regulations of the local animal house authorities and Committee of Animal Use of UFRGS (project number 24472).

Preparation and incubation of hippocampal slices

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. 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 replaced every 15 min with fresh saline medium at room temperature. Following a 120-min equilibration period, the medium was removed and replaced with basal or specific treatments for 60 min at 30°C on a warm plate (Nardin et al. 2009).

Slices were incubated with the following treatments: muscimol (5, 10 and 20 μM), pentylenetetrazol (PTZ) (5, 10 and 15 mM), bicuculline (5, 10 and 20 μM), methylglyoxal (MG) (1, 10, 100 and 500 μM), β-hydroxybutyrate (1, 5 and 10 mM), high potassium (20 mM), kainic acid (KA) (50 μM) or tetrodoxin (TTX) (1 μM). Fluocitrate (FLC) was used at 100 μM and diluted in HCl 0.1 M. Therefore, experiments with FLC were performed always with control of vehicle.

Astrocyte culture

Primary astrocyte cultures from *Wistar* rats were prepared as previously described (Gottfried et al., 2003). Procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local authorities. Briefly, the cerebral cortex of newborn *Wistar* rats (1-2 days old) were removed and mechanically dissociated in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS), pH 7.2, containing (in mM) 137.93 NaCl, 2.66 KCl, 8.09 Na_2HPO_4, 1.47 KH_2PO_4, and 5.55 glucose. The cortex was cleaned of meninges and mechanically dissociated by sequential passage through a Pasteur pipette. After centrifugation at 1400 rpm for 5 min, the pellet was resuspended in DMEM (pH 7.6) supplemented with 8.39 mM HEPES, 23.8 mM $NaHCO_3$, 0.1% amphotericin B, 0.032% garamycin, and 10% fetal bovine serum. Approximately 300,000 cells were seeded in each well in 24-well plates, respectively, and maintained in DMEM containing 10% fetal bovine serum in 5% CO_2/95% air at 37°C. Cell were

then allowed to grow to confluence and used at 21 days in vitro. The medium was replaced by DMEM without fetal bovine serum in the absence or presence of PTZ (15 mM) or bicuculline (10 μM), coincubated with FLC (100 μM) or muscimol (10 μM).

Lactate dehydrogenase assay

Slice integrity was evaluated by Lactate dehydrogenase (LDH) kinetics activity using a commercial kit (BioClin, Brazil). Assay was performed by according to the manufacturer's instructions. Results were expressed as percentages of the control.

MTT reduction assay

A slice our cell viability assay was performed using the colorimetric MTT method (Hansen et al., 1989). Slices were incubated with 0.5 mg/mL MTT for 30 min in 5% CO_2/95% air at 37°C. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide (DMSO) and measured at 560 and 650 nm. The reduction of MTT was calculated by the following formula: [(abs 560 nm) − (abs 650 nm)]. Results were expressed as percentages of the control.

S100B measurement

S100B content in the supernatant was measured by an enzyme-linked immunosorbent (ELISA) assay (Leite et al., 2008). Briefly, 50 μL of sample (previously diluted when necessary) plus 50 μL of Tris buffer were incubated for 2 h on a microtiter plate that was previously coated overnight with monoclonal anti-S100B (SH-B1) antibody. Polyclonal anti-S100 was incubated for 30 min, and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min, at 37°C. The color reaction with OPD was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng/mL. Data were calculated as ng/mL and were expressed as percentages of the control.

Statistical Analysis

All results were expressed as mean ± standard error mean (SEM) and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. The level of statistical significance set at $P<0.05$. All analysis were performed using the Prism 5.0 (GraphPad).

Results

GABA*ergic receptors activity modulates S100B secretion in acute hippocampal slice*

Hippocampal slices model preserves cellular architecture similar to *in vivo*, allowing to study the involvement of neurons –astrocyte interaction on S100B secretion

Muscimol, an agonist of $GABA_A$ receptor, did not affect S100B secretion (figure 1A). However, classically known $GABA_A$ receptor antagonists, PTZ and bicuculline affect S100B secretion in opposite directions; PTZ increased S100B secretion in a dose dependent manner and at 15 mM the difference was statistically significant (figure 1D, P=0.0003). Bicuculline at 10 μM decreased S100B secretion (figure 1G, P<0.0001). These treatments did not change cellular viability (MTT assay) and integrity (LDH assay) (Figure 1 B-C, E-F, H-I).

Interestingly, co-incubation of muscimol with these $GABA_A$ receptor antagonists inhibited the effect on S100B secretion; muscimol prevented the increment in extracellular S100B levels promoted by PTZ (figure 2A, P<0.0001) and the decreased of S100B secretion caused by bicuculline (figure 2B, P=0.0021). Cellular viability and integrity did not change (data from MTT and LDH assays were not shown).

TTX and FLC prevented S100B alteration caused by PTZ, but not by bicuculline in hippocampal slice

We also investigated whether the effects of GABAergic antagonists depend on astrocytes activity, as well as the communication of these cells with neurons. Therefore, we co-incubated FLC, an astrocyte inhibitor of aconitase, and TTX, a voltage gated Na^+ channel blocker in neurons, with PTZ and bicuculline. Firstly, we observed that FLC decreased S100B secretion (figure 3A, P=0.0103), on the other hand, TTX did not affect S100B secretion (figure 3A, P=0.9606). Co-incubation of FLC or TTX with PTZ was able to prevent PTZ effect on S100B secretion (figure 3B, P<0.0001). However, co-incubation of FLC or TTX did not affect the decreased in S100B secretion from bicuculline treatment (figure 3C, P<0.0001). Cellular viability and integrity did not change (data from MTT and LDH assays were not shown).

S100B secretion was modulated by different metabolites in hippocampal slice

To further investigate the effect of other $GABA_A$ agonists on S100B secretion we treated hippocampal slices with MG and β-hydroxybutyrate. MG and β-

hydroxybutyrate, physiological metabolites described as GABAergic agonists, could modulate S100B secretion and depends of interaction with neurons. MG at 10 μM increased S100B secretion (figure 4A, $P<0.0001$); however β-hydroxybutyrate, in different concentrations tested, was not able to alter significantly S100B secretion (figure 4B). Co-incubation with TTX (at 1 μM) prevented the augmentation of S100B secretion by MG (figure 5A, $P<0.0001$).

Depolarizing condition usually reduced S100B secretion. In fact, high potassium medium and kainic acid incubation decreased S100B secretion (figure 5B and C, $P=0.0025$ and $P=0.0003$, respectively). Interestingly, co-incubation with TTX inhibited the reduction of S100B secretion from high potassium and kainic acid treatments (figure 5B and C, $P=0.0025$ and $P=0.0003$, respectively). Cellular viability and integrity did not change (data from MTT and LDH assays were not shown).

PTZ and FLC effects on astrocyte's S100B secretion

We have known that depolarization conditions reduced the S100B secretion in hippocampal slices (Nardin et al., 2009) and that the GABAergic antagonists have opposite effects on S100B secretion in hippocampal slice. Therefore, we also investigated S100B secretion in astrocyte cultures incubated with PTZ, bicuculline, FLC and muscimol in order to elucidate if the alterations on S100B secretion depend manly of astrocytes activity. As well as in slices, FLC decreased S100B secretion (figure 6A and C, $P=0.0003$ and $P=0.0046$, respectively) and muscimol did not affect the S100B secretion in astrocyte cultures (figure 6B and D, $P=0.7141$ and $P=0.8515$, respectively). S100B secretion was greater by PTZ (figure 6A, $P=0.0003$), however bicuculline did not affect the secretion (figure 6C, $P=0.8515$). The increased in S100B secretion caused by PTZ was prevented by FLC (figure 6A, $P=0.0003$) and by muscimol co-incubations (figure 6B, $P=0.0234$). Cellular viability and integrity did not change (data from MTT and LDH assays were not shown).

Discussion

Several studies have demonstrated the importance of the role of astrocytes in neuronal activity (Araque et al., 1999; Perea et al., 2009). Astrocytes are involved in synaptic regulation and the crosstalk with neurons by up take and release neurotransmitters (Garnier et al., 2016). GABA is predominantly binding to $GABA_A$ receptor. It is well known the effect of the activation of $GABA_A$ receptor on neurons,

which is an ionotropic receptor that gate chloride channels, mediateing inhibitory signaling in brain (Sigel and Steinmann, 2012). Any dysfunction in these receptors are associated with neuronal disorders (Chebib et al., 2004; Wong et al., 2003).

In the past years, there are growing evidance of the role of astrocytes in GABAergic signaling. Astrocytes express GABA transporter (Kersanté et al., 2013), $GABA_A$ receptors (Lee et al., 2011) and enzymes that convert GABA cytosolic (Angulo et al., 2008). The present work proposes to study the effect of GABAergic signaling on S100B secretion, a protein predominantly present in astrocytes. S100B is a calcium binding protein predominantly synthesized and secreted by astrocytes in brain. This protein is involve in several intracellular and extracellular mechanisms (Donato et al., 2009). In extracellular space, modulates neuron and glia cells and these effects depends on the S100B concentration (Van Eldik and Wainwright, 2003).

Muscimol has been used as selective agonist of $GABA_A$ receptor (Chebib et al., 2004). On the other hand, bicuculline, picrotoxin and PTZ have been known as antagonists of $GABA_A$ receptor. They block the receptor and reverse the decrease total $GABA_A$ current. These antagonists have been studied as epileptic drugs and have been used to screening the most drugs anti-epileptics (Kasthuri et al., 2013). Interestingly, in our study an hyperpolarization caused by the agonist muscimol did not affect S100B secretion in hippocampal slice and astrocyte cultures. However, depolarization induced by antagonists PTZ and bicuculline affect the S100B secretion in hippocampal slice in opposite ways. In acute hippocampal slice, depolarization condition induced by high potassium or inhibitor of potassium uptake by Kir 4.1 channel reduced the S100B secretion (Nardin et al., 2009; Zanotto et al., 2013). These discrepancies in hippocampal slice may be due to different actions of the drugs.

PTZ acts similarly to picrotoxin on $GABA_A$ receptor. PTZ via competitive binds in $\alpha1\beta2\gamma2$ $GABA_A$ receptor domain (Huang et al., 2001). Nonetheless, bicuculline acts as allosteric inhibitor of GABAA receptor (Ueno et al., 1997) and in solution can be converted into bicucine, a metabolic with less convulsive effect (Johnston, 2013). These different mechanisms of action may be related with the different effect observed in S100B secretion, once that only PTZ changed S100B secretion in astrocyte cultures. Therefore, it is possible to consider that PTZ acts directly on astrocyte activity.

The agonist muscimol prevented the effect on S100B secretion promoted by PTZ and bicuculline in acute hippocampal slices. It is possible to hipothesize that

muscimol interacts with $GABA_A$ receptor and influences the depolarization condition of the medium preventing the action of antagonists to these domains.

Previous study demonstrated that communication between neurons and astrocytes is necessary to modulation S100B secretion and the role of receptor for advanced glycation endproducts (RAGE) in the interaction (Sakatani et al., 2008). Our results also demonstrated that PTZ effect of S100B secretion depends on the communication between neurons and astrocytes in hippocampal slice. Furthemore, is necessary the maintenance of astrocytes activity to PTZ signaling. However, the effect on S100B secretion by bicuculline was not affect by co-incubation of TTX or FLC in hippocampal slice. In fact, differently of PTZ, bicuculline did not alter S100B secretion in astrocyte culture. Apparently, bicuculline acts by another way on S100B secretion.

Several metabolites modulated S100B secretion (Gonçalves et al., 2008). β-hydroxybutyrate is the most predominant ketone body synthetized during ketogenic diet. Even without demonstrating the effect of β-hydroxybutyrate on changes current at GABA receptors, β-hydroxybutyrate has been considered a GABAergic agonist (Hartman et al., 2007) and antiepileptic (or anticonvulsant) (Rho et al., 2002). In astrocyte culture incubated for one hour with β-hydroxybutyrate S100B secretion increased (Leite et al., 2004). Nevertheless, in our study in acute hippocampal slices β-hydroxybutyrate did not alter S100B secretion. The absence of effect might be the due to medium composition, because the slices were co-incubated with glucose and β-hydroxybutyrate. In this sense, in GABAergic neuron cultures the presence of β-hydroxybutyrate together with glucose did not affect the total GABA content (Lund et al., 2011).

Other physiological metabolite is MG, which is produced as a by-product of glycolysis metabolism and is a precursor of advanced glycation endproducts (AGEs). MG has been considered a partial competitive $GABA_A$ agonist receptor and, possibly, acts on $GABA_A$ receptors contains α6 subunits (Distler et al., 2012). MG at 10 μM increased S100B secretion in acute hippocampal slice. However, previous study in slice showed that MG at 0.5 mM reduced S100B secretion in extracellular medium (Hansen et al., 2016). MG is involved in physiological and pathological mechanisms (Allaman et al., 2015). These discrepancies may be explained by MG concentration tested and could suggest that lower concentrations of MG, close to physiological range (as 10 μM MG), was able to increase S100B secretion. Moreover, as we can see with depolarization condition (high potassium, kainate acid and PTZ), the action of MG on S100B secretion

in hippocampal slices is also dependent of the communication between neurons and astrocytes.

Conclusions

The mechanism of S100B secretion is not completely understood. However, based on our results in acute hippocampal slice and astrocyte cultures, modulation of $GABA_A$ receptor promotes changes on S100B secretion and depends on astrocyte activity and interaction with neurons. The opposite effects on S100B secretion by GABAergic antagonists in hippocampal slice was probably due to directly action of PTZ in astrocyte's S100B secretion. The activity of astrocytes can be regulated by GABAergic signaling and through S100B it communicates with other neuronal cell types in the brain.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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Figure legends

Figure 1: *Effects of gabaergic agonist and antagonists on S100B secretion in hippocampal slices*. Agonist $GABA_A$ muscimol did not affect S100B secretion (A). Antagonists $GABA_A$, PTZ and bicuculline, altered S100B secretion in opposite directions (D, G). These treatments did not affect slice viability (MTT) and integrity (LDH) (B-C, E-F, H-I). Values were expressed by mean ± SEM, of 6-8 animals per group, independent experiments were performed in triplicate, assuming the control value as 100%. Data were analyzed by ANOVA, followed by the Tukey's test. Bars without a common letter differ significantly, assuming $P<0.05$.

Figure 2: *Muscimol prevented the effects of* GABA*ergic antagonists on S100B secretion in hippocampal slices*. Muscimol (10 μM) prevented the increased of S100B secretion promoted by PTZ (15 mM) (A) and the decreased of S100B level caused by bicuculline (10 μM) (B). Values were expressed by mean ± SEM, of 6-8 animals per group, independent experiments were performed in triplicate, assuming the control value as 100%. Data were analyzed by ANOVA, followed by the Tukey's test. Bars without a common letter differ significantly, assuming $P<0.05$.

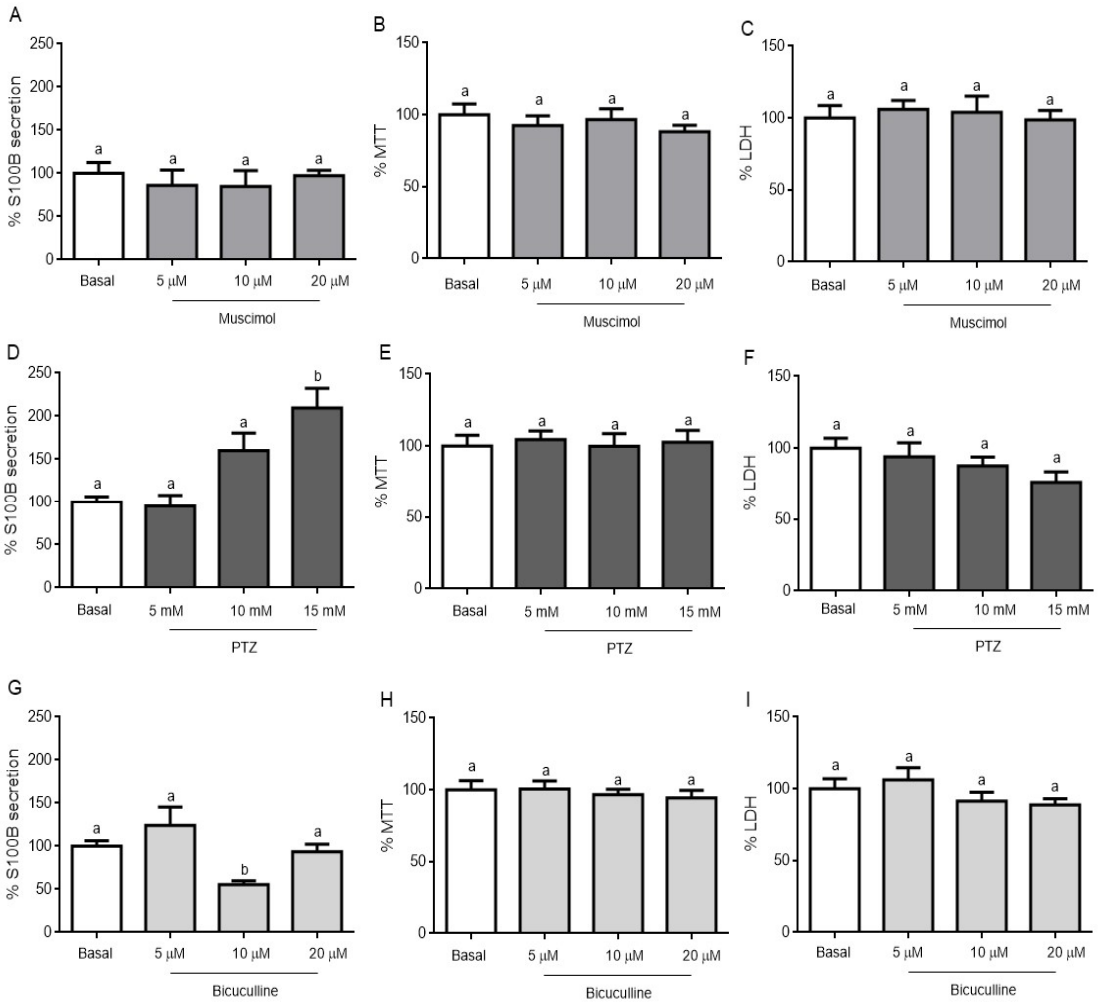
Figure 3: *TTX and FLC prevented S100B alteration caused by PTZ, but not by bicuculline.* FLC decreased S100B secretion (A). Co-incubation of TTX (1 μM) or FLC (100 μM) (B, C) prevented the increased in S100B caused by PTZ (15 mM) (B); however, did not affect the bicucculine (10 μM) action (C). Values were expressed by mean ± SEM, of 6-8 animals per group, independent experiments were performed in triplicate, assuming the control value as 100%. Data were analyzed by ANOVA, followed by the Tukey's test. Bars without a common letter differ significantly, assuming $P<0.05$.

Figure 4: *MG increased S100B secretion, but not β-hydroxybutyrate*. MG increased S100B secretion (A). β-hydroxybutyrate did not affect S100B secretion. Values were expressed by mean ± SEM, of 6-8 animals per group, independent experiments were performed in triplicate, assuming the control value as 100%. Data were analyzed by ANOVA, followed by the Tukey's test. Bars without a common letter differ significantly, assuming $P<0.05$.

Figure 5: *TTX prevented the effects of S100B secretion modulators*. TTX (1 μM) prevented the S100B levels augmentation promoted by MG (10 μM) (A). TTX (1 μM) also prevented the S100B levels diminished caused by high potassium concentration (20 mM) (B) and kainate acid (KA 50 μM) treatment (C). Values were expressed by mean ± SEM, of 6-8 animals per group, independent experiments were performed in triplicate, assuming the control value as 100%. Data were analyzed by ANOVA, followed by the Tukey's test. Bars without a common letter differ significantly, assuming $P<0.05$.

Figure 6: *Effects of* GABA*ergic modulators on S100B secretion in astrocyte culture*. PTZ (15 mM) increased S100B secretion (A, B). FLC (100 μM) and muscimol (10 μM) prevented the effect of PTZ (A, B). However, bicuculline (10 μM) did not affect S100B secretion (C, D). The S100B reduction by FLC was prevented by bicuculline (C). Values were expressed by mean ± SEM of 6 independent experiments performed in triplicate, assuming the control value as 100%. Data were analyzed by ANOVA, followed by the Tukey's test. Bars without a common letter differ significantly, assuming $P<0.05$.

Figure 1


A
% S100B secretion
250
200
150
100
50
0
a
a
a
a
Basal
5 μM
10 μM
20 μM
Muscimol
B
% MTT
150
100
50
0
a
a
a
a
Basal
5 μM
10 μM
20 μM
Muscimol
C
% LDH
150
100
50
0
a
a
a
a
Basal
5 μM
10 μM
20 μM
Muscimol
D
% S100B secretion
250
200
150
100
50
0
a
a
a
b
Basal
5 mM
10 mM
15 mM
PTZ
E
% MTT
150
100
50
0
a
a
a
a
Basal
5 mM
10 mM
15 mM
PTZ
F
% LDH
150
100
50
0
a
a
a
a
Basal
5 mM
10 mM
15 mM
PTZ
G
% S100B secretion
250
200
150
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50
0
a
a
b
a
Basal
5 μM
10 μM
20 μM
Bicuculline
H
% MTT
150
100
50
0
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a
a
a
Basal
5 μM
10 μM
20 μM
Bicuculline
I
% LDH
150
100
50
0
a
a
a
a
Basal
5 μM
10 μM
20 μM
Bicuculline

Figure 2

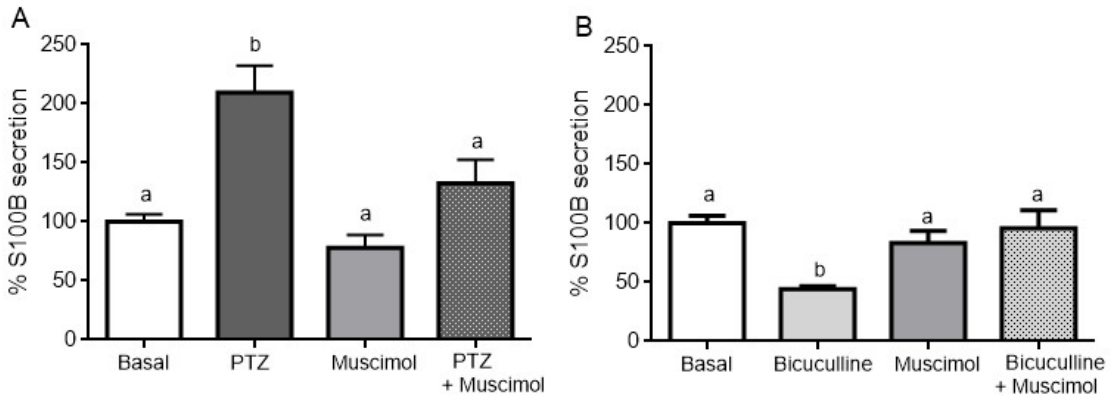

A
% S100B secretion
250
200
150
100
50
0
a
b
a
a
Basal
PTZ
Muscimol
PTZ
+ Muscimol
B
% S100B secretion
250
200
150
100
50
0
a
b
a
a
Basal
Bicuculline
Muscimol
Bicuculline
+ Muscimol

Figure 3

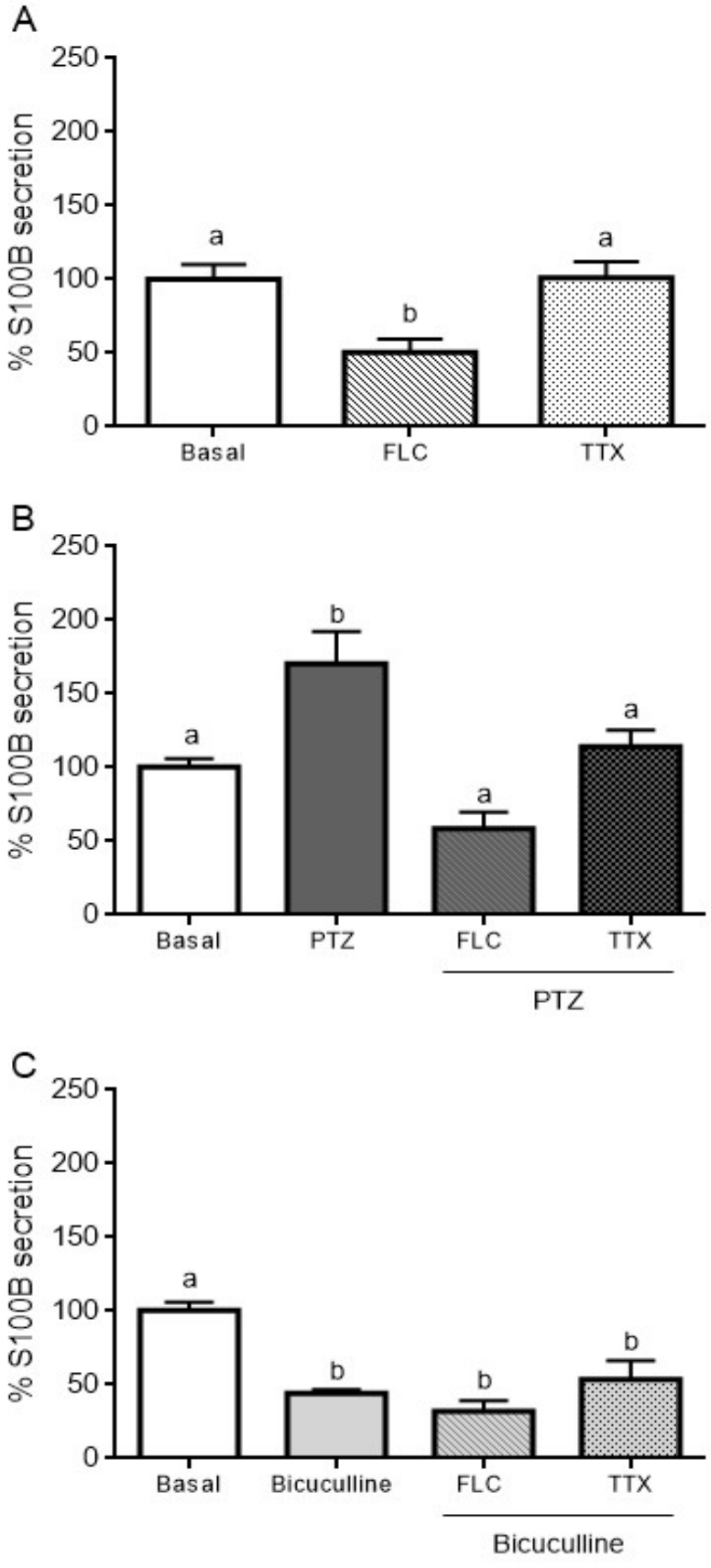

A
% S100B secretion
250
200
150
100
50
0
a
b
a
Basal
FLC
TTX
B
% S100B secretion
250
200
150
100
50
0
a
b
a
a
Basal
PTZ
FLC
TTX
PTZ
C
% S100B secretion
250
200
150
100
50
0
a
b
b
b
Basal
Bicuculline
FLC
TTX
Bicuculline

Figure 4

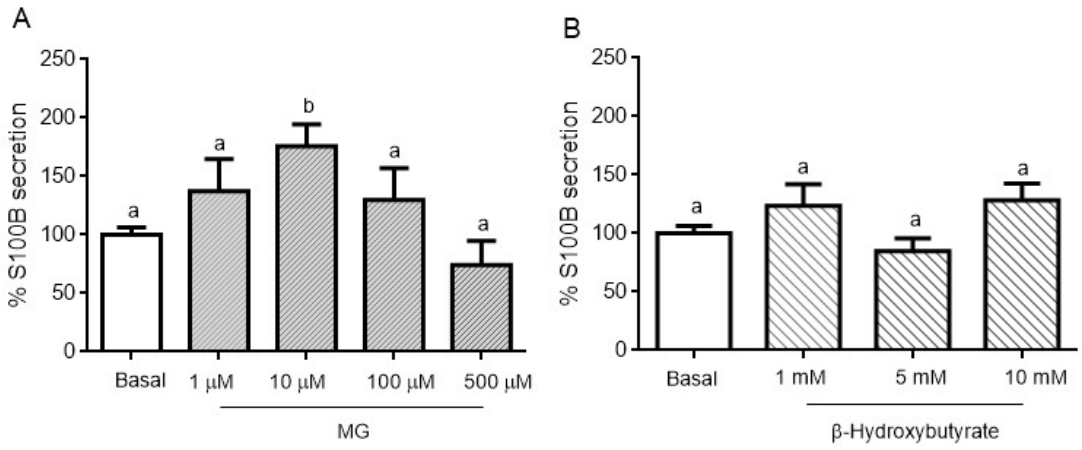

A
% S100B secretion
250
200
150
100
50
0
a
a
b
a
a
Basal
1 μM
10 μM
100 μM
500 μM
MG
B
% S100B secretion
250
200
150
100
50
0
a
a
a
a
Basal
1 mM
5 mM
10 mM
β-Hydroxybutyrate

Figure 5

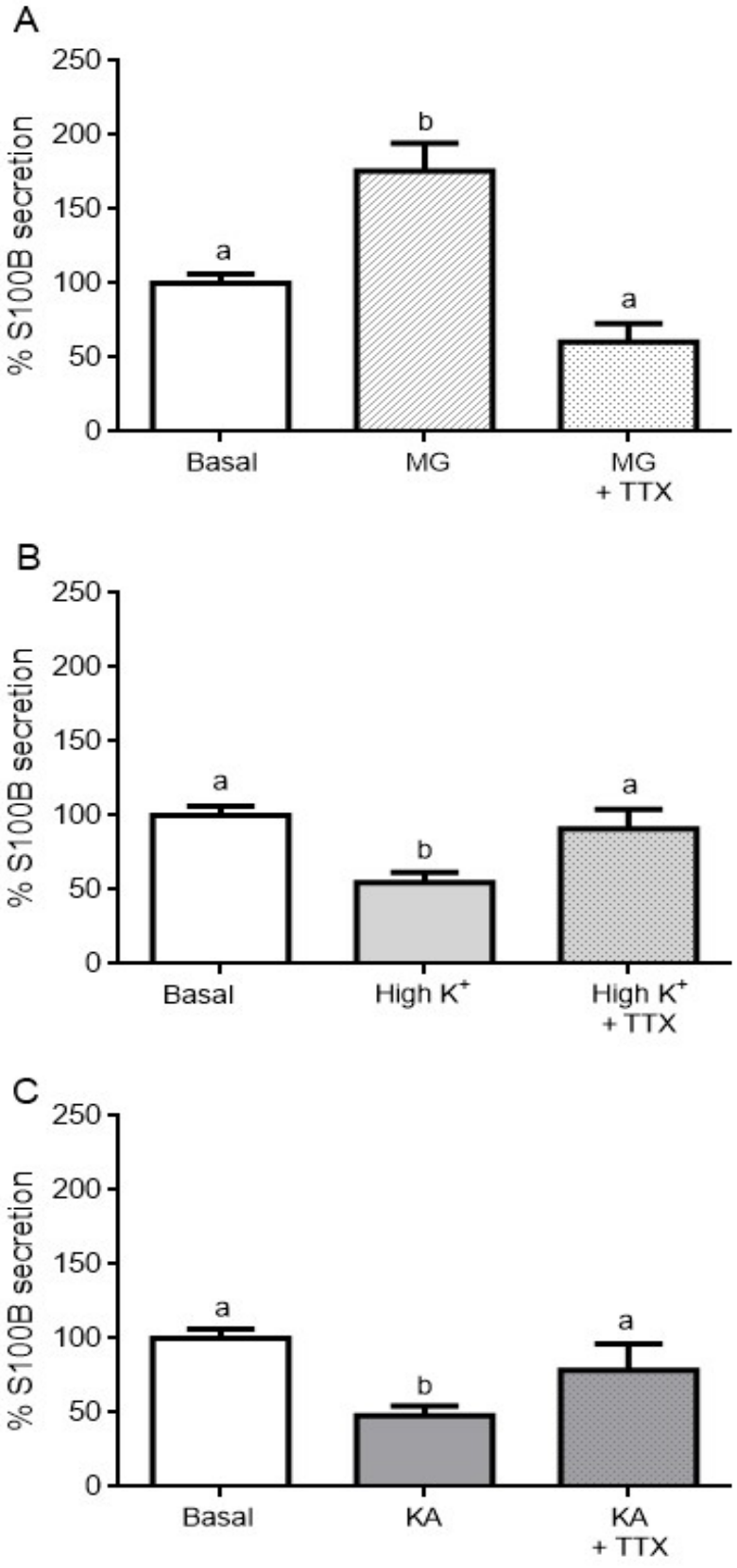
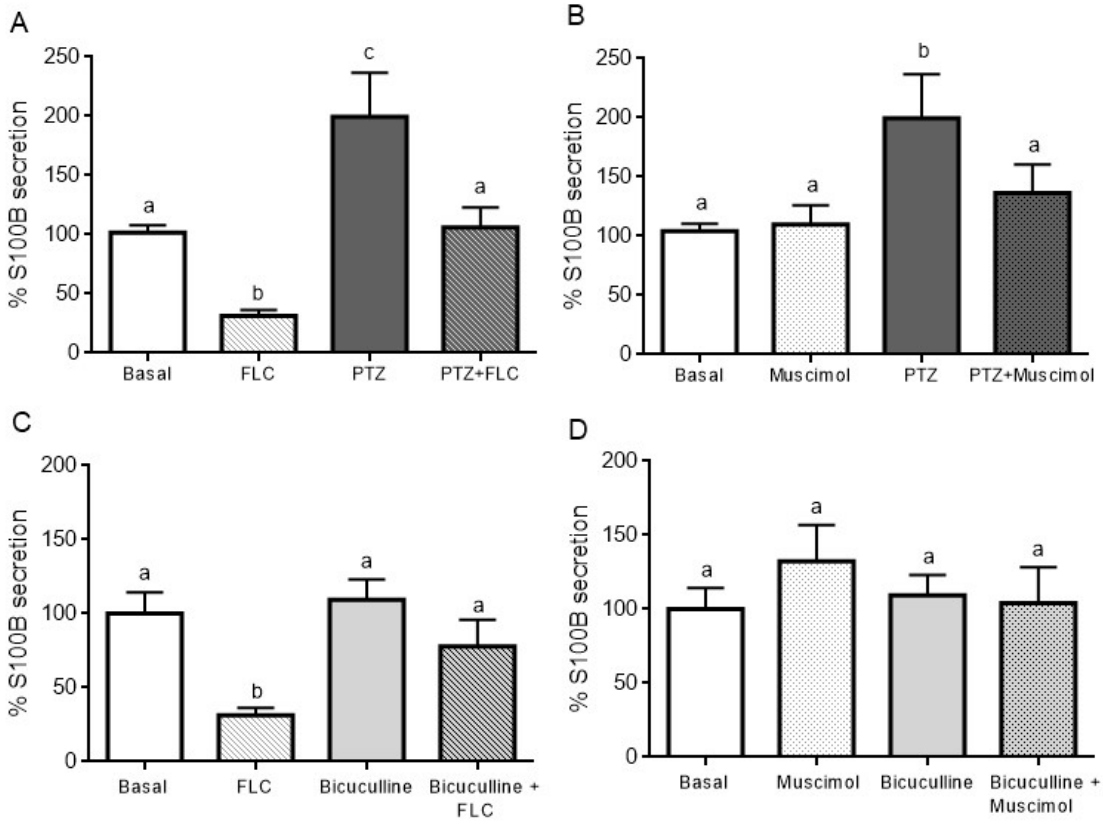

A
% S100B secretion
250
200
150
100
50
0
a
b
a
Basal
MG
MG
+ TTX
B
% S100B secretion
250
200
150
100
50
0
a
b
a
Basal
High K+
High K+
+ TTX
C
% S100B secretion
250
200
150
100
50
0
a
b
a
Basal
KA
KA
+ TTX

Figure 6


A
% S100B secretion
250
200
150
100
50
0
a
b
c
a
Basal
FLC
PTZ
PTZ+FLC
B
% S100B secretion
a
a
b
a
Basal
Muscimol
PTZ
PTZ+Muscimol
C
% S100B secretion
200
150
100
50
0
a
b
a
a
Basal
FLC
Bicuculline
Bicuculline + FLC
D
% S100B secretion
a
a
a
a
Basal
Muscimol
Bicuculline
Bicuculline + Muscimol

DISCUSSÃO

A epilepsia de lobo temporal (ELT) é um dos tipos mais frequentes de epilepsia em humanos, afetando regiões do sistema límbico como hipocampo, amígdala e córtex entorrinal (Engel, 1996, 2011). É caracterizada por crises epilépticas límbicas recorrentes e espontâneas, resultantes da atividade excessiva, anormal e hipersincrônica de neurônios iniciando nas regiões do lobo temporal (Banerjee, 2009; Dalby & Mody, 2001; Dichter, 1994).

As alterações neuronais têm sido extensivamente estudadas na epilepsia e a maioria dos fármacos anti-epilépticos são moduladores da atividade neuronal e muitos atuam sobre canais iônicos do receptor $GABA_A$. Entretanto, sabe-se que cerca de 30% dos pacientes com epilepsia desenvolvem refratariedade às drogas antiepilépticas (Engel, 2011; Leite et al., 2002). Diversos mecanismos têm sido propostos para o desenvolvimento da refratariedade. Um deles é relacionado à modificação dos alvos de ação dos farmacos antiepilépticos. Outro é alteração genética em proteínas que podem ser usadas como transportadores dos fármacos. E por fim, o desenvolvimento de tolerância à droga (Wahab et al., 2010). Desta forma, torna-se de extrema importância o desenvolvimento de tratamentos alternativos com foco em outras células nervosas, como as células gliais, a fim de amenizar o quadro clínico bem como melhorar a qualidade de vida dos pacientes.

Estudos vêm demonstrando o papel das células gliais e da neuroinflamação na epileptogênese (Arisi et al., 2011a; Shapiro et al., 2008). Hipotetizamos que alterações gliais específicas e dependentes do tempo podem ocorrer durante o desenvolvimento do modelo de epilepsia. Inicialmente, no primeiro estudo deste trabalho, utilizamos o modelo de Li-pilocarpina em ratos a fim de mimetizar as alterações neuroquímicas de ELT em humanos (Engel, 2011; Leite et al., 2002), e observamos alterações gliais ao longo do tempo relacionadas com inflamação, morte neuronal e com as mudanças de comportamento associada a este modelo. Por conseguinte, também estudamos a modulação da secreção de S100B pelo anti-inflamatório dexametasona e seus efeitos biológicos em animais epilépticos. Por fim, observamos a modulação do receptor $GABA_A$ através de agonistas e antagonistas sobre a secreção de S100B.

A indução de *SE* foi realizada através da administração intraperitoneal de pilocarpina em ratos jovens (P28), 12-18h pós injeção de LiCl (3mEq/kg). Os animais nesta idade são pós-desmame; portanto, não cetogênicos. Também neste período a barreira hemato-encefálica, o metabolismo energético e de neurotransmissão estão

completamente desenvolvidos e maduros (Ben-Ari, 2002; Engelhardt, 2003; Nehlig et al., 1989). Além disso, esta idade corresponde com à fase infantil em humanos (Andersen, 2002), período comum de desenvolvimento de ELT.

Neste modelo de epilepsia, os animais foram divididos nos tempos 1, 14 e 56 dias pós indução de status epilepticus (*SE*). A escolha destes tempos seguiu alguns dados da literatura de modelos de epilepsia induzidos por pilocarpina. O modelo de pilocarpina devido a mudanças comportamentais e bioquímicas é caracterizado por fase aguda, latente e crônica (Cavalheiro et al., 1991; Leite et al., 2002; Scorza et al., 2009). A fase aguda é logo após a administração do fármaco e indução do *SE*, com mudanças bioquímicas e comportamentais caracterizadas na escala 4-5 de Racine (Lüttjohann et al., 2009; Pinel & Rovner, 1978). A fase latente é também denominada de fase silenciosa em relação ao comportamento epiléptico do animal, entretanto, neste período há mudanças neuroquímicas que possivelmente levem ao desenvolvimento da fase crônica. O período latente é bem variável, podendo ser de 4-44 dias pós indução de *SE* (Scorza et al., 2009), 14-15 dias pós *SE* (Cavalheiro et al., 1991) ou entre 5-7 dias pós indução de *SE* (Goffin et al., 2007). A fase crônica é caracterizada por crises epilépticas espontâneas e recorrentes e pode ser observada até 2 meses pós indução de *SE* (Holtman et al., 2013).

Em nosso estudo, classificamos SE1 como o período de 24 horas pós indução de *SE*, isto é, a fase aguda do modelo. Somente os animais que desenvolveram *SE*, isto é, que desenvolveram comportamento de clonias de patas posteriores e perda de postura (elevação e queda) foram utilizados neste trabalho. O tempo médio de desenvolvimento deste comportamento foi de 13 minutos após a injeção de pilocarpina e a média de taxa de sobrevivência foi de 87% de animais. Após vídeo-monitoramento dos animais, observamos o comportamento da escala 2-3 de Racine, assim como comportamento de pulos e corridas (Furtado, 2011) entre 18-27 dias pós indução de *SE*. Estes sinais clínicos indicam o desenvolvimento de crises epilépticas espontâneas e recorrentes caracterizando que este modelo torna os animais epilépticos. Portanto, através da análise do comportamento, caracterizamos a fase latente como 14 dias pós *SE* (SE14) e a fase crônica como 56 dias pós indução de *SE* (SE56). Este período da fase crônica foi escolhido a fim de garantir que todos animais estivessem com crises recorrentes e para mimetizar o acúmulo de modificações neuronais e gliais ao longo do tempo em indivíduos epilépticos.

Em relação à inflamação periférica, foi observado níveis séricos de TNF-α diminuídos nos diferentes tempos após a injeção de pilocarpina. Estudo anterior não encontrou alterações significativas nos níveis séricos de TNF-α no início do *SE* em ratos adultos submetidos ao modelo Li-pilocarpina (Marchi et al., 2007). Além disso, um estudo que empregou a administração sistêmica de diferentes agonistas de receptores muscarínicos apresentou uma diminuição de TNF-α em homogenatos cardíacos após a injeção de pilocarpina (Freeling et al., 2007). Outro estudo, demonstrou que a ativação do sistema nervoso periférico parassimpático através de agonistas colinérgico atenua a inflamação sistêmica e inibi a expressão de TNF-α durante a injeção sistêmica de pilocarpina (Altavilla et al., 2006). No SNC foi observada uma sinalização inicial e transitória de inflamação. TNF-α é predominantemente sintetizado e secretado pela microglia, entretanto, astrócitos e neurônios também são capazes de produzir essa citocina (Welser-alves & Milner, 2013). No hipocampo, os níveis de TNF-α aumentaram apenas em SE1, não havendo diferença entre os animais controle e tratados nos tempos SE14/SE56. Estes resultados sugerem um mecanismo de sinalização inflamatória aguda no início da epileptogênese neste modelo. Em outros modelos animais de epilepsia foram observados a elevação dos níveis de citocinas pró-inflamatórias na região do hipocampo e a sensibilidade da resposta inflamatória é aparentemente dependente da idade dos animais (Pernot et al., 2011; Ravizza et al., 2005; Somera-Molina et al., 2007; Voutsinos-porche et al., 2004). Em modelo de epilepsia por ácido caínico, o aumento de TNF-α é transitório, enquanto, os níveis de IL-1β permanecem elevados ao longo da epileptogênese (De Simoni et al., 2000). Em pacientes ELT, a expressão de ARNm de TNF-α é elevada na esclerose do hipocampo (Teocchi et al., 2013).

A neurodegeneração está relacionada com eventos de neuroinflamação e astrogliose (Castillo-Ruiz et al., 2007; Nascimento, 2012). Sabe-se que TNF-α atua sobre os astrócitos, inibindo a captação de glutamato, aumentando a liberação de glutamato (Zhu et al., 2010), assim como diminuindo a expressão de receptores $GABA_A$ (McCoy & Tansey, 2008), promovendo um desequilíbrio da neurotransmissão excitatória/inibitória o que resulta na excitoxicidade glutamatérgica e morte neuronal (Zhu et al., 2010). Nosso estudo analisou a morte neuronal através da coloração de Fluoro-Jade C (FJC). FJC é um fluorocromo derivado de fluoresceína usado na marcação de neurônios em degeneração, entretanto, o mecanismo de morte celular avaliado não é conhecido (Schmued et al., 1997) e é independente do insulto. Em estudo

de morte neuronal induzida pelo modelo de epilepsia através da pilocarpina, observou-se que as células FJC positivas eram neurônios que co-localizavam com marcadores da via de apoptose (citocromo C, caspase-9 e caspase-3) e que a degeneração ocorria em neurônios GABAérgicos e diminuía ao longo do tempo (Wang et al., 2008).

Em nosso estudo esperávamos observar morte neuronal nas fases aguda e latente. Entretanto, células FJC-positivas foram observadas em SE14 em diferentes regiões do hipocampo: CA1 e giro dentado. Outro estudo também detectou perdas significativas entre 1 e 3 semanas após a indução da *SE* (Poirier & Koninck, 2000). No entanto, trabalhos anteriores observam outros resultados. Em ratos neonatos, a perda neuronal no hipocampo foi observada nas primeiras horas após a *SE* (Druga et al., 2010) e em camundongos submetidos ao modelo de pilocarpina, foram observadas células FJC-positivas de 12h até 7-14 dias nas regiões CA1 e CA3 do hipocampo (Wang et al., 2008). Essas discrepâncias na literatura podem ser atribuídas a diferenças metodológicas de indução de *SE* e avaliação de perda neuronal.

Paralelamente à lesão neuronal e à inflamação, observamos sinais claros de disfunção astrocitária no hipocampo. Nossos resultados baseados no imunoconteúdo de GFAP e S100B em SE14 e SE56 e na imunofluorescência de GFAP indicam astrogliose durante a epileptogênese. Astrogliose é uma rápida resposta dos astrócitos a um ambiente nocivo agudo ou crônico em virtude da liberação de moléculas que sinalizam a lesão. Astrogliose está associada com a neurodegeneração (Eng et al., 1992; Hol & Pekny, 2015; Maragakis & Rothstein, 2006). A hiperplasia e hipertrofia de astrócitos vêm sendo descritas como sinais precoces de ativação glial que contribuem para o mecanismo da epileptogênese (Arisi et al., 2011b; Shapiro et al., 2008), células GFAP positivas e S100B positivas foram observadas em diferentes regiões do hipocampo após 5 dias indução de *SE*. Em tecido de neocórtex de pacientes com epilepsia intratável há um aumento de células S100B positivas e de processos astrocíticos (Griffin et al., 1995).

Cabe ressaltar, que também é observado ação do lítio sobre os astrócitos, aumentando os níveis de GFAP e um declínio deste marcador nos animais sham ao longo do tempo. Sabe-se que o lítio *per se* potencializa a indução de astrogliose (Rocha et al., 1998), o qual é reduzido pelo fármaco gabapentina (Ramos et al., 2013).

S100B é uma proteína ligante de cálcio predominantemente sintetizada e secretada por astrócitos no SNC, exercendo efeitos parácrinos e autócrinos em neurónios e células da glia (Donato et al., 2009). Alterações extracelulares dos níveis de

S100B vêm sendo empregadas como sinais de ativação ou disfunção astroglial (Gonçalves et al., 2008). Neste trabalho, foi detectado um aumento precoce desta proteína no líquido cefalorraquidiano (LCR) em SE1 e também tardiamente em SE56. Estudo prévio do nosso grupo demonstrou níveis elevados de S100B no LCR em ratos adultos 2 semanas após *SE* (de Oliveira et al., 2008). Elevação do conteúdo de S100B no LCR pode estar relacionado com o aumento de resposta astrocitária de secreção devido à morte ou disfunção neuronal. Concentração nanomolar de S100B é benéfica e possui efeito neuroprotetor, sendo um fator neurotrófico (Van Eldik & Wainwright, 2003). Entretanto, concentração maior (micromolar) de S100B medeia efeitos tóxicos (Donato et al., 2009; Gonçalves et al., 2008)

Sabe-se que há uma elevação da secreção de S100B no SNC em resposta a condições deletérias. A indução de ambiente inflamatório em animais, fatias agudas hipocampais e cultura de astrócitos eleva a secreção e o conteúdo de S100B no meio extracelular (de Souza et al., 2013; Guerra et al., 2011). Mais recentemente, S100B é conhecida como proteína associada à dano celular (conhecida pela sigla em inglês DAMP) ou alarmina (Schaefer, 2014; Sorci et al., 2010). Proteína do grupo de alta mobilidade 1 (conhecida pela sigla em inglês HMGB1) é outra molécula considerada DAMP, vêm se destacando como marcador de dano celular em modelo de epilepsia e em tecidos de pacientes epilépticos (Maroso et al., 2010; Schaefer, 2014). Nossos resultados demonstram uma elevação de S100B no LCR em SE1 e SE56, períodos considerados de fase aguda e crônica, em que os animais apresentam crises induzidas e espontâneas, respectivamente. Portanto, nosso estudo sugere a avaliação de S100B no LCR como marcador de *SE*, assim como tem sido proposto em outras condições de lesão cerebral aguda, como lesão cerebral traumática e acidente vascular cerebral (Gonçalves et al., 2008; Kleindienst et al., 2010; Kleindienst et al., 2010).

S100B pode agir sobre receptores de produtos finais de glicação avançada (conhecidos pela sigla em inglês, RAGE). RAGE é um receptor transmembrana de 35KDa pertencente a família de imunoglobulina de superfície celular. Inicialmente foi descrito como receptor para produtos avançados de glicação. Entretanto, atualmente diversos ligantes já foram descritos, como S100B, HMGB1, proteína β-amiloide, anfoterinas e fosfatidilserina (Lee & Park, 2013; Ramasamy et al., 2011). A ação de S100B sobre os receptores RAGE são consideradas fisiológicas ou patológicas, sinalizando de forma trófica ou tóxica. Entretanto, não se sabe ao certo o mecanismo de ativação de RAGE que acarreta esta dualidade de resposta celular (Huttunen et al.,

2000). Em modelos experimentais de morte ou alteração de circuitos neuronais, tanto a expressão de RAGE quanto os níveis de S100B estão elevados, ativando a via do fator nuclear kappa B (NF-kB) e, consequentemente, a liberação de fatores pró-inflamatórios que desencadeiam morte neuronal e astrogliose (Angelo et al., 2014; Bianchi et al., 2010; Vilarrreal et al., 2014). Sabe-se que uma hiperativação de receptores RAGE através da elevação do seu ligante (S100B) promove apoptose neuronal através da via da cinase ERK 1/2 e da alta produção de espécies reativas de oxigênio (Huttunen et al., 2000).

Curiosamente, os níveis de S100B diminuem no soro em SE1, enquanto que os níveis desta proteína estão elevados no LCR. Há elevação de S100B no LCR nos tempos SE1 e SE56 o que pode sugerir a ativação astroglial. Sabe-se que os níveis periféricos de S100B não estão necessariamente relacionados com as alterações do LCR e que outras células (por exemplo, os adipócitos) contribuem para os níveis de S100B no sangue (Gonçalves et al., 2010, 2008; Kleindienst, 2010). Nossos resultados confirmam esta independência. Essa independência pode ser devido às mudanças específicas na barreira hematoencefálica (BHE) durante e após o *SE* (Friedman et al., 2009; Gorter et al., 2015; Milesi et al., 2015). Alterações da BHE induzidas por lesão cerebral têm sido observadas sob condições experimentais. A indução de *SE* através da administração de pilocarpina promove alteração da permeabilidade da BHE (Marchi et al., 2007). No entanto, apesar desta maior permeabilidade cerebrovascular, os níveis elevados de S100B no LCR não foram acompanhados por extravazamento de S100B do LCR para o soro em SE1 e SE56. De fato, nestes períodos ocorre uma queda da concentração de S100B no soro. No período SE14, há aumento dos níveis de S100B no soro, sem afetar seus níveis no LCR. Não podemos determinar quanto desta diminuição sérica de S100B é devido à "retenção cerebral" (Kleindienst et al., 2010a; Kleindienst et al., 2010b) ou se é um efeito dos fármacos administrados na sinalização periférica. Outro resultado interessante é a observação da redução de S100B sérica nos animais sham ao longo do tempo. Seria interessante uma investigação futura para observar um possível efeito do lítio na sinalização de S100B periférica. Sabe-se que o tecido adiposo é fonte de S100B na periferia (Gonçalves et al., 2010) e o lítio é capaz de regular respostas neste tecido (Löffler et al., 2016).

Independentemente dessa limitação, observamos a relação de LCR/soro de S100B. Em SE1 e SE56 esta relação está elevada, enquanto que em SE14 está baixa. A elevação da taxa LCR/soro é um evento precoce que pode desencadear ou marcar o

processo epileptogênico e também pode ser um marcador da fase crônica da doença em que se desenvolvem as crises recorrentes e espontâneas. Este dado reforça a ideia de que S100B pode representar um marcador útil para *SE*. A análise da relação LCR/soro auxilia na observação da ativação astroglial e/ou alterações a longo prazo na permeabilidade cerebrovascular. Para avaliar a utilidade da relação LCR/soro de S100B, também foi feita uma correlação não paramétrica entre este índice e a severidade da *SE*, isto é, a latência para o desenvolvimento de comportamento epiléptico (fase crônica). De fato, houve uma correlação entre o tempo de latência do desenvolvimento de crises epilépticas espontâneas (em SE56) e a alta relação LCR/soro (correlação de Spearman R=-0,9487 e P<0,0001). No entanto, este é um resultado preliminar devido à pequena amostra (n = 4).

Outros marcadores astrocitários também foram estudados neste modelo ao longo do tempo, como a glutamina sintetase (GS). Encontramos um decréscimo da atividade de GS no hipocampo em SE1, seguida de um aumento em SE14. Entretanto, nenhuma diferença de atividade foi encontrada em SE56. Curiosamente, a atividade de GS não se correlacionou com o conteúdo de GS analisado por *Western blot*. O conteúdo de GS foi menor em SE1 e SE56, e maior em SE14, quando comparado com os respectivos controles. Já foi observado em amostras de hipocampo de pacientes com epilepsia do lobo temporal mesial (ELTM) e ELT com desenvolvimento de esclerose hipocampal diminuição do conteúdo e da atividade da GS, demonstrando eventos de astrogliose e disfunção astrocitária na epileptôgenese (Eid et al., 2004; Van Der Hel, 2005). Além disso, modelos experimentais de inibição da atividade GS através de metionina sulfoximina resultaram em deficiência de GS e crises epilépticas recorrentes (Eid et al., 2008).

Também foi realizada a relação atividade/conteúdo da proteína GS. Esta relação está elevada nos tempos SE1 e SE56, sugerindo também a existência de astrogliose após *SE*. A elevação desta taxa pode ser considerado um mecanismo compensatório a fim de remover o excesso de glutamato na fenda sináptica, devido ao desequilíbrio de neurotransmissores excitatórios e inibitórios imediatamente após *SE* e crises epilépticas espontâneas na fase crônica.

Outro destino para o glutamato nos astrócitos é a síntese de glutationa (GSH) (Dickinson & Forman, 2002). No entanto, sabe-se que a excitotoxicidade glutamatérgica decorrente do estresse oxidativo consome GSH e, devido à interferência na captação de cisteína, há um prejuízo na síntese de GSH (Dringen & Hirrlinger,

2003). Em SE1 e SE56 há uma baixa dos níveis de GSH. Esta diminuição também foi observada 24 horas após a administração de pilocarpina (Freitas & Fonteles, 2005) e em pacientes com epilepsia (Mueller et al., 2001). Estes resultados nos permitem inferir que durante a astrogliose induzida por *SE* ocorre diminuição das defesas antioxidantes, favorecendo um cenário de estresse oxidativo e de neurodegeneração.

Sabe-se ainda que a astrogliose induzida por *SE* é acompanhada por mudanças na expressão, localização, distribuição e atividade dos canais iônicos e de água (Hubbard et al., 2016; Kim et al., 2009; Lee et al., 2012). AQP-4 e Kir 4.1são canais comumente localizados nos astrócitos e são funcionalmente importantes para a homeostase e captação de potássio e água, respectivamente, durante a intensa atividade despolarizante induzidas por *SE* (Butt & Kalsi, 2006; Lee et al., 2012; Seifert & Steinhäuser, 2011; Strohschein et al., 2011). Os níveis de AQP-4 diminuíram em SE1 e aumentaram em SE14, quando comparados com animais sham. Este padrão foi bastante semelhante ao observado para o conteúdo de GS nos animais *SE*. Por outro lado, o conteúdo de canais Kir 4.1 apresentaram-se reduzidos em todos os períodos de tempo analisados. A queda dos níveis de Kir 4.1 durante todas as fases da epileptogênese revela o comprometimento a longo prazo do mecanismo de liberação e captação de potássio, assim como a manutenção de um ambiente excitante que promova atividade neuronal despolarizante anormal neste modelo de Li-pilocarpina.

Neste primeiro estudo cabe ressaltar algumas limitações. Primeiramente, seria interessante relacionar registros de eletroencefalograma (EEG) com o comportamento dos animais baseados na escala de Racine observados por vídeo monitoramento. Esta observação permitiria uma melhor caracterização das diferentes fases do modelo de Li-pilocarpina. Em segundo lugar, o emprego de períodos de tempo adicionais em futuros estudos seria valioso para a compreensão de alguns parâmetros astrogliais. Além disso, algumas alterações astrogliais podem ser subestimadas, uma vez que estas foram comparadas a um grupo controle que foi submetido a uma exposição ao lítio que *per se* altera alguns parâmetros gliais. E por fim, observamos astrogliose em SE14 e SE56 induzida por Li-pilocarpina. Apenas em SE14 foi observada células FJC positiva nas regiões do giro dendato e CA1. E em SE56 há atividade anormal e hipersincrônica dos neurônios. Pode-se sugerir que as alterações astrogliais dependentes do tempo podem estar ligadas à excitabilidade neuronal e/ou à morte neuronal, entretanto, deve-se melhor detalhar em novos experimentos a relação da astrogliose com esses eventos.

Em virtude dos resultados dos níveis de S100B no LCR, nos perguntamos se a indução de *SE* altera a secreção de S100B nos astrócitos e se a modulação deste mecanismo pode promover neuroproteção à epileptogênese. Além disso, pensamos também que se regularmos a resposta de neuroinflamação e de astrogliose poderíamos promover uma melhora nos animais epilépticos. No segundo trabalho, analisamos a modulação da secreção de S100B nos tempos 1, 14 e 56 dias após a indução de *SE* em fatias hipocampais agudas em modelo *ex vivo*. As fatias de animais controle e *SE* foram incubadas com o anti-inflamatório dexametasona e também expostas a um ambiente despolarizante, isto é, com alto nível de potássio.

A escolha por um anti-inflamatório esteroideo deve-se ao fato que os receptores GABA são sensíveis à fármacos esteroides, tornando estes drogas adjuvantes ao tratamento da epilepsia (Joshi et al., 2013; Rogawski, 2006; Sun et al., 2007). Anti-inflamatórios também modulam astrócitos e neuroinflamação. É crescente as evidências do papel dos astrócitos e da microglia na epilepsia sendo considerados potenciais alvos para desenvolvimento de novos fármacos para tratamento doenças epilépticas (Vezzani et al., 2015).

Como mencionado anteriormente, S100B pode ser utilizada como marcador de distúrbios epilépticos (Chen et al., 2015; Portela et al., 2003) e sua secreção é modulada por LPS (Guerra et al., 2011) e drogas anti-inflamatórias (Leite et al., 2009).

Sabe-se que a incubação de fatias hipocampais agudas em meio com alto potássio diminui a secreção de S100B (Nardin et al., 2009; Zanotto et al., 2013). Nós também confirmamos que fatias provindas de animais controle, em diferentes idades, também secretam menos S100B em meio com alto potássio. No entanto, em ratos submetidos ao modelo de epilepsia por Li-pilocarpina, a secreção de S100B não respondeu ao estímulo do alto potássio. O mecanismo envolvido nesta diferença não é claro no momento (Nardin et al., 2009), bem como o mecanismo de secreção de S100B (Gonçalves et al., 2008). No entanto, como observamos uma diminuição nos canais de potássio retificadores de entrada Kir 4.1 em animais SE1, SE14 e SE56 (Vizuete et al., 2017), isso pode estar interferindo em um menor influxo de potássio pelos astrócitos (Butt & Kalsi, 2006; Strohschein et al., 2011) e alterando a secreção de S100B nestes animais.

Na literatura, muitos moduladores da secreção de S100B já foram descritos (Donato et al., 2009). Escolhemos a dexametasona para testar a regulação da secreção de S100B por ser anti-inflamatório esteroideo. O mecanismo de neuroproteção da

dexametasona não é completamente esclarecido. Sabe-se que este esteroide é capaz de diminuir edema e ativação de microglia e astrócitos, assim como reduzir citocinas pró-inflamatórias em modelos de neuroinflamação (Gamache & Ellis, 1986; Murray et al., 2011). Dexametasona também reduz morte neuronal e inflamação induzida pela exposição à proteína β-amiloide (Previti et al., 2006). Um possível mecanismo de ação deste anti-inflamatório é a repressão da enzima ciclooxigenase-2 (COX-2). Na inflamação há um aumento da expressão e da atividade de COX-2 e, consequentemente, maior produção de prostaglandinas. Dexametasona é capaz de modular de forma pós-transcricional COX-2, reprimindo a expressão desta enzima e consequentemente diminuindo a via de sinalização do ácido araquidônico e a produção de prostaglandinas (Newton et al., 1998).

Em nosso estudo, dexametasona *per se* diminuiu a secreção de S100B em fatias de hipocampo agudas de animais controle (em todos os tempos analisados) e em animais SE1 e SE56. Nenhum efeito *in vitro* da dexametasona ocorreu em animais 14 dias após a indução de *SE*, o que corresponde ao período latente deste modelo (Curia et al., 2008). O efeito agudo da dexametasona sobre a atividade epileptiforme de fatias do hipocampo já foi relatado (Duport et al., 1997), entretanto o mecanismo deste efeito ainda permanece obscuro.

Baseados no efeito da dexametasona sobre a secreção de S100B em fatias de hipocampo agudas em animais SE1, nós decidimos testar se a dexametasona possui efeitos de reversão da neuroinflamação e da astrogliose durante a epileptogênese. Para investigar a neuroproteção deste anti-inflamatório no modelo de epilepsia, foi administrado intraperitonealmente dexametasona 1 dia após injeção de pilocarpina e indução de *SE*. Foram realizadas 2 injeções de 2mg/kg em intervalos de 12 horas e posteriormente analisados marcadores de atividade astrocitária, de astrogliose e de neuroinflamação em hipocampo nos tempos 1 e 56 dias após administração de dexametasona. Nós optamos por não utilizar o tempo 14 dias a fim de reduzir o número de animais durante a experimentação.

Interessantemente, observamos efeito a longo prazo da dexametasona sobre parâmetros astrogliais e inflamatórios. A dexametasona foi capaz de reverter diversos marcadores de neuroinflamação durante a epileptogênese. De fato, a dexametasona reverteu o aumento dos níveis de IL-1β e TNF-α no hipocampo de animais *SE* 1 dia após a administração da dexametasona. Deve-se observar que nos animais *SE* tratados com dexametasona, o nível de TNF-α reduziu a níveis inferiores aos dos animais *SE*,

entretanto, não fizemos medições de citocinas em animais controle injetado com dexametasona. Este grupo (controle+dexametasona) não foi realizado a fim de reduzir o número de animais durante a experimentação.

Sabe-se que a neuroinflamação participa da epileptogênese e diversas citocinas pró-inflamatórias estão elevadas após a indução de *SE* (De Simoni et al., 2000; Rizzi et al., 2003; Vizuete et al., 2017). Diversos estudos focam o papel da IL-1β na epilepsia (Ravizza et al., 2005; Somera-Molina et al., 2007; Vezzani et al., 2008). Sabe-se que receptores IL-1R1 associado ao receptor *toll like* estão ativos nas crises epilépticas. Esta sinalização pode ser devida a ligação de moléculas consideradas DAMPs, como por exemplo HMGB1 (Maroso et al., 2010). Drogas pró-convulsivas promovem a liberação de IL-1β e HMGB1 pelos astrócitos antes e durante o comportamento de crises epilépticas (Chiavegato, 2014; Maroso et al., 2010). IL-1β além de induzir a formação de descargas espontâneas, é capaz de reduzir a latência para a geração do primeiro evento ictal (Chiavegato, 2014). A modulação desta citocina como potencial alvo farmacológico, pode ser feita pela administração do antagonista do receptor IL-1R1, a IL-1Ra. IL-1Ra apresenta efeitos antiepiléptico modulando a ativação astrocitária e inibe a formação de crises epilépticas induzidas pela bicuculina (Vezzani et al., 2000). Mesmo a dexametasona não possuindo um efeito específico sobre IL-1β, nossos resultados demonstram uma redução desta citocina nos animais *SE*. Esta redução dos níveis de IL-1β no hipocampo pode ser entendida como uma diminuição da neuroinflamação no início da epileptogênese, assim como uma diminuição da excitação e geração de novas crises espontâneas.

Também podemos inferir que a redução de TNF-α nos animais *SE* pode melhorar a sinalização GABAérgica visto que esta citocina age sobre receptores $GABA_A$ (McCoy & Tansey, 2008) e desta forma promover um melhor equilíbrio no sistema de neurotransmissão excitatório/inibitório.

Os níveis de COX-1 não alteraram ao longo do tempo em nenhum grupo experimental, isto se deve ao fato desta enzima ter uma expressão constitutiva nas células. O conteúdo da COX-2, enzima ativada positivamente na inflamação, diminuiu nos dois tempos analisados e, consequentemente, um dos produtos da via da COX-2, a PGE2 foi reduzida 24h após injeção de dexametasona.

Outro efeito a longo prazo da dexametasona é o aumento de IL-10 nos animais *SE* 56 dias após a injeção do anti-inflamatório. IL-10 é uma citocina anti-inflamatória. Em pacientes neonatos epilépticos, os níveis de IL-10 estão elevados 24-72h após o

início da crise. A IL-10 possui ação neuroprotetora e antiepiléptica através da inibição de citocinas pró-inflamatórias (Youn et al., 2013).

Esses resultados sugerem um cenário não inflamatório promovido pela dexametasona no hipocampo de animais tratados com Li-pilocarpina a curto e longo prazo e, possivelmente, uma condição menos despolarizante, diminuindo a geração de crises epilépticas.

Além de regular a resposta de neuroinflamação, dexametasona foi capaz de reverter a astrogliose decorrente do *SE*. Baseados em marcadores de astrogliose clássicos, GFAP e S100B, verificou-se que a administração de dexametasona 24 horas após a indução de *SE* foi capaz de reverter a astrogliose. Sabe-se que dexametasona diminui a expressão de GFAP e de S100B em culturas gliais (Avola et al., 2004; Niu et al., 1997) e que a administração *in vivo* deste corticoide tem sido utilizada para reduzir resposta de inflamação e gliose reativa (Bruccoleri et al., 1999; Jaquins-Gerstl et al., 2011). Além disso, embora a dexametasona não tenha sido capaz de reduzir os níveis de S100B no LCR a curto prazo, observamos um efeito tardio após 56 dias da injeção de dexametasona. Esta redução dos níveis de S100B neste período pode ser um reflexo da redução da morte e/ou uma melhora da atividade neuronal. Portanto, este efeito da dexametasona é de certa relevância. A cronicidade de altos níveis de S100B no LCR contribuem para o desenvolvimento de doenças neurodegenerativas (Donato et al., 2009; Gonçalves et al., 2008).

Foi observado a redução dos níveis de S100B no soro logo após a indução de *SE*, sendo que a dexametasona não apresentou efeito neste parâmetro, mantendo o nível de S100B sérico baixo. O nível de S100B sérico pode ser devido à "retenção" cerebral dessa proteína, como proposto em alguns casos de lesão cerebral aguda (Kleindienst et al., 2010b) ou pode ser devido à sua alteração periférica (independente da fonte cerebral) . É importante mencionar que, em outro modelo de indução de *SE*, utilizando-se a escopolamina e pilocarpina, foi observado aumento de S100B sérica e que a administração de dexametasona prévia à indução de *SE* impediu esse aumento (Marchi et al., 2011).

Também foi observado efeitos da dexametasona sobre as funções dos astrócitos ao longo da epileptogênese. Neste estudo, detectamos uma precoce diminuição da atividade de GS que persistiu até a fase crônica. Sabe-se que em pacientes com ELT esta enzima está reduzida no hipocampo (Eid et al., 2012) e no modelo Li-pilocarpina sua expressão é reduzida 2 semanas após *SE* (Van Der et al., 2014). A dexametasona

per se reduziu a atividade de GS logo após a injeção e não foi capaz de reverter a baixa da atividade de GS induzida pelo *SE*. Entretanto, é bem conhecido que a dexametasona induz a expressão desta enzima (Patel et al., 1983). No entanto, citocinas pró-inflamatórias são capazes de bloquear esta indução em culturas de astrócitos (Huang & Banion, 1998). Assume-se que a queda de GS no hipocampo no modelo Li-pilocarpina é devido à inflamação, mas não está claro no momento porque a dexametasona não foi capaz de inverter este efeito. É possível que este efeito possa depender da dose e do tempo de administração deste corticóide.

Estresse oxidativo é observado no hipocampo em modelos de epilepsia (Freitas & Fonteles, 2005; Waldbaum & Patel, 2010). Nosso estudo, observou a diminuição de GSH. Dexametasona foi capaz de reverter completamente a redução de GSH promovida pela indução de *SE* em todos os tempos. Rosiglitazona, um agonista do receptor gamma ativado por proliferador de peroxissomos (PPARγ), possui atividade anti-inflamatória, também é capaz de evitar desequilíbrio de GSH no hipocampo após indução de *SE* pela pilocarpina (Hong et al., 2012).

A excitabilidade neuronal é altamente dependente dos níveis extracelulares de K^+ (Devinsky et al., 2013). Dexametasona reverteu a redução dos canais Kir 4.1 aos 56 dias após a indução da *SE*, mas não no dia seguinte ao tratamento do anti-inflamatório. Esta mudança do conteúdo de Kir 4.1 pode refletir em uma melhora na captação do K^+ extracelular. Isto sugeriria que a dexametasona pode alterar a condição excitante e despolarizante do meio, contribuindo para uma atividade e sinalização normal dos neurônios o que é refletido na redução da neuroinflamação, da astrogliose e da disfunção astrocitária. Dexametasona ao ser utilizada para tratar edema macular de retina seletivamente aumentou a expressão dos canais Kir 4.1 (mas não AQP-4) (Zhao et al., 2011). Também observamos esta independência da ação da dexametasona não ocorrendo alterações na expressão dos canais AQP-4 neste modelo.

A terapia com uso de corticosteroide em epilepsia é motivo de debate, devido aos efeitos pró- e anti-convulsivos já observados. Estudos experimentais que administraram dexametasona antes da indução da *SE*, observaram alterações comportamentais, do período de latência de indução de *SE* e da mortalidade, assim como, alterações bioquímicas, neurológicas e inflamatórias (Al-Shorbagy et al., 2012; Fazekas et al., 2006; Pieretti et al., 1992). No entanto, a administração de dexametasona durante a indução de *SE*, não altera período de latência e até aumenta a taxa de mortalidade e o edema cerebral (Duffy et al., 2014). Os dados relativos à expressão de

COX-2 em modelos experimentais de epilepsia sugerem que o efeito da terapia com corticoide depende da dose, do tempo de administração, do tipo de inibidor (seletivo ou não seletivo) e das diferenças entre os modelos de indução de *SE* (Rojas et al., 2014). Este estudo demonstrou que a administração de dexametasona (24h após indução de *SE*) promove efeitos tanto nas fases aguda quanto crônica da epilepsia. Nossos resultados mostram uma redução da neuroinflamação, astrogliose e disfunção astroglial, bem como melhorias no comportamento epiléptico (dados não mostrados). Em conjunto, os nossos dados reforçam o papel neuroprotetor da dexametasona nos distúrbios da epilepsia e as doenças neurodegenerativas resultantes.

Nos dois primeiros estudos observamos que S100B no LCR pode ser considerada um marcador de epilepsia e que a modulação não específica da sua secreção através do anti-inflamatório dexametasona promove efeitos benéficos na epileptogênese à longo prazo. Diversos estudos vêm demonstrando a importância do papel dos astrócitos na atividade dos neurônios (Araque et al., 1999; Perea et al., 2009). Os astrócitos estão envolvidos na regulação da atividade sináptica e interagem com os neurônios regulando a neurotransmissão (Garnier et al., 2016). Sabe-se que há diversos moduladores da secreção de S100B (Gonçalves et al., 2008). Entretanto, há pouca informação na literatura sobre os efeitos da sinalização GABAérgica sobre os astrócitos e a secreção de S100B. A partir dessas informações nos perguntamos se condições despolarizantes induzidas pela regulação do receptor $GABA_A$ *in vitro* modulam a secreção de S100B.

GABA é o principal neurotransmissor inibitório do SNC adulto e é liberado tanto por neurônios quanto por células gliais, como gliotransmissor (Angulo et al., 2008). Os astrócitos estão envolvidos na sinalização GABAérgica e consequentemente modulam a condutância celular no SNC (Kaczor et al., 2015; Vélez-Fort et al., 2012). Recentes estudos observaram a colocalização de GABA com células GFAP positivas com (Yoon & Lee, 2014), com a enzima ácido glutâmico decarboxilase (isoforma GAD67) e monoamina oxidase, enzimas responsáveis pela síntese de GABA nos astrócitos a partir de glutamato e pela via alternativa de putrescina, respectivamente (Angulo et al., 2008). As células astrocitárias também expressam receptores $GABA_A$ e $GABA_B$ (Lee et al., 2011) e são responsáveis pela captação extracelular de GABA através do transportador GABA 3 (GAT-3) localizado em CA3 e giro dentado (Kersanté et al., 2013).

O neurotransmissor GABA liga-se predominantemente no receptor $GABA_A$. Os receptores $GABA_A$ são ionotrópicos e modulam a abertura de canais de cloreto, sinalizando a inibição no SNC. Muscimol tem sido utilizado como agonista seletivo de receptores $GABA_A$ (Chebib et al., 2004). Enquanto que bicuculina, picrotoxina e PTZ são caracterizados como antagonistas desse receptor. Esses antagonistas bloqueiam o receptor e são capazes de reverter a diminuição da corrente promovida por GABA. São considerados fármacos pró-convulsivos e são utilizados para testar a maioria dos fármacos anti-epilépticos (Kasthuri et al., 2013).

A hiperpolarização promovida pelo agonista muscimol não afetou a secreção de S100B. No entanto, a despolarização induzida pelos antagonistas PTZ e bicuculina altera a secreção de forma oposta. PTZ eleva a secreção de S100B, enquanto que bicuculina diminui. Em fatias hipocampais agudas a condição de despolarização induzida por alto potássio ou por inibidor da captação de potássio pelo canal Kir 4.1 reduz a secreção de S100B (Nardin et al., 2009; Zanotto et al., 2013). A discrepância dos resultados dos antagonistas pode ser em decorrência das diferentes ações das drogas. PTZ age de forma semelhante à picrotoxina no receptor $GABA_A$. PTZ liga-se de forma competitiva no domínio $\alpha1\beta2\gamma2$ do receptor $GABA_A$ (Huang et al., 2001). Bicuculina atua como inibidor alostérico do receptor $GABA_A$ (Ueno et al., 1997) e em solução pode ser convertida em bicucina, um metabólito com menor efeito convulsivante (Johnston, 2013).

Quando co-incubado o agonista muscimol inverte o efeito sobre a secreção de S100B promovida por PTZ e bicuculina. Possivelmente o muscimol influencia a condição de despolarização do meio, impedindo e interagindo com o receptor $GABA_A$ impossibilitando a ação dos antagonistas nos seus domínios.

Sabe-se que a comunicação entre neurônios e astrócitos é necessária para a modulação da secreção de S100B e esta interação é dependente do receptor RAGE (Sakatani et al., 2008). E a despolarização excessiva dos neurônios afeta a função astrocítica e promove a neuroinflamação (Xanthos & Sandkühler, 2014). Nossos resultados demonstram que o efeito da ação de PTZ também depende da interação entre neurônios e astrócitos. Além disso é necessária a manutenção da atividade de astrócitos na sinalização de PTZ. Contudo, a ação de bicuculina na secreção de S100B ocorre por outro mecanismo, tanto a co-incubação com TTX quanto com o fluorocitrato não alteraram a redução da secreção de S100B.

Outros metabólitos também modulam a secreção de S100B. β-hidroxibutirato (βOHB) é o corpo cetônico predominantemente sintetizado durante a dieta cetogênica. βOHB, mesmo sem alterar corrente elétrica induzida pela ativação dos receptores $GABA_A$, é considerado um agonista GABAérgico (Hartman et al., 2007) e um antiepiléptico (Rho et al., 2002). Em cultura de astrócitos a incubação por uma hora com βOHB aumenta a secreção de S100B (Leite et al., 2004). Entretanto, βOHB não alterou a secreção de S100B em fatias agudas de hipocampo. A ausência de ação de βOHB pode ser devida à composição do meio de incubação, as fatias hipocampais foram co-incubadas com glicose e βOHB. Em cultura de neurônios GABAérgicos, meios de incubação com a presença de βOHB e de glicose não alteram o conteúdo total de GABA (Lund et al., 2011).

Metilglioxal (MG) é produzido como um subproduto do metabolismo da glicólise e é um precursor de produtos finais de glicação avançada (AGEs). MG está envolvido em mecanismos celulares fisiológicos e patológicos (Allaman et al., 2015). Estudo anterior, considerou MG um agonista parcial do receptor de $GABA_A$, possivelmente atuando sobre os receptores $GABA_A$ contendo subunidades α6 (Distler et al., 2012). Apenas a concentração de 10 μM de MG é capaz de elevar a secreção de S100B em fatias hipocampais agudas. Importante ressaltar que estudo anterior em fatias demonstrou que MG a 0,5 mM reduz os níveis de S100B no meio extracelular (Hansen et al., 2016). Esta discrepância da modulação da secreção de S100B pelo MG deve ser devido às diferentes concentrações de MG no meio de incubação e sugerem que a concentração fisiológica de MG (MG 10 μM) aumenta a secreção de S100B. A ação de MG sobre a secreção é dependente da comunicação de neurônios e astrócitos, assim como outras condições de incubação (alto teor de potássio, ácido cainato e PTZ).

Este último estudo possui algumas limitações. Não há clareza do mecanismo de ação dos antagonistas PTZ e bicuculina sobre os astrócitos. A perspectiva futura é realizar controles dos fármacos em cultura de astrócitos e melhor identificar a ação desses antagonistas sobre a secreção de S100B.

CONCLUSÕES

O modelo de epilepsia induzido por Li-pilocarpina produz *SE* em ratos de 27 dias de idade e o desenvolvimento de crises recorrentes espontâneas (epilepsia) foi confirmado por vídeo-monitoramento. A inflamação no hipocampo (baseada no conteúdo de TNF-α) é precoce e transitória logo após a indução de *SE*. Astrogliose e disfunção astrocitária ocorrem na epileptogênese e são dependentes do tempo. Estas alterações sugerem que a resposta astrocitária induzida por Li-pilocarpina desencadeia e/ou exacerba danos neuronais, possivelmente através da redução da defesa antioxidante e da capacidade de regular o ambiente iônico (particularmente os níveis extracelulares de K^+). Esta insuficiência astroglial agrava a excitotoxicidade glutamatérgica já conhecida neste modelo e a morte neuronal. A proteína S100B pode ser vista como um marcador de *SE*, visto que constatamos um aumento claro, precoce e persistente de seu conteúdo no LCR, bem como alterações séricas de S100B após indução de *SE*. Ao analisar a relação LCR/soro de S100B observamos um aumento proeminente logo após *SE* e persistente em SE56. A elevação da relação LCR/soro de S100B possivelmente correlaciona-se com a severidade de *SE*. Entretanto, não é completamente claro os mecanismos associados as alterações de S100B no LCR e soro.

Dexametasona foi capaz de modular a secreção de S100B em fatias de hipocampo *ex vivo* de animais controle e *SE*. Dexametasona injetada intraperitonealmente 1 dia após a indução de *SE* alterou a curto e longo prazo parâmetros inflamatórios (citocinas, COX2 e PGE2), bem como a astrogliose (GFAP e S100B) e indicadores de disfunção astrocitária (atividade GS, conteúdo de GSH, canal de potássio Kir4.1 no hipocampo. A redução dos níveis extracelulares de S100B *ex vivo* em SE1 pode ser um dos mecanismos da redução da astrogliose e neuroinflamação em animais *SE* induzidos. A administração *in vivo* de dexametasona, 24h após a indução de *SE*, reverteu a maioria dos parâmetros analisados, reforçando a importância da terapia anti-inflamatória esteróide na epilepsia. Nossos dados sobre alterações específicas em astrócitos neste modelo claramente contribuem para a nossa compreensão da importância dessas células na epilepsia, bem como sugerem que a modulação dos astrócitos é um alvo terapêutico potencial para o desenvolvimento de novas drogas anti-epilépticas.

Em fatias hipocampais agudas a modulação do receptor $GABA_A$ promove alterações da secreção de S100B e é dependente da comunicação entre neurônios e astrócitos, assim como a manutenção da atividade dos astrócitos.

Em conjunto nossos resultados demonstram o papel dos astrócitos e da neuroinflamação na epileptogênese. Assim como, apontam para a proteínas S100B como um possível marcador de epilepsia e alvo farmacológico de tratamento.

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