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EFEITOS COMPORTAMENTAIS E NEUROQUÍMICOS DO STATUS EPILEPTICUS INDUZIDO POR LiCl-PILOCAPINA EM RATOS JOVENS.

Porto Alegre, maio de 2008.

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EFEITOS COMPORTAMENTAIS E NEUROQUÍMICOS DO STATUS EPILEPTICUS INDUZIDO POR LiCl-PILOCAPINA EM RATOS JOVENS.

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Bioquímica, como requisito parcial obtenção do título de Doutor em Ciências Biológicas – Bioquímica.

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Co-Orientador: Prof. Diogo Onofre Gomes de Souza

Porto Alegre, maio de 2008.

"Tudo neste mundo tem uma resposta. O que leva é tempo para se formular as perguntas."

José Saramago.

Às duas pessoas mais importantes da minha vida,

meus pais.

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SUMÁRIO

RESUMO

Convulsões prolongadas, *status epilepticus* (SE), durante o desenvolvimento cerebral podem afetar a estrutura e a função neuronal, levando a morte celular e alterações comportamentais na idade adulta. Nesta tese, investigamos os efeitos neuroquímicos e comportamentais, a curto e longo prazo, do SE induzido por LiCl-pilocarpina em animais jovens. O SE induzido por LiCl-pilocarpina reduz em 50% a atividade da Na^+/K^+ ATPase 1,5 h após a indução do insulto. Doze e vinte e quatro horas após a administração de pilocarpina, a atividade da Na^+/K^+ ATPase retornou aos níveis do controle. A diminuição da atividade da Na^+/K^+ ATPase foi acompanhada por uma diminuição significativa na captação de glutamato em concentrações fisiológicas (1 μM). No entanto, em concentrações elevadas (100 μM) não observou-se alteração da captação de glutamato. Nos animais tratados com LiCl-pilocarpina observou-se uma diminuição dos níveis de fosforilação das proteínas PKB e GKS-3β de 35,84% e 38,79%, respectivamente, 1,5 h após a indução do SE na região CA1 do hipocampo. No entanto, 24 h após a indução do SE, a fosforilação da PKB e GKS-3β retornaram aos níveis do controle. No giro denteado, observou-se uma redução de aproximadamente 30% nos níveis de fosforilação tanto da PKB e GKS-3β em 1,5 e 24 h após a indução do SE. Um elevado número de neurônios em degeneração foi observado 24 h após a indução do SE nas regiões CA1 e hilo do hipocampo. Na idade adulta, os animais tratados com LiCl-pilocapina apresentaram um severo déficit de memória-aprendizado na tarefa de esquiva inibitória, sendo que os animais que apresentaram menor latência para descida da plataforma apresentaram um maior escore para brotamento de fibras musgosas no hipocampo. No teste do claro-escuro, os animais submetidos ao SE retornaram menos e permaneceram menos tempo no compartimento claro quando comparados aos animais controles. Além disso, os animais tratados com LiCl-pilocarpina apresentaram elevados níveis da proteína S100B no líquor, bem como apresentaram uma correlação positiva entre o escore para brotamento de fibras musgosas e o imunoconteúdo de GFAP na região CA1. Portanto, nossos resultados mostraram que o SE induzido por LiCl-pilocarpina diminui a atividade da Na^+/K^+ ATPase, bem como a captação de glutamato em hipocampo 1,5 h após a indução do insulto. Estes efeitos foram acompanhados por uma diminuição da fosforilação das proteínas PKB e GKS-3β nas regiões CA1 e giro denteado, os quais podem estar relacionados com a morte neuronal observada 24 h após a indução do SE. Além disso, na idade adulta, o SE induzido aos 14 dias pós-natal induziu alterações no processo de memória-aprendizado e no comportamento emocional. As alterações comportamentais parecem estar relacionadas ao brotamento de fibras musgosas, visto que os animais que apresentaram déficit de memória na tarefa de esquiva inibitória e elevados níveis de ansiedade no teste claro-escuro apresentaram maior escore para brotamento de fibras musgosas no hipocampo. Além disso, os animais tratados com LiCl-pilocarpina apresentaram elevados níveis da proteína S100B no líquor bem como uma correlação positiva entre o imunoconteúdo de GFAP na região CA1 e o escore para brotamento de fibras musgosas, sugerindo uma resposta glial ao dano neuronal. Estes dados indicam que o *status epilepticus* induzido em períodos iniciais do desenvolvimento cerebral é danoso ao sistema nervoso central, alterando sua maturação e levando a alteração neuroquímicas, morfológicas e comportamentais na idade adulta.

ABSTRACT

Prolonged seizure activity, i.e. status epilepticus (SE), or repeated, brief seizures affect neuronal structure and function in the developing nervous system leading to neuronal death and behavioral impairment at adulthood. In this study, we investigated the short- and long-term effects of early-life LiCl-pilocarpine-induced SE on neurochemical and behavioral parameters. LiCl-pilocarpine-induced SE decreased Na+/K+ ATPase activity by 42% in hippocampal plasma membranes 1.5 h after SE induction when compared with control group. However, at 12 and 24 h after SE induction the activity of pump returned to control levels. At physiologic concentration of glutamate (1 μM), LiCl-pilocarpine treated animals showed decreased levels of glutamate uptake 1.5 h after SE induction. However, LiCl-pilocarpine-induced SE did not alter the glutamate uptake 12 and 24 h after SE induction. At higher concentration of extracellular glutamate (100 μM), no alterations were observed in glutamate uptake between control and LiCl-pilocarpine-induced SE groups in all times tested. LiCl-pilocarpine-induced SE decreased the phosphorylation of PKB by 35.84% and GSK-3β by 38.79% 1.5 h after SE induction in CA1 subfield. However, 24 h after LiCl-pilocarpine-induced SE the phosphorylation of PKB and GSK-3β returned to control levels. At dentate gyrus, the phosphorylation of PKB was reduced by 35.18% and 31.19% at 1.5 and 24 h after SE induction, respectively. GSK-3β phosphorylation was reduced by 38.05% and 33.12% at 1.5 and 24 h, respectively, after LiCl-pilocarpine administration. Twenty four h after SE, it was observed an increased number of degenerating neurons in the CA1 subfield and in the hilus. At adulthood, SE group presented an aversive memory deficit in an inhibitory avoidance task and the animals that presented lower latency to the step down showed a higher score for mossy fiber sprouting. In the light-dark exploration task, SE rats returned less and spent less time in the light compartment and present an increased number of risk assessment behavior (RA). There was a negative correlation between the time spent in the light compartment and the score for mossy fiber sprouting and a positive correlation between score for mossy fiber sprouting and number of RA. LiCl-pilocarpine-treated animals showed higher levels of S100B immunocontent in the CSF as well as a positive correlation between the score for sprouting and the GFAP immunocontent in the CA1 subfield, suggesting an astrocytic response to neuronal injury. Our results showed that early-life LiCl-pilocapine-induced SE reduce hippocampal Na+/K+ ATPase as well as glutamate uptake 1.5 h after SE induction. These effects were accompanied by a reduced phosphorylation of Akt and GSK-3β at CA1 and dentate gyrus, which may be related to neuronal death observed 24 h after SE induction. Moreover, at adulthood the animals submitted to LiCl-pilocapine induced SE displayed memory impairment and increased anxiety-like behavior. The behavioral alterations appear to be associated with the mossy fiber sprouting, since the animals that presented impairment in the performance of inhibitory avoidance task and higher levels of anxiety in adulthood showed higher scores for mossy fiber sprouting in the hippocampus. Moreover, LiCl-pilocarpine-treated animals showed higher levels of S100B immunocontent in the CSF as well as a positive correlation between the score for mossy fiber sprouting and the GFAP immunocontent in the CA1 subfield, suggesting an astrocytic response to neuronal injury. These data indicate that LiCl-pilocarpine-induced SE early in life might harmfully affect brain maturation, leading to behavioral, morphological and neurochemical alterations in adulthood.

APRESENTAÇÃO

Esta Tese é constituída de seis partes:

Parte I. Introdução e Objetivos;

Parte II. Os Resultados que fazem parte desta Dissertação estão apresentados sob a forma de artigos científicos, subdivididos em: Introdução, Material e Métodos, Resultados, Discussão, Conclusão e Referências Bibliográficas;

Parte III. Discussão final;

Parte IV. Conclusões;

Parte V. Perspectivas;

Parte VI. Referências Bibliográficas referentes à Introdução e Discussão final.

LISTA DE ABREVIATURAS

AMPA	α-amino-3-hidroxi-5-metil-4-isoxazolepropionato
EAAC 1	carreador de aminoácidos excitatórios 1
EAAT 1-5	transportador de aminoácidos excitatórios 1-5
PKB	proteína quinase B
GABA	ácido gama-aminobutírico.
GFAP	proteína ácida fibrilar glial
GLAST	transportador de glutamato e aspartato
GLT-1	transportador glial 1
GluRs	receptores glutamatérgicos
GSK-3β	glicogênio sintase quinase – 3β
iGluRs	receptores glutamatérgicos ionotrópicos
LiCl	cloreto de lítio.
SNC	sistema nervoso central
SE	*status epilepticus*

Parte I. Introdução

I.1 EPILEPSIAS

As epilepsias referem-se a um grupo diverso, etiologica e clinicamente, de transtornos neurológicos caracterizados por crises epilépticas recorrentes, as quais resultam da atividade neuronal excessiva, anormal e hipersincrônica (Engel, 2001). Trata-se de uma disfunção cerebral caracterizada clinicamente por alterações subjetivas ou comportamentais súbitas (crises epilépticas), com tendência a se repetirem por toda a vida do paciente. Estas crises refletem uma atividade nervosa anormal, de início súbito, acometendo uma ou mais regiões cerebrais (Nabbout and Dulac, 2008).

Aproximadamente 50 milhões de pessoas no mundo são portadores de epilepsia, sendo que 40 milhões estão em países subdesenvolvidos. A incidência das epilepsias é extremamente alta nos primeiros anos de vida, declinando conforme a criança chega à adolescência (Sidenvall *et al.*, 1993). Em crianças, os tipos de epilepsias podem ser altamente variáveis, desde simples crises de ausência até convulsões tônico-clônicas e crises parciais complexas. Múltiplos tipos de crises, evolução de um tipo a outro e remissão espontânea das crises são características marcantes das epilepsias em crianças (Cowan, 2002).

Alguns estudos demonstram que há uma maior incidência de epilepsias em crianças do sexo masculino do que naquelas do sexo feminino (Hauser *et al.*, 1991; Sidenvall *et al.*, 1993). No entanto, esta diferença sexo-específica varia com a idade e com o tipo de síndrome. Aos cinco anos, as taxas de incidência são 30%-60% maiores em indivíduos do sexo feminino, enquanto que na adolescência tendem a ser 10%-20% maiores em indivíduos do sexo masculino (Annegers *et al.*, 1999; Hauser *et al.*, 1991). Estas flutuações refletem, principalmente, diferenças na predominância de um ou outro tipo de epilepsia, diferenças na exposição a fatores de risco, diferenças de susceptibilidade sexo-específicas e também variações no diagnóstico (Cowan, 2002).

Somente 25% a 45% das epilepsias pediátricas são atribuídas a fatores de risco específicos, o restante possui etiologia desconhecida (Berg *et al.*, 1999; Waaler *et al.*, 2000). Os fatores, com consistente associação ao aumento do risco de epilepsia em crianças, são: malformações congênitas e desordens metabólicas, convulsões febris, convulsões nos primeiros 28 dias de vida (convulsões neonatais), trauma crânio-encefálico moderado ou severo, infecções do SNC e história familiar de epilepsias (Cowan, 2002).

O prognóstico das epilepsias infantis depende principalmente de sua etiologia e da presença de outras patologias neurológicas associadas (Berg and Shinnar, 1994; Sillanpaa, 2000), sendo que a maioria das crianças com epilepsia na infância poderá apresentar remissão total das crises quando atingirem a idade adulta (Sillanpaa, 2000). No entanto, segundo Austin et al. 1992, para epilepsias seguidas de lesões estruturais no sistema nervoso, cerca de 60% das crianças epilépticas apresentam algum tipo de desordem neurológica ou psiquiátrica na idade adulta. Diversos autores têm observado que crises epilépticas ocorridas durante a infância estão associadas a um maior risco de prejuízo intelectual quando comparadas a crises epilépticas iniciadas durante a adolescência e ou idade adulta. No entanto, ainda não está claro se estes efeitos adversos são secundários ao dano causado pelas crises ou são um reflexo do insulto responsável por elas (Dikmen *et al.*, 1975).

I.1.1 Epilepsia do lobo temporal

A epilepsia do lobo temporal é a forma mais comum de epilepsia na população adulta, sendo responsável por 40% dos casos (Gastaut *et al.*, 1975; Lowe *et al.*, 1998). Sua importância clínica decorre não somente de sua elevada prevalência, mas também por ser refratária a maioria dos tratamentos farmacológicos disponíveis atualmente. Dentre as síndromes epilépticas refratárias, a epilepsia do lobo temporal destaca-se por ser a mais freqüente em pacientes adultos, sendo que,

neste caso, a remoção cirúrgica do foco epiléptico torna-se a única alternativa de tratamento (Engel, 1993).

Dentre os agentes etiológicos, a esclerose hipocampal é encontrada em 50-70% dos pacientes portadores de epilepsia do lobo temporal (Engel *et al.*, 1997). Do ponto de vista anatomopatológico a esclerose hipocampal caracteriza-se por perda neuronal e gliose reativa, especialmente das células das regiões CA1 e da região hilar, com relativa preservação de região CA2, subiculum e giro denteado (McNamara, 1994). Associado à perda neuronal, observa-se também a dispersão das células granulares. Esta é caracterizada pela perda da justaposição habitual das células granulares na camada granular do giro denteado, produzindo aumento da sua espessura e dos espaços intercelulares (El Bahh *et al.*, 1999; Houser, 1990). Adicionalmente, observa-se uma importante reorganização axonal, caracterizada por brotamentos de sinapses colaterais das células granulares (as fibras musgosas) na região da camada molecular interna do giro denteado (Babb *et al.*, 1991; Tauck and Nadler, 1985).

Apesar da relação entre esclerose hipocampal e a epilepsia do lobo temporal já estar bem estabelecida, o mecanismo exato pelo qual a esclerose hipocampal participa da gênese das crises epilépticas ainda é tema de grande debate. Já em 1954 havia a proposta de que a esclerose hipocampal poderia estar associada a uma história prévia de injúria precipitante inicial ocorrida em fases precoces do desenvolvimento cerebral (Meyer *et al.*, 1954). Dentre as injúrias, destacam-se as crises epilépticas prolongadas (*status epilepticus*) e as convulsões febris (Mathern *et al.*, 2002). Atualmente, sabe-se que cerca de 80% dos pacientes portadores de epilepsia do lobo temporal possuem história de convulsões febris e/ou *status epilepticus* durante os primeiros anos de vida (Falconer, 1971; French *et al.*, 1993; Mathern *et al.*, 1995b; VanLandingham *et al.*, 1998).

I.1.2 Status epilepticus

O *status epilepticus* é definido como uma crise epiléptica prolongada, a qual persiste por tempo suficiente ou repete-se com uma freqüência tal que não permite a recuperação da consciência entre os ataques (Chen and Wasterlain, 2006). A incidência de *status epilepticus* em crianças, durante os primeiros anos de vida, é de 156 casos por 100000 habitantes, diminuindo para 38 por 100000 habitantes na infância e 27 casos por 100000 habitantes na idade adulta (DeLorenzo *et al.*, 1995). A elevada incidência de *status epilepticus* durante os perídos iniciais do desenvolvimento cerebral deve-se ao fato de o cérebro em desenvolvimento possuir uma maior pré-disponibilidade a atividade epiléptica do que o cérebro maturo (Holmes, 1997). Isto se deve principalmente a um desbalanço entre os sistemas excitatórios e inibitórios, visto que o ácido gama-aminobutírico (GABA), o principal aminoácido inibitório no cérebro adulto, possui uma ação excitatória sobre o cérebro em desenvolvimento (Holmes, 1997).

Atualmente, há um grande debate com relação ao fato de uma atividade epiléptica pronlongada (como o *status epileticus*), quando corrida durante do desenvolvimento cerebral, alterar e prejudicar a maturação do sistema nervoso central (Sankar and Rho, 2007). Convulsões febris têm sido citadas como um exemplo de crises epilépticas prologadas de natureza benigna (Maytal *et al.*, 1989). No entanto, estudos retrospectivos indicam que 80% dos pacientes com epilepsia refratária, principalmente epilepsia do lobo temporal, apresentaram *status epilepticus* ou convulsões febris prolongadas durante a infância (Falconer, 1971; French *et al.*, 1993; Mathern *et al.*, 1995b; VanLandingham *et al.*, 1998). Além disso, crianças acometidas de *status epilepticus* apresentam, na idade adulta, alterações comportamentais, esclerose hipocampal, epilepsia secundária, déficit cognitivo e alterações no comportamento emocional (van Esch *et al.*, 1996).

Inúmeras abordagens têm sido realizadas na tentativa de elucidar os mecanismos celulares e moleculares envolvidos em tais alterações, tanto á nível do tecido epiléptico humano, obtido após

remoção cirúrgica, quanto através do estudo de tecidos cerebrais, provenientes de animais submetidos a diferentes modelos de *status epilepticus*.

Os modelos mais comumente usados para indução de *status epilepticus* são: o modelo da pilocarpina (Turski *et al.*, 1983) e o modelo do ácido caínico (Ben-Ari, 1985).

A aministração sistêmica de altas doses de pilocarpina, um agonista colinérgico, induz a uma série de alterações comportamentais e eletrográficas, indicativas de *status epilepticus*. A indução da crise se dá pelo efeito agonista colinérgico e sua manutenção se deve a mecanismos excitatórios do tipo glutamatérgicos (McDonough and Shih, 1997). O pré-tratamento com lítio potencia a ação epileptogência da pilocarpina, reduzindo a mortalidade e evitando os efeitos colinomiméticos periféricos (Clifford *et al.*, 1987). O *status epilepticus* induzido por LiCl-pilocarpina é caracterizado divido em três fases distintas.

Na primeira fase, denominada de fase aguda, os animais apresentam hiperatividade, tremores e clonismos de patas nos primeiros 5 min (Hirsch *et al.*, 1992). Este comportamento é seguido pelo aumento na atividade motora culminando em *status epilepticus* 30-40 min após a administração. Durante o *status epilepticus*, os animais apresentam automatismos orofaciais repetidos, salivação, clonismos de patas e perda de equilíbrio. Eletrograficamente, o *status epilepticus* foi caracterizado por uma intensa atividade elétrica poliponta tanto cortical quanto hipocampal a qual estendeu-se por aproximadamente 6 h (Hirsch *et al.*, 1992). Vinte e quatro horas após a indução do insulto, observa-se uma intensa morte celular em diversas regiões do sistema nervoso central, como córtex, tálamo e hipocampo (Sankar *et al.*, 1997). Na formação hipocampal, o dano celular é mais proeminente nas região CA1 e hilo quando comparadas as regiões CA2 e CA3 (Sankar *et al.*, 1998).

A segunda fase (3-60 dias após o insulto) é denominada de fase latente, sendo caracterizada principalmente por uma normalização comportamental. Nesta fase os animais não aprensentam nenhuma alteração comportamental evidente, bem como não são encontradas alterações nos padrões eletroencefalográficos (Leite *et al.*, 2002). No entanto, acredita-se que os eventos de plasticidade sináptica observadas na fase crônica ocorram durante a fase latente (Leite *et al.*, 2002).

A fase crônica é caracterizada principalmente pela ocorrência de convulsões espontâneas de origem límbica, sendo que aproximadamente 80% dos animais apresentaram crises epilépticas espontâneas na idade adulta (Leite *et al.*, 2002). Além disso, observa-se uma importante reorganização axonal, caracterizada por brotamentos de sinapses colaterais das células granulares (as fibras musgosas), na região da camada molecular interna do giro denteado (Babb *et al.*, 1991; Tauck and Nadler, 1985). Tais alterações são similares aquelas encontradas em pacientes com esclerose hipocampal seguida de epilepsia do lobo temporal (Babb *et al.*, 1991; Mathern *et al.*, 1995a; Tauck and Nadler, 1985).

I.2 SISTEMA GLUTAMATÉRGICO

O glutamato é o aminoácido encontrado em maior concentração no SNC de mamíferos onde desempenha um importante papel metabólico (Krebs, 1935).

O glutamato exerce uma potente ação excitatória sobre neurônios da medula espinhal (Curtis *et al.*, 1959). Atualmente, o glutamato é considerado o principal aminoácido excitatório do SNC de mamíferos, onde participa de inúmeros eventos fisiológicos e plásticos tais como: memória e aprendizado (Izquierdo *et al.*, 2006), desenvolvimento e envelhecimento (Segovia *et al.*, 2001), adaptação ambiental (Ozawa *et al.*, 1998), proliferação e migração celular (McDonald and Johnston, 1990). No entanto, quando presente em altas concentrações na fenda sináptica por um longo período, pode ser uma potente neurotoxina (Obrenovitch *et al.*, 2000) e sua excitotoxicidade

tem sido implicada na patogênese de inúmeros transtornos agudos e crônicos do SNC (Maragakis and Rothstein, 2001).

As diversas ações do glutamato, tanto fisiológicas quanto patológicas, resultam da presença de receptores glutamatérgicos (GluRs), tanto neuronais como gliais (Ozawa *et al.*, 1998). Os GluRs são divididos em duas classes: ionotrópicos (iGluRs) e metabotrópicos (mGluRs) (Tanabe et al., 1992). Os iGluRs contêm um canal iônico cátion-específico e são subdivididos em três subtipos: α-amino-3-hidroxi-5-metil-4-isoxazolepropionato (AMPA), cainato and N-metil-D-aspartato (NMDA). Por outro lado, os mGluRs são acoplados a proteínas G e estão subdivididos em 8 subtipos ($mGluR_{1-8}$).

Uma terceira classe de proteínas, igualmente importantes para a função glutamatérgica, são os transportadores de glutamato. Sua principal atividade é recaptar o glutamato liberado na fenda a fim de terminar a transmissão sináptica (Danbolt, 2001). Os transportadores, portanto, desempenham importante papel na manutenção dos níveis extracelulares de glutamato abaixo daqueles considerados tóxicos (Danbolt, 2001; Gether *et al.*, 2006).

I.2.1 Captação de glutamato

A captação de glutamato é o principal mecanismo responsável pela manutenção dos níveis extracelulares de glutamato abaixo dos níveis tóxicos (Danbolt, 2001), sendo realizada por transportadores de glutamato presentes na membrana plasmática de neurônios e células gliais, principalmente em astrócitos (Rothstein *et al.*, 1996).

Atualmente, já foram descritos 5 tipos de transportadores: GLAST/EAAT1 (Storck et al., 1992), GLT-1/EAAT2 (Pines et al., 1992), EAAC1/EAAT3 (Kanai e Hediger, 1992), EAAT4 (Fairman et al., 1995) e EAAT5 (Arriza et al., 1997). Estes cinco transportadores de glutamato apresentam entre 50 e 60% de semelhança na seqüência de aminoácidos (Gether *et al.*, 2006).

GLAST e GLT-1 foram somente detectados em astrócitos (Rothstein et al., 1994; Lehre et al., 1995; Lehre e Danbolt, 1998), tanto no corpo celular quanto nos processos das células astrocitárias (Danbolt, 2001). Uma exceção é a expressão do GLT-1 em retina, onde é encontrado em diferentes tipos de células bipolares, mas não na glia de Müller ou outras células gliais (Rauen *et al.*, 1992). Estes dois transportadores são quantitativamente os principais transportadores de glutamato, sendo responsáveis pela maior parte de sua captação no SNC (Anderson and Swanson, 2000; Danbolt, 2001). Sua distribuição e concentração no SNC é região dependente (Danbolt, 2001; Gegelashvili and Schousboe, 1998) e varia conforme o estágio de desenvolvimento cerebral (Furuta *et al.*, 1997; Maragakis and Rothstein, 2001).

Os demais transportadores de glutamato, EAAC1, EAAT4 e EAAT5, são encontrados predominantemente em neurônios (Gegelashvili and Schousboe, 1998); entretanto, a expressão do EAAT4 foi recentemente descrita em astrócitos (Hu *et al.*, 2003).

O EAAC1 é encontrado na maioria dos neurônios do sistema glutamatérgico, assim como nos neurônios do sistema gabaérgico (células de Purkinje no cerebelo) (Danbolt, 2001; Nedergaard *et al.*, 2002), mas quando comparado com GLAST e GLT-1, sua expressão é insignificante (Tanaka *et al.*, 1997). O EAAT4 está localizado nas células de Purkinje no cerebelo (Tanaka *et al.*, 1997), mas pode também ser encontrado em concentrações muito baixas no telencéfalo (Danbolt, 2001). Encontra-se em maiores concentrações na parte da membrana espinhal que se defronta com a astroglia, desaparecendo gradualmente através do corpo celular (Dehnes *et al.*, 1998). Além disso, é transitoriamente expresso durante o desenvolvimento, com pico em 10 dias pós-natal (Furuta *et al.*, 1997).

Poucos estudos sobre a localização do transportador de glutamato EAAT5 em mamíferos foram realizados até o presente momento; entretanto, alguns relatos têm demonstrado sua presença em células da retina (Arriza *et al.*, 1997).

É indiscutível a importância dos transportadores de glutamato na manutenção dos níveis extracelulares deste aminoácido abaixo daqueles neurotóxicos, a fim de evitar excessiva ativação dos receptores glutamatérgicos e conseqüente excitotoxicidade. Recentes estudos em humanos (Maragakis and Rothstein, 2001) e em ratos transgênicos (Meldrum *et al.*, 1999) indicaram que alterações nos transportadores de glutamato podem resultar em fenótipos epilépticos. Ratos knock-out para o GLT-1 apresentam convulsões letais nas primeiras horas de vida (Tanaka *et al.*, 1997). Paralelamente, estes animais apresentam uma maior suscetibilidade à injúria cerebelar e cortical, assim como uma redução da coordenação motora (Watase *et al.*, 1998). A perda do transportador neuronal EAAC1 produziu neurotoxicidade moderada, resultando em epilepsia (Rothstein *et al.*, 1996) e anormalidades de comportamento (Danbolt, 2001). Animais epilépticos (Ingram *et al.*, 2001) e ratos com epilepsia crônica induzida por ácido caínico (Ueda *et al.*, 2001) apresentaram uma redução na expressão de transportadores gliais (GLT-1 and GLAST). Estes resultados indicam que disfunções na captação de glutamato podem desempenhar um importante papel no processo epileptogênico.

I.3 SINALIZAÇÃO INTRACELULAR E FATORES TRÓFICOS

I.3.1 Proteína cinase b (akt/pkb)

Akt é uma cinase de serina e treonina também denominada proteína cinase B (PKB). Está altamente expressa no SNC, constituindo um promotor de sobrevivência e neuroproteção de neurônios embrionários. A Akt é ativada por insulina e vários fatores de crescimento, por intermédio da PI3K (Brunet *et al.*, 2001). Quando ativa, a Akt inibe a apoptose por fosforilar e inativar uma variedade de substratos pertencentes a maquinaria apoptótica, tais como a BAD – proteína pró-apoptótica pertencente a família das Bcl-2 (Downward, 1999), GSK-3b (Cross *et al.*, 1995), capase-9 e a família de fatores de transcrição em forquilha (Forkhead, FKHR) (Brunet *et al.*,

2001). A Akt é ativada por fosforilação por intermédio de duas cinases dependentes de fosfoinositóis, as PDKs1 e 2 (Chan *et al.*, 1999). Os principais reguladores negativos da Akt são duas fosfatases: a fosfatase da PI3K (phosphatase and tensin homolog deleted on chromossome 10, PTEN) (Leslie and Downes, 2002) e a proteína fosfatase 2ª (PP2A), que diretamente a desfosforila e inativa (Ugi *et al.*, 2004).Apesar da relevância deste sistema defensivo celular, o papel da via PI3K/Akt não há trabalhos na literatura mostrando o envolvimento desta via se sinalização no dano celular induzido pelo *status epilepticus*.

I.3.2 Glicogênio sintase cinase-3β (GSK-3b)

A enzima glicogênio sintase cinase-3b (GSK-3b) foi originalmente identificada como um modulador do metabolismo do glicogênio (Wagman *et al.*, 2004). Atualmente se sabe do importante papel regulatório desta enzima em uma variedade de vias intracelulares, incuindo iniciação da síntese de proteínas, proliferação e diferenciação celular, desenvolvimento embrionário e apoptose (minhas referencias). A GSK-3b é considerada uma enzima pró-apoptótica por inibier a ativação de uma variedade de fatores de transcrição importantes para a sobrevivência celular (Li *et al.*, 2002). A GSK-3b é constitutivamente ativa, mas pode ser ativada or aumento transitório de cálcio intracelular (Hartigan and Johnson, 1999), e inibida pelas vias de sinalização da Wnt/b-catenina (Li *et al.*, 2002) e através da ativação da cascata da fosfatisilinositol 3-cinase/Akt (PI3K/Akt) (Cross *et al.*, 1995).

I.3.3 Proteína S100B

A proteína S100B é uma proteína de 21 kDa, ligante de cálcio do tipo “EF-hand”. É integrante de uma família de proteínas denominadas S100, assim determinadas por serem solúveis em solução 100% de sulfato de amônio (Moore, 1965). No SNC, a S100B é expressa e secretada por astrócitos (Donato, 2001). O mecanismo pelo qual a S100B é secretada ainda permanece

desconhecido (Davey *et al.*, 2001), porém estudos demonstram que sua concentração no meio extracelular pode ser modulada por diversos fatores, tais como: ativação de receptores de adenosina (Ciccarelli *et al.*, 1999), elevados níveis extracelulares de glutamato (Goncalves *et al.*, 2002), estimulação de receptores de serotonina (Whitaker-Azmitia *et al.*, 1990) e aumento de cálcio intracelular (Davey *et al.*, 2001). As ações extracelulares da proteína S100B, em culturas de células, dependem de sua concentração no meio extracelular. Em concentrações nanomolares exerce um efeito neurotrófico estimulando a extensão de neuritos, facilitando a sobrevivência de neurônios durante o desenvolvimento, além de estimular a proliferação de astrócitos e a captação glial de glutamato (Donato, 2001). Em níveis micromolares, a S100b é capaz de estimular a expressão de citocinas pró-inflamatórias e induzir apoptose (Donato, 2001; Van Eldik and Wainwright, 2003). Além dos efeitos extracelulares, a proteína S100B apresenta um papel intracelular, atuando na modulação da plasticidade do citoesqueleto e da proliferação celular (Donato, 2001; Van Eldik and Wainwright, 2003).

Em lesões do SNC, como isquemias, e também em doenças neurodegenerativas, como a Doença de Alzheimer, há um aumento dos níveis extracelulares da proteína S100B (Heizmann, 1999). Estudos realizados em animais de laboratório têm demonstrado um aumento dos níveis extracelulares da proteína S100B em modelos de injúria cerebral (Busnello *et al.*, 2006; Oses *et al.*, 2004). Além disso, níveis aumentados de S100B foram detectados em pacientes com epilepsia do lobo temporal (Steinhoff *et al.*, 1999). Embora o significado fisiológico e patológico das variações nos níveis extracelulares da proteína S100B ainda permaneça desconhecido, ela tem sido usada como um marcador da atividade astrocítica, provavelmente como uma conseqüência a injúria neuronal (Donato, 2001; Van Eldik and Wainwright, 2003).

I.4 OBJETIVOS

I.4.1 Objetivo geral

Investigar os efeitos a curto e longo prazo de crises epilépticas prolongadas induzidas por LiCl-pilocarpina sobre parâmetros comportamentais e neuroquímicos.

I.4.2 Objetivos específicos

Parâmetros comportamentais:

- Investigar os efeitos de crises epilépticas prolongadas induzidas durante o desenvolvimento cerebral sobre a memória e aprendizado; sobre o comportamento emocional (ansiedade) e a atividade locomotora.

Parâmetros neuroquímicos:

- Avaliar os efeitos do *status epilepticus* sobre a expressão das proteínas S100b e GFAP; sobre a atividade da Na+/K+ ATPase; sobre a capatação de glutamato; e sobre as vias de sinalização da Akt e GSK-3b.

Parte II. Resultados

CAPÍTULO I

Effects of early-life LiCl-Pilocarpine-induced *status epilepticus* on memory and anxiety in adult rats are associated with mossy fiber sprouting and elevated CSF S100B protein.

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FULL-LENGTH ORIGINAL RESEARCH

Effects of early-life LiCl-pilocarpine-induced status epilepticus on memory and anxiety in adult rats are associated with mossy fiber sprouting and elevated CSF S100B protein

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SUMMARY

Purpose: This study investigated putative correlations among behavioral changes and: (1) neuronal loss, (2) hippocampal mossy fiber sprouting, and (3) reactive astrogliosis in adult rats submitted to early-life LiCl-pilocarpine-induced status epilepticus (SE).

Methods: Rats (P15) received LiCl (3 mEq/kg, i.p.) 12–18 h prior pilocarpine (60 mg/kg; s.c.). At adulthood, animals were submitted to behavioral tasks and after the completion of tasks biochemical and histological analysis were performed.

Results: In SE group, it was observed an increased number of degenerating neurons in the CA1 subfield and in the hilus of animals 24 h after SE. At adulthood, SE group presented an aversive memory deficit in an inhibitory avoidance task and the animals that presented lower latency to the step down showed a higher score for mossy fiber sprouting. In the light-dark exploration task, SE rats returned less and spent less time in the light compartment and present an increased number of risk assessment behavior (RA). There was a negative correlation between the time spent in the light compartment and the score for mossy fiber sprouting and a positive correlation between score for mossy fiber sprouting and number of RA. LiCl-pilocarpine-treated animals showed higher levels of S100B immunocontent in the CSF as well as a positive correlation between the score for sprouting and the GFAP immunocontent in the CA1 subfield, suggesting an astrocytic response to neuronal injury.

Conclusions: We showed that LiCl-pilocarpine-induced SE during development produced long-lasting behavioral abnormalities, which might be associated with mossy fiber sprouting and elevated CSF S100B levels at adulthood.

KEY WORDS: Status epilepticus, Development, Learning and memory, Anxiety, S100B protein, Mossy fiber sprouting.

Epilepsy is a common neurological disorder that affects children much more often than adults (Cowan, 2002). There is a clinical debate about brain damage and behavioral impairment induced by epileptic activity in infants and children (Camfield, 1997; Wasterlain, 1997; Raspall-Chaure et al., 2006). Febrile convulsions have been cited as a classic example of the benign nature of seizures in children with no previous brain pathology (Maytal et al., 1989). However, retrospective studies indicate that up to 80% of adult patients with drug refractory temporal lobe epilepsy, presented childhood status epilepticus (SE) or prolonged febrile seizures (Cendes & Andermann, 2002). Moreover, an early-life febrile SE can cause brain damage, resulting in long-term behavioral impairment and permanent susceptibility to future seizures (van Esch et al., 1996).

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In animal models, one of the earliest brain consequences of SE early in life is the selective neuronal loss observed in specific brain areas. Rats with LiCl-pilocarpine-induced SE during the second week of life presented damage in the hippocampus, amygdala, thalamus, and septum as well as an elevation in the serum levels of neuron specific enolase (NSE) 24 h after SE induction (Sankar et al., 1997). Further analysis of hippocampal CA1 pyramidal cells showed DNA fragmentation (TUNEL analysis) and apoptotic bodies in electron micrographs of damaged neurons, supporting the idea that apoptosis is involved in neuronal cell death (Sankar et al., 1998).

Another morphological alteration induced by SE during development is anomalous synapse formation. The most well-documented and well-studied synaptic rearrangement in the hippocampus is mossy fiber sprouting, which is a pronounced expansion of a small normal projection of granule cell mossy fiber axons into the supragranular layer of the dentate gyrus (Tauck & Nadler, 1985; Houser et al., 1990) and within the hilus (Sutula et al., 1998).

Reactive astrocytes have been found in the hippocampus, amygdala, thalamus, and cortex of rats subjected to pilocarpine-induced SE (Garzillo & Mello, 2002), pointing to an astrocytic response to neuronal injury. This astrocytic response includes morphological alterations, proliferation, gene expression, glial fibrilary acidic protein (GFAP) over expression, and S100B protein (S100B) secretion (Steinhoff et al., 1999; Jankowsky & Patterson, 2001).

Moreover, SE induced early in life can result in a long-term cognitive impairment. Flurothyl-induced SE in rats 10 days old (P10) causes memory deficits in the Morris water maze at P82–85 (Huang et al., 1999). Cilio et al. (2003) showed that LiCl-pilocarpine-induced SE on either P16 or P20, but not on P12, induces cell loss and mossy fiber sprouting within the hippocampus as well as cognitive impairment on P55. More recently, Kubova et al. (2004) showed that rats with LiCl-pilocarpine-induced SE on P25 also display memory impairment in the water maze and higher anxiety levels in the plus maze 3 months after the SE induction. Moreover, animals with kainic acid-induced SE on P1, P7, P14, and P24 had impaired long-term memory in the water maze and higher anxiety in the plus maze at P90–100 (Sayin et al., 2004).

Therefore, the aim of this study was to investigate whether the behavioral changes observed in adult rats submitted to early-life LiCl-pilocarpine-induced SE, are associated with neuronal loss, hippocampal mossy fiber sprouting and reactive astrogliosis.

METHODS

Materials

Polyclonal anti-S100 and anti-GFAP antibodies were purchased from Dako, Carpinteria, CA, U.S.A. Monoclonal anti-S100B (SH-B1) antibody was purchased from Sigma (St. Louis, MO, U.S.A.) The standard human GFAP protein was purchased from Calbiochem (San Diego, CA, U.S.A.) Lithium chloride and other chemicals for Neo-Timm staining were purchased from Nuclear, Brazil (Diademg, São Paulo). Pilocarpine hydrochloride was purchased from Sigma-RBI (St. Louis, MO, U.S.A.), and Fluoro-Jade B was purchased from Chemicon-Millipore (Billerica, MA, U.S.A.).

Animals

Thirty-four male Wistar rats were used. The day of birth was defined as day 0 and the animals were weaned on postnatal day 21 (P21). The litters were culled to eight pups. Rats were housed under a controlled environment (temperature of 21 ± 1°C, standard light/dark cycle of 12 h) with food and water ad libitum. The handling and care of the animals were conducted according to the guidelines of the Guide for the Care and Use of Laboratory Animals, Brazilian School of Animal Research. All procedures in the present study were approved by the Committee of Ethics from the Universidade Federal do Rio Grande do Sul.

Induction of status epilepticus

Rat pups 15 days old (P15) were injected i.p. with solution of LiCl (3 mEq/kg, Nuclear) 12–18 h prior to s.c. pilocarpine hydrochloride administration (60 mg/kg, Sigma-RBI) (Hirsch et al., 1992; Sankar et al., 1998). Control animals were handled and housed in the same manner as the treated animals and received an equal volume of saline solution (0.9% NaCl). Rats were put in individual plastic cages at 34°C (nest temperature) for seizure observation. The duration of status epilepticus was evaluated only by behavioral measures. The rats were allowed to spontaneously recover from SE. Each experimental group contained pups from several litters. To quantify spontaneous recurrent seizures (SRS), a video monitoring system was used for continuous behavioral observation of the animals in the following 55 days, for 23 h a day (Cavalheiro et al., 1991). This system allowed simultaneous video-monitoring of 8–12 subjects. The monitoring was stopped for periods ranging from 15 to 30 min for daily care. SRS were counted when the rats exhibited forelimb clonus with rearing and falling. The body weight was checked daily from P15 to P21, as well as on P45 and P70.

Behavioral procedures

For behavioral procedures, a group of 14 rats were equally distributed in control and treated animals. Behavioral sessions were carried out in an experimental room with constant temperature (21 ± 2°C) and light conditions (60-W light), except for the light-dark exploration task (see details below). Before the sessions, the animals were allowed to adapt to the experimental room for at least 1 h. All tasks were performed between 9:00 and 12:00 a.m. The open-field exploration, the inhibitory avoidance and

light-dark exploration tasks were carried out on P60, P65, and P70, respectively.

Open-field exploration

In order to verify whether animals present or not gross motor impairments, the rats (P60) were gently placed in the corner of a 40 × 50 × 60-cm box, the floor of which was divided into 3 × 4 squares, and left free to explore it for 2 min. The following parameters were quantified: (1) locomotor activity: the total number of squares crossed; (2) exploratory activity: the total number of rearings; and (3) the whole-body groomings. Latency for leaving the first square was also measured. After each trial, the apparatus was cleaned with an ethanol solution (70%).

Step-down inhibitory avoidance task

On P65, rats were gently placed on a 2.5-cm high, 7.0-cm wide, 25.0-cm long platform at the left side of a 50 × 25 × 25-cm apparatus, the floor of which contained a series of parallel 0.1-cm caliber stainless steel bars spaced 1.0 cm apart. In the training session, after the rats step down placing the four paws on the grid, they received a 3.0-s, 0.4-mA foot shock and were returned to the home cage. The test session was carried out 24 h after training session. Testing session was procedurally identical to the training session except that no foot shock was given and the step-down latency was cut off at 180 s, i.e., test session values higher than 180 s were counted as 180 s. The difference between test and training sessions latencies to step down was considered as a measurement of retention (avoidance memory). After each trial, the apparatus was cleaned with an ethanol solution (70%).

Light-dark exploration task

The task was performed in a 40 × 50 × 60-cm box divided equally into two compartments, connected by a small opening (10.0 cm × 7.5 cm). The light compartment was illuminated under a 60-W light. The dark compartment received only part of the room illumination (at 20 W). The floor of each compartment was divided into 3 × 2 squares. Rodents are nocturnal animals preferring darker environments, and the decrease in the exploratory activity in the light area is considered an index of anxiety. On P70, the animals were gently placed in the corner of the light compartment and left free to explore for 5 min. The following parameters were scored: (1) the frequency of crossings and rearings in the light or in the dark compartment; (2) the number of entries into the light compartment (putting the four paws inside); (3) latency for the first entry into the dark compartment; and (4) the total time spent in the light compartment. In addition, the risk assessment behavior index/min (RA, i.e., the number of exploration of the light compartment by placing some but not all paws) was also recorded. After each trial, the apparatus was cleaned with an ethanol solution 70%.

Brain tissue, cerebrospinal fluid (CSF), and serum sampling

Samples were collected from young and adult rats. Twenty-four hours after the SE induction (P17; 5 controls and 5 treated rats) or after completion of the last behavioral task (P70; 7 controls and 7 treated rats), rats were deeply anesthetized by an i.p. injection of ketamine (90 mg/kg) and xylazine (12 mg/kg) and positioned in a stereotaxic apparatus. CSF was obtained by cisterna magna puncture using a 0.33-mm diameter needle and a maximum volume of 30 μL was collected to minimize the risk of brainstem damage. CSF samples were frozen (–20°C) for S100B measurement. Cardiac blood samples were drawn and approximately 1 ml was allowed to coagulate at room temperature and then centrifuged at 3000 rpm. The serum was collected and frozen (–20°C) for S100B assay. The animals were then decapitated and the brain removed. Transverse hippocampal slices (0.4 mm) taken from one of the hemispheres were prepared with a McIlwain tissue chopper. Micro slices (1 mm in diameter) were obtained from hippocampal subfields CA1, CA3, and dentate gyrus using a stainless steel punch. The brain microslices were homogenized in 0.1 M sodium phosphate buffer, pH 7.4, containing 1 mM EGTA and 1 mM PMSF and stored at −20°C for S100B and GFAP measurement.

S100B measurement

The S100B immunocontent was determined in hippocampal microslices, CSF, and serum samples. ELISA for S100B was carried out as previously described (Tramontina et al., 2000), with modifications. Briefly, 50 μL of samples plus 50 μL of 1.5 mM Tris-HCl buffer, pH 8.8, were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B (SH-B1, from Sigma). Polyclonal anti-S100 (from DAKO) was incubated for 30 min and then peroxidase-conjugated antirabbit antibody was added for a further 30 min. The color reaction with o-phenylenediamine was measured at 492 nm. A standard curve of S100B ranged from 0.025 to 2.5 ng/ml.

GFAP measurement

The GFAP immunocontent was determined in hippocampal microslices (Ziegler et al., 2004). ELISA for GFAP was carried out by coating the microtiter plate with 100 μL samples, containing 20 ng of protein, for 24 h at 4°C. Incubation with a polyclonal anti-GFAP from rabbit for 1 h was followed by incubation with a secondary antibody conjugated with peroxidase for 1 h, at room temperature. A colorimetric reaction with o-phenylenediamine was measured at 492 nm. The standard human GFAP (from Calbiochem) curve ranged from 0.1 to 5 ng/ml.

Fluoro–Jade B staining

The Fluoro–Jade B staining was performed as described by Schmued et al. (1997). Briefly, 24 h after the SE induction, another set of rats (5 control and 5 treated rats),

that were not used for GFAP and S100B measurement, were deeply anesthetized by i.p. injection of ketamine (90 mg/kg) and xylazine (12 mg/kg) and sequentially perfused through the heart with 200 ml of ice-cold 0.1 M sodium phosphate buffer, pH 7.4, followed by ice-cold fixative solution, 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The brains were removed and immersed in fixative solution plus 30% sucrose until the brains sank to the bottom of the chamber. Frozen 30-μm coronal sections were mounted onto gelatin-coated slides and dried at 37°C overnight. They were then immersed in absolute alcohol for 3 min, followed by 70% ethanol for 2 min, and distilled water for 2 min. The slides were transferred to 0.06% potassium permanganate for 15 min. After rinsing with distilled water for 2 min, the slides were incubated for 30 min in 0.001% Fluoro–Jade B solution (Chemicon, Inc.) made in 0.1% acetic acid, rinsed in water, dried at 37°C, dehydrated in xylene, and cover slipped. Sections were examined using a Nikon Eclipse TE300 fluorescent microscope (I3 filter cube for FITC, excitation band 450–490 nm) for presence of Fluoro–Jade B-labeled fluorescent neurons.

Neo-Timm staining

The mossy fiber sprouting was evaluated in the dentate gyrus and CA3 subfield. The other hemisphere (that was not used for GFAP and S100B measurement) was immersed in 100 ml sodium sulfide fixative 0.1% in 0.12 M Millonig's buffer (97 mM NaOH, 138 mM NaH2PO4, 0.18 mM CaCl2) for 5–8 h and then changed to 100 ml of glutaraldehyde 3% plus 30% sucrose until the brains sank to the bottom of the chamber. Frozen 30-μm coronal sections corresponding to Swanson's plates 28–38 [interval between 2.6 and 5.6 mm from bregma (Swanson, 1992)] were collected, comprising a total sectioned extent of 3 mm per animal. The processing solutions consisted of 240 ml of 50% gum arabic with 10.25 g of citric acid, 9.45 g sodium citrate in 30 ml of H_2O, 3.73 g hydroquinone in 60 ml of H_2O, and 2 ml solution of 0.51 g silver nitrate in 3 ml H_2O. The developing time was 40–50 min for heavy staining. The slides were washed twice in distilled water for 5 min, dehydrated through alcohol to xylene, and coverslipped with Canada balsam. Timm staining was analyzed in a semiquantitative scale for terminal sprouting in CA3 and supragranular region. The scales used for visual analysis are given in Table 1. This scoring system is an adaptation of the scale used by Tauck and Nadler (1985). Timm staining in the pyramidal and infrapyramidal CA3 regions and supragranular region was assessed on each section from the septal area, where the two blades of the dentate were equal and formed a "V" shape (2.8-mm posterior from the bregma) to a point approximately 3.8-mm posterior to the bregma (Paxinos & Watson, 1986). Assessment of the Timm score in the supragranular region was done in the inferior blade of the dentate, avoiding the edge and crest of the gyrus. Each plate was analyzed by two independent observers who were blind to the experimental conditions.

Statistics

Data from body weight, GFAP and S100B immunocontent and from the open-field exploration are expressed as mean ± SD. Body weight data were analyzed by the repeated-measure analysis of variance (ANOVA) followed by the Tukey's post hoc test for unequal samples. Data from the open-field exploration and GFAP and S100B immunocontent were analyzed by the independent-sample *t*-test.

Since controls were not normally distributed (Kolmogorov—Smirnov goodness-of-fit test, $p < 0.05$), nonparametric statistical analyses were used in the inhibitory avoidance and in the light-dark exploration tasks. Data are expressed as median (interquartile range). In the inhibitory avoidance task, the Wilcoxon signed ranks test was used to compare the performance between the training and test sessions in each group and the Mann-Whitney *U*-test was used to compare the training performance between groups. Data from light-dark exploration task were analyzed by the Mann-Whitney *U*-test.

The intensity of sprouting in the hippocampus is expressed as the percentage of animals presenting Timm scores of 0, 1, 2, or 3 and was analyzed by the Mann-Whitney *U*-test. The correlation between the Timm's score for dentate gyrus and other behavioral parameters was analyzed by the Pearson's correlation coefficient.

For all parameters, $p < 0.05$ was considered significant.

Results

Status epilepticus

The behavioral pattern of SE correlated well with the description by Hirsch et al. (1992), lasting up to 3–4 h. Systemic administration of LiCl-Pilocarpine produced defecation, salivation, body tremor, and scratching within 5 to 20 min. This behavioral pattern progressed within 30 to 45 min to increased levels of motor activity and culminated in SE in all animals. SE was characterized by sustained orofacial automatisms, salivation, chewing, forelimb clonus, loss of the righting reflex, and falling. The mortality rate 24 h after the status epilepticus induction was 10%. The LiCl-pilocarpine group did not develop spontaneous seizures at adulthood.

Body weight

There was a significant difference in the interaction group x day [$F(8,104) = 3.10$; $p = 0.0036$] in the body weight (Fig. 1). The LiCl-pilocarpine group showed a lower weight from P17 until P45 ($p < 0.01$); this difference disappeared on P70 ($p = 0.10$).

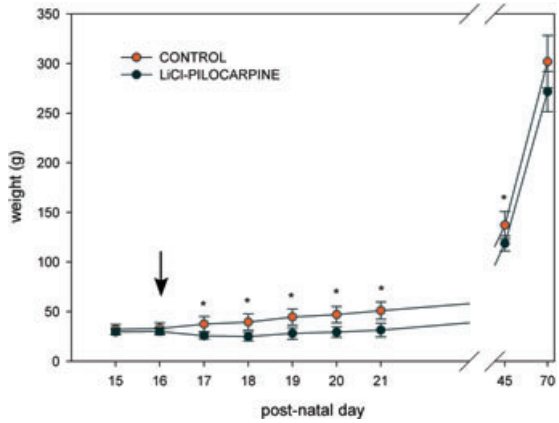

Figure 1.
Body weight mean ± SD of control and LiCl-pilocarpine-induced SE groups. The arrow indicates the time of SE induction. An asterisk (∗) indicates a difference between groups ($p < 0.05$ by Tukey's post hoc test for unequal samples).
Epilepsia © ILAE

Degeneration of hippocampal neurons

In the control group, a small number of Fluoro Jade B-positive neurons were found in CA1 subfield and in the hilus. However, in the LiCl-pilocarpine group, an increased number of labeled neurons were found in the stratum pyramidale (S. PYR) from CA1 subfield and in the hilus (Fig. 2).

Mossy fiber synaptic reorganization

All control animals had a mossy fiber sprouting score of 0 or 1 (71.4% and 28.6%, respectively). The mossy fiber sprouting pattern in rats subjected to LiCl-pilocarpine-induced SE differed from control rats ($Z < -3.044$, $p < 0.05$; Fig. 3). LiCl-pilocarpine-treated animals presented the following scores for mossy fiber sprouting: score 1 (21.3% of animals), score 2 (21.5%), and score 3 (57.2%) in dentate gyrus; score 1 (7.1% of animals), score 2 (35.7%), and score 3 (57.2%) in CA3 region.

Open-field exploration

There was no difference between control and LiCl-pilocarpine-induced SE groups regarding the total number of squares crossed, the total number of rearings, the whole-body groomings and the time for leaving the first square, as shown in Table 2.

Step-down inhibitory avoidance task

The latency to the step down from the platform was significantly higher in the test session compared with the training session in the control group ($Z = -2.366$; $p = 0.018$) but not in the LiCl-pilocarpine group ($Z = -1.270$; $p = 0.204$) (Fig. 4). No difference between groups was found in the training session ($Z = -1.415$; $p = 0.165$).

The animals that presented lower latency to the step down showed a higher score for mossy fiber sprouting ($r = -0.995$; $p < 0.001$; Pearson's correlation coefficient), i.e., there was a negative correlation between the latency to the step down and the score for mossy fiber sprouting in the LiCl-pilocarpine-treated animals (insert Fig. 4). There was no correlation between the score for mossy fiber sprouting and the latency to the step down in the control animals (insert Fig. 4; $r = -0.700$; $p = 0.08$).

Light-dark exploration task

Compared with control rats, LiCl-pilocarpine-induced SE rats returned less often to the light compartment ($Z = -2.568$; $p = 0.011$; Fig. 5A) and spent less time ($Z = -2.118$; $p = 0.036$; Fig. 5B) in the light compartment. Moreover, the number of RA was higher in the LiCl-pilocarpine group ($Z = -2.738$; $p = 0.006$; Fig. 5C). The frequency of crossings was significantly lower in the LiCl-pilocarpine group only in the light compartment ($Z = -2.117$; $p = 0.036$; Fig. 5D). There was no difference between groups in the frequency of rearings in both compartments (light: $Z = -0.724$; $p = 0.481$; dark: $Z = -1.443$; $p = 0.167$; Fig. 5E). The latency for the first entry into the dark compartment was not different between groups ($Z = -0.145$; $p = 0.888$; Fig. 5F).

There was a negative correlation between the time spent in the light compartment and the score for mossy fiber sprouting in the LiCl-pilocarpine-induced SE ($r = -0.930$; $p = 0.001$; insert Fig. 5B) and the animals that showed a higher score for mossy fiber sprouting also presented a higher number of RA ($r = -0.930$; $p = 0.001$; insert Fig. 5C). However, there was no correlation between the score for mossy fiber sprouting and the number of entries into the light compartment in the LiCl-pilocarpine group ($r = -0.127$; $p = 0.764$; insert Fig. 5A). There was no correlation between the score for mossy fiber sprouting and the above-mentioned parameters in the control group.

GFAP and S100B immunocontent

There was no difference in GFAP and S100B immunocontent between the control and LiCl-pilocarpine-induced SE groups in the hippocampal subfields from both young (Figs 6A and C) and adult rats (Figs 6B and D). However, there was a significant positive correlation between the score for mossy fiber sprouting and the GFAP immunocontent in the CA1 subfield in the LiCl-pilocarpine-treated animals in adulthood ($r = -0.995$; $p < 0.001$; insert Fig. 6B). Moreover, LiCl-pilocarpine-treated animals showed persistent elevated levels of CSF S100B in adulthood ($t = -4.593$; $p = 0.01$; Fig. 6F).

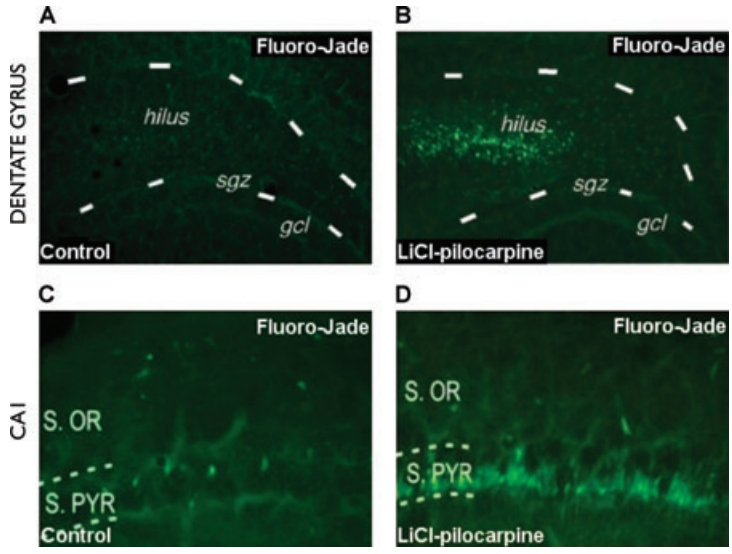

Figure 2.
Distribution of Fluoro Jade B-labeled degenerating neurons in dentate gyrus (**A–B**) and CA1 subfield (**C–D**) 24 h after SE induction. Note more damaged neurons in the dentate hilus after LiCl-pilocarpine-induced SE (**B**) as compared with control (**A**). There were a markedly increased number of Fluoro Jade B-labeled neurons in the stratum pyramidale (S. PYR) from CA1 subfield in the LiCl-pilocarpine group (**D**) when compared with control group (**C**). Abbreviations: sgz, subgranular zone; gcl, granule cell layer.
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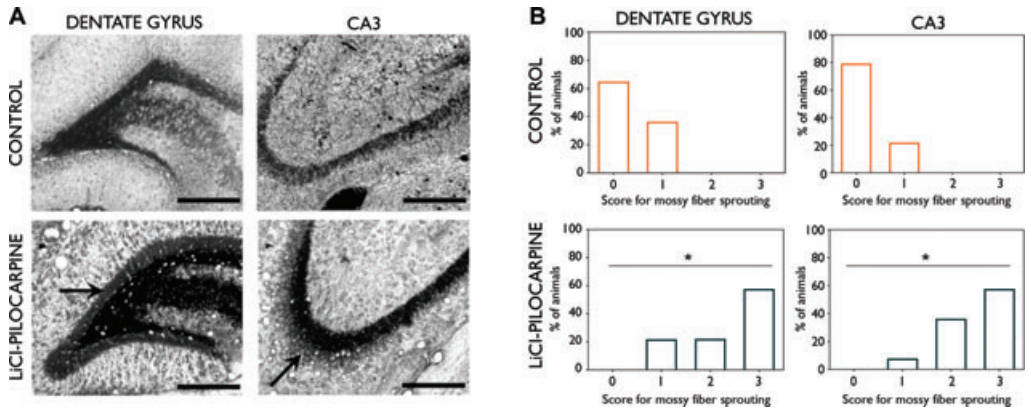

Figure 3.
(A) Mossy fiber sprouting in dentate gyrus and CA3 subfield in control and LiCl-pilocarpine groups. The Neo-Timm staining was performed on P70 in the other hemisphere that was not used for GFAP and S100B measurement. **(B)** The graphs show the percentage of animals presenting scores for mossy fiber sprouting of 0, 1, 2, or 3 in control and LiCl-pilocarpine groups. Assessment of mossy fiber sprouting in the supragranular layer was evaluated in the blade of the dentate, avoiding the edge and the crest of the gyrus. Mossy fiber sprouting in the CA3 region was assessed in the pyramidal cell region. Neo-Timm staining in both CA3 and dentate gyrus was analyzed by using a subjective gradation score propose by Tauck and Nadler (1985) which varies from zero (no staining) to three (intense staining), by two independent observers who were blind to the experimental conditions. The Timm-staining pattern in the CA3 and dentate gyrus (arrows) hippocampal subfields in rats subjected to LiCl-pilocarpine-induced SE differed from that in control rats ($Z = -3.044$; $p = 0.04$ by Mann-Whitney *U*-test). An asterisk ($*$) indicates a difference between control and LiCl-pilocarpine groups. Scale bars = 100 μm.
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Table 1. Scoring system for mossy fiber sprouting

CA3		Dentate gyrus	
Score	Criteria	Score	Criteria
0	No granules in the stratum pyramidal and stratum oriens along any portion of the CA3	0	No granules noted between crest and tips in the supragranular region
1	Occasional granules in the stratum pyramidal and stratum oriens occurring in discrete bundles	1	Occasional granules in the supragranular region occurring in patchy distribution
2	Prominent granules in the stratum pyramidal and stratum oriens	2	Granules in the supragranular region occurring near-continuous distribution
3	Continuous dense laminar band of granules in the stratum pyramidal and stratum oriens along the entire CA3	3	Continuous dense laminar band of granules from the crest to tip of the dentate

Discussion

Pediatric neurologists have repeatedly described neuronal damage with subsequent mental impairment and development of epilepsy after SE in children. In the present study, we evaluated the effects of LiCl-pilocarpine-induced SE early in life on memory and anxiety, and whether these effects are associated with hippocampal neuronal degeneration, mossy fiber sprouting, and reactive astrogliosis in adulthood.

The behavioral duration of the SE was 3–4 h and this pattern correlated well with the description of Hirsch et al. (1992). As described by these authors none LiCl-pilocarpine-treated animal showed behavioral manifestations of SE without electrographic manifestations of SE (established by simultaneous behavioral observation and electroencephalographic recordings); however, it is possible that EEG seizures might have likely occurred still after the cessation of the observed behavioral signs. Therefore the time spent in SE in our work may be underestimated.

SE produced expressive neuronal damage in specific hippocampal areas 24 h after SE induction (Fig. 2). Although previous studies demonstrated that the developing brain may be relatively resistant to seizure-induced morphological alterations when compared with the mature brain (Haas et al., 2001), recent studies have been indicating that SE induced early in life may cause neuronal death. Sankar et al. (1997) showed that rat pups (1–4 weeks of age) submitted to a LiCl-pilocapine-induced SE had a higher serum levels of s-NSE compared to controls animals and this increase was accompanied by neuronal damage in specific brain areas such as cortex, thalamus, amygdala, septum, and hippocampus. The hippocampal damage seen at this age appears to involve both necrosis, as evidenced by the eosinophilic cells, and apoptosis as demonstrated by various techniques that included TUNEL staining, visualization of fragmented nuclei under fluorescence microscopy of ethidium bromide-stained sections, and electron microscopy (Sankar et al., 1998). Hamon and Heinemann (1988) had shown that during the second postnatal week, the apical dendrites of pyramidal neurons

Table 2. Mean + SD of crossings, rearings, and time for leaving the first square in the open field

Group	N	Number of crossings	Number of rearings	Time for leaving the first square
Control	7	43.89 ± 13.95	9.67 ± 4.09	3.72 ± 1.89
LiCl-pilocarpine	7	35.00 ± 18.99	10.67 ± 7.84	4.45 ± 2.41
P		0.311	0.761	0.224

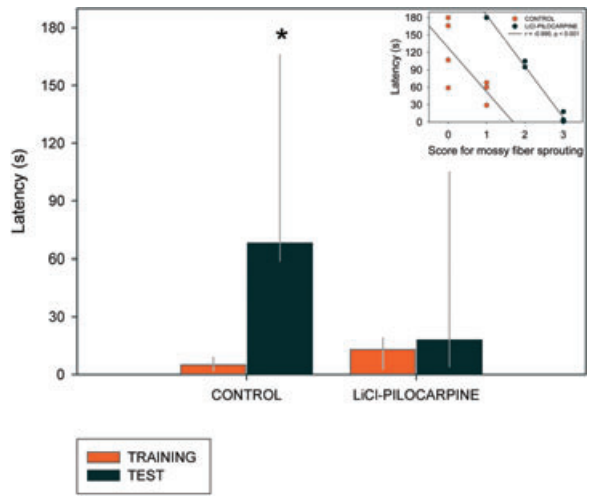

Figure 4.
Median (interquartile range) of step-down latencies in training and test sessions of step-down inhibitory avoidance task. The task was performed on P65. An asterisk (∗) indicates a difference between training and test sessions ($Z = -2.366$; $p = 0.018$; Wilcoxon signed ranks test). ***Inset:*** Correlation between the score for mossy fiber sprouting in the dentate gyrus and latency to the step down from the platform in control [medium dash (——) line; $r = -0.700$; $p = 0.08$] and LiCl-pilocarpine-induced SE [solid dash (—) line; $r = -0.995$; $p < 0.001$] groups.
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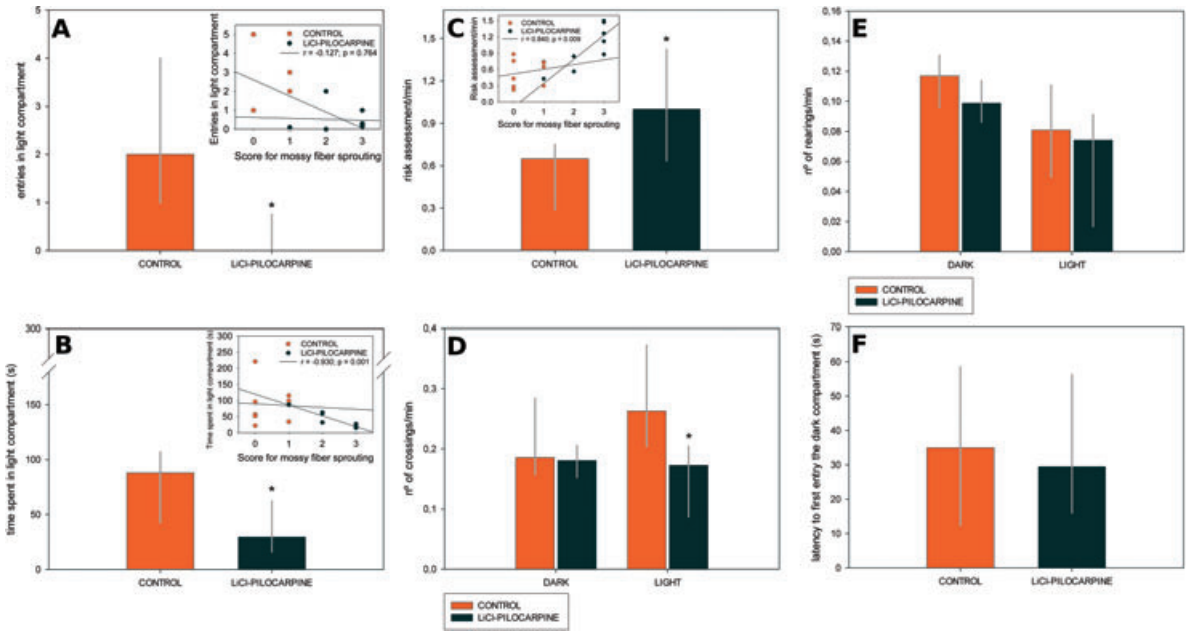

Figure 5.
Light-dark exploration task. Data show median (interquartile range) of the: **(A)** the number of entries into the light compartment and **(B)** the total time spent in the light compartment; **(C)** the risk assessment behavior index/min (see Material and Methods); **(D)** the frequency of crossings and **(E)** rearings either in the light or in the dark compartment; **(F)** the latency for the first entry into the dark compartment. The light-dark exploration task was performed on P70. As asterisk (∗) indicates $p < 0.05$ as compared with the control group (Mann–Whitney *U*-test). ***Insets:*** Correlation between the score for mossy fiber sprouting in the dentate gyrus and light-dark exploration task parameters in control [medium dash (——) line] and LiCl-pilocarpine [solid dash (—) line] groups.

in area CA1 become more sensitive to NMDA, which is expressed by big influxes of calcium at this level. Harris and Teyler (1984) showed that long-term potentiation in area CA1 of the rat was maximal at P15. Excitability comparable to that seen in fully mature animals could be demonstrated in the CA1 region after postnatal day 14, whereas the inhibitory processes did not reach an adult stage of maturation until several weeks later (Michelson & Lothman, 1989). Our observation of preferential CA1 damage at P16 is consistent with the observations of the development of synaptic inhibition lagging in CA1 compared with other hippocampal areas (Swann et al., 1989).

In addition to neuronal damage, we found that animals with a history of SE had significant memory deficits in an inhibitory avoidance task, which is a task widely used to evaluate behavioral tasks that depend on the integrity of the dorsal hippocampus (Izquierdo et al., 2006). These findings indicate that early-life prolonged seizures can cause long-standing cognitive impairment; however, the cellular and molecular mechanisms involved it is unclear. It is possible that the sprouting of mossy fibers following the prolonged seizures (Fig. 3) alters learning and memory, whereas we showed a significantly negative correlation between the score for mossy fiber sprouting and the latency to step down from the platform in the LiCl-pilocarpine-treated animals (insert Fig. 4). Lipp et al. (1988) compared the number of trials for rats to learn to avoid a 10-s electrical shock by moving from one compartment to another following a conditioning stimuli (two-way avoidance learning) with magnitude of the stratum pyramidale projections of mossy fibers. Learning was directly related to extent of mossy fiber projections to the intra- and infrapyramidal layers of CA3, with animals having more CA3 mossy fiber terminals doing less well than animals with fewer terminals. Furthermore, Wimer et al. (1983) noted a negative correlation between granule cell density in the dentate gyrus and two-way avoidance conditioning in the mouse. However, the relationship between the size of the hippocampal mossy fiber projections and learning and memory may be task dependent (Crusio et al., 1987, 1993). Neill et al. (1996) found that rats with recurrent seizures during the first weeks of life were impaired in the water maze and in an auditory location learning task, but not in a quality discrimination task. While it is tempting to speculate that the altered connectivity as demonstrated

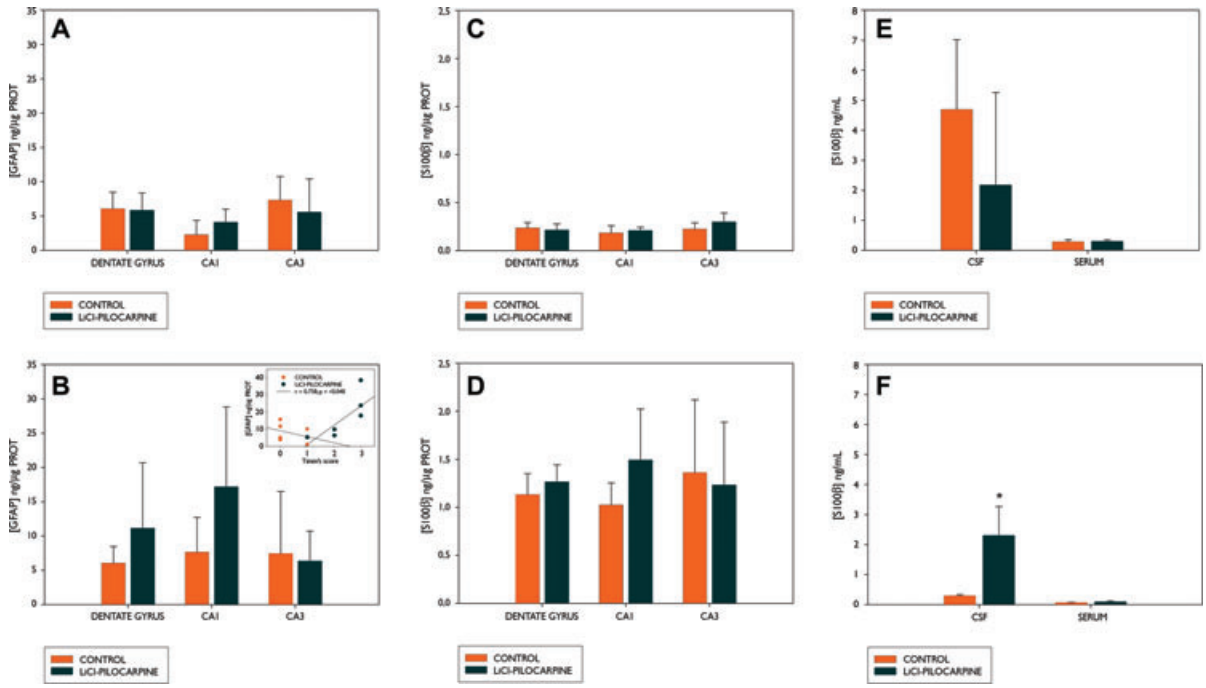

Figure 6.
GFAP and S100B immunocontent of control and LiCl-pilocarpine-induced SE groups. The samples were collected in young (24 h after the SE induction; figures **A**, **C,** and **E**) and adult rats (after finishing the light-dark exploration task; figures **B**, **D**, and **F**). An asterisk (∗) indicates $p < 0.001$ as compared with the control group (Student's *t*-test). ***Inset* Figure *6B*:** Correlation between the score for mossy fiber sprouting and the GFAP immunocontent in the CA1 subfield in the LiCl-pilocarpine-treated animals [solid dash (—) line; $r = 0.758$; $p < 0.048$].
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with the Timm stains provides the physiological substrate for learning impairments, we have no direct proof that this hypothesis is correct. Future studies will need to address the pathophysiological basis for these changes in cognition.

Another long-lasting consequence observed in rats submitted to early-life LiCl-pilocarpine-induced SE is an increase in anxiety-like behavior (Fig. 5). Experimental studies have reported that SE at an early age can induce permanent abnormalities in emotional behavior. Kubova et al. (2004) showed that rats with LiCl-pilocarpine-induced SE on P25, but not on P12, displayed higher levels of anxiety in the plus maze 3 months after the SE induction. Animals with kainic acid-induced SE on P1, P7, P14, and P24 showed a greater degree of anxiety in the plus maze at P90–100 (Sayin et al., 2004). It is possible that these elevated anxiety levels observed in LiCl-pilocarpine-treated animals are associated with CA3 and dentate gyrus aberrant mossy fiber sprouting. This is supported by (1) a negative correlation between the time spent in the light compartment and the score for mossy fiber sprouting (insert Fig. 5B) as well as by (2) a positive correlation between the number of risk assessment behavior and score for mossy fiber sprouting (insert Fig. 5C). Recent studies have suggested that the ventral hippocampus may have an important role in brain processes associated with anxiety-related behaviors. The ventral subregion projects to the prefrontal cortex and is closely connected to the bed nucleus of the stria terminalis and the amygdala, as well as other subcortical structures which are associated with the hypothalamic-pituitary-adrenal axis. Most of the amygdalar nuclei have some reciprocal projections with the hippocampal formation, although this is most pronounced for the basal and lateral nuclei (Bannerman et al., 2004). The strong connectivity between ventral hippocampus and both the hypothalamus and the amygdala, makes it tempting to propose a role for the ventral subregion in fear and/or anxiety, and thus potentially account for some of the hippocampal lesion effects on emotionality.

The effects of LiCl-pilocarpine-induced SE seen in both the inhibitory avoidance task and the light-dark exploration task were not due to gross motor alterations or to a difference in body weight, since there was no difference in the number of crossings in the open field and in the dark compartment in the light-dark exploration task as well as in the body weight on P70.

SE did not alter the immunocontent of GFAP in all hippocampal subfields from both P16 and P65 rats (Figs 6A–D). However, there was a positive correlation between the score for mossy fiber sprouting and the GFAP immunocontent in the CA1 subfield in the LiCl-pilocarpine-treated animals in adulthood (insert Fig. 6D). The reactive astrogliosis only in LiCl-pilocarpine-treated animals may be related with the massive neuronal degeneration observed by Fluoro–Jade B staining.

LiCl-pilocarpine-treated animals showed also higher levels of S100B in the CSF in adulthood (Fig. 6F) and this alteration was not accompanied by any change in serum S100B. Serum S100B levels appear to present a distinct pattern when compared with CSF S100B levels. In fact, serum and brain S100B levels are poorly correlated, with serum levels dependent primarily on the integrity of the blood–brain barrier, and not the level of S100B in the brain (Kleindienst & Ross Bullock, 2006). There are some previous studies pointing that S100B levels in CSF present an early increase after brain injury in rats (Oses et al., 2004; Busnello et al., 2006). In addition, an increase in S100B level was detected in CSF in patients with temporal lobe epilepsy (Steinhoff et al., 1999). Although the meaning of these level variations is not clear (if it presents beneficial or detrimental effects), CSF S100B levels are used as a marker of astrocytic activity alteration, probably as a consequence to a neuronal injury (Donato, 2001; Van Eldik & Wainwright, 2003).

In conclusion, we showed that LiCl-pilocarpine-induced SE during development produced long-lasting behavioral abnormalities. These effects might be associated with the mossy fiber sprouting since the animals that presented impairment in the performance of inhibitory avoidance task and higher levels of anxiety in adulthood showed higher scores for mossy fiber sprouting in the hippocampus. Moreover, LiCl-pilocarpine-treated animals showed higher levels of S100B immunocontent in the CSF as well as a positive correlation between the score for mossy fiber sprouting and the GFAP immunocontent in the CA1 subfield, suggesting an astrocytic response to neuronal injury. These data indicate that LiCl-pilocarpine-induced SE early in life might harmfully affect brain maturation, leading to behavioral, morphological, and neurochemical alterations in adulthood.

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Conflicts of interest: The authors have no conflicts of interest.

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CAPÍTULO II

Early life LiCl-pilocarpine-induced status epilepticus reduces hippocampal glutamate uptake and Na^+/K^+ ATPase activity.

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Running title

Effect of status epilepticus on glutamate uptake and Na^+/K^+ ATPase.

Key Words

Development; glutamate uptake; Na+ K+ ATPase; status epilepticus.

Abstract

Prolonged seizure activity, i.e. status epilepticus, or repeated, brief seizures affect neuronal structure and function in the developing nervous system. The status epilepticus-induced neuronal loss is associated with excitotoxicity induced by increased levels of extracellular glutamate which is normally neutralized by high-affinity uptake mechanism. The energy source for the glutamate uptake is the electrochemical Na^+ gradient maintained by Na^+/K^+ ATPase. In this study, we demonstrated that the early-life LiCl-pilocarpine-induced SE decreases hippocampal Na^+/K^+ ATPase activity and glutamate uptake. Na^+/K^+ ATPase activity and glutamate uptake was determined 1.5, 12 and 24 h after the SE induction. At physiologic concentration of glutamate (1 μM), LiCl-pilocarpine treated animals showed decreased levels of glutamate uptake 1.5 h after SE induction. However, LiCl-pilocarpine-induced SE did not alter the glutamate uptake 12 and 24 h after SE induction. At a higher concentration of extracellular glutamate (100 μM), there was no alterations in glutamate uptake between control and treated groups in all times tested. LiCl-pilocarpine-induced SE decreased Na^+/K^+ ATPase activity by 42% in hippocampal plasma membranes 1.5 h after SE induction when compared with control group. However, at 12 and 24 h after SE induction the activity of pump returned to control levels. Moreover, LiCl-pilocarpine-induced SE early in life significantly increased hippocampal thiobarbituric acid-reactive substances production 24 h after SE induction. These alterations were followed by an increased number of degenerating neurons in the CA1 subfield 24 h after SE induction. In conclusion, in this study we showed that LiCl-pilocarpine-induced SE during development decreases Na^+/K^+ ATPase activity and glutamate uptake in hippocampus 1.5 h after SE onset. These effects might be associated with the elevation of lipid peroxidation levels and neuronal damage found in the stratum pyramidale from hippocampal CA1 subfield 24 h later. These alterations may lead to behavioral, morphological and neurochemical alterations in adulthood.

1. Introduction

Epilepsy is a common neurological disorder that occurs more frequently in children than in adults. Prolonged seizure activity, i.e. status epilepticus (SE), or repeated, brief seizures affect neuronal structure and function in the developing nervous system which lead to short-term brain damage and long-term behavioral impairments (Holopainen, 2008). One of the earliest brain consequences of SE early in life is the selective neuronal loss observed in specific brain areas. Rats with LiCl–pilocarpine-induced SE during the second week of life presented damage in the hippocampus, amygdala, thalamus, and septum as well as an elevation in the serum levels of neuron specific enolase (NSE) 24 h after SE induction (Sankar et al., 1997). Further analysis of hippocampal CA1 pyramidal cells showed DNA fragmentation (TUNEL analysis) and apoptotic bodies in electron micrographs of damaged neurons (Sankar et al., 1998). The SE-induced neuronal loss is associated with excitotoxicity induced by increased levels of extracellular glutamate and excessive activation of ionotropic glutamate receptors whereas systemic administration of ionotropic glutamate receptors antagonists protects against SE-induced neuronal damage (Clifford et al., 1990; Fujikawa, 1995).

The excess of extracellular glutamate is normally neutralized by high-affinity uptake mechanism executed by a family of glutamate transporter proteins: GLAST/EAAT1, GLT1/EAAT2, EAAC1/EAAT3, EAAT4 and EAAT5 (Gether et al., 2006). Studies in humans and in transgenic mice indicate that alterations in glutamate transporters can lead to epileptic phenotypes. ‘‘Knock-out’’ mice lacking astroglial transporters exhibit lethal spontaneous seizures (Tanaka et al., 1997). Intracerebroventricular administration of antisense oligonucleotide to EAAC1 produced epilepsy, characterized initially by facial twitches and freezing behavior that began 3-5 days of treatment. By 7 days, the animals showed tonic forepaws extension and clonic seizures (Rothstein et al., 1996).

The energy source for the glutamate uptake is the electrochemical Na+ gradient maintained by Na^+/K^+ ATPase (adenosinetriphosphatase – EC 3.6.1.3) (Danbolt, 2001). Na^+/K^+ ATPase is a plasma membrane-embedded enzyme responsible for the active transport of sodium and potassium ions in most animal cells. It is present in higher concentrations in the brain and other nervous tissue, where it plays several roles in the complex and finely tuned control of the ionic environment which underlies nerve activity. It maintains the ion gradient of sodium and potassium which are the energy source for the nerve impulse. It is highly concentrated in glial cells, which are involved in the uptake and clearance of extracellular potassium after a volley of nerve activity (Sweadner, 1992). It is an electrogenic pump, transporting 3Na+ ions for every 2K+ ions, and its activity can hyperpolarize a nerve cell, modulating the fling threshold for nerve impulse initiation (Erecinska and Silver, 1994). Due to its high importance in the maintenance of resting membrane potential and the propagation of neuronal impulse, the malfunction of this enzyme has been associated with the neuronal hyperexcitability (Donaldson et al., 1971).

Reduced Na^+/K^+ ATPase activity has been found in cerebral tissue of epileptic patients (Rapport et al., 1975) as well as in the hippocampus of kainate-injured rats (Anderson et al., 1994), although higher enzyme activity levels were observed in pilocarpine-lesioned rats (Fernandes et al., 1996). In addition, ouabain, an irreversible inhibitor of the Na^+/K^+ ATPase pump, induces focal spikes discharges as well as contralateral focal seizures when applied locally on the cortical surface of rats (Lewin, 1971).

The aim of this study was to investigate the effect of early-life LiCl-pilocarpine-induced SE on hippocampal Na^+/K^+ ATPase activity and glutamate uptake.

2. Experimental procedures

2.1. Materials

L-[2, 3-3H] glutamate (specific activity 30 Ci/mmol) was purchased from Amersham International, UK. ATP was purchased from from Sigma Chemical Co., St. Louis, MO, USA. Pilocarpine hydrochloride was purchased from RBI, USA, and Fluoro-Jade B was purchased from Chemicon, Inc., USA. Lithium chloride and other chemicals for glutamate uptake were purchased from Nuclear, Brazil.

2.2. Animals

Ninety male Wistar rats were used. The day of birth was defined as day 0 and the litters were culled to 8 pups. Rats were housed under a controlled environment (temperature of 21±1°C, standard light/dark cycle of 12 h) with food and water *ad libitum*. The handling and care of the animals were conducted according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 80-23, revised 1978). All procedures in the present study were approved by the Committee of Ethics from the Universidade Federal do Rio Grande do Sul.

2.3. Induction of status epilepticus

Rat pups 15 days old (P15) were injected i.p. with solution of LiCl (3 mEq/kg – Nuclear, Brazil) 12-18h prior to s.c. pilocarpine hydrochloride administration (60 mg/kg) (Hirsch et al., 1992; Sankar et al., 1998). Control animals were handled and housed in the same manner as the treated animals and received an equal volume of saline solution (0.9% NaCl). Rats were put in individual plastic cages at 34°C (nest temperature) for seizure observation. The status epilepticus was evaluated only by behavioral observations. The rats were allowed to spontaneously recover from SE. Each experimental group contained pups from several litters.

2.4. Glutamate uptake

Glutamate uptake was performed according to a previous report with modifications

(de Oliveira et al., 2004). Animals were decapitated 1.5, 12 and 24 h after the SE induction and brains were immediately removed and humidified with Hank's balanced salt solution (HBSS) containing (mM): 137 NaCl, 0.63 Na2HPO4, 4.17 NaHCO3, 5.36 KCl, 0.44 KH2PO4, 1.26 CaCl2, 0.41 MgSO4, 0.49 MgCl2 and 1.11 glucose, pH 7.2, 35°C. Hippocampal slices (0.4 mm) were obtained using a McIIwain tissue chopper. For each animal, five hippocampal slices (3 for total and 2 for sodium-independent uptake) were transferred to 24-well dishes containing 0.3 mL of HBSS. For total uptake, the slices were pre-incubated at 35°C for 30 min. The uptake assay was assessed by adding 20 μL of a solution containing 0.33 μCi/mL L-[2, 3-3 H] glutamate with 1 μM or 100 μM unlabeled glutamate at 35°C. Incubation was stopped after 5 min by two washes with 1 mL ice-cold HBSS immediately followed by addition of 0.5N NaOH. Aliquots of lysates were taken for determination of intracellular content of L-[2, 3-3 H] glutamate by scintillation counting. Sodium-independent uptake was determined by using an ice-cold (4°C) HBSS containing N-methyl-D-glucamine instead of sodium chloride. The results were subtracted from the total uptake to obtain the sodium-dependent uptake. Protein content was measured following the method described by Peterson (1977).

2.5. Na^+/K^+ ATPase activity

For *in vitro* and *in vivo* assays, naïve and treated animals were decapitated and the brains rapidly removed. For in vivo assay, the animals were sacrificed by decapitation 1.5, 12 and 24 h after the SE induction. Hippocampi were isolated on ice and homogenized in 10 v/w of an ice-cold solution containing: 0.32 M sucrose, 5.0 mM HEPES and 0.1 mM EDTA (pH 7.4). Membranes were prepared according to the method of (Jones and Matus, 1974) with modifications (Wyse et al., 1998). All centrifugation steps were conducted at 4°C. The homogenate was centrifuged at 1000xg for 10 min, the resultant pellet was discarded and the supernatant was centrifuged at 12,000xg for 20 min. The pellet was resuspended in hypotonic buffer (5.0 mM Tris–HCl buffer, pH 8.1) at 0°C for 30 min, and gently transferred onto a

three-step discontinuous sucrose density gradient (2 mL each of 0.3, 0.8 and 1.0 M). Tubes were centrifuged at 69,000 g for 120 min. The turbid layer between 0.8 and 1.0 M sucrose was collected as membrane enzyme samples.

The samples were incubated for 10 min at 37° C in 40 mM Tris–HCl buffer (pH 7.4) containing: 5 mM MgCl2, 80 mM NaCl, 20 mM KCl and 3 mM ATP, in the presence or absence of 1 mM ouabain. Na+, K+ ATPase activity was calculated by the difference between the values obtained in the presence and absence of ouabain. Released inorganic phosphate (Pi) was determined by the method of (Chan et al., 1986). Specific activity of the enzyme was expressed as nmol Pi released per mg of protein per min. All samples were run in duplicate.

2.6. Thiobarbituric acid-reactive substances (TBA-RS)

The lipid peroxidation was assessed by thiobarbituric acid-reactive substances production according to method described by (Ohkawa et al., 1979). Another set of rats were treated for TBA-RS (5 control and 5 treated rats). Both hippocampi were homogenized in an ice-cold 1.15% KCl and mixed with a solution containing 20% trichloroacetic acid and 0.8% thiobarbituric acid. The samples were heated in a boiling water bath for 60 min. Thiobarbituric acid-reactive substances were determined by the absorbance at 535 nm and were expressed as malonaldehyde equivalents (nmol/mg protein).

2.7. Fluoro-Jade B staining

The Fluoro-Jade B staining was performed as described by (Schmued et al., 1997). Twenty four hours after the SE induction, another set of rats (5 control and 5 treated rats), that were not used for glutamate uptake and Na^+/K^+ ATPase measurements, were deeply anesthetized by i.p. injection of ketamine (90 mg/kg) and xylazine (12 mg/kg) and sequentially perfused through the heart with 200 mL of ice-cold 0.1 M sodium phosphate buffer, pH 7.4, followed by ice-cold fixative solution, 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The brains were removed and immersed in fixative solution plus 30% sucrose until the brains sank to the bottom of the chamber. Frozen 30-μm coronal

sections were mounted onto gelatin-coated slides and dried at 37° C overnight. They were then immersed in absolute alcohol for 3 min, followed by 70% ethanol for 2 min, and distilled water for 2 min. The slides were transferred to 0.06% potassium permanganate for 15 min. After rinsing with distilled water for 2 min, the slides were incubated for 30 min in 0.001% Fluoro-Jade B solution made in 0.1% acetic acid, rinsed in water, dried at 37° C, dehydrated in xylene, and cover slipped. Sections were examined using a Nikon Eclipse TE300 fluorescent microscope (I3 filter cube for FITC, excitation band 450–490 nm) for presence of Fluoro Jade B-labeled fluorescent neurons.

2.8. Statistical analysis

Data from glutamate uptake and Na^+/K^+ ATPase activity were expressed as mean $\pm$ SD and were analyzed by the one-way ANOVA followed by the Tukey´s *post hoc* test for unequal samples. For all parameters, $p < 0.05$ was considered significant.

3. RESULTS

3.1. Status epilepticus

The behavioral pattern of SE correlated well with the description by Hirsch *et al.* (1992), lasting up to 3-4 h. Systemic administration of LiCl-Pilocarpine produced defecation, salivation, body tremor, and scratching within 5 to 20 min. This behavioral pattern progressed within 30 to 45 min to increased levels of motor activity and culminated in SE in all animals. SE was characterized by sustained orofacial automatisms, salivation, chewing, forelimb clonus, loss of the righting reflex and falling. The mortality rate 24 h after the status epilepticus induction was 20%.

3.2. Na^+/K^+ ATPase activity

The hippocampal activities of Na^+/K^+ ATPase are shown in Fig. 1. LiCl-pilocarpine-induced SE decreased Na^+/K^+ ATPase activity by 42% in hippocampal plasma membranes 1,5h after SE induction when compared with control group. However, at 12 and 24 h after SE induction the activity of Na^+/K^+ ATPase returned to control levels. Application of 100 μM of pilocarpine in vitro did not alter the activity of Na^+/K^+ ATPase from hippocampal membranes (control: 1331.5 $\pm$ 314.2 nmol Pi/min.mg Prot; pilocarpine: 1339 $\pm$ 264.8 nmol Pi/min.mg Prot; data not show).

3.3. Thiobarbituric acid-reactive substances (TBA-RS)

LiCl-pilocarpine-induced SE early in life significantly increased hippocampal thiobarbituric acid-reactive substances production only 24 h after SE induction (Fig 1).

3.4. Glutamate uptake

At physiologic concentration of glutamate (1 μM), LiCl-pilocarpine treated animals showed decreased levels of glutamate uptake 1.5 h after SE induction (Fig. 2). However, LiCl-pilocarpine-induced SE did not alter the glutamate uptake 12 and 24 h after SE induction. At a higher concentration of extracellular glutamate (100 μM), no alterations were observed in glutamate uptake between control and LiCl-pilocarpine-induced SE groups in all

times tested.

3.5. Degeneration of hippocampal neurons

In the control group, a small number of Fluoro Jade B-positive neurons were found in CA1 subfield and in the hilus. However, in the LiCl-pilocarpine group, an increased number of labeled neurons were found in the stratum pyramidale (S. PYR) from CA1 subfield (Fig. 3).

4. Discussion

Pediatric neurologists have repeatedly described neuronal damage with subsequent mental impairment and development of epilepsy after status epilepticus in children. However, the cellular and molecular mechanisms involved in this processes are still under investigation. In order to contribute to this question, in the present study we investigated the effect of early-life LiCl-pilocarpine-induced SE on hippocampal Na^+/K^+ ATPase activity and glutamate uptake.

SE produced a reduction of Na^+/K^+ ATPase activity in hippocampus 1.5 h after pilocarpine administration (Fig. 1). Malfunction of this enzyme has been associated with the neuronal hyperexcitability (Donaldson et al., 1971) and subsequent development of epilepsy (McNamara, 1994). Vaillend et al. (2002) showed that inhibition of Na^+/K^+ ATPase by low-affinity cardiac glycoside, dihydroouabain, reversibly caused the appearance of robust extracellular epileptiform burst potentials in hippocampal CA1 in response to single stimuli to stratum radiatum. The bursts resembled interictal-like burst potentials recorded in other models of epileptiform bursting. Morevoer, application of ouabain, another pump inhibitor, cause a massive efflux of K+ ions and cell depolarization (Haglund and Schwartzkroin, 1990), epileptic activity, and ultimately, cell death (Lees and Leong, 1994). This neuronal hyperexcitability may be related with an elevation of extracellular K+ levels. (Rutecki et al., 1985) show that the frequency of spontaneous epileptiform discharges is a function of extracellular K+ levels. Increasing extracellular K+ levels from 5 to 10 mM caused a fivefold increase in the rate of spontaneous discharges in hippocampus. Moreover, recently Slais et al. (2008) showed that animals submitted to pilocarpine-induced SE presented elevated extracellular K+ levels (3.07 to 13.3 mM) 80 min after SE onset. Therefore, taken together these data tempting to speculate that decrease in Na^+/K^+ ATPase activity may be a contributing factor to keep the sustained seizure activity during LiCl-pilocarpine-induced SE.

In order to determine whether the inhibition of Na^+/K^+ ATPase was related to plasma membrane damage, the production of thiobarbituric acid-reactive substances was evaluated 0.5, 1.5 and 12 h after SE onset. No alterations were observed between SE and control group in lipid peroxidation levels 0.5, 1.5 and 12 h after SE onset. However, 24 h after SE the LiCl-pilocarpine treated animals displayed elevated levels of lipid peroxidation. This may be associated to neuronal damage found in the stratum pyramidale from hippocampal CA1 subfield.

The effect of LiCl-pilocarpine-induced SE appear don't involve a direct effect of pilocarpine on pump structure, whereas pilocarpine at 100 μM failed to exert any significant effect on Na^+/K^+ ATPase activity *in vitro*. The reduced activity of Na^+/K^+ ATPase may be due a reduction of extracellular glucose levels and subsequent intracellular ATP concentrations. During SE, metabolism is markedly increased, resulting in depletion of adenosine triphosphate and energy reserves (Lothman, 1990). Slais et al. (2008) showed that after pilocarpine administration glucose dialysate concentrations increased, reaching a maximum of 3.49 mmol/L 30 min later. Eighty minutes after, glucose concentrations decreased, reaching a value of 1.25 mmol/L. This effect is accompanied to a rise in lactate dialysate levels (0.61 mmol/L to 2.92 mmol/L). Similar results were obtained by (Fernandes et al., 1999). They showed that after 1 hour of SE, the cerebral lactate concentrations increased 3-fold after 1 hour of SE at P10 and 4.5-fold in P21 and adult rats. After 90min of SE onset the cerebral lactate levels decreased at P10 and stayed stable in P21 rats. Moreover, lithium-pilocarpine-induced SE did lead to decrease in cerebral glucose concentration by 30 to 43% in both ages.

Another neurochemical consequence observed in rats submitted to early-life LiCl-pilocarpine-induced SE is a reduction of hippocampal glutamate uptake 1.5h after SE onset (Fig. 2). However, this decreased uptake was observed only at lower extracellular glutamate concentration 1 μM. At higher concentration (100 μM), glutamate can stimulate the

translocation of glutamate transporters from the cytoplasm to the plasma membrane. (Gegelashvili et al., 2007) showed that cultures submitted to extracellular concentration of glutamate below 3 mM, a bulk of GLAST immunoreactivity was located in the cytoplasmic compartment. However, administration of L-glutamate (10–50 μM) during 10 min caused dramatic redistribution of GLAST from the cytoplasm to the plasma membrane, resulting in the increased uptake capacity without affecting the quantity of total pool. Since in our study the hippocampal slices were exposed to 100 μM of glutamate during 5 min and GLAST is highly expressed in hippocampus, it is possible that this high concentration stimulates the translocation of glutamate transporter GLAST to the plasma membrane, leading to a compensatory effect on glutamate uptake capacity.

Glutamate uptake decrease may be related with a reduction of Na^+/K^+ ATPase activity, whereas disruption of pump activity can lead to an impaired glutamate uptake. Loss of Na+ and K+ homeostasis by inhibition of Na^+/K^+ ATPase with ouabain in dorsal spinal white matter slices (LoPachin et al., 1999) and cortical prisms (Nanitsos et al., 2004) induce release of glutamate by reversal of Na+-dependent transporters from intracellular compartments and decrease glutamate uptake, respectively. Moreover, it has been showed that animals submitted to LiCl-pilocarpine-induced SE displayed an increased extracellular levels of glutamate 40 min after SE induction which returned to control values 120 min later (Slais et al., 2008). Taken together, these findings support the notion that during the SE a decreased or reversed transmembrane Na+ and K+ gradients may induce a release of intracellular glutamate through reverse operation of Na+-dependent glutamate transporters, which in turn can lead to uncontrolled efflux of this excitatory amino acid to extracellular space.

An elevation of extracellular glutamate concentrations by uncontrolled efflux can lead to neuronal damage through activation of ionotropic glutamate receptors (Fujikawa, 1995). This mechanism may be responsible for neuronal damage found in the present study in

the stratum pyramidale from CA1 subfield 24h after SE induction (Fig. 3). Recent studies have been indicating that SE induced early in life may cause neuronal death and this appears associated to overstimulation of glutamate receptors. Sankar et al. (1997) showed that rat pups (1-4 weeks of age) submitted to a LiCl-pilocapine induced SE had a higher serum levels of s-NSE compared to control animals and this increase was accompanied by neuronal damage in specific brain areas such as cortex, thalamus, amygdala, septum and hippocampus. Systemic administration of NMDA receptor antagonists, even when given after the onset of SE, is remarkably neuroprotective against status epilepticus-induced neuronal damage. Ketamine, phencyclidine, and MK-801, which are noncompetitive antagonists of the N-methyl-D-aspartate (NMDA), prevented kainic acid-induced brain damage in the amygdala, piriform cortex, thalamus, and CA1 region of the hippocampus but conferred little or no protection in the lateral septum and CA3 region of the hippocampus (Clifford et al., 1990). Morevoer, the ketamine administration had significant neuroprotection 15 min after LiCl-pilolcarpine-induced SE onset (Fujikawa, 1995).

In conclusion, in this study we showed that LiCl-pilocarpine-induced SE during development decreases Na^+/K^+ ATPase activity and glutamate uptake in hippocampus 1.5 h after SE onset. These effects might be associated with the elevation of lipid peroxidation levels and neuronal damage found in the stratum pyramidale from hippocampal CA1 subfield 24 h later. Therefore, SE induced early in life cause a short-term disruption in glutamate uptake and Na^+/K^+ ATPase activity, which can be harmful to brain and neurons. These alterations may lead to behavioral, morphological and neurochemical alterations in adulthood.

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FIGURE LEGENDS

Fig. 1. Hippocampal Na+, K+ ATPase activity and thiobarbituric acid-reactive substances production in control and LiCl-pilocarpine-induced SE rats. Animals were sacrificed by decapitation 90 min, 12 h and 24 h after the SE induction for Na+, K+ ATPase assay and 30 min, 90 min, 12 h and 24 h for thiobarbituric acid-reactive substances production. The data expressed as mean ± SD. * Indicates $p < 0.01$ as compared with the control group (one-way ANOVA followed by the Tukey´s *post hoc* test).

Fig. 2. Glutamate uptake profile in hippocampal slices from control and LiCl-pilocarpine-induced SE rats. The uptake was performed 90 min, 12 h and 24 h after the SE induction. For each animal, five hippocampal slices (3 for total and 2 for sodium-independent uptake) were transferred to 24-well dishes containing 0.3mL of HBSS. The uptake assay was assessed by adding 20 uL of a solution containing 0.33 uCi/mL L-[2, 3-3 H] glutamate with 100 μM or 1 μM unlabeled glutamate at 35°C. The data expressed as mean ± SD. * Indicates $p < 0.01$ as compared with the control group (one-way ANOVA followed by the Tukey´s *post hoc* test).

Fig. 3. Distribution of Fluoro Jade B-labeled degenerating neurons in CA1 subfield 24 h after SE induction. There was found a markedly increased number of Fluoro Jade B-labeled neurons in the stratum pyramidale (S. PYR) from CA1 subfield in the LiCl-pilocarpine group when compared with control group.

Figure 1.

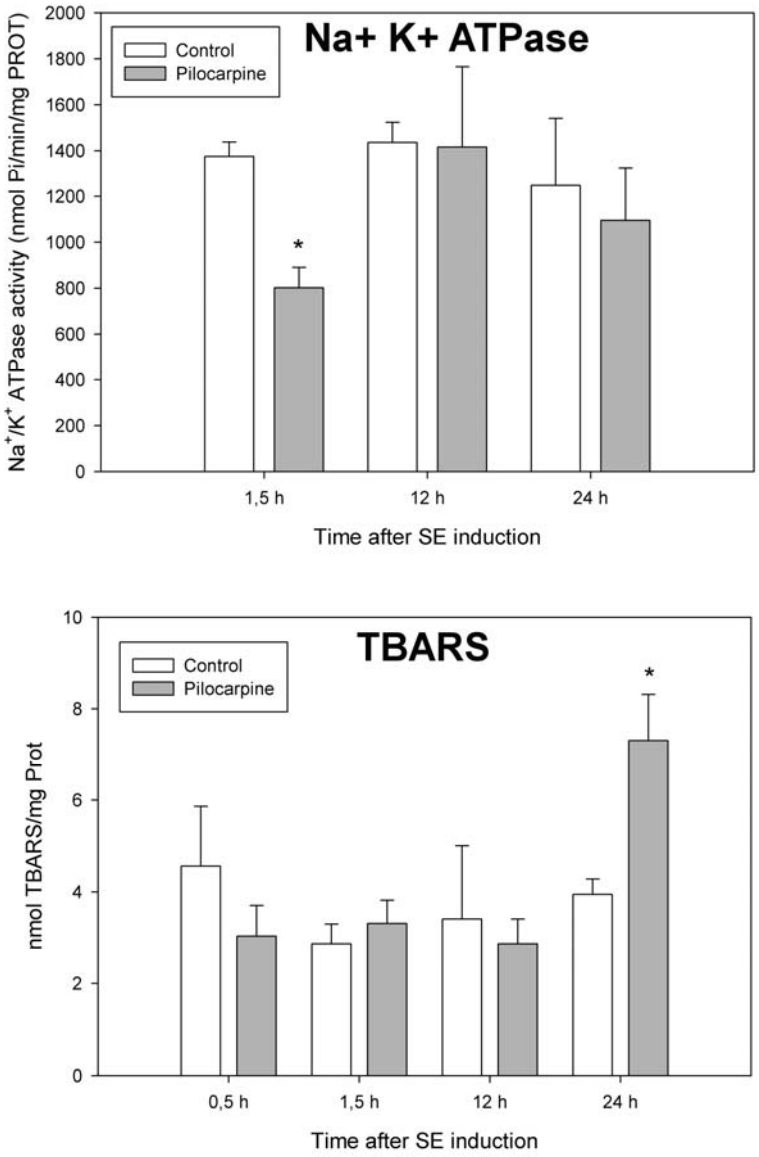

Na+ K+ ATPase
Control
Pilocarpine
Na+/K+ ATPase activity (nmol Pi/min/mg PROT)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
1,5 h
12 h
24 h
Time after SE induction
TBARS
Control
Pilocarpine
nmol TBARS/mg Prot
10
8
6
4
2
0
*
0,5 h
1,5 h
12 h
24 h
Time after SE induction

Figure 2.

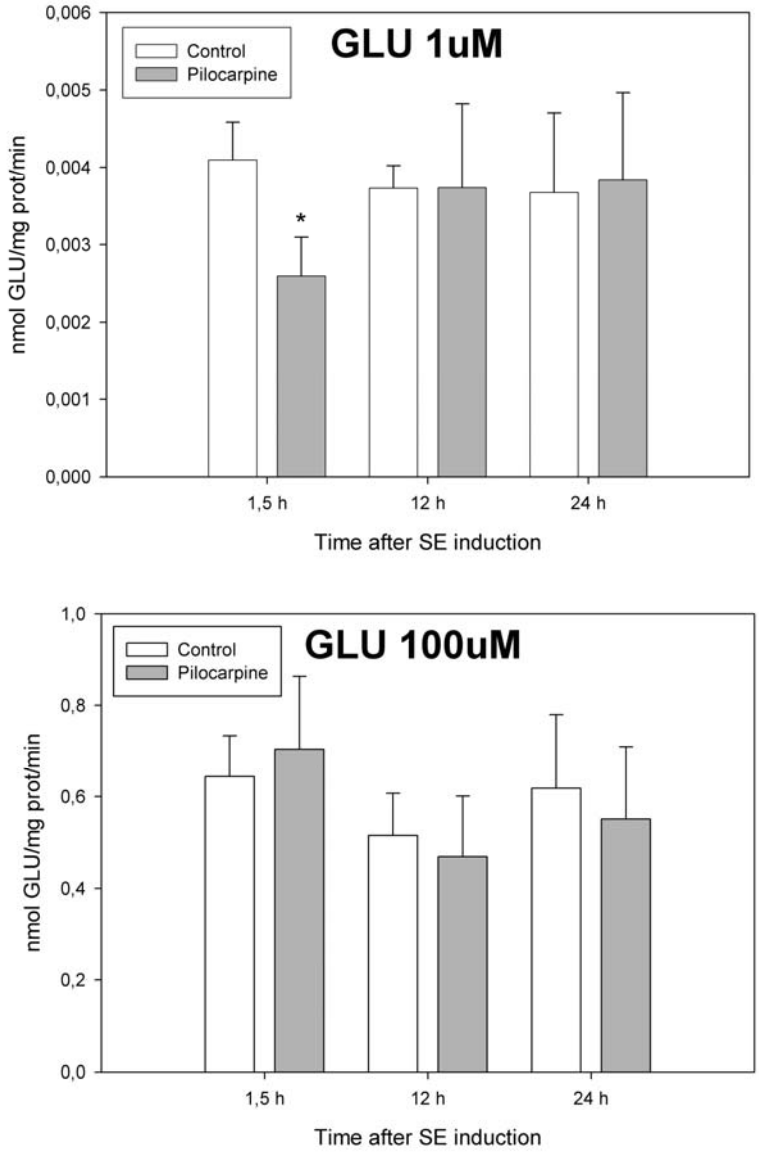
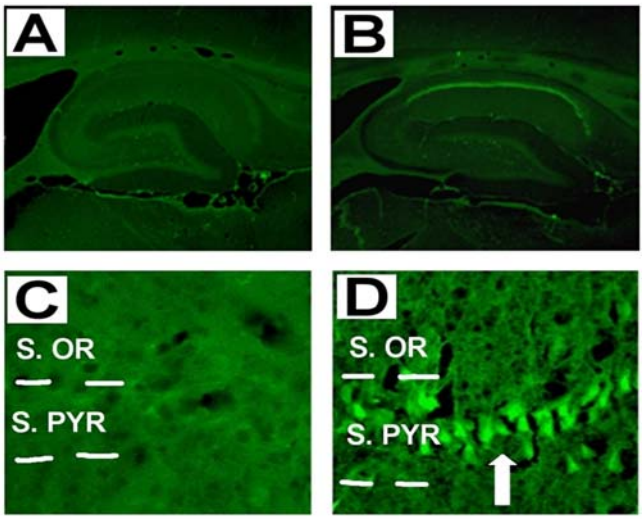

GLU 1uM
Control
Pilocarpine
nmol GLU/mg prot/min
0,006
0,005
0,004
0,003
0,002
0,001
0,000
*
1,5 h
12 h
24 h
Time after SE induction
GLU 100uM
Control
Pilocarpine
nmol GLU/mg prot/min
1,0
0,8
0,6
0,4
0,2
0,0
1,5 h
12 h
24 h
Time after SE induction

Figure 3.



CAPÍTULO III

Status epilepticus induced by LiCl-pilocarpine early in life decreases the phosphorylation of cell survival proteins PKB/AKT and GSK-3beta in hippocampus.

Diogo Losch de Oliveira *, Fabrício Simão, Ben Hur Mussulini, Alice Fischer, Diogo Souza, Christianne Salbego and Susana Wofchuk.

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Status epilepticus induced by LiCl-pilocarpine early in life decreases the phosphorylation of cell survival proteins PKB/AKT and GSK-3β in hippocampus.

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Running title: SE decreases phosphorylation of hippocampal AKT and GSK.

Number of text pages: 15.

Number of figures: 2.

SUMMARY

Programmed cell death mechanisms triggered by pro-apoptotic proteins, such as p53 and Bcl-2 family members, are frequently implicated in SE-induced neuronal death. In contrast, the role of other proteins, such as Akt/PKB and GSK-3β has not been addressed. The aim of this study was to investigate the effect of early life LiCl-pilocarpine-induced SE on phophorylation of Akt/PKB and GSK-3β in hippocampal subfields CA1 and dentate gyrus. Rats (P15) received LiCl (3mEq/kg, i.p.) 12-18h prior pilocarpine (60 mg/kg; s.c.). Primary antibodies against the following proteins were used: anti-phospho Akt/PKB, anti-Akt, anti-phospho GSK-3β, anti-GSK-3β. LiCl-pilocarpine-induced SE decreased the phosphorylation of Akt/PKB by 35.84% and GSK-3β by 38.79% 1.5 after SE induction in CA1 subfield. However, 24 h after LiCl-pilocarpine-induced SE the phosphorylation of Akt/PKB and GSK-3β returned to control levels. At dentate gyrus, the phosphorylation of Akt/PKB was reduced by 35.18% and 31.19% at 1.5 and 24 h after SE induction, respectively. GSK-3β phosphorylation was reduced by 38.05% and 33.12% at 1.5 and 24 h, respectively, after LiCl-pilocarpine administration.

Key words: Status epilepticus, development, AKT, GSK.

INTRODUCTION

One of the earliest brain consequences of SE early in life is the selective neuronal loss observed in specific brain areas. Rats with LiCl–pilocarpine-induced SE during the second week of life presented damage in the hippocampus, amygdala, thalamus, and septum as well as an elevation in the serum levels of neuron specific enolase (NSE) 24 h after SE induction (Sankar, et al. 1997). Further analysis of hippocampal CA1 pyramidal cells showed DNA fragmentation and apoptotic bodies (Sankar, et al. 1998). The precise cellular mechanism by which status epilepticus induces hippocampal neuronal damage is still under investigation. Programmed cell death mechanisms, such as p53 activation, activation of cell death-promoting Bcl-2 family members, and endonuclease-induced DNA laddering, occur in SE-induced neuronal death. Caspase-independent excitotoxic mechanisms, such as NMDA-induced calpain I activation, with activation and translocation of the cell death-promoting Bcl-2 family member Bid from cytoplasm to mitochondria, and subsequent translocation of apoptosis-inducing factor and endonuclease G to nuclei (which cause large-scale and internucleosomal DNA cleavage, respectively), may be triggered by SE (Fujikawa 2005).

PI3-K pathway is believed to be an important anti-apoptotic signaling pathway in neurons (Yuan and Yankner 2000). Akt/PKB, also known as protein kinase B, is the main downstream kinase of PI3-K that promotes cell survival, because of its ability to phosphorylate and inactivate several pro-apoptotic targets, including the Bcl-2 family member BAD, the fork head transcription factors, and the glycogen synthase kinase-3b (GSK-3β) (Brazil, et al. 2004). Akt/PKB is activated through phosphorylation mediated by two phosphoinositidedependent kinases, PDK 1 and 2. The downregulation of these serine/threonine kinases by phosphatases that catalyze Akt/PKB dephosphorylation is still not fully understood. Recently, Akt/PKB has been shown to be dephosphorylated and inactivated in vitro by the protein phosphatase 2A (PP2A) and by the PI3-K phosphatase PTEN (Brazil,

et al. 2004).

The aim of this study was to investigate the effect of early life LiCl-pilocarpine-induced SE on phophorylation of Akt/PKB and GSK-3β in hippocampal subfields CA1 and dentate gyrus.

METHODS

Animals

Thirty four male Wistar rats were used. The day of birth was defined as day 0. The litters were culled to 8 pups. Rats were housed under a controlled environment (temperature of 21±1 °C, standard light/dark cycle of 12 h) with food and water *ad libitum*. The handling and care of the animals were conducted according to the guidelines of the Guide for the Care and Use of Laboratory Animals, Brazilian School of Animal Research. All procedures in the present study were approved by the Committee of Ethics from the Universidade Federal do Rio Grande do Sul.

Induction of status epilepticus

Rat pups 15 days old (P15) were injected i.p. with solution of LiCl (3 mEq/kg – Nuclear, Brazil) 12-18h prior to s.c. pilocarpine hydrochloride administration (60 mg/kg – Sigma, St. Louis, MO, USA). Control animals were handled and housed in the same manner as the treated animals and received an equal volume of saline solution (0.9% NaCl). Rats were put in individual plastic cages at 34°C (nest temperature) for seizure observation. Each experimental group contained pups from several litters.

Western blotting assay

The animals were killed by decapitation 1.5 and 24 h after SE induction and the brain removed. Transverse hippocampal slices (0.4 mm) were prepared with a McIlwain tissue chopper. Micro slices (1 mm in diameter) were obtained from hippocampal subfields CA1 and dentate gyrus using a stainless steel punch. The brain microslices were homogenized in lysis buffer (4% sodium dodecylsulfate (SDS), 2.1 mM EDTA, 50 mM Tris). Aliquots were taken for protein determination and b-mercaptoethanol was added to a final concentration of 5%. Samples containing 35 mg of protein were resolved by 10% SDS-PAGE. Proteins were electro transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad,

Trans-Blot SD). After 2 h incubation at 4^{o} C in blocking solution containing 5% powdered milk and 0.1% Tween-20 in Tris–buffered saline (TBS; 50 mM Tris–HCl, 1.5% NaCl, pH 7.4), membranes were incubated overnight with the appropriate primary antibody diluted in the same blocking solution. Primary antibodies (Cell Signaling Technology, Beverly, MA, USA) against the following proteins were used: anti-phospho Akt (Ser473) (pAkt, 1:1000), anti-Akt (1:1000), anti-phospho GSK-3β (Ser9) (pGSK-3β, 1:1000), anti-GSK-3β (1:1000) (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:1000). The chemioluminescence (ECL) was detected using X-ray films (Kodak X-Omat). Films were scanned and the percentage of band intensity was analyzed using Optiquant software. For each experiment, SE group were compared to control group which were considered 100%. Data are expressed as percentage of phosphorylated protein for Akt/PKB and GSK-3β, which was obtained by the ratio of the phospho-protein (pAkt or pGSK-3β) with its whole amount (Akt/PKB or GSK-3β). Data are expressed as mean $\pm$ SD and were analyzed by the one-way ANOVA followed by the Tukey´s *post hoc* test for unequal samples.

RESULTS

The behavioral pattern of SE correlated well with the description by Oliveira *et al.* (2008), lasting up to 3-4 h. Systemic administration of LiCl-Pilocarpine produced defecation, salivation, body tremor, and scratching within 5 to 20 min. This behavioral pattern progressed within 30 to 45 min to increased levels of motor activity and culminated in SE in all animals. SE was characterized by sustained orofacial automatisms, salivation, chewing, forelimb clonus, loss of the righting reflex and falling. The mortality rate 24 h after the status epilepticus induction was 20%.

Effect of SE on the phosphorylation/dephosphorylation of Akt/PKB and GSK-3β

LiCl-pilocarpine-induced SE decreased the phosphorylation of Akt/PKB by 35,84% and GSK-3β by 38.79% 1.5 after SE induction in CA1 subfield (Fig. 1). However, 24 h after LiCl-pilocarpine-induced SE the phosphorylation of Akt/PKB and GSK-3β returned to control levels.

At dentate gyrus, SE reduced the phosphorylation of Akt/PKB and GSK-3β in both times evaluated (Fig. 2). The phosphorylation of Akt/PKB was reduced by 35.18% and 31.19% at 1.5 and 24 h after SE induction, respectively. GSK-3β phosphorylation was reduced by 38.05% and 33.12% at 1.5 and 24 h, respectively, after LiCl-pilocarpine administration.

DISCUSSION

LiCl-pilocarpine-induced SE early in life can produce expressive neuronal damage in specific brain areas 24h after SE induction (Sankar, et al. 1998). The cellular mechanisms by which status epilepticus induces selective neuronal damage are still under investigation. Programmed cell death mechanisms triggered by pro-apoptotic proteins, such as p53, Bcl-2 family members, and endonuclease-induced DNA laddering, are frequently implicated in SE-induced neuronal death (Fujikawa 2005). In contrast, the role of other proteins, such as Akt/PKB and GSK-3β has not been addressed.

Western blotting analysis determined that Akt/PKB was rapidly dephosphorylated at Ser473 residues after SE onset both in dentate gyrus and CA1 subfields. Akt/PKB is the main downstream kinase of PI3-K that promotes cell survival due to its ability to phosphorylate and inactivate several pro-apoptotic targets. Therefore, the dephosphorylation of Akt/PKB during SE may lead to activation of these pro-apoptotic targets and consequently triggering neuronal death. (Henshall, et al. 2002) showed that intracerebroventricular administration of an inhibitor of phosphatidylinositol 3-kinase (LY294002), thought to be an upstream activator of Akt/PKB, exacerbated cortical apoptosis after kainic acid-induced seizures. Our study provides the first demonstration that the activity of Akt/PKB is decreased in the hippocampus of rats submitted to early-life LiCl-pilocarpine-induced SE.

When active, Akt/PKB is able to phosphorylate and inactivate several pro-apoptotic targets, including the Bcl-2 family member BAD, the fork head transcription factors, and GSK-3β. Here we showed that the decrease in Akt/PKB phosphorylation is accompanied by a decrease in GSK-3β phosphorylation both in CA1 and dentate gyrus.

Our study provides the first demonstration that the activity of Akt/PKB and GKS-3b is altered in the hippocampus of young rats submitted to early-life LiCl-pilocarpine-induced SE.

ACKNOWLEDGEMENTS

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DISCLOSURE OF CONFLICT OF INTEREST

None of the authors has any conflict of interest to report in relation to this paper.

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FIGURE LEGENDS

Fig. 1. Effect of LiCl-pilocarpine-induced status epilepticus on phosphorylation of Akt/PKB in hippocampal slices. The animals were killed by decapitation 1.5 and 24 h after SE induction and the brain removed. Transverse hippocampal slices (0.4 mm) were prepared with a McIlwain tissue chopper. Micro slices (1 mm in diameter) were obtained from hippocampal subfields CA1 and dentate gyrus using a stainless steel punch. The data expressed as mean ± SD. * Indicates $p < 0.01$ as compared with the respective control group (one-way ANOVA followed by the Tukey´s *post hoc* test).

Fig. 2. Effect of LiCl-pilocarpine-induced status epilepticus on phosphorylation of GSK-3β in hippocampal slices. The animals were killed by decapitation 1.5 and 24 h after SE induction and the brain removed. Transverse hippocampal slices (0.4 mm) were prepared with a McIlwain tissue chopper. Micro slices (1 mm in diameter) were obtained from hippocampal subfields CA1 and dentate gyrus using a stainless steel punch. The data expressed as mean ± SD. * Indicates $p < 0.01$ as compared with the respective control group (one-way ANOVA followed by the Tukey´s *post hoc* test).

Figure 1.

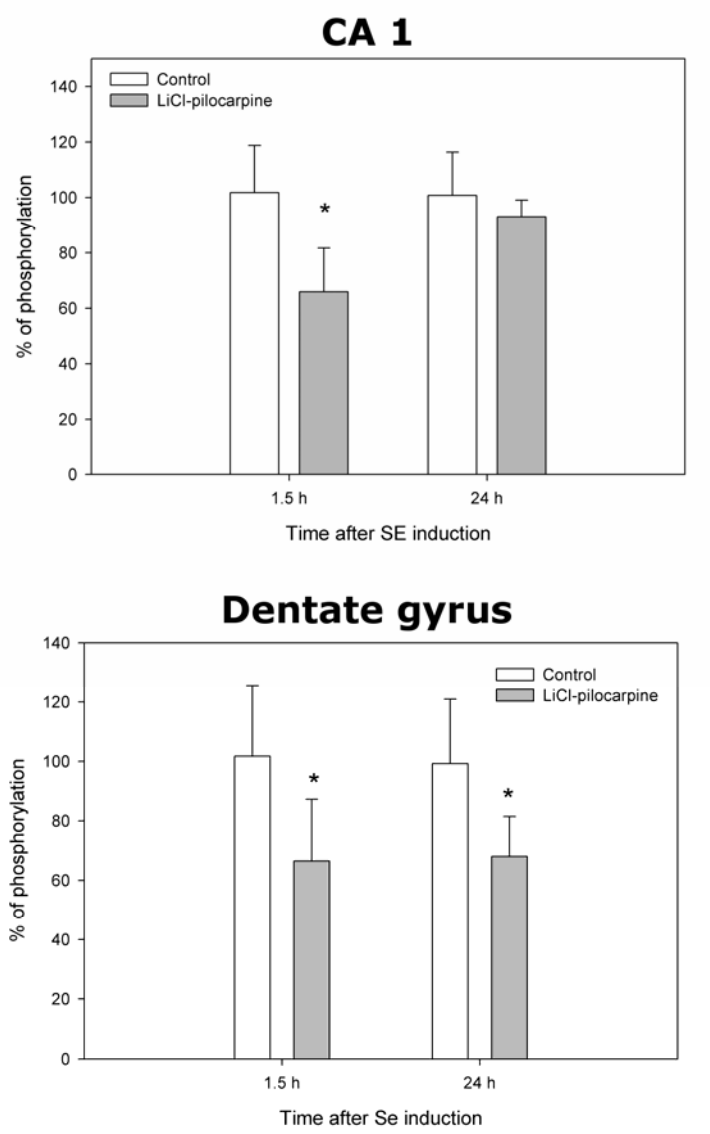
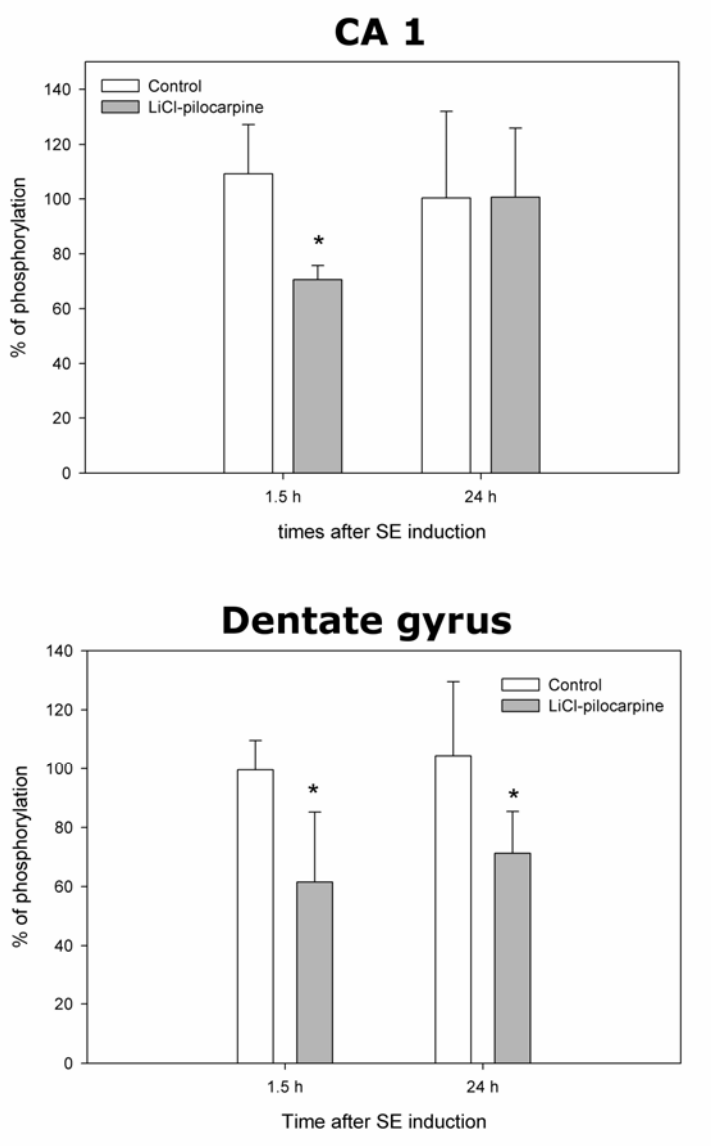

CA 1
Control
LiCl-pilocarpine
% of phosphorylation
0
20
40
60
80
100
120
140
*
1.5 h
24 h
Time after SE induction
Dentate gyrus
Control
LiCl-pilocarpine
% of phosphorylation
0
20
40
60
80
100
120
140
*
*
1.5 h
24 h
Time after Se induction

Figure 2.


CA 1
Control
LiCl-pilocarpine
% of phosphorylation
140
120
100
80
60
40
20
0
*
1.5 h
24 h
times after SE induction
Dentate gyrus
Control
LiCl-pilocarpine
% of phosphorylation
140
120
100
80
60
40
20
0
*
*
1.5 h
24 h
Time after SE induction

Parte III. Discussão

Inúmeros trabalhos clínicos têm demonstrado danos neuronais com subseqüente alterações neurológicas em crianças acometidas de *status epilepticus* (ver a revisão de Sankar et al., 2007). No presente trabalho, nós demonstramos que o *status epilepticus* induzido por LiCl-pilocarpina durante períodos iniciais do desenvolvimento cerebral pode levar a alteração neuroquímicas e anormalidades comportamentais a curto e longo prazo.

O *status epilepticus* produziu uma redução de aproximadamente 50% na atividade da Na^+/K^+ ATPase em hipocampo 1,5 h após a administração de pilocarpina. Disfunções na atividade da Na^+/K^+ ATPase têm sido associadas com hiperexcitabilidade neuronal e subseqüente desenvolvimento de epilepsia (McNamara, 1994). A inibição da bomba Na^+/K^+ ATPase pela administração de di-hidro-ouabaína, um inibidor de baixa afinidade, produz uma atividade epiléptica na região CA1 do hipocampo. Além disso, o tratamento de fatias hipocampais com ouabaína, um inibidor de alta afinidade da bomba, causa um grade efluxo de íons K^+ para o meio extracelular bem como despolarização (Haglund and Schwartzkroin, 1990), atividade epiléptica e morte celular (Lees and Leong, 1994). Portanto, uma diminuição da atividade da bomba Na^+/K^+ ATPase pode levar a um acúmulo de íons K^+ no meio extracelular e conseqüentemente induzir a um aumento na atividade epiléptica. Rutecki et al. (1985), demonstraram que a elevação da concentração de K^+ extracelular de 5 para 10 mM causa um aumento de 5x na atividade epiléptica em fatias hipocampais. Além disso, recentemente Slais et al. (2008) demonstraram que animais submetidos ao *status epilepticus* induzido por LiCl-pilocarpina apresentam um aumento nas concentrações extracelulares de K^+ (3,07 para 13,3 mM) 80 min após a indução do insulto. Estes altos níveis de K^+ extracelular podem estar relacionados à baixa atividade da bomba Na^+/K^+ ATPase observada neste estudo, sendo, portanto, um fator determinante para a manutenção da atividade epiléptica excessiva e anormal observada durante o *status epilepticus* induzido por LiCl-pilocarpina (Hirsch et al., 1992). A diminuição da atividade da bomba parece não estar relacionada

com uma ação direta da pilocarpina sobre a estrutura protéica, visto que o tratamento *in vitro* com pilocarpina não alterou a atividade da Na^+/K^+ ATPase. Além disso, não observou-se alteração nos níveis de lipoperóxidos, o que indica de forma indireta que a membrana plasmática manteve-se íntegra.

A diminuição na atividade da Na^+/K^+ ATPase 1,5 h após a administração de pilocarpina foi acompanhada de uma diminuição significativa da captação de glutamato. A diminuição da captação de glutamato pode estar relacionada com a diminuição da atividade da bomba, visto que a energia necessária para o transporte de glutamato provém do gradiente eletroquímico de Na^+, gerado pela atividade da Na^+/K^+ ATPase. A perda da homeostase do Na^+ e do K^+ por inibição da bomba induz liberação de glutamato por inversão da atividade dos transportadores em fatias de medula (LoPachin et al., 1999) e também uma diminuição da captação de glutamato em fatias de córtex (Nanitsos et al., 2004). Além disso, Slais et al. (2008) demonstraram que, 40 min após indução de *status epilepticus* por LiCl-pilocarpina, ocorre aumento nos níveis de glutamato extracelular, o qual permanece elevado até 120 min após a indução do insulto.

A diminuição da captação de glutamato, no entanto, foi observada somente em concentrações fisiológicas de glutamato (~1 μM). Em concentrações elevadas (100 μM), os níveis de captação de glutamato permaneceram iguais àqueles observados nos animais controles. Em altas concentrações, o glutamato estimula a translocação de transportadores provenientes de estoques intracelulares para a membrana celular. Gegelashvili et al. (2007), demonstraram que culturas submetidas a concentrações extracelulares de até 3 μM, apresentam uma grande imunoreatividade para GLAST nos compartimentos intracelulares. No entanto, a administração de glutamato em concentrações de 10-50 μM produz uma redistribuição de GLAST do citoplasma para a membrana plasmática, 15 min após a administração. Visto que em nosso estudo as fatias hipocampais foram submetidas a 100 μM de glutamato durante um período de 5 min, é possível que esta elevada

concentração de glutamato extracelular tenha recrutado transportadores de glutamato de estoques intracelulares para a membrana celular, levando a um efeito compensatório na captação de glutamato.

Uma diminuição da captação e subseqüente elevação nas concentrações extracelulares de glutamato podem levar a morte neuronal devido à excessiva ativação de receptores ionotrópicos e elevação do influxo de íon Ca^{+2}. O Ca^{+2} é bastante conhecido por ser um importante segundo mensageiro intracelular, capaz de desencadear a ativação ou inibição de diversas cascatas de sinalização celular. Recentemente, Dong et al. (2007) demonstrou que o complexo Ca+2/calmodulina pode ser co-imunoprecipitado com PKB e esta interação pode regular a atividade cinásica da enzima. Neste trabalho observamos uma diminuição dos níveis de PKB fosforilada nas regiões CA1 e giro denteado 1,5 h após a indução do *status epilepticus*. No giro denteado, a diminuição da fosforilação da PKB permaneceu até 24 h após a indução do insulto. Quando ativa (fosforilada), PKB fosforila e inativa uma série de proteínas pró-apoptóticas, incluindo membros da família da Bcl-2 como BAD, fatores de transcrição "fork head" e a GSK-3β. Neste trabalho, 1,5 h após a indução do *status epilepticus*, observamos uma redução significativa da fosforilação da GSK-3β tanto em CA1 quanto no giro denteado. Da mesma forma com que observado com a PKB, esta diminuição manteve-se até 24 h após a indução do insulto no giro denteado. É possível que uma diminuição da fosforilação da PKB leve a uma diminuição de sua atividade cinásica e conseqüentemente uma diminuição da fosforilação da GSK-3β. Quando desfosforilada, a proteína GSK-3β está ativa, levando a uma cascata de eventos intracelulares como, por exemplo, a inibição de alguns fatores de transcrição importantes para a sobrevivência celular (Li et al., 2002). Além disso, pode ser ativada por aumento transitório de cálcio intracelular (Hartigan and Johnson, 1999), e inibida pelas vias de sinalização da Wnt/β-catenina (Li et al., 2002) e através da ativação da cascata PI3K/PKB (Cross et al., 1995).

A diminuição da fosforilação da PKB, bem como da GSK-3β, pode estar relacionada com a intensa morte neuronal observada neste trabalho 24 após a administração de pilocarpina. O *status epilepticus* produziu uma expressiva morte celular, 24 h após a indução do insulto, nas regiões CA1 e giro denteado. Embora estudos prévios tenham demonstrado que o cérebro em desenvolvimento é relativamente menos vulnerável ao dano celular que o cérebro maturo, estudos recentes têm demonstrado que o *status epilepticus* induzido em períodos iniciais do desenvolvimento cerebral pode levar a morte neuronal em diversas regiões cerebrais. Sankar et al. (1997) demonstraram que o *status epilepticus* induzido por LiCl-pilocarpina, em animais de 14 dias pós-natal, induz a uma expressiva morte neuronal 24 h após a indução do insulto no córtex, tálamo, amígdala e hipocampo. Estas alterações são acompanhadas por elevação dos níveis plasmáticos de N*status epilepticus* (enolase específica de neurônios). Em estudo posterior, Sankar et al. (1998) demonstraram que a morte celular observada no hipocampo envolve tanto necrose quanto apoptose.

A morte celular observada em hipocampo dos animais submetidos ao *status epilepticus* induzido por LiCl-pilocarpina pode levar a alterações comportamentais e plasticidade sináptica na idade adulta. Neste trabalho, os animais submetidos ao *status epilepticus* apresentaram um elevado brotamento de fibras musgosas na região CA3 e giro denteado do hipocampo. Além disso, esta plasticidade sináptica parece estar relacionada com as alterações cognitivas e comportamentais observadas nos animais tratados com pilocarpina. Observamos um significativo déficit cognitivo nos animais submetidos ao *status epilepticus* na tarefa de esquiva inibitória. Além disso, aqueles animais que apresentaram menor latência para descida da plataforma apresentaram os maiores escores para brotamento de fibras musgosas. Lipp et al. (1988) compararam o número de "trials" para ratos se esquivarem de choques na tarefa de esquiva ativa com intensidade de fibras musgosas na região CA3 do hipocampo. Este trabalho demonstrou uma relação negativa, ou seja, os animais

que possuíam um maior brotamento de fibras musgosas apresentaram um pior desempenho na tarefa.

Os animais submetidos ao *status epilepticus* durante do desenvolvimento também apresentaram elevados níveis de ansiedade na tarefa do claro-escuro na idade adulta. Esta alteração no comportamento emocional parece estar relacionada ao brotamento de fibras musgosas no hipocampo. Isto é suportado pela forte correlação positiva entre os níveis de ansiedade e a intensidade do brotamento de fibras musgosas observados nos animais submetidos ao *status epilepticus*. Estudos recentes têm sugerido que a porção ventral do hipocampo pode desempenhar um importante papel nos processos cerebrais associados com comportamentos de ansiedade. A região ventral do hipocampo projeta-se para o córtex pré-frontal e também para a amígdala. A maioria dos núcleos amigdalares possui algumas conexões recíprocas com a formação hipocampal (Bannerman et al., 2004), o que nos leva a propor que alterações nas conexões sinápticas intrahipocampais podem alterar a conectividade com outras estruturas cerebrais e levar a alterações no comportamento emocional.

Além das alterações comportamentais, os animais submetidos ao *status epilepticus*, induzido por LiCl-pilocarpina, apresentaram elevados níveis da proteína S100B no líquor. Estudos têm demonstrado elevados níveis da proteína S100B após injúria cerebral (Busnello et al., 2006, Oses et al., 2004). Além disso, elevados níveis de S100B também foram detectados em líquor de pacientes portadores de epilepsia do lobo temporal (Steinhoff et al., 1999). Embora o real significado destas variações não esteja claro (se apresenta efeitos prejudiciais ou benéficos), a proteína S100B tem sido amplamente utilizada como um marcador de injuria cerebral (Donato, 2001).

Portanto, nossos resultados mostraram que o *status epilepticus* induzido por LiCl-pilocarpina diminui a atividade da Na^+/K^+ ATPase bem como a captação de glutamato em hipocampo 1,5 h após a indução do insulto. Estes efeitos foram acompanhados por uma diminuição

da fosforilação das proteínas PKB e GSK-3β nas regiões CA1 e giro denteado, os quais podem estar relacionados com a morte neuronal observada 24 h após a indução do *status epilepticus*. Além disso, na idade adulta, o *status epilepticus* induzido aos 14 dias pós-natal induziu alterações no processo de memória-aprendizado e no comportamento emocional. As alterações comportamentais parecem estar relacionadas ao brotamento de fibras musgosas, visto que os animais que apresentaram déficit de memória na tarefa de esquiva inibitória e elevados níveis de ansiedade no teste claro-escuro apresentaram maior escore para brotamento de fibras musgosas no hipocampo. Além disso, os animais tratados com LiCl-pilocarpina apresentaram elevados níveis da proteína S100B no líquor bem como uma correlação positiva entre o imunoconteúdo de GFAP na região CA1 e o escore para brotamento de fibras musgosas, sugerindo uma resposta glial ao dano neuronal.

Diante dos resultados obtidos, podemos constatar que o *status epilepticus* induzido em períodos iniciais do desenvolvimento cerebral afeta o SNC, alterando sua maturação e levando a alterações neuroquímicas, morfológicas e comportamentais na idade adulta.

Parte IV. Conclusões

O *status epilepticus*, a curto prazo, induziu significativas alterações à nível celular e neuroquímico, como a diminuição da atividade da Na^+/K^+ ATPase e subseqüente redução da captação de glutamato. Um desbalanço na atividade da Na^+/K^+ ATPase associado a uma elevação dos níveis extracelulares de glutamato (decorrentes da diminuição da sua captação), pode levar a uma processo patológico denominado de excitotoxicidade e conseqüentemente a morte neuronal (a qual foi observada 24 horas após a indução do insulto).

A morte celular observada em hipocampo pode estar relacionada às alterações comportamentais (déficit cognitivo e aumento na ansiedade) e a plasticidade sináptica (brotamento de fibras musgosas e aumento do imunoconteúdo da proteína GFAP) observadas na idade adulta nos animais submetidos ao *status epilepticus*.

Diante destes resultados, podemos constatar que o *status epilepticus* induzido em períodos iniciais do desenvolvimento cerebral pode causar danos ao SNC, alterando seu desenvolvimento e maturação e levando à alterações neuroquímicas, morfológicas e comportamentais na idade adulta.

Parte V. Perspectivas

Considerando que o *status epilepticus* é umas das crises epilépticas de maior incidência na infância e que altera o desenvolvimento e maturação do SNC, torna-se de extrema importância avaliar o efeito de antagonistas de receptores glutamatérgicos ionotrópicos sobre a morte celular e as alterações comportamentais induzidas pelo *status epilepticus*. Pretendemos também investigar de forma mais aprofundada o envolvimento da via de sinalização da PI3K-PKB, bem como os efeitos de ativadores e inibidores desta via sobre o processo de morte celular desencadeado pelo *status epilepticus*.

Visto que alguns trabalhos têm demonstrado que o exercício físico e a exposição a ambientes enriquecido melhoram o desempenho de ratos nas tarefas de memória-aprendizado, é interessante avaliarmos o efeito de tais intervenções não farmacológicas sobre os prejuízos de memória e aprendizado causados pelo *status epilepticus* durante o desenvolvimento do SNC.

Com relação à captação de glutamato, pretendemos investigar, a curto e longo prazo, os efeitos do *status epilepticus* induzido por LiCl-pilocarpina sobre a expressão de transportadores glutamatérgicos em hipocampo e córtex.

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