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PAPEL DA MODULAÇÃO DE GLICOSE HIPOCAMPAL APÓS STATUS EPILEPTICUS INDUZIDO POR PILOCARPINA

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde, da Universidade Federal de Alagoas, em cumprimento às exigências para a obtenção do título de Doutor pela referida Instituição.

Orientador: Prof. Dr. Olagide Wagner de Castro

Coorientador: Prof. Dr. Alexandre Urban Borbely

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Papel da modulação de glicose hipocampal após Status epilepticus induzido por pilocarpina

Tese submetida ao corpo docente do Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Alagoas e aprovada em 3 de agosto de 2020.

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"Tenho posto o SENHOR continuamente diante de mim; por isso que ele está à minha mão direita, nunca vacilarei."

(Salmo 16:8)

“O cavalo prepara-se para o dia da batalha, mas a vitória vem do SENHOR.”

(Provérbios 21:31)

RESUMO

Status Epilepticus (SE) é definido como crises contínuas e autossustentadas, que desencadeia neurodegeneração, disfunção mitocondrial, estresse oxidativo e falha energética. Durante o SE, os neurônios ficam hiperexcitados, levando ao aumento do consumo energético. Sabe-se que a captação de glicose aumenta via cotransportador de sódio e glicose tipo 1 (SGLT1) no hipocampo sob condições epilépticas. Além disso, o suprimento de glicose pode prevenir o dano neuronal causado pelo SE. O propósito do presente estudo foi avaliar o efeito do aumento da disponibilidade de glicose no comportamento das crises límbicas, disfunção da memória, processo de neurodegeneração, atividade neuronal e expressão do SGLT1.Veículo (VEH, salina 0.9%, 1μL) ou glicose (GLU; 1, 2 ou 3mM) foram administrados no hipocampo antes ou depois da pilocarpina (PILO), usada para induzir o SE. A análise comportamental das crises foi feita durante 90 minutos do SE, de acordo com a escala de Racine. Os processos de memória e aprendizagem foram analisados pelo teste de esquiva inibitória. Após 24h do SE, o processo de neurodegeneração, a atividade neuronal e a expressão do SGLT1 foram avaliados em regiões hipocampais e extra-hipocampais por meio da histoquímica de Fluoro-Jade C (FJ-C) e imunofluorescência para cFos e SGLT1, respectivamente. A administração de glicose após a PILO reduziu a gravidade das crises, bem como o número e tempo total das crises límbicas de classes 3-5. Adicionalmente, a modulação de glicose hipocampal protegeu da disfunção da memória causada pelo SE. Similarmente, glicose após a PILO atenuou o número de neurônios FJ+ e cFos+ no hipocampo, subículo, tálamo e áreas corticais. Finalmente, a expressão do SGLT1 foi elevada no hipocampo após o aumento dos níveis de glicose. Estes resultados sugerem que possivelmente a administração intra-hipocampal de glicose protege o cérebro na fase aguda da epileptogênese por meio um importante suporte energético garantido pelo SGLT1.

PALAVRAS-CHAVE: Sodium glucose cotransporter. Epileptogenic. Glucose. Hippocampus.

ABSTRACT

Status Epilepticus (SE) is defined as continuous and self-sustaining seizures, which trigger hippocampal neurodegeneration, mitochondrial dysfunction, oxidative stress and energy failure. During SE, the neurons become overexcited, increasing energy consumption. Glucose uptake is increased via the sodium glucose cotransporter 1 (SGLT1) in the hippocampus under epileptic conditions. In addition, a supply of glucose can prevent neuronal damage caused by SE. We evaluated the effect of increased glucose availability in behavior of limbic seizures, memory dysfunction, neurodegeneration process, neuronal activity and SGLT1 expression. Vehicle (VEH, saline 0.9%, 1μL) or glucose (GLU; 1, 2 or 3mM) were administered into hippocampus before or after pilocarpine (PILO) to induce SE. Behavioral analysis of seizures was performed for 90 minutes during of SE, according to Racine scale. The memory and learning processes were analyzed by the inhibitory avoidance test. After 24 hours of SE, neurodegeneration process, neuronal activity and SGLT1 expression were evaluated in hippocampal and extrahipocampal regions by Fluoro-Jade C (FJ-C histochemistry, c-Fos and SGLT1 immunofluorescence, respectively. The administration of glucose after PILO reduced the severity of seizures, as well as the number of limbic seizures classes 3-5. In addition, modulation of hippocampal glucose protected memory dysfunction followed by SE. Similarly, glucose after PILO attenuated the number of FJ+ and c-Fos+ neurons in hippocampus, subiculum, thalamus and cortical areas. Finally, SGLT1 expression was elevated in hippocampus after increasing glucose levels. These data suggest that possibly the administration of intrahippocampal glucose protects brain in the earlier stage of epileptogenic processes via an important support of SGLT1.

KEYWORDS: Sodium glucose cotransporter. Epileptogenic. Glucose. Hippocampus.

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LISTA DE ABREVIATURAS

μg	Micrograma
μL	Microlitro
2-DG	2-deoxi-D-glicose
AC	Ácido caínico
AEDs	Drogas antiepilépticas
AIP	Córtex insular agranular
ANOVA	Análise de Variância
AP	Antero-posterior
ATP	Adenosina trifosfato
AuD	Córtex auditivo
CA1	Corno de Ammon 1
CA2	Corno de Ammon 2
CA3	Corno de Ammon 3
CEUA	Comissão de Ética no Uso de Animais
CHS	Estimulação hipocampal contínua
CL	Núcleo talâmico centrolateral
CREs	Crises recorrentes e espontâneas
DG	Giro dentado
dH2O	Água destilada
DLG	Núcleo talâmico geniculado lateral dorsal
DS	Subículo
DV	Dorso-ventral
Ect	Córtex ectorhinal
ELT	Epilepsia do Lobo Temporal
EPM	Erro padrão da média
FDG-PET	Tomografia de emissão de prótons de fluorodeoxiglicose
FJ	Fluoro-Jade
fMRI	Imagem de ressonância magnética funcional
GD	Giro denteado
GLU	Glicose
GLUT1	Transportadores de glicose por difusão facilitada tipo 1

GLUT2	Transportadores de glicose por difusão facilitada tipo 2
GLUT3	Transportadores de glicose por difusão facilitada tipo 3
GLUT4	Transportadores de glicose por difusão facilitada tipo 4
GLUT5	Transportadores de glicose por difusão facilitada tipo 5
GLUT8	Transportadores de glicose por difusão facilitada tipo 8
GLUTs	Família dos transportadores de glicose por difusão facilitada
H-PILO	Administração de pilocarpina intrahipocampal
IAT	Teste de esquiva inibitória
ip	Intraperitoneal
KA	Ácido caínico
Kg	Quilograma
LaDL	Núcleo amigdaloide lateral, parte dorsolateral
LPMR	Núcleo talâmico lateral posterior, mediorostral
M1	Córtex motor primário
mg	Miligrama
ML	Medio-lateral
mM	Milimolar
Na	Sódio
NMDA	Receptor ionotrópico de glutamato – *N-methyl-D-aspartate*
PBS	Tampão Fosfato Salina
PFA	Paraformaldeido
PILO	Pilocarpina
Pir	Córtex piriforme
PLEDs	Descargas epileptiformes laterais
PRh	Córtex perirhinal
PVP	Núcleo talâmico paraventricular, parte posterior
PZN	Florizina
RCGU	Utilização de glicose cerebral regional
RSGc	Córtex retrosplenial granular
S1	Córtex somatossensorial
SAL	Salina
SAL+SAL	Ratos que receberam apenas microinjeção de salina
SE	Status Epilepticus

SGLT1	Cotransportador Na^+/ glicose/ água tipo 1
SGLT2	Cotransportador Na^+/ glicose/ água tipo 2
SGLTs	Família dos cotransportadores de glicose acoplados ao sódio
SLC5A1	Gene que codifica a proteína SGLT1
SLC5A2	Gene que codifica a proteína SGLT2
SNC	Sistema nervoso central
SNR	Substância nigra reticular
SSLSE	Status Epilepticus límbico autossustentado
STZ	Estreptozotocina
Suppl	Suplementar
TCA	Ciclo do ácido tricarboxílico
TCA	Ácido tricarboxílico
TLE	Epilepsia do lobo temporal
VEH	Veículo
WDS	Wet dog shake

SUMÁRIO

1 INTRODUÇÃO

A Epilepsia do Lobo Temporal (ELT) é caracterizada por crises parciais e complexas, iniciando-se por lesões ou alterações fisiológicas decorrentes de diversos insultos, tais como *Status Epilepticus* (SE), traumas ou infecções cerebrais. Estes insultos são geralmente seguidos de um período de latência que podem variar entre dias e semanas em modelo animal (Pierson e Liebmann, 1992) e meses a anos em humanos, antes do início das crises recorrentes e espontâneas (CREs) (Sharma et al., 2007; Bae et al., 2010; O'Dell et al., 2012), capazes de promover uma série de eventos neurobiológicos, bem como alterações bioquímicas e histológicas, que incluem fenômenos como neurodegeneração e neurogênese (Andres-Mach et al. 2011; Liefferinge et al., 2013).

Durante a crise epiléptica ocorre o aumento da captação de glicose (Poppe et al., 1997). Sabe-se que a glicose é o principal substrato metabólico utilizado pelo cérebro, sendo capaz de atravessar a barreira hematoencefálica e atingir o líquido extracelular do sistema nervoso central (SNC), tornando-se disponível para neurônios e células da glia (Siesjö, 1978). Por ser uma molécula polar, a glicose não atravessa a bicamada lipídica, assim proteínas carreadoras realizam o seu transporte, como os transportadores de glicose por difusão facilitada (GLUTs) e os cotransportadores de Na+/glicose (SGLTs).

No SNC, o transportador de glicose 1 (GLUT1) é expresso na barreira hematoencefálica e células gliais (Devaskar et al., 1991), enquanto o transportador de glicose 3 (GLUT3), nos neurônios (Mantych et al., 1992). Os SGLTs são transportadores que pertencem à família do gene SLC5A, que aproveitam o gradiente de ions sódio através da membrana plasmática para dirigir a glicose e galactose nas células (Scafoglio et al., 2015; Wright et al., 2011). Os membros mais estudados são SGLT1 e SGLT2, que estão envolvidos no transporte de glicose em regiões especializadas do cérebro (Yu et al., 2013, 2010).

A proteína SGLT1, codificada pelo gene SLC5A1, é responsável por transportar uma molécula de glicose a favor do influxo de dois íons de sódio, incluindo 264 moléculas de água (Sabino-Silva et al., 2010). O SGLT1 é expresso em muitas áreas do cérebro, incluindo as regiões CA1 e CA3 e giro denteado do hipocampo (Poppe et al., 1997; Yu et al., 2013, 2010). Vale ressaltar que a presença de glicose ativa o heterodímero T1R2/T1R3, via adenilatociclase-AMPc-PKA, o que leva ao aumento da expressão do SGLT1 na membrana luminal de enterócitos (Dyer et al., 2003). Além disso, sabe-se que o heterodímero T1R2/T1R3 está expresso no hipocampo e em áreas extrahipocampais (Ren et al., 2009).

O SGLT2, codificado pelo gene SLC5A2, tem sido observado no hipocampo e cerebelo. Os ensaios funcionais com fatias de cérebro de rato sugere que o SGLT2 é responsável por captar 20% do total metil-4-[F-18]fluoro-4-desoxi-D-glucopiranosídeo (Me-

4FDG), um substrato de SGLT altamente específico e não transportado por GLUTs (Yu et al., 2010).

O foco epiléptico induzido por penicilina no córtex frontal promove aumento da captação de [14C]AMG, um substrato de SGLT marcado com isótopos, no cérebro de ratos (Poppe et ai., 1997). Alguns autores sugeriram que o aumento da captação de glicose via SGLTs funciona como um mecanismo de neuroproteção do cérebro epiléptico (Yu et al., 2010; Yu et al., 2013). Além disso, outros autores administraram glicose intraperitoneal e observaram uma diminuição da morte neuronal seguida do SE induzido por cainato. Diante disso, o controle glicêmico pode ser uma estratégia importante para proteger o SNC dos danos causados pelo SE (Schauwecker, 2012).

Dados anteriores do nosso grupo de pesquisa sugeriram que a inibição de SGLTs com florizina pode aumentar a gravidade das crises límbicas do SE. Além disso, demonstramos que este bloqueio foi capaz de aumentar, em diversas regiões do hipocampo, a quantidade de células Fluoro-Jade positiva (FJ+), que estão em processo de degeneração (Melo et al., 2016). Em conjunto, nossos dados corroboram a literatura (Poppe et al., 1997; Yu et al., 2010; Yu et al., 2013), indicando que os SGLTS podem contribuir com a sobrevivência dos neurônios diante das crises epilépticas. No entanto, os efeitos de substâncias que promovam o aumento de SGLTs no cérebro de ratos sob condições epilépticas foram estudados pela primeira vez.

Diante disso, o objetivo do presente estudo foi avaliar o papel do controle glicêmico hipocampal na fase aguda da epileptogênese. Por isso, administramos glicose diretamente no hipocampo antes ou após a infusão intra-hipocampal de pilocarpina (PILO). Inicialmente, investigamos a quantidade total de WDS, o tempo e as classes das crises límbicas, a fim de detectar se o aumento da disponibilidade de glicose interfere na gravidade das crises. Usando o método de histoquímica de Fluoro-Jade C (FJ-C) e imunofluorescência de c-Fos, respectivamente, analisamos também o processo de neurodegeneração e atividade neuronal no hipocampo e em áreas adjacentes após 24h de SE induzida por PILO seguido de infusão de glicose. Além disso, avaliamos a memória e os processos de aprendizagem por meio do teste de esquiva inibitória, a fim de observar se a modulação de glicose protege contra o déficit cognitivo causado pelo SE. Finalmente, a translocação de SGLT1 foi analisada por imunofluorescência para detectar se está relacionada aos efeitos do controle glicêmico do hipocampo.

2 ARTIGO 1

Modulation of glucose availability and effects of hypo- and hyperglycemia on status epilepticus: what we do not know yet?

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ABSTRACT

Status epilepticus (SE) can lead to serious neuronal damage and act as an initial trigger for epileptogenic processes that may lead to temporal lobe epilepsy (TLE). Besides promoting neurodegeneration, neuroinflammation and abnormal neurogenesis, SE can generate an extensive hypometabolism in several brain areas and, consequently, reduce intracellular energy supply, such as adenosine triphosphate (ATP) molecules. Although some antiepileptic drugs show efficiency to terminate or reduce epileptic seizures, approximately 30% of TLE patients are refractory to regular antiepileptic drugs (AEDs). Modulation of glucose availability may provide a novel and robust alternative for treating seizures and neuronal damage that occurs during epileptogenesis, however, more detailed information remains unknown, especially under hipo- and hyperglycemic conditions. Here we review several pathways of glucose metabolism activated during and after SE, as well as the effects of hypo- and hyperglycemia in the generation of self-sustained limbic seizures. Furthermore, this study suggests the control of glucose availability as a potential therapeutic tool for SE.

KEYWORDS: Glucose. Hypometabolism. Hypoglycemia. Hyperglycemia. Status Epilepticus. Epilepsy.

2.1 Introduction

Status epilepticus (SE) is a clinical emergency characterized by either a continuous self-sustained seizure or a sequence of short seizures with no return to baseline, unleashing a high mortality rate (Cole et al., 2002; Leite et al., 1990; Meldrum and Brierley, 1973; Meldrum and Horton, 1973; Mohapel et al., 2004; Sánchez and Rincon, 2016; Sloviter, 1999; Turski et al., 1983a). SE can be convulsive or nonconvulsive (Castro et al., 2011; Islas-Espinoza et al., 2018; Kršek et al., 2004; Melo et al., 2016; Pari et al., 2014; VanLandingham and Lothman, 1991a; Wang et al., 2008; Wong et al., 2003) and it is capable of promoting neuronal death, gliosis and wide molecular changes in several brain areas (Castro et al., 2011; Leite et al., 1990; Melo et al., 2016; Rami et al., 2018; Trinka and Kälviäinen, 2017). Furthermore, SE leads to temporal lobe epilepsy (TLE), which is characterized by spontaneous recurrent seizures, abnormal synaptic reorganization, mossy fiber sprouting, hippocampal neurodegeneration and neurogenesis (Furtado et al., 2002; Sharma et al., 2007; Van Liefferinge et al., 2013; Castro et al., 2017; Upadhya et al., 2018). SE induction in rodents by electrical or chemical stimulation have been used to model epileptogenesis process and TLE clinical features (Castro et al., 2011; Cavalheiro et al., 1987; Danzer et al., 2009; Hester et al., 2016; Islas-Espinoza et al., 2018; Leite et al., 2002, 1990; Melo et al., 2016; Mishra et al., 2015; Rodrigues et al., 2005; Turski et al., 1984, 1983a, 1983b, VanLandingham and Lothman, 1991a, 1991b). Local or systemic administration of the pilocarpine (PILO) or kainic acid leads to a pattern of repeated limbic seizures and/or SE, which can endure for many hours (De Furtado et al., 2002; Leite et al., 2002, 1990, Turski et al., 1984, 1983a).

Recent [^{18}F]fluorodeoxyglucose positron emission tomography (FDG-PET) studies with patients and animal models showed that SE induces secondary hypometabolism in the epileptogenic areas, including hippocampus, cortex and striatum (Ding et al., 2014; García-

García et al., 2017; Goffin et al., 2009; Guo et al., 2009; Kumar and Chugani, 2013; Lee et al., 2012; Shiha et al., 2015; Wong et al., 2010). Secondary hypometabolism has also been associated to specific patterns of SE in studies with humans and animal models (Farooque et al., 2017; Fernández-Torre et al., 2006; García-García et al., 2017; Jupp et al., 2012). During an epileptic seizure or SE, the overexcited neurons must increase glucose uptake (Chugani and Chugani, 1999; McDonald et al., 2017; Vielhaber et al., 2003) above organism's supply capacity, resulting in adenosine triphosphate (ATP) deficit in the nervous tissue (Schauwecker, 2012), where energy consumption is much higher than in several other body tissues (Lundgaard et al., 2015). Glucose is taken up into brain cells by two major groups of transporters: 1) glucose transporters (GLUTs) for facilitated diffusion and 2) sodium/glucose cotransporters (SGLTs) for its secondary active transport (Maher et al., 1996; Mantych et al., 1992; Poppe et al., 1997; Yu et al., 2013, 2010; Zhao et al., 2010). Glucose transporter 1 (GLUT1) is expressed in both blood–brain barrier and glial cells (Devaskar et al., 1991), while glucose transporter 3 (GLUT3) presents high glucose affinity and transport capacity in neurons (Dakic et al., 2018; Maher et al., 1991; Mantych et al., 1992; Simpson et al., 2008, 2007). Sodium/glucose cotransporters 1 and 2 (SGLT1 and SGLT2) have been observed in hippocampus (Yu et al., 2013, 2010). Despite the evidence of glucose availability modulating brain damage, it has been difficult to define the role of glucose transporters and associated glucose metabolism in isolated epileptic seizures or during and after SE.

Here, we review recent advances regarding regulation of glucose supply during self-sustained epileptic seizures. Our purpose was to survey the main data associated with the modulation of glucose in seizures, to discuss the possible pathways that could explain the alterations on seizures susceptibility in hypo- and hyperglycemic conditions. Furthermore, we summarize these findings and draw an overview to contribute for new insights in treatment

strategies that could effectively reduce neuronal damage associated with TLE, in the absence of counteractive side effects.

2.2 Intracellular metabolism of glucose and SE

The physiological pattern of the brain requires high amounts of energy supply, in constant demand, comprising an average of 20% of the body's energetic consumption (Magistretti and Allaman, 2015). Glucose is converted into glucose-6-phosphate (glucose-6P) immediately after been transported to the intracellular environment. Glucose-6P is processed through glycolysis, resulting in two molecules of pyruvate which are metabolized during the tricarboxylic acid cycle and the oxidative phosphorylation in the mitochondria (Allaman and Magistretti, 2013).

Functional brain imaging techniques, such as FDG-PET and functional magnetic resonance imaging (fMRI), allowed for the conduction of integrative studies between regional brain energy metabolism (energy delivery and use) and neural cell activity (Logothetis et al., 2001; Magistretti, 2000; Raichle, 1998, 1983). FDG-PET detects alterations in regional cerebral blood flow, as well as in glucose and oxygen consumption (Frackowiak et al., 1980; Pari et al., 2014; Phelps et al., 1979; Zhang et al., 2015); while fMRI provides data on regional blood oxygenation levels (Ogawa et al., 1992). It was demonstrated that cerebral glucose metabolism is altered in epilepsy as well as in other neurological disorders (Bathina and Das, 2018; Contreras and Gutiérrez-García, 2017; Galeano et al., 2018; Kang et al., 2018; Liguori et al., 2019; Piquet et al., 2018).

2.2.1 Glucose metabolism in clinical studies

SE promotes a sharp increase of regional cerebral blood flow and oxygen consumption correlated with enhancement in glucose utilization (Franck et al., 1986). In an

11-year-old girl, an intense hypometabolism was observed in most of the left hemisphere, six weeks, and eight months after one SE episode. The right hemisphere, however, showed a heterogeneous increase in the metabolic rate of glucose only at eight months after the SE occurred (Van Bogaert et al., 1994). Similarly, a severe hypometabolism was observed in the left hemisphere of a 48-year-old right-handed man with Alien hand syndrome after a complex partial SE. Notably, the periodic lateralized epileptiform discharges (PLEDs) generated during the SE were only detected in the left hemisphere, especially in areas such as basal ganglia and thalamus (Kim et al., 2012). Moreover, a patient presenting right hemispheric PLEDs due to SE showed right hypometabolism in the temporal, parietal and occipital lobes (Sakakibara et al., 2014). The same pattern has been seen in an elderly patient with permanent sensorimotor dysphasia after SE (Fernández-Torre et al., 2006). These authors suggest that both lateralized seizures and hypometabolism are directly correlated with the pattern of neuronal death observed in hippocampal formation and adjacent areas (Castro et al., 2011; Melo et al., 2016; Wasterlain et al., 1993).

In other clinical studies, FDG-PET showed scattered areas of regional hypometabolism in pediatric and adult patients with refractory and nonconvulsive SE (Barros et al., 2014; Duane et al., 2004; Pari et al., 2014). A recent study showed hypometabolism in cortical areas, such as frontal, parietal and posterior cingulate cortices, and hypermetabolism in thalamic, caudate and dentate nuclei, and cerebellar vermis, of a 17-year-old girl with absence SE (Shimogori et al., 2017). According to the authors, these findings indicate a relevance of the thalamus in absence SE, as well as modulation of cortical and cerebellar regions associated with the control of epileptiform discharges (Figure 1).

2.2.2 Glucose metabolism in animal models

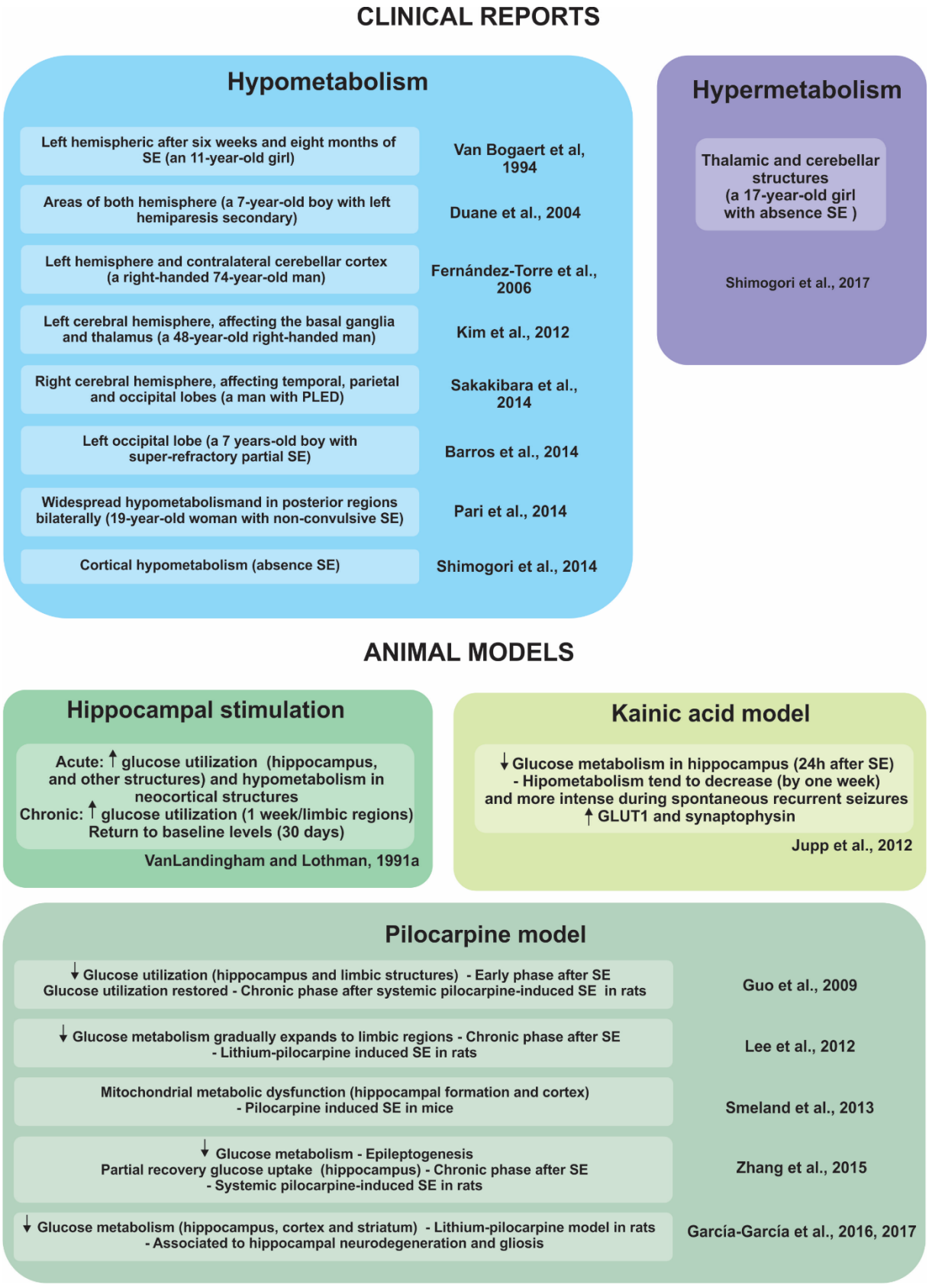
Since the 1990s, distinct animal models of SE have shown alterations in the metabolism of cerebral glucose during or after self-sustained seizures (VanLandingham and Lothman, 1991a, 1991b). In 1991, VanLandingham and Lothman, using continuous hippocampal stimulation (CHS), developed a nonconvulsive model of self-sustained limbic SE (SSLSE). This model was used to evaluate the regional cerebral glucose utilization (RCGU) after 90 minutes of CHS (VanLandingham and Lothman, 1991a, 1991b). RCGU was observed in acute (1 hour after induction) and chronic (1 week or 1 month) periods. During the acute period, RCGU was elevated in some brain regions, such as hippocampus, retrohippocampal area, and limbic and nonlimbic structures, while hypometabolism was observed in neocortical structures. In the chronic period, one week after SE, an increase of RCGU was recorded in some limbic regions, but RCGU levels returned to baseline at 30 days after SE (VanLandingham and Lothman, 1991a). On another study, rats were submitted to the same protocol with or without hippocampal commissurotomies, in order to test whether hippocampal commissures were necessary for the initiation and maintenance of SSLSE. These studies showed RCGU was bilaterally and symmetrically enhanced in hippocampus and other areas (retrohippocampal, limbic and nonlimbic structures) in the group with preserved commissures, while both groups presented bilateral hypometabolism in neocortical regions (VanLandingham and Lothman, 1991b).

Glucose hypometabolism was also shown in rodent SE models where SE was induced by kainic acid or PILO. In lithium-PILO model of SE in rats, a severe glucose hypometabolism occurred in hippocampus, cortex and striatum during epileptogenesis, and this alteration was associated to other pathological changes, such as hippocampal, atrophy, neuronal death and gliosis (García-García et al., 2017, 2016). This hypometabolism was

partially recovered in the chronic phase, maybe due to increased cell number associated with astrogliosis (Zhang et al., 2015). Similarly, in the early phase after systemic PILO-induced SE, glucose utilization was reduced severely in limbic structures, such as hippocampus, but glucose levels were restored during the chronic period (Guo et al., 2009). Controversially, other authors have reported that reduced glucose metabolism gradually expands to limbic regions in the chronic phase (Lee et al., 2012). Furthermore, in the kainic acid-induced SE model in rats, FDG-PET demonstrated hippocampal hypometabolism after 24h of SE, which tended to decrease in the early phase of epileptogenesis and then stood out again with the emergence of CREs, what was accompanied by an increase in the expression of GLUT1 and synaptophysin in the same area (Jupp et al., 2012). These authors concluded that the glucose hypometabolism precedes neuronal loss and CRE. In addition, in a mouse model of PILO-induced SE, mitochondria were metabolically dysfunctional in hippocampal formation and cortex, 3.5 to 4 weeks after SE (Smeland et al., 2013). Moreover, a recent study showed more than 40% increase in glucose uptake in rat hippocampus after 4 and 24 h of SE induced by lithium-PILO, later returning to baseline levels (Bascuñana et al., 2018). Diversely, these same authors observed a hippocampal hypometabolism in chronic epileptic rats (Figure 1).

Taken together, these clinical and experimental findings support the idea that SE compromises intracellular glucose metabolism, which may contribute to the activation of inflammatory mechanisms, neuronal loss and gliosis, and may also predispose the brain to develop epilepsy (Zilberter and Zilberter, 2017). Therefore, restoring glucose metabolism during and after continuous self-sustained seizures may be an effective way of dealing with epileptogenesis and its complications.

Figure 1. Illustration of clinical reports and animal models on hypo- and hypermetabolism.



Fonte: Autor

2.3 Hypo- and hyperglycemic mechanisms associated with SE generation

Metabolic disorders are often associated with epileptic seizures; however their relationship is poorly understood. The importance of glucose balance has emerged from studies demonstrating that epileptic seizures can be accentuated under conditions of hyper- or hypoglycemia (Schauwecker, 2012).

2.3.1 Hypoglycemia and SE

Hypoglycemia is a clinical condition characterized by low plasma glucose concentration affecting both diabetic and non-diabetic patients. Hypoglycemia and hypoglycemic seizures may occur due to an increase in the amount of insulin in the plasma, being common in patients who are under treatment with insulin (Falip et al., 2014).

Neuronal excitation is tightly tied to brain energy metabolism. Severe hypoglycemia represents a serious threat for normal brain metabolism causing unbalance between inhibitory and excitatory neuronal networks, what leads to increased seizure susceptibility and risk for brain damage. The functional and structural injuries induced by severe hypoglycemia may persist when normal glucose levels are restored, leading to permanent cognitive dysfunction, EEG abnormalities, and predisposition for unprovoked seizures (Hyllienmark et al., 2005).

According to several studies, there is a not fully understood relationship between hypoglycemia and epileptic seizures (Chapman et al., 1987; Chin et al., 2006; Leckie et al., 2005; Maheandiran et al., 2013; Moseley et al., 2013). It is well established that low glucose concentrations can change cortical excitability (Verrotti et al., 2012) and stimulate glutamate release, inducing cerebral hyperexcitability and, consequently, NMDA receptor-mediated excitotoxicity (McCall, 2004), with hippocampus showing particular susceptibility to these effects (Auer, 2004).

Studies aiming to describe epileptic manifestations associated with hypoglycemia have been carried out since the 1980s. Sapolsky and Stein (1989), following a kainic acid-induced SE in rats, showed that hippocampus of hypoglycemic animals presented more damage when compared to normoglycemic or hyperglycemic ones, suggesting that limited energy compromises the survival of neurons in seizures. Severe acute cases of hypoglycemia in humans as a complication of therapy for insulin-dependent diabetes mellitus (Leckie et al., 2005) or due to the excessive consumption of alcohol (Hart and Frier, 1998) are often associated with neurological side-effects (Limbert et al., 1993; MacLeod et al., 1993), resulting frequently in generalized seizures (Davis et al., 1997; Malouf and Brust, 1985). A study evaluated causes of symptomatic convulsive SE in children, and found that most of them had metabolic disorders, such as electrolyte imbalance, hypoglycemia, hypocalcemia, or hypomagnesemia (Chin et al., 2006). Other authors found that 11 % of adult patients with SE presented a metabolic disfunction as cause of convulsive SE (Towne et al., 1994). Unfortunately, these studies did not separate the isolating effect of blood glucose from other metabolic disturbances (Neil and Hemmen, 2011). In rats, insulin-induced hypoglycemia also leads to generalized seizures (Chapman et al., 1987; Panickar et al., 1998).

There is relative difficulty in finding studies that have evaluated the relationship between hypoglycemia and epilepsy in animal models. One such study investigated this relationship by evaluating the modulation, by glycemic levels, of kainate-induced seizure susceptibility, as well as its neuropathological consequences (Schauwecker, 2012). These authors found no difference in the severity of seizures between hypoglycemic mice and other groups, as evidenced by similar latency at the onset of the first severe seizure and its duration. However, their results demonstrated that mice with insulin-induced hypoglycemia had an increase in hippocampal neurodegeneration, with significant loss of cells in three hippocampal subfields (dentate hilus, CA3 and CA1).

Diabetes is not directly related to seizures, however the hypoglycemic conditions eventually generated in this process may be associated. Seizures can occur in diabetic and non-diabetic rats (Maheandiran et al., 2013), and seizures may be associated with acute hypoglycemia, as well as the death of animals is related to the frequency of seizures and not to blood glucose levels. This suggests that hypoglycemia would be a predisposing factor to seizures, therefore a morbidity and not a mortality factor. Furthermore, the same authors showed that severe hypoglycemia as a precondition for seizures was associated with animal deaths, and suggest that this is probably due to the brainstem involvement in the seizures, which may affect the cardiorespiratory system and lead to mortality (Maheandiran et al., 2013; Moseley et al., 2013).

Unlike animal model studies, in the last years several, mostly cohort, studies in human patients have been conducted to elucidate the relationship between hypoglycemia and epilepsy. In 2015, Halawa and colleagues investigated an association between different levels of hypoglycemia and the occurrence of epileptic seizures in patients without previous diagnosis of epilepsy; as a result, coma was reported as the neurological symptom mostly caused by hypoglycemia, with convulsions being a rare event. In addition, other authors evaluated more than 2,000 patients with type 1 diabetes and identified an increased risk for epilepsy in patients with type 1 diabetes with a history of hypoglycemia (16,5%), compared to patients without hypoglycemia (2,67%) (Chou et al., 2016). Moreover, an elegant study showed that seizures associated with hypoglycemia occurred in 90 of 170 patients aged 0 to 4 years; in 68% of the patients, the first hypoglycemic seizure was brief and fast, whereas the remaining 32% had more severe conditions and evolved to SE or coma (Gataullina et al., 2015). The evaluation of such events according to the age of the patients demonstrated that brief seizures were more frequent than SE in both age groups studied: 63% versus 37% in neonates and 71% versus 29% in infants/children; in fact, blood glucose levels seem to be

critical for the type of seizures presented by the patients, as they were significantly lower in SE than in brief seizures events (Gataullina et al., 2015).

Comparing neonatal period with childhood, the sequelae of SE are more intense in early ages (Gataullina et al., 2015). Hypoglycemia in the neonatal period is relatively more common than in the older age groups and is considered a possible cause of seizures in the first year of life (Cross, 2015). As we can see in the study conducted by Gataullina et al. (2015), in which the relationship between the first hypoglycemic event and seizures was observed, most of the association occurred below 3 years of age. In the first years of life, seizures may initially appear as spasms (Kumaran et al., 2010).

It is important to note that SE is not necessarily associated with systemic factors, including hypoglycemia, in human patients, therefore hypoglycemia does not work as an etiological agent of SE (Fujikawa, 1996). Although this study was performed through medical records and electroencephalograms of only three patients without systemic complications who had died between 11 and 27 days after triggering SE, other authors found a weak correlation between hypoglycemia and epileptic seizures (Falip et al., 2014; O'Connell et al., 2008). For example, in a case of 229 diabetic patients, only 2 presented hypoglycemia and epileptic seizures concomitantly, representing a total of 0.8% of the diabetic patients evaluated (Falip et al., 2014) (Figure 2).

2.3.2 Hyperglycemia and SE

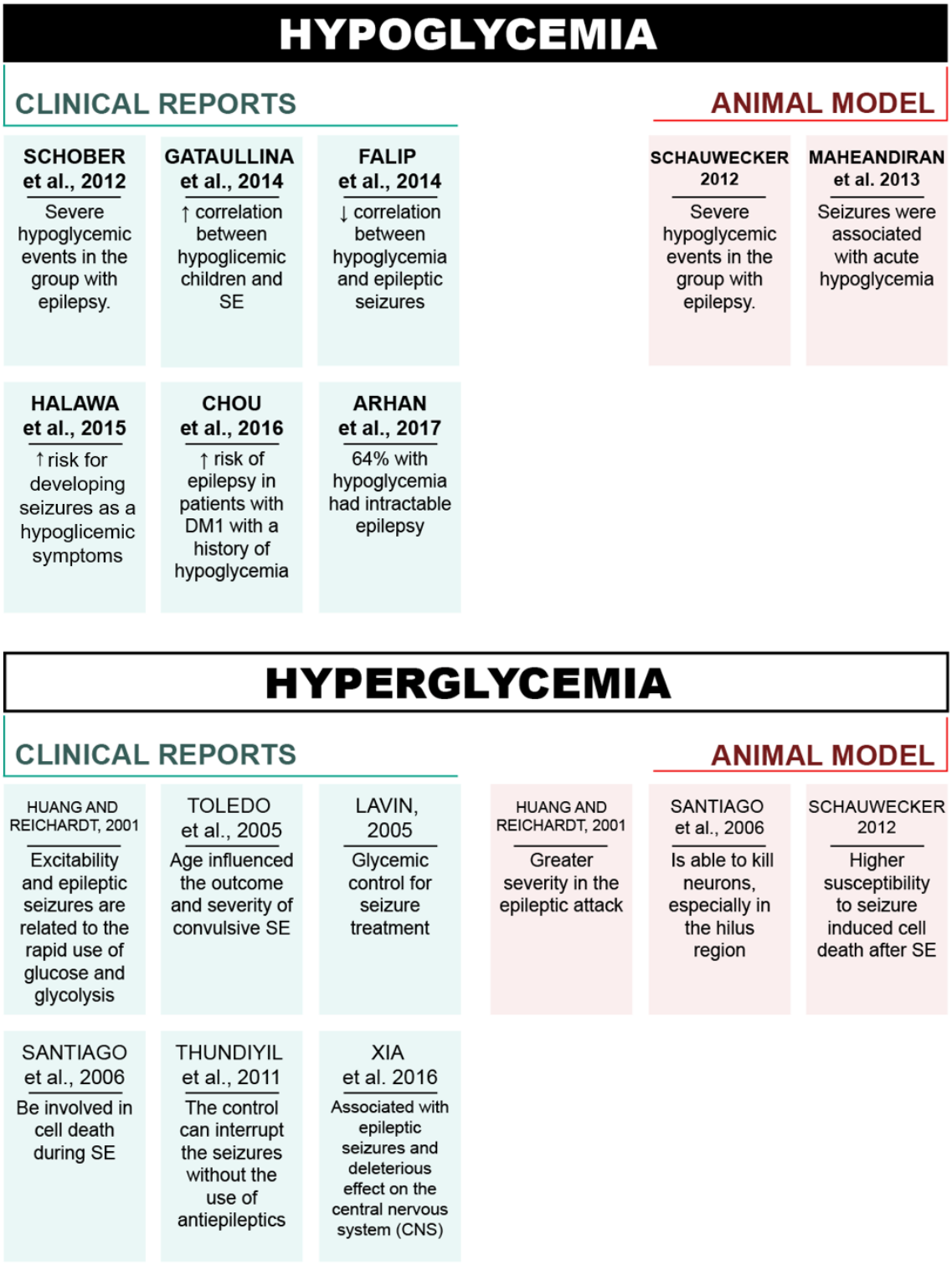
Among metabolic disturbances, hyperglycemia has been frequently associated with deleterious effects on the CNS induced by epileptic seizures (Xia et al., 2016). Both type I and type II diabetes increase the susceptibility to epileptic seizures in these patients, emphasizing the importance of glycemic control for seizure treatment (Lavin, 2005; Lee et al., 2014).

Neuronal excitability and epileptic seizures are related to the rapid use of glucose and glycolysis (Greene et al., 2003; Huang and Reichardt, 2001). Hyperglycemia may also be associated with other factors to become the cause of SE. It is known that age can influence the outcome and severity of convulsive SE (Rathakrishnan et al., 2009; Toledo et al., 2005).

As in the clinical studies, it has been shown that diabetic animals frequently develop seizures, depending on the severity of an ischemic insult and blood glucose concentration (Li et al., 1998; Xia et al., 2016). Additionally, rats with diabetic hyperglycemia had a higher severity of seizures which induced a greater damage of the hippocampus after SE, followed by a higher mortality rate, or worsening of the cognitive capacity for learning in surviving animals (Huang and Reichardt, 2001). This corroborates with another study that showed an increase of kainate-induced cell loss after SE in mice with non-ketotic hyperglycemia and diabetes-induced hyperglycemia (Schauwecker, 2012).

Currently, there are some studies that point out that hyperglycemia can facilitate the entry of glucose into the brain and be involved in cell death during SE (Santiago et al., 2006), also triggering morphological changes at the presynaptic terminals of mossy fibers that play important roles in increasing neuronal damage. Acute and chronic hyperglycemia produces increased susceptibility to excitotoxic cell death, the same effect observed as a consequence of seizures (Magariños and McEwen, 2000; Schauwecker, 2012). High levels of glucose in the brain may increase the amount of ATP, facilitating ATP-dependent brain reactions. These results show that hyperglycemia by itself is able to kill neurons, especially in the hilus region of hippocampus (Santiago et al., 2006). On the other hand, infusing a certain concentration of glucose after kainic acid-induced SE can be profoundly neuroprotective against seizure-induced neuronal damage (Santiago et al., 2006; Schauwecker, 2012). In other words, this brain glucose modulation can protect neurons of specific hippocampi regions (Figure 2).

Figure 2. Illustration of clinical reports and animal models on hypo- and hyperglycemia.



Fonte: Autor

2.4 Glucose transporters in the brain and their involvement in SE modulation

Although the brain accounts for only 2% of body mass, it receives 15% of cardiac output and 25% of glucose supply, being responsible for 20% of the organism's oxygen consumption (Sokoloff, 1981). Glucose is transported through the blood-brain barrier to the cerebrospinal fluid via glucose transporters or by the capillaries of the circumventricular organs which do not have tight junctions nor exert barrier properties (Magistretti and Allaman, 2015; Rahner-Welsch et al., 1995; Young and Chung, 1990; Zeller et al., 1996). Glucose passes through the cell membrane by a specific transport system, which includes two types of glucose transporters: 1) the facilitated diffusion GLUTs that transport glucose in favor of its concentration gradient and 2) the SGLTs that transport glucose in favor of the sodium concentration gradient (Sabino-Silva et al., 2010).

2.4.1 Glucose transporters

GLUT1 is expressed in the basal and luminal membranes of the blood-brain barrier endothelial cells, as well as in astrocytes and cell bodies of neurons, but it has not been described in microglia (Devaskar et al., 1991). GLUT3 is the most abundant in the brain and is expressed in neurons, mainly in axons and dendrites, having a transport capacity five times greater than that of GLUT1 (McEwen and Reagan, 2004). Furthermore, GLUT3 adapts to the demands of neuronal metabolism (Maher et al., 1996). Currently, it is known that other types of GLUTs are expressed in the brain. GLUT2 protein is present in hypothalamic neurons and serves as a glucose sensor in the regulation of food intake (Levin et al., 2001); moreover, GLUT2 regulates synaptic activity and contributes to neurotransmitters release in hippocampal neurons. Besides, GLUT5 has been described only in microglia as a hexose

transporter and its regulation is still poorly understood. Finally, GLUT4 and GLUT8 are insulin-regulated glucose transporters and these transporters, although expressed in cell bodies of cortex and cerebellum neurons, are mainly found in the hippocampus and amygdala (Jurcovicova, 2014).

2.4.2 Sodium/glucose cotransporters

SGLTs are transmembrane proteins that contribute to cellular homeostasis by performing secondary active glucose transport in favor of the electrochemical gradient of sodium ions. These proteins were initially identified in the brush border membrane of enterocytes and in the proximal kidney tubule cells (Wright et al., 2011). SGLT1 is encoded by the SLC5A1 gene and is composed of 14 transmembrane segments, whose N-terminal face is directed towards the interstitial and the C-terminal face is anchored inside the plasma membrane (Wright and Turk, 2004). A powerful role of water transport was associated with SGLT1, once the stoichiometric relationship of transport capacity was observed to be 2 Na^+: 1 glucose: 264 H_2O molecules (Zeuthen, 2000). Sodium ions initially bind to the extracellular side of SGLT1, promoting a conformational change that allows glucose to attach to the binding site. A new conformational change of the protein allows the release of sodium ions and glucose in the intracellular environment, after what the transport cycle is completed, allowing for the return of the protein to its initial conformation (Wright et al., 2011). SGLT1 is mainly expressed in the intestine, but is also present in the kidneys, salivary glands, trachea, skeletal muscle, heart, liver, testis, prostate and brain (Balen et al., 2008; Poppe et al., 1997; Takata and Kasahara, 1992; Wright et al., 2011). Recently, expression of SGLT1 has been shown in various regions of the central nervous system, such as hippocampus (CA1 and

CA3), parietal and frontal cortices, putamen, paraventricular nucleus of the hypothalamus, amygdala and in the Purkinje cells of the cerebellum (Yu et al., 2013).

SGLT2 isoform is encoded by the SLC5A2 gene and carries the transport of only one sodium ion for each glucose molecule (Zhao and Keating, 2007). SGLT2 has been observed in the hippocampus and cerebellum. Functional assays with mouse brain slices suggest that SGLT2 is responsible for capturing 20 % of total methyl-4- [F-18] fluoro-4-deoxy-D-glucopyranoside (Me-4FDG), a highly specific substrate of SGLT not transported by GLUTs (Yu et al., 2010).

2.5 Functional role of brain SGLTs regulation in limbic seizures and neurodegeneration after PILO-induced SE

In conditions of normoglycemia and adequate oxygen perfusion, glucose transport is probably mediated by GLUT3 and facilitated glucose diffusion may be sufficient for the energy supply of neurons (Wright et al., 2011). However, reality may be different when energy supply is reduced and/or energy consumption is enhanced in pathological situations such as ischemia, hypoxemia, hypoglycemia or epileptic seizures. The concentration of glucose in the firing microenvironment of the neurons can decrease greatly, being lower than the Km value of GLUT3 (Poppe et al., 1997). These authors induced an epileptic focus with penicillin in the frontal cortex and observed increased uptake of glucose via SGLTs, reflecting the upregulation of this protein in neurons. These findings show that on a low glucose concentration, as occurs during seizures, SGLTs may be essential for the survival of neurons.

Few studies have associated the SGLTs in SE modulation (Melo et al., 2016; Poppe et al., 1997; Yu et al., 2013, 2010). Our group has recently demonstrated for the first time an intrinsic association of SGLTs with neuronal survival during limbic self-sustained seizures

induced by intrahippocampal PILO microinjection (Melo et al., 2016). In this study, SGLTs were blocked by intrahippocampal administration of phlorizin (PZN), a non-specific inhibitor of SGLTs, 30 minutes prior to PILO administration. Inhibition of SGLTs increased the number of wet dog shakes, that occur during SE and can serve as an indicator of SE severity. In addition, aggravated self-sustained limbic seizures occurred during the 90 minutes of SE, showing a higher frequency of the most severe behaviors described by the Racine's scale (class 5) (Racine, 1972) in the PZN group. These behavioral findings indicate that the blockage of SGLTs intensify limbic seizures during SE.

Additionally, 24h after SE, the PZN increased the number of Fluoro-Jade positive neurons, a marker of neural cells in degeneration (Castro et al., 2011; Nascimento et al., 2012), in the regions of the dentate gyrus (DG), the hilus of the DG, CA3 and CA1 of hippocampus (Melo et al., 2016). Taken together, these behavioral and histopathological results support the idea that SGLTs are fundamental tools in low glucose concentration and high metabolic demand conditions, such as during prolonged limbic seizures, thus protecting neurons against degeneration.

2.6 New insights for action mechanism of glucose in the SE

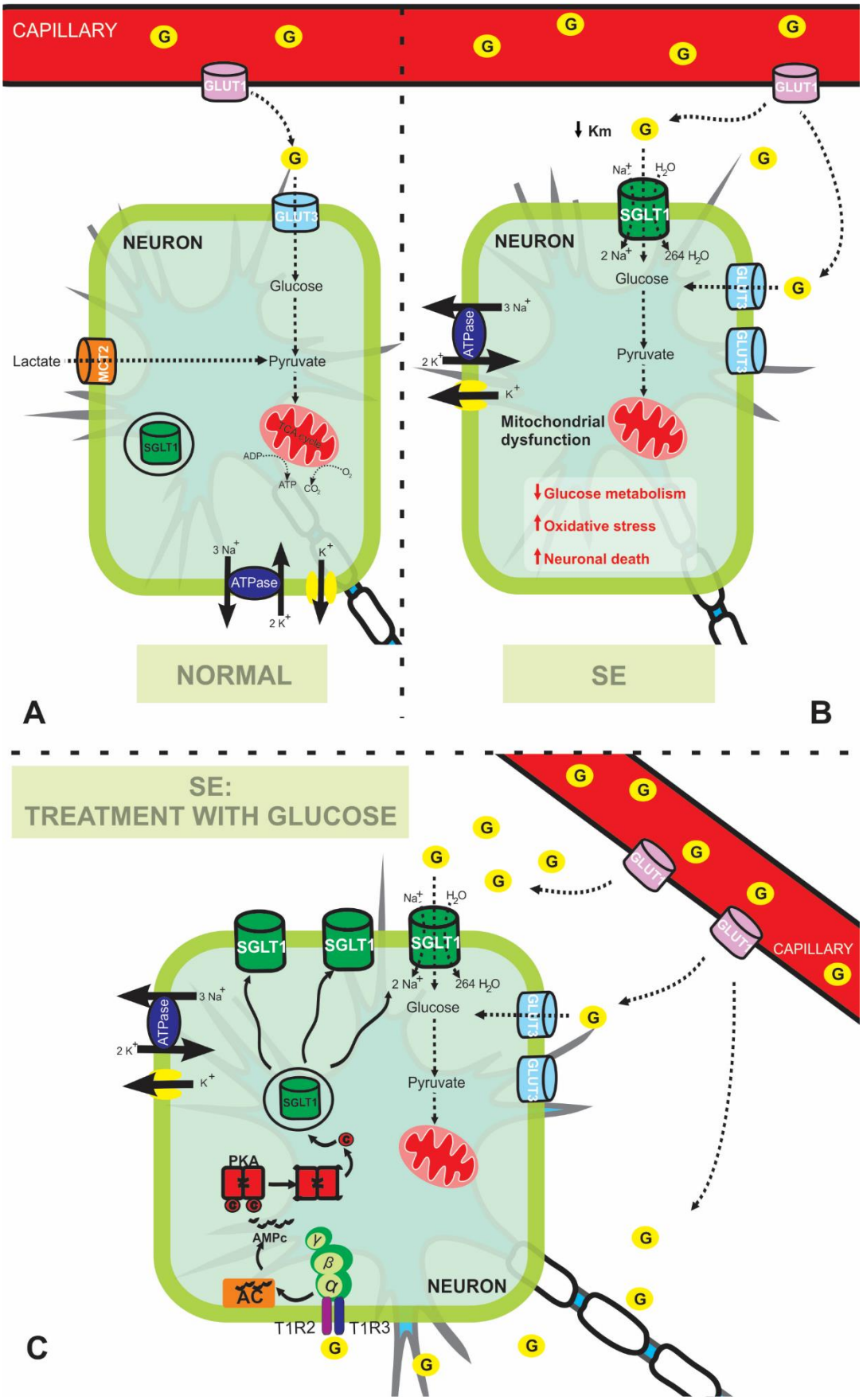
Under normal physiological situation, glucose crosses the blood-brain barrier through GLUT1 and can enter directly into neurons through GLUT3. Glucose is metabolized in cytoplasm by conversion into molecules of pyruvate, which pass through the tricarboxylic acid cycle and oxidative phosphorylation in mitochondria (Magistretti and Allaman, 2015) (Fig. 3A).

On the other hand, during prolonged epileptic seizures, such as seen in SE, glucose metabolism is compromised, which is directly associated with mitochondrial dysfunction, as well as increased reactive oxygen species levels. This metabolic deficit impairs cell survival,

aggravating cell death rates. In an attempt to protect neurons against neuronal death, according to some authors (Melo et al., 2016; Yu et al., 2013, 2010), the affected brain areas increase uptake of glucose via SGLT1 (Poppe et al., 1997), possibly by enhancing the expression of this transporter. Although greater GLUT3 expression also occurs during SE (Jupp et al., 2012) to support the increased energy demand and glucose metabolism (Barros et al., 2014; García-García et al., 2017, 2016; Shimogori et al., 2017; Zhang et al., 2015), SGLT1 expression is necessary in order to transport glucose in lower concentrations, that are below the Km of GLUT3 (Poppe et al., 1997). Therefore, increased expression of SGLT1 may function as an additional support to GLUT3, contributing to the survival of neurons in hypoglycemic conditions (Fig. 3B).

So far, control of glucose availability by modulating their transporters may be a form of protection against damage from SE. It is known that glucose sensors, such as T1R2/T1R3 heterodimer, are expressed in the hippocampus (Ren et al., 2009) and these sensors transduce signals for the translocation of SGLTs into enterocytes (Dyer et al., 2003). Our hypothesis is that increased hippocampal glucose concentration activates a higher SGLT translocation in neurons via T1R2/T1R3, protecting the neurons from degeneration (Fig. 3C).

Figure 3. Schematic drawing on glucose modulation during SE.



In a physiological condition, glucose enters neurons through GLUT3, and it is metabolized to pyruvate, following the citric acid (TCA) cycle to generate ATP (A). However, neuronal hyperexcitability associated with hypermetabolism occurs during SE settlement, activating a compensatory pathway mediated by SGLTs' translocation to transport glucose in lower concentrations and attempt to protect neurons against degeneration (B). As this epileptogenic insult is continuous and self-sustaining, a mitochondrial dysfunction is triggered followed by oxidative stress, culminating in excitotoxic neuronal death. Possibly, increased SGLTs' translocation through modulation of brain glucose levels may protect neurons in the acute phase of epileptogenesis (C). Fonte: Autor

2.7 Conclusion and future perspectives

During the early epileptogenesis, shortly after SE, glucose metabolism is elevated, suffering a significant decrease in the chronic phase (Bascuñana et al., 2018). Furthermore, glycemic disorders may increase the susceptibility to the genesis of epileptic seizures. As seen previously, hypo- and hyperglycemia can function as crucial factors for the SE generation, although the intrinsic pathophysiological mechanisms responsible for this association remain unclear. In addition, hypo- and hyperglycemia are able to accentuate SE-induced hippocampal damage in animal models (Schauwecker, 2012). Interestingly, these authors showed that adequate glucose supply protects hippocampal cells against seizure-induced excitotoxic cell death. Considering these, regulation of glucose availability appears to be a promising pathway capable of attenuating the severity of seizures as well as the seizure-induced brain damage in the earlier phase of epileptogenesis generated by SE. Likewise, the intracellular mechanisms of hypo- and hyperglycemia and glucose modulation associated with SE as possible therapeutic targets need to be further investigated.

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Conflicts of Interest

There is no conflict of interest.

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3 OBJETIVOS

3.1 Geral

Analisar o efeito da modulação de glicose hipocampal após *Status Epilepticus* induzido por pilocarpina.

3.2 Específicos

Utilizando animais controle e com SE, pré-tratados ou tratados com salina ou glicose, propomos os seguintes objetivos específicos:

1) Identificar o período de latência para o SE;
2) Analisar o número de Wet Dog Shake total, antes e durante o SE;
3) Avaliar as diferentes classes das crises epilépticas durante o SE;
4) Determinar a consolidação da memória de longo prazo após o SE;
5) Descrever o padrão de neurodegeneração em regiões hipocampais e extra-hipocampais após SE;
6) Avaliar a atividade neuronal em regiões hipocampais e extra-hipocampais após SE;
7) Determinar a expressão da proteína SGLT1 em regiões hipocampais após SE.

4 ARTIGO 2

Role of modulation of hippocampal glucose following pilocarpine-induced Status Epilepticus

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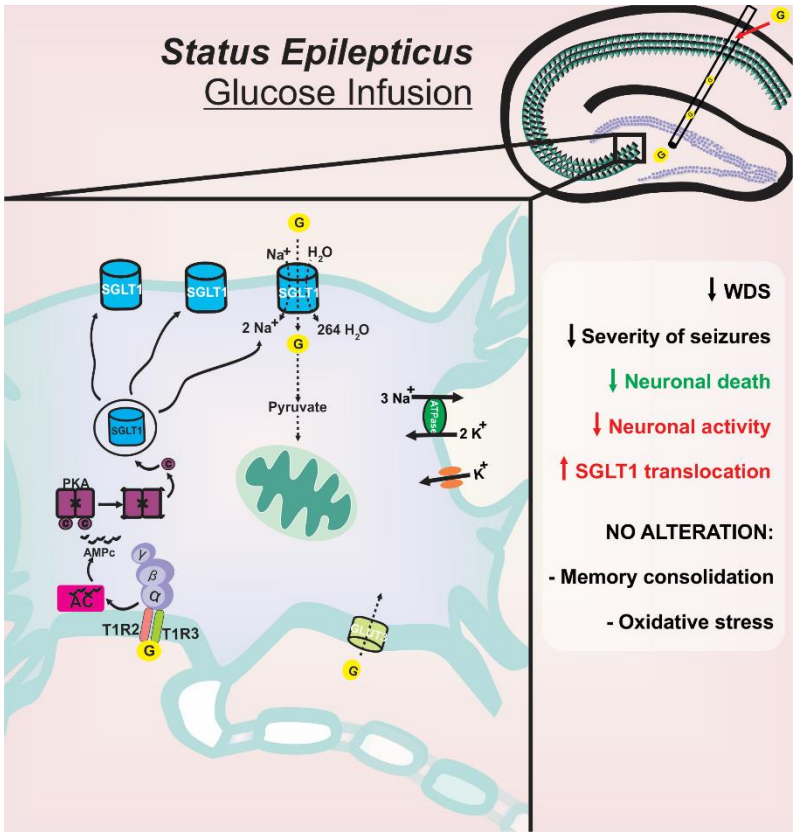
Graphical abstract



In brief

We showed that intrahippocampal glucose supply reduces the severity of seizures but does not change memory dysfunction and oxidative stress. Cell death and neuronal activity are attenuated in the hippocampus and extrahippocampal areas because of this glucose modulation, via elevation of sodium-glucose cotransporter (SGLT1) translocation in hippocampus.

Highlights

- Seizures severity is decreased after the infusion of intrahippocampal glucose
- Glucose modulation reduces neuronal death in the hippocampus and extrahippocampal areas
- Neuronal activity in the hippocampus is attenuated by glucose infusion
- SGLT1 translocation is increased after intrahippocampal glucose supply

Context and Significance

Naturally, glucose is the main source of energy for the brain and hypo or hyperglycemia can lead to the generation of epileptic seizures. Researchers at the Federal University of Alagoas and their colleagues observed that glucose control in the hippocampus holds great promise for improving the repercussions of continuous and self-sustaining epileptic seizures. They modulated the glucose level before and after the induction of epileptic seizures. The supply of hippocampal glucose repaired cell machinery breakdowns and relieved the severity of epileptic seizures, paving the way for new experimental and clinical trials to test whether glucose control improves the quality of life of an epileptic patient.

SUMMARY

Status Epilepticus (SE) is defined as continuous and self-sustaining seizures, which trigger hippocampal neurodegeneration, oxidative stress, and energy failure. During SE, the neurons become overexcited, increasing energy consumption. Glucose uptake is increased via the sodium glucose cotransporter 1 (SGLT1) in the hippocampus under epileptic conditions. In addition, modulation of glucose can prevent neuronal damage caused by SE. Here, we evaluated the effect of increased glucose availability in behavior of limbic seizures, memory dysfunction, neurodegeneration process, neuronal activity and SGLT1 translocation. Glucose supply reduced the severity of seizures but did not protect memory dysfunction followed by SE. Similarly, glucose modulation reduced cell death and neuronal activity in hippocampus, *subiculum*, thalamus, and cortical areas. Finally, SGLT1 translocation was elevated in hippocampus after increasing glucose levels. Taken together, our data suggest that intrahippocampal glucose supply protect brain in the earlier stage of epileptogenic processes via important support of SGLT1.

KEYWORDS: Sodium glucose cotransporter, epileptogenic, glucose, hippocampus.

4.1 INTRODUCTION

Status epilepticus (SE) is defined as continuous and self-sustaining seizures lasting >30min, reaching a significant number of patients (Lowenstein et al., 1999; Sánchez and Rincon, 2016; Santos et al., 2019; Sloviter, 1999). Because it is an epileptogenic insult, SE is capable of leading to temporal lobe epilepsy (TLE) and promoting severe damage to the central nervous system (CNS), such as activation of a recurrent excitatory circuit, neurodegeneration, aberrant neurogenesis, and mossy fiber sprouting (Castro et al., 2017; De Furtado et al., 2002; Upadhya et al., 2018). Despite treatment with AEDs, about 30% of patients may be refractory to standard drugs, experiencing frequent and lasting seizures capable of promoting brain damage (Ho et al., 2019; Kälviäinen and Reinikainen, 2019; Trinka and Kälviäinen, 2017).

Putative neuroprotective substances have been increasingly identified using animal models of seizures. Intrahippocampal (H) administration of pilocarpine (PILO) has typically been used to induce TLE in rodents, mimicking epileptic seizures in humans, which initiate as focal and then evolve to generalized (Castro et al., 2011; Furtado et al., 2011; Melo et al., 2016). After the infusion of H-PILO, the animal behavior is altered, presenting wet dog shake (WDS), forelimb myoclonus, rearing, and falling (Lai et al., 2018; Rodrigues et al., 2005; Wu and Wang, 2018). As a consequence of PILO-induced SE, especially after 24h, selective cell death occurs in DG hilus, CA3 and CA1 hippocampal subareas, as well as in extrahippocampal regions, including *subiculum*, thalamus, amygdala, substantia nigra and cortical areas (Jung et al., 2009; Loss et al., 2012; Oliveira et al., 2016; Scholl et al., 2013; Zenki et al., 2018). Furthermore, PILO-induced SE increases reactive oxygen species (ROS) production, directly associated with neuronal degeneration, increasing malondialdehyde (MAD) levels and decreasing catalase (CAT) and superoxide dismutase (SOD) antioxidant enzymes activity (Santos et al., 2008; dos Santos et al., 2011; Shakeel et al., 2017; Xue et al., 2011). In addition, cell death in the hippocampus and adjacent limbic areas can lead to memory and learning impairments (Khalil et al., 2017; Long et al., 2017; Shetty, 2014).

Glucose is the main source of energy for the mammalian brain and energy deficit can lead to neuronal dysfunction (Simpson et al., 2007). During epileptic seizures, glucose uptake increases in hyperexcited neurons above the body's supply capacity (McDonald et al., 2017; Nehlig et al., 2006; Poppe et al., 1997; Vielhaber et al., 2003). Furthermore, oxidative stress, mitochondrial dysfunction, energy failure and tricarboxylic acid cycle (TCA) failure typically occur in the hippocampus and nearby area after SE, working together contribute to neuronal

damage (Folbergrová et al., 2016; McDonald et al., 2017; Smeland et al., 2013). Interestingly, both hypo- and hyperglycemia lead to aggravated epileptic seizures and, consequently, compromised the physiology of CNS (Chou et al., 2016; Maheandiran et al., 2013; Moseley et al., 2013; Schauwecker, 2012; Xia et al., 2016). Therefore, cerebral glycemic control may be an interesting approach to protect against damage following PILO-induced SE, but its underlying mechanisms of action remain uncertain. Since the neuronal membranes are impermeable to glucose, the transport of glucose into the neuron is mediated by facilitated diffusion and secondary active transport, via glucose transporters (GLUTs) and sodium/glucose cotransporters (SGLTs), respectively (Sabino-Silva et al., 2010; Wright et al., 2011). SGLT1 isoform is expressed in the hippocampus and other brain regions, including amygdala, hypothalamus, basal ganglia and cortical areas (Poppe et al., 1997; Yu et al., 2010, 2013). We have previously observed that SGLTs play a crucial role in protecting against pilocarpine-induced SE damage. When we inhibited hippocampal SGLTs with phlorizin, a nonspecific inhibitor, there was an increase in WDS number, seizure severity, and neuronal death pattern after SE (Melo et al., 2016). In addition, other authors have shown that glycemic index control was able to suppress neuronal death following kainate-induced SE, indicating that hippocampal glucose modulation may be a critical therapeutic target (Schauwecker, 2012).

Starting from this, we tested the hypothesis that increased glucose availability upregulate SGLTs translocation, which is correlated to the neuroprotective effect in the acute phase of epileptogenesis.

4.2 RESULTS

4.2.1 Intrahippocampal glucose supply does not change latency for seizures but increases the number of WDS

Typically, as expected, after intrahippocampal administration of pilocarpine, the animals had a change in behavior, including immobility, facial movements, head nodding, and myoclonic movements of the limbs that evolved to continuous tonic clonic convulsive seizures, indicating the onset of SE.

In the initial periods after pilocarpine microinjection, animals had a latency interval without manifesting epileptic seizures. In order to verify if the increase in glucose supply at different concentrations (1, 2 or 3mM) before and after PILO interferes with the generation time of SE, the animal behavior was carefully evaluated before SE (Figure 1A and B). The

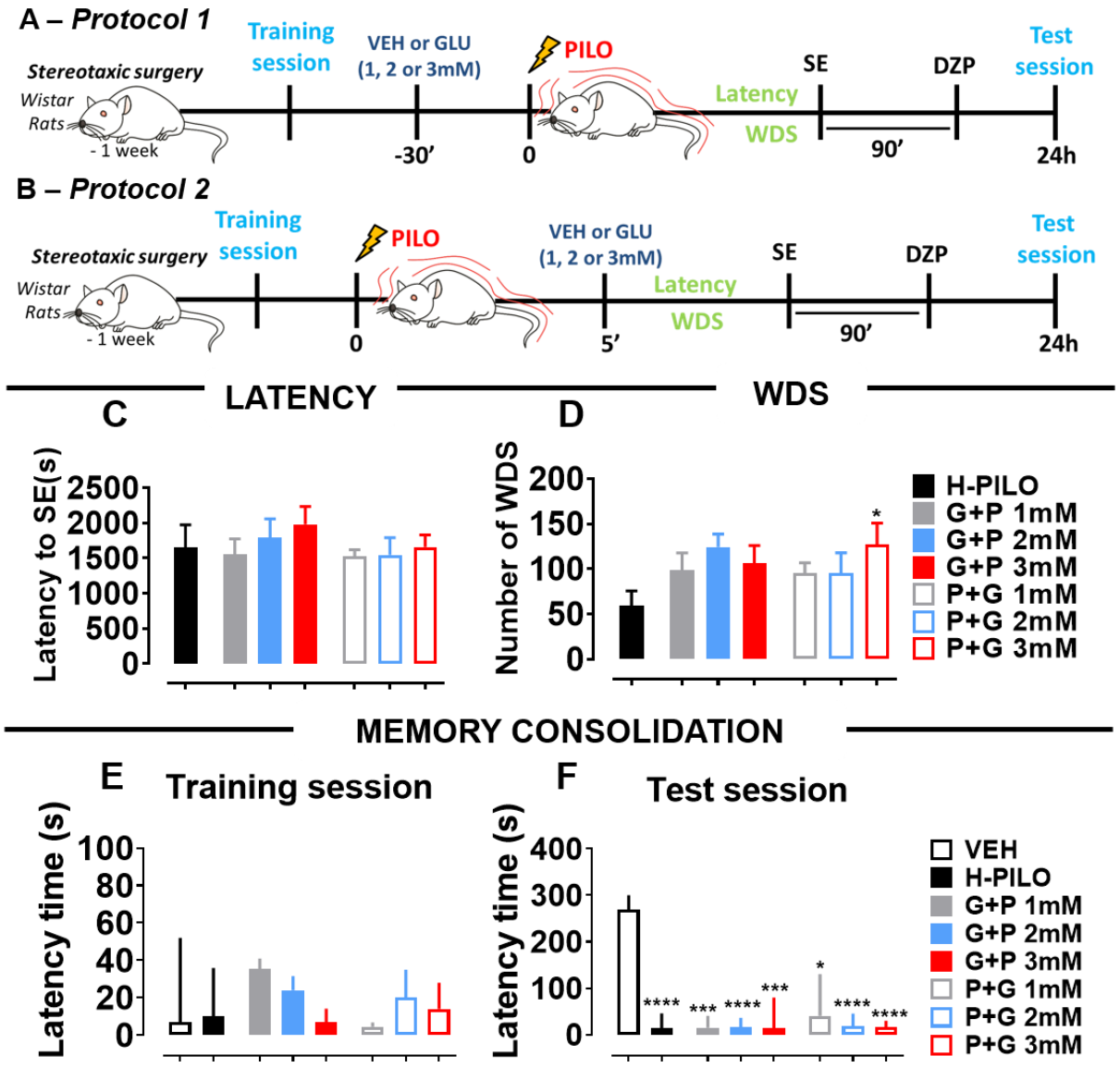
latency for SE was similar in all groups that received the different concentrations of glucose in relation to the control (one-way ANOVA, $F_{(6, 64)} = 0.4881$, $P = 0.8149$; Figure 1C).

In intrahippocampal PILO models, it is common to observe the presence of repetitive movements in the head and neck of rodents, motor pattern that stereotypes the shaking of a wet dog. As expected, wet dog shake (WDS) was quite common and frequent during the latency period in all animals, reducing drastically after the beginning of the SE. Increased glucose supply (3 mM) after PILO increased the number of total WDS when compared to the control (*t-test*, $t_{16} = 2.252$, $P = 0.0387$; Figure 1D).

4.2.2 The impairment in memory consolidation is maintained after increased glucose availability

In order to evaluate the effect of increased hippocampal glucose availability on memory consolidation, the inhibitory avoidance test was performed (Figure 1A and B). During the training period, all animals spent little time on the platform (Figure 1E). In contrast, in the test period, the group that did not have SE consolidated a long-term memory. Typically, the group that received only H-PILO had impaired memory consolidation, as expected (Kruskal-Wallis test, p = 0.0006 vs VEH; Figure 1F). Increased glucose availability (1, 2 or 3 mM) before and after PILO was not able to reverse memory dysfunction following PILO-induced SE (Kruskal-Wallis test, p = 0.0006 vs VEH; Figure 1F).

Figure 1: Glucose control does not alter latency for seizures and memory dysfunction but increases the number of WDS after pilocarpine-induced SE



(A) shows the experimental scheme. Rats received glucose microinjections 30 min before (A1) or 5 min after PILO (B). Glucose infusion prior and after PILO do not change the latency to SE (one-way ANOVA, $F_{(6, 64)} = 0.5160$, $P = 0.7941$; C). The increased availability of glucose (3mM) increased the number of total WDS (one-way ANOVA, $F_{(6, 64)} = 0.8919$, $P = 0.5063$; D). After 24h of SE, long-term memory consolidation was analyzed by inhibitory avoidance test in both rats receiving glucose before (A) or after (B) PILO. Initially, all animals were submitted to an aversive stimulus (training session) prior to SE induction (E). Memory consolidation was not protected from increased glucose (1, 2 or 3mM) administration before (grey, blue and red bar) and after (grey, blue and red bar outline) PILO (F) (Kruskal-Wallis test, p = 0.0006 vs VEH). Error bars indicate the SEM. Latency and WDS data represent the mean ± S.E.M. of 10-11 rats. *P<0.05; one-way ANOVA with Dunnett's post-hoc test or unpaired t-test. Memory dysfunction data represent the median with interquartile range. *P<0.05, ***P<0.001 and ****P<0.0001 compared with VEH; one-way ANOVA with Kruskal-Wallis test with Dunn's post-hoc test. VEH, vehicle; H-PILO, pilocarpine and saline; G+P, glucose followed by pilocarpine infusion; P+G, pilocarpine followed by glucose infusion; DZP, diazepam; SE, *Status epilepticus*; SEM, standard error of the mean. Fonte: Autor

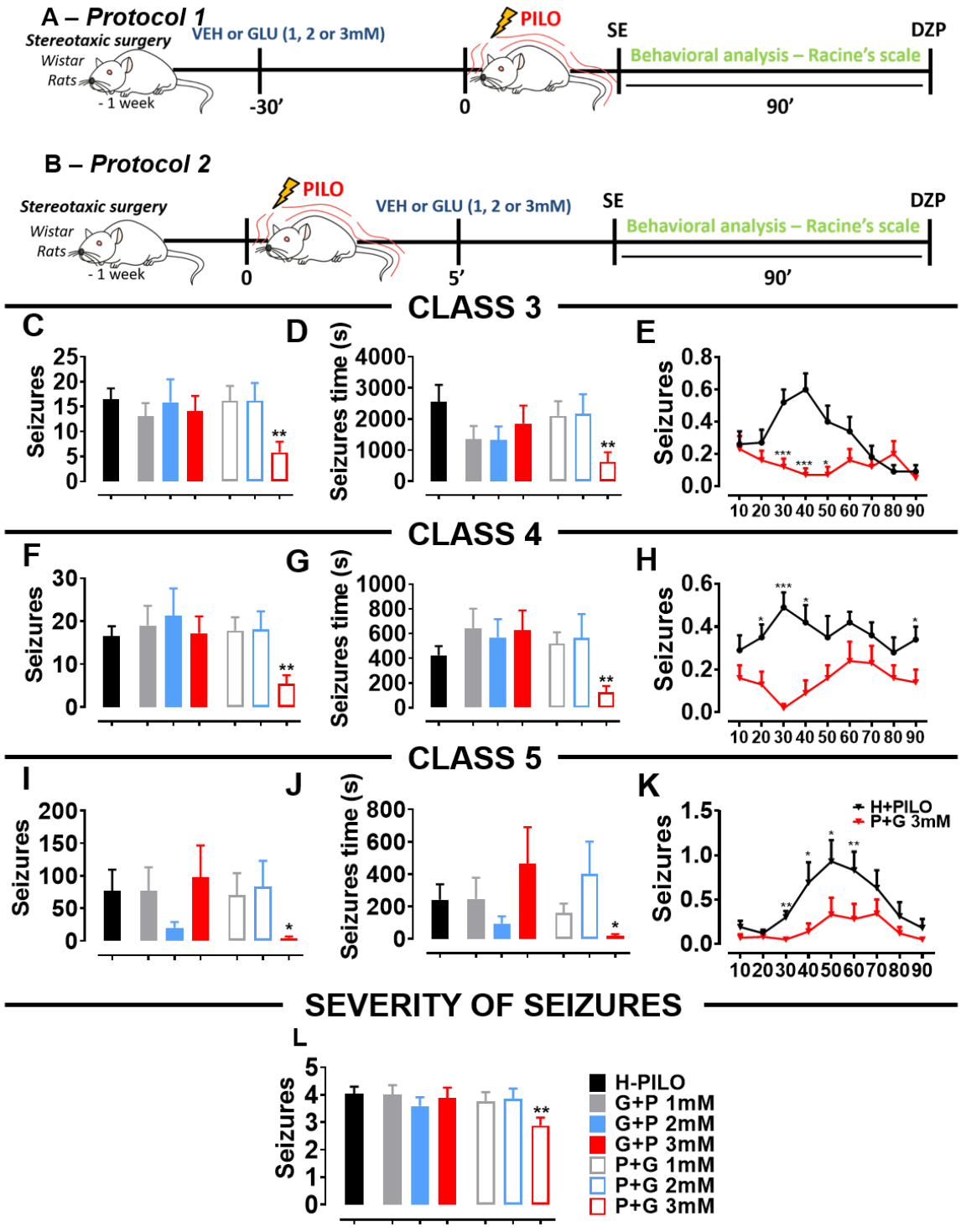
4.2.3 Intrahippocampal glucose supply attenuates the severity of seizures after SE

To assess the impact of glucose modulation on the severity of seizures, epileptic seizures were analyzed during 90 minutes of SE according to Racine's scale (1972) (Figure 2A and B). The number and total time of classes 3, 4 and 5 seizures remained unchanged after administration of the different glucose concentrations (1, 2 and 3 mM) before and (1 or 2mM) after PILO ($p>0.05$). However, the higher glucose concentration (3mM) after PILO was able to reduce the frequency and total time of classes 3, 4 and 5 seizures compared to control (Figure 2B1-2, C1-2, D1-2; $p<0.05$).

When analyzed in detail the effect of increased glucose concentration (3 mM) in the evolution of the seizures over the SE, we observed that in the intervals of 20-40 and 30-50 minutes, classes 3 and 4 seizures remained reduced, respectively (Figure 2 B3 and C3, $p<0.05$). In all other final times, classes 3 and 4 seizures of the PILO+GLI (3mM) group remained like those of the control, except class 3 seizure that decreases again at 90 min (Figure 2 B3, $p<0.05$). In addition, class 5 seizure is decreased in the range of 30-60 minutes ($p<0.05$), remaining the same to the control until the end of the SE (Figure 2 D3).

Using the average of the most severe seizures along the 18 windows of SE, the seizure severity was evaluated. Corroborating previous behavioral findings, only the high availability of glucose (3 mM) after PILO was able to reduce the severity of seizures compared to the H-PILO group (one-way ANOVA, $F_{(6, 64)} = 1.65$, $P = 0.04$; Figure 2E). In other words, glucose availability may exert an anticonvulsive effect on the SE.

Figure 2: Increased glucose availability reduces seizure severity following pilocarpine-induced SE



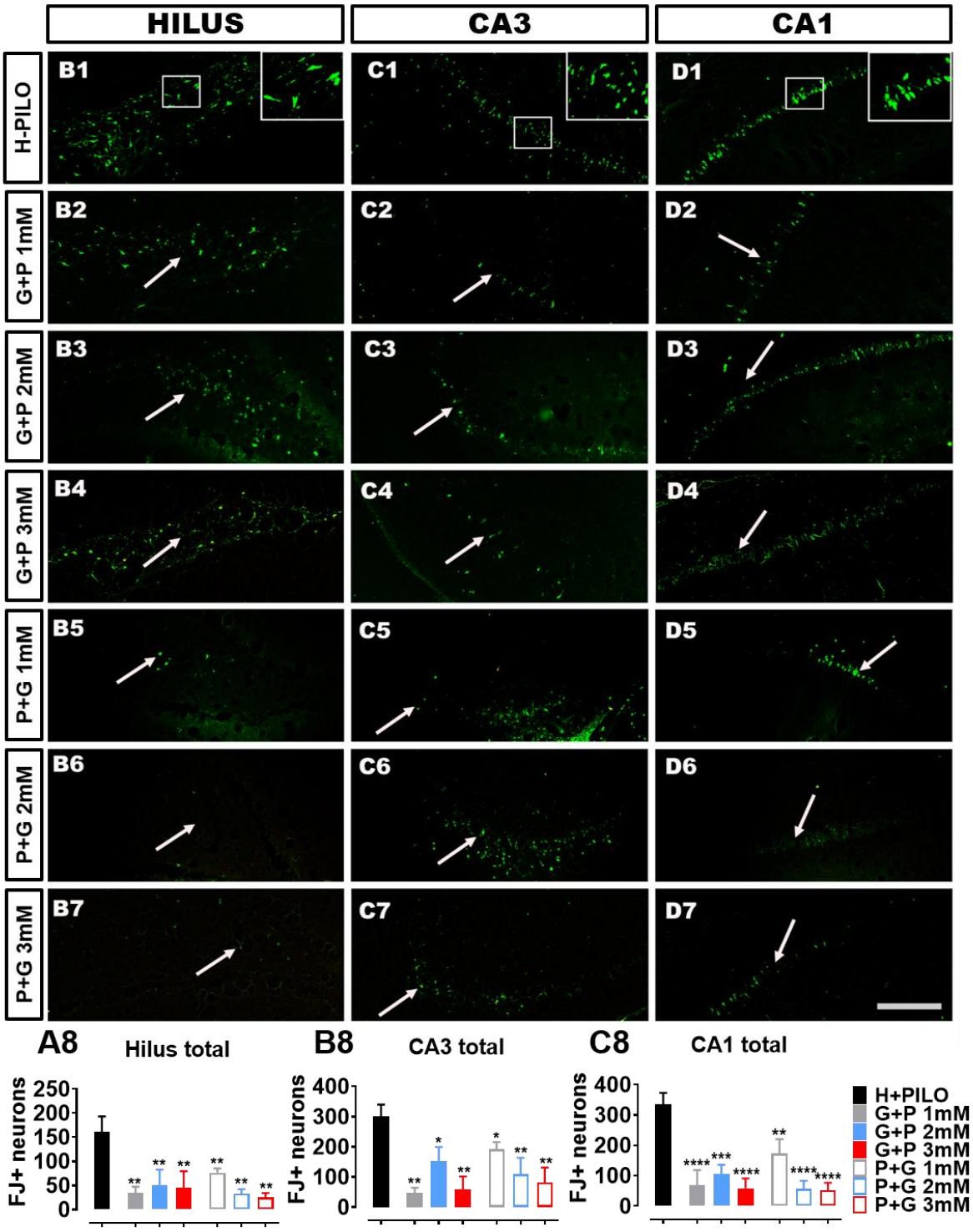
(A) shows the experimental scheme. Rats received glucose microinjections 30 min before (A1) or 5 min after PILO (A2). The epileptic seizures were analyzed during the 90 minutes of SE, according to the Racine scale. Over the 90 minutes of SE, the total number (one-way ANOVA, $F_{(6, 64)} = 1.716$, $P = 0.04$; B1) and time (one-way ANOVA, $F_{(6, 64)} = 2.035$, $P = 0.01$; B2) of class 3 seizures (head and neck myoclonus) were decreased when administered glucose (3mM; red bar outline) into hippocampus. Class 3 was reduced at the beginning and at the end of SE (B3). Glucose administration (3 mM; red bar outline) reduced the total number (one-way ANOVA, $F_{(6, 64)} = 2.714$, $P = 0.01$; C1)

and time (one-way ANOVA, $F_{(6, 63)} = 2.328$, $P = 0.02$; C2) of class 4 seizures, decreasing significantly in the 30-50 time range (C3). Similarly, the total number (one-way ANOVA, $F_{(6, 63)} = 1.742$, $P = 0.03$; D1) and time (one-way ANOVA, $F_{(6, 63)} = 1.744$, $P = 0.02$; D2) of class 5 was attenuated by increased glucose availability (3mM; red bar outline). During the evolution of class 5, glucose interfered strongly between times 30-60 (D3). Increased glucose supply (3mM; red bar outline) was able to decrease the severity of seizures (one-way ANOVA, $F_{(6, 64)} = 1.65$, $P = 0.04$; E). Error bars indicate the SEM. Data represent the mean ± S.E.M. of 10-11 rats. To normalize data that did not follow a normal distribution, the values were transformed from a logarithmic calculation [log 10 (x+1), being "x" equal to the value of the sample]. *P<0.05, **P<0.01 and ***P<0.001; one-way ANOVA with Dunnett's post-hoc test or unpaired t-test. H-PILO, pilocarpine and saline; G+P, glucose followed by pilocarpine infusion; P+G, pilocarpine followed by glucose infusion; DZP, diazepam; SE, *Status epilepticus*; SEM, standard error of the mean. Fonte: Autor

4.2.4 Brain glucose supply reduces neuronal death in the hippocampus and other brain areas

Classically, pilocarpine-induced SE triggers neuronal death in several brain areas, especially in the hippocampus (Castro et al., 2011; Melo et al., 2016). In order to evaluate whether brain glucose modulation can prevent neuronal damage caused by SE, the neurodegeneration process was evaluated by histochemistry of FJ. When glucose was administered prior to PILO at all concentrations (1-3mM), the number of FJ + neurons was reduced in the DG hilus (one-way ANOVA, $F_{(6, 33)} = 4.985$, $P = 0.001$), CA3 (one-way ANOVA, $F_{(6, 33)} = 4.976$, $P = 0.001$) and CA1 (one-way ANOVA, $F_{(6, 33)} = 9.216$, $P < 0.0001$) subfields of hippocampus compared to the control (Figure 4 A1-4, B1-4 and C1-4). Similarly, all concentrations of glucose after PILO were able to attenuate neuronal death in the same areas of the hippocampus ($P < 0.001$, Figure 3 A5-8, B5-8 and C5-8). In other words, besides promoting an anticonvulsive role, hippocampal glucose modulation prevents the neuronal damage characteristic of SE. The highest concentration of glucose (3mM) administered after PILO showed better efficiency in the behavior of seizures and in the neurodegenerative process, therefore it was chosen for the analysis of the other methodological approaches.

Figure 3: Increased glucose availability attenuates neuronal death in the hippocampus following pilocarpine-induced SE

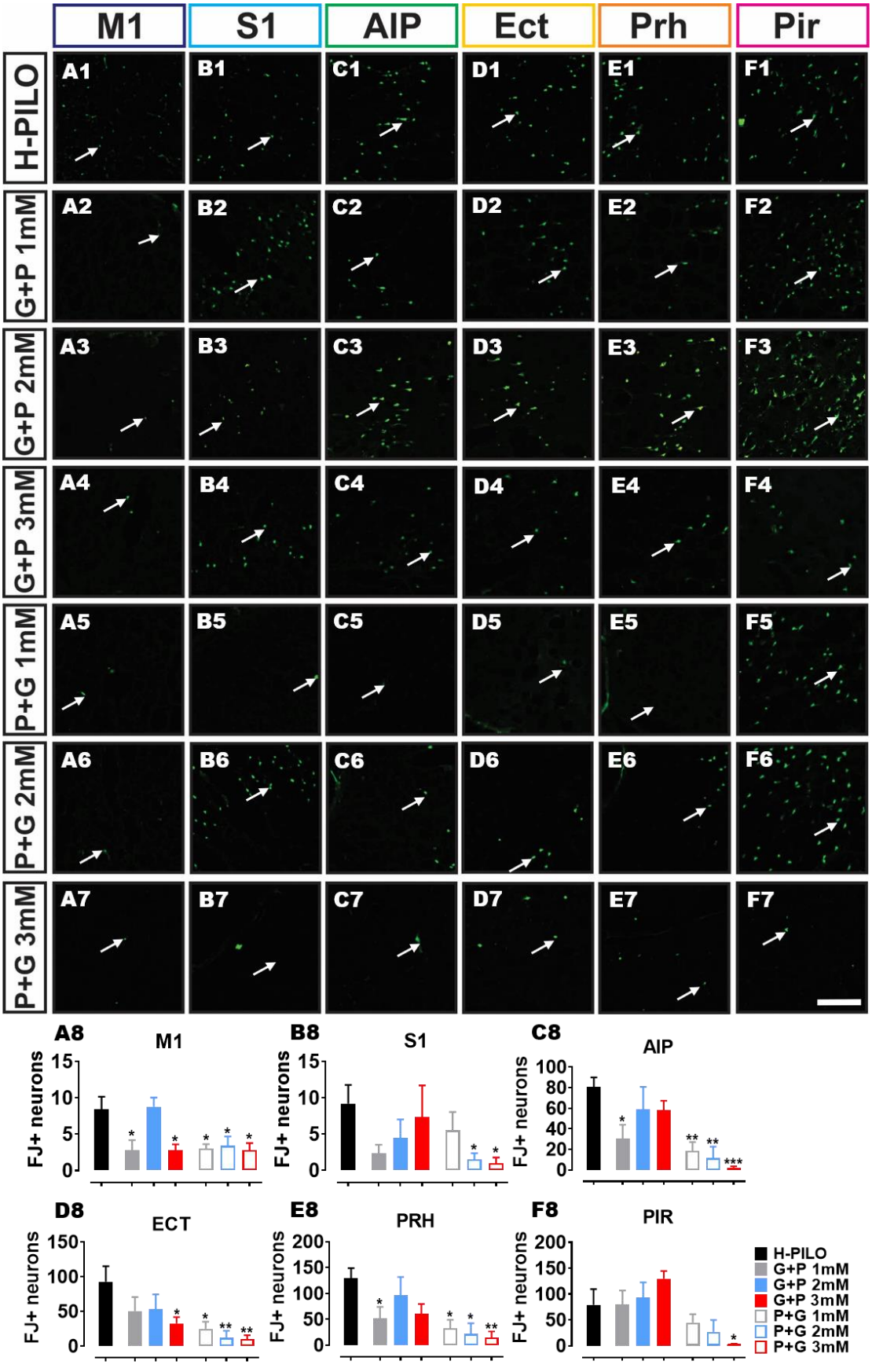


After 24h of SE, the neurodegeneration process was evaluated by Fluoro-Jade C (FJ-C) histochemistry in both rats receiving glucose 30 min before or 5 min after PILO. Hilar interneurons (A1-7) and pyramidal neurons of the CA3 (B1-7) and CA1 (C1-7) regions were labeled with FJ (FJ+, green). Increased glucose administration at all concentrations before and after PILO was able to reduce the number of FJ + neurons in the DG hilus (one-way ANOVA, $F(6, 33) = 4.985$, $P = 0.001$; A8), as well as in the CA3 (one-way ANOVA, $F(6, 33) = 4.976$, $P = 0.001$; B8) and CA1 (one-way ANOVA, $F(6, 33) = 9.216$, $P < 0.0001$; C8) regions. Representative digital zoom was done on the photomicrographs

of the control (A1, B1 and C1; see squares) Arrows represent the DG hilus, CA3 or CA1 regions. Magnification, 100x; scale bar, 100 μm. Error bars indicate the SEM. Data represent the mean ± S.E.M. of 5-7 rats. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 compared with H-PILO; one-way ANOVA with Dunnett's post-hoc test or unpaired t-test. H-PILO, pilocarpine and saline; G+P, glucose followed by pilocarpine infusion; P+G, pilocarpine followed by glucose infusion; DZP, diazepam; SE, Status epilepticus; SEM, standard error of the mean. See also Figures S1. Fonte: Autor

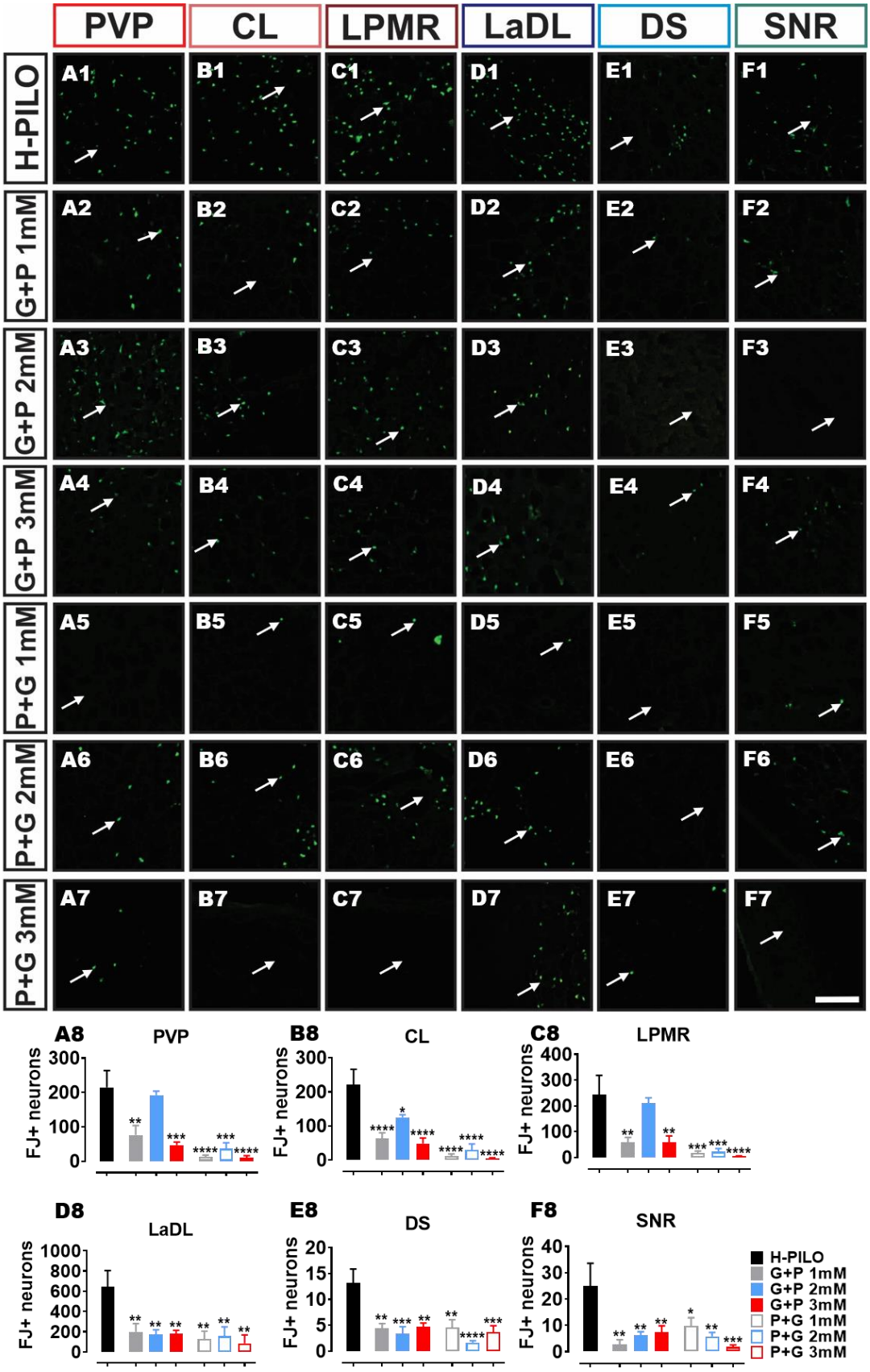
Some infusion of glucose administered before and after PILO were also able to attenuate the number of FJ+ neurons in motor (one-way ANOVA, F (6, 30) = 4.81, *P* = 0.0015), somatosensory (one-way ANOVA, F (6, 19) = 2.17, *P* = 0.09), insular (one-way ANOVA, F (6, 26) = 6.16, *P* = 0.0004), ectorhinal (one-way ANOVA, F (6, 29) = 3.79, *P* = 0.006), perirhinal (one-way ANOVA, F (6, 26) = 3.73, *P* = 0.008) and piriform (one-way ANOVA, F (6, 23) = 4.36, *P* = 0.004) cortices compared to control (Figure 4 A-F 1-8). In addition, thalamic [lateral posterior (one-way ANOVA, F (6, 27) = 8.66, P < 0.0001), centrolateral (one-way ANOVA, F (6, 26) = 15.06, *P* < 0.0001) and posterior paraventricular (one-way ANOVA, F (6, 27) = 10.96, *P* < 0.0001)] and amygdaloid lateral (one-way ANOVA, F (6, 27) = 4.27, *P* = 0.004) nuclei were protected due to high glucose administration (Figure 5 A-D 1-8). Similarly, the supply of cerebral glucose was able to reduce neuronal damage in the subiculum (one-way ANOVA, F (6, 33) = 6.38, *P* = 0.0002) and the substantia nigra (one-way ANOVA, F (6, 30) = 4.48, *P* = 0.002) (Figure 5 E-F 1-8). Besides, presenting an anticonvulsive effect, modulation of hippocampal glucose plays a neuroprotective role in several brain regions.

Figure 4: Increased glucose supply decreases the neurodegeneration process in cortical areas after pilocarpine-induced SE



The concentration of 3mM glucose had a more significant potential in protecting neuronal damage following SE. Some concentrations of hippocampal glucose (1, 2 or 3mM) before and after PILO reduced FJ+ neurons in M1 (one-way ANOVA, $F(6, 30) = 4.81$, $P = 0.0015$; A1-8; dark blue rectangle), S1 (one-way ANOVA, $F(6, 19) = 2.17$, $P = 0.09$; B1-8; light blue rectangle), AIP (one-way ANOVA, $F(6, 26) = 6.16$, $P = 0.0004$; C1-8; green rectangle), Ect (one-way ANOVA, $F(6, 29) = 3.79$, $P = 0.006$; D1-8; yellow rectangle), PRh (one-way ANOVA, $F(6, 26) = 3.73$, $P = 0.008$; E1-8; orange rectangle) and Pir (one-way ANOVA, $F(6, 23) = 4.36$, $P = 0.004$; F1-8; pink rectangle) areas compared with control (H-PILO, black bar). Arrows represent the FJ+ neurons in each cortical area. Magnification, 100x; scale bar, 100 μm. Error bars indicate the SEM. Data represent the mean ± S.E.M. of 5-7 rats. *$P<0.05$ and **$P<0.01$ compared with H-PILO; one-way ANOVA with Dunnett's post-hoc test. H-PILO, pilocarpine and saline; G+P, glucose followed by pilocarpine infusion; P+G, pilocarpine followed by glucose infusion; M1, motor; S1, somatosensory; AIP, insular; Ect, ectorhinal; PRh, perirhinal; Pir, piriform; SEM, standard error of the mean. Fonte: Autor

Figure 5: Glucose control reduces the neuronal death in the thalamus, amygdala, subiculum, and substantia nigra after pilocarpine-induced SE

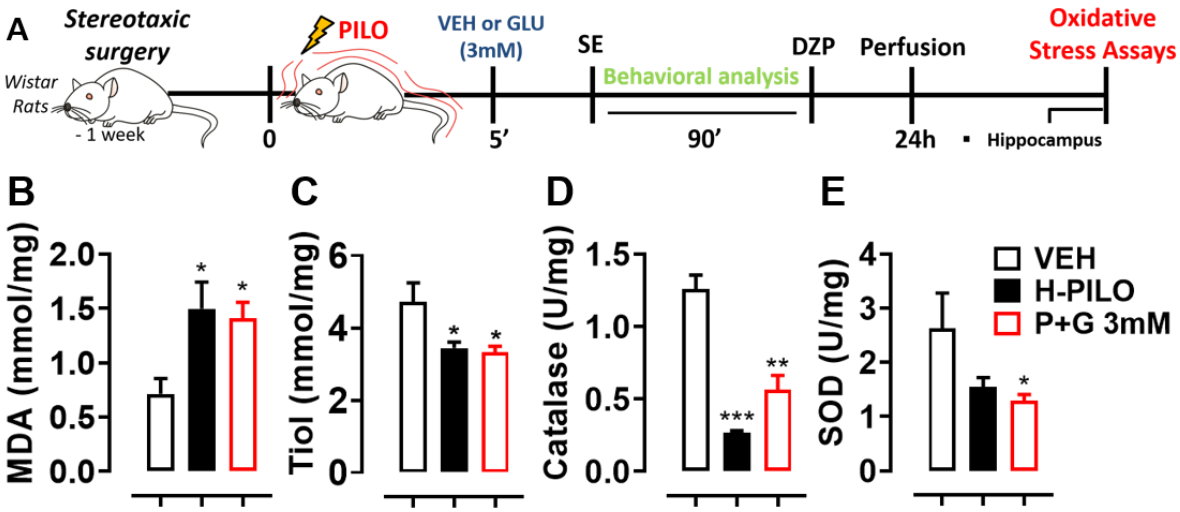


The concentration of 3mM glucose had a more significant potential in protecting neuronal damage following SE. Most hippocampal glucose concentrations (1, 2 or 3mM) before and after PILO decreased FJ+ neurons in PVP (one-way ANOVA, F (6, 27) = 10.96, P < 0.0001; A1-8; red rectangle), CL (one-way ANOVA, F (6, 26) = 15.06, P < 0.0001; B1-8; pink rectangle), LPMR (one-way ANOVA, F (6, 27) = 8.66, P < 0.0001; C1-8; dark red rectangle), LaDL (one-way ANOVA, F (6, 27) = 4.27, P = 0.004; D1-8; blue rectangle), DS (one-way ANOVA, F (6, 33) = 6.38, P = 0.0002; E1-8; light blue rectangle) and SNR (one-way ANOVA, F (6, 30) = 4.48, P = 0.002; F1-8; green rectangle) areas compared with control (H-PILO, black bar). Arrows represent the FJ+ neurons in each brain area. Magnification, 100x; scale bar, 100 μm. Error bars indicate the SEM. Data represent the mean ± S.E.M. of 5-7 rats. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 compared with H-PILO; one-way ANOVA with Dunnett's post-hoc test. H-PILO, pilocarpine and saline; G+P, glucose followed by pilocarpine infusion; P+G, pilocarpine followed by glucose infusion; PVP, posterior paraventricular th ncl; CL, centrolateral th ncl; LPMR, lateral posterior th ncl; LaDL, lateral amygdaloid ncl; DS, subiculum; SNR, substantia nigra; ncl, nucleus; th, thalamic; SEM, standard error of the mean. Fonte: Autor

4.2.5 Effects of brain glucose supply on oxidative stress markers and antioxidants enzymes activity in the hippocampus

Typically, pilocarpine-induced SE exacerbates reactive oxygen species (ROS) levels in hippocampus. In order to analyze whether hippocampal glucose control interferes with oxidative stress promoted by SE, oxidative stress markers and antioxidants enzymes activity were assessed (Figure S1A). As the higher concentration of glucose (3 mM) administered after PILO had a more significant result, it was chosen for all subsequent analyzes. MDA formation was significantly increased in H-PILO hippocampus when compared with VEH, but increased glucose (3mM) supply after PILO was not able to prevent elevated MDA levels (one-way ANOVA, $F_{(2, 8)} = 5.182$, $P = 0.036$) (Figure S1B). In addition, as a result of pilocarpine-induced SE, the total thiol number was markedly reduced in the H-PILO hippocampus and hippocampal glucose infusion did not change this condition (one-way ANOVA, $F_{(2, 10)} = 6.048$, $P = 0.019$) (Figure S1C). Concordant with the elevated MDA level, we found significant decreases in the activity of antioxidant enzymes CAT (one-way ANOVA, $F_{(2, 8)} = 24.57$, $P = 0.0004$) and SOD (one-way ANOVA, $F_{(2, 9)} = 4.947$, $P = 0.0355$) in P+G 3mM and H-PILO animals, compared with VEH (Figure S1D and E). Specifically, hippocampal glucose modulation did not reverse decreased antioxidant enzymatic activity. Taken together, these data indicate that increased glucose availability was not able to interfere with oxidative stress caused by pilocarpine-induced SE.

Figure S1: Effects of increased glucose availability on status of oxidative stress levels in rat hippocampus after 24 h of pilocarpine-induced SE.

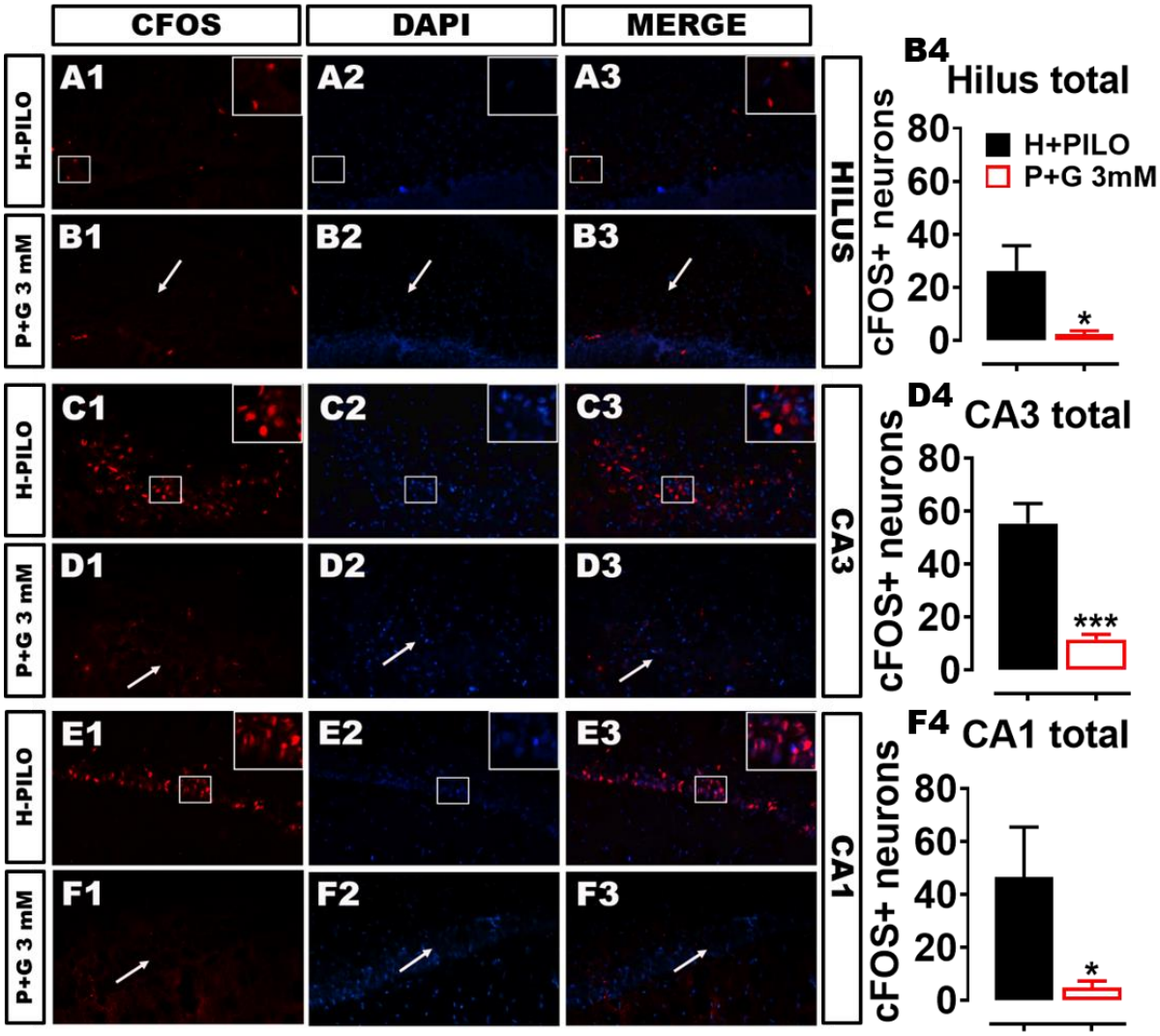


Modulated levels of malondialdehyde (MDA), total thiol, catalase (CAT), and superoxide dismutase (SOD) were assessed (A). Glucose-(3mM)-treated rats showed a significant increase in MDA levels (B) and a significant decrease in total thiol (C), CAT (D) and SOD (E) levels compared with saline-treated rats. Error bars indicate the SEM. Data represent the mean ± S.E.M. of 4-6 rats. *P<0.05, **P<0.01, and ***P<0.001 compared with VEH; one-way ANOVA with Dunnett's post-hoc test. VEH, saline-treated vehicle; H-PILO, pilocarpine and saline; P+G, pilocarpine followed by glucose infusion; DZP, diazepam; SE, *Status epilepticus*; SEM, standard error of the mean. Fonte: Autor

4.2.6 Increased glucose availability attenuates neuronal activity in the hippocampus and other brain areas

Neuronal hyperexcitability is a typical characteristic of pilocarpine-induced SE (Castro et al., 2011; Sanabria et al., 2002; Tejada et al., 2014). In order to identify whether modulation of hippocampal glucose interferes with neuronal hyperexcitability, cellular activity was evaluated by cFOS immunofluorescence. In hippocampus, increased glucose (3mM) after PILO reduced the total number of cFOS+ neurons in the DG hilus (*t-test*, t_8 = 2.481, P = 0.0380), CA3 (*t-test*, t_{10} = 5.651, P = 0.0002) and CA1 (*t-test*, t_8 = 2.735, P = 0.0257) subfield compared to control (Figure 6 A-F 1-4).

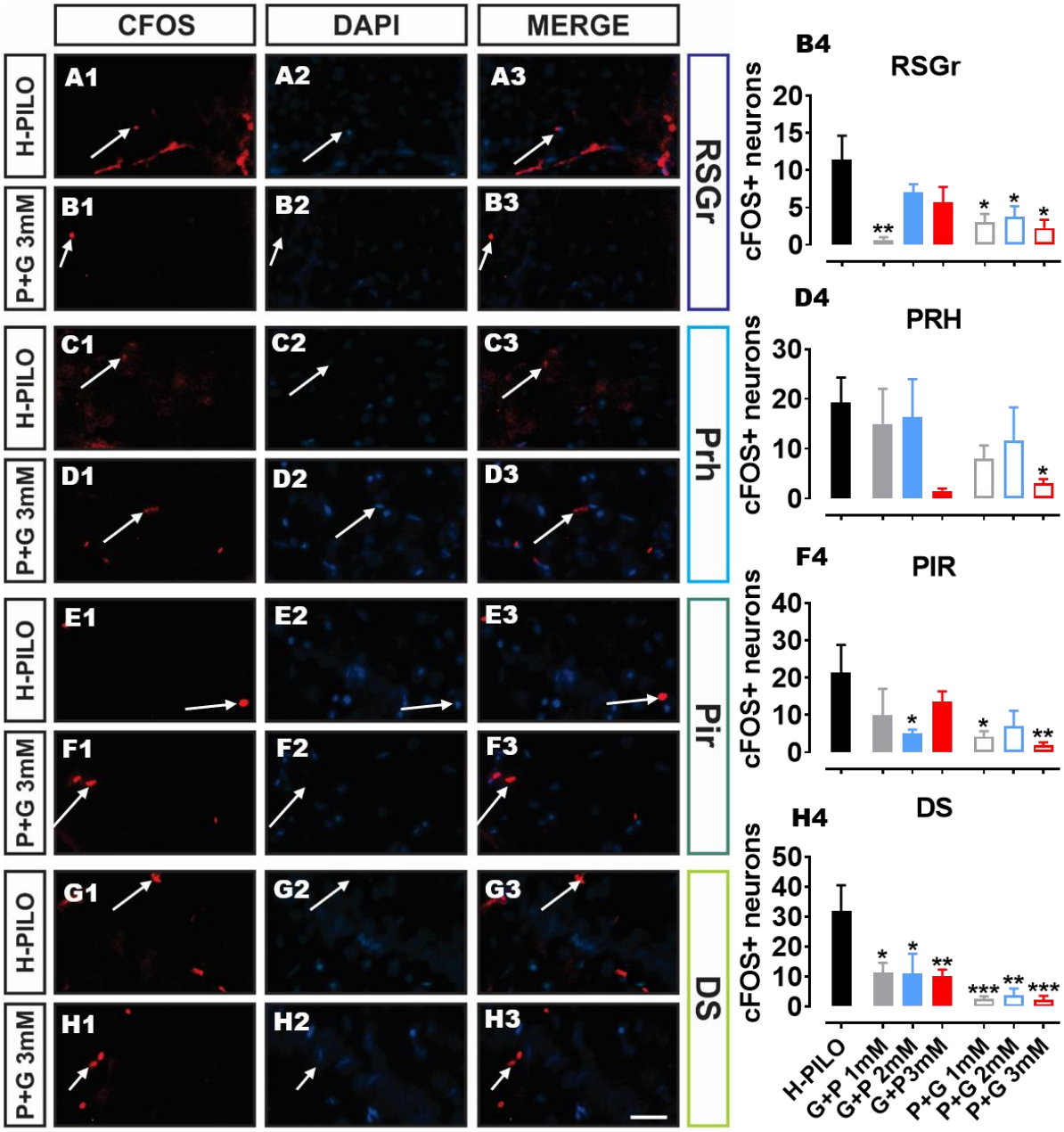
Figure 6: Glucose modulation decreases the cFOS expression in hippocampal neurons after pilocarpine-induced SE



After 24h of SE, the neuronal activity was analyzed by cFOS immunofluorescence in both rats receiving glucose 5 min after PILO. The nuclei were labeled with DAPI (blue, middle panels). Fluorescent labeling of the hippocampus shows strong cFOS immunoreactivity (red, left panels) in hilar interneurons (DG hilus, A1) and pyramidal neurons (CA3, C1; and CA1, E1) in H-PILO rats. Merge of cFOS and DAPI is shown in right panels. Quantitative analysis of cFOS immunofluorescent labeling of pyramidal neurons and interneurons of the H-PILO (black bars) and P+G (red bar outline) rats shown in B4, D4 and F4. Glucose control (3mM) after PILO reduced the number of cFOS + neurons in the DG hilus (*t-test*, t_{10} = 2.521, P = 0.0303; B4), as well as in the CA3 (*t-test*, t_{10} = 5.750, P = 0.0002; D4) and CA1 (*t-test*, t_9 = 2.581, P = 0.0296; F4) regions. Representative digital zoom was done on the photomicrographs of the control (A1-3, C1-3 and E1-3; see squares) Arrows represent the DG hilus, CA3 or CA1 regions. Magnification, 200x; scale bar, 50 μm. Error bars indicate the SEM. Data represent the mean ± S.E.M. of 5-6 rats. *P<0.05 and ***P<0.001 compared with H-PILO; unpaired t-test. H-PILO, pilocarpine and saline; P+G, pilocarpine followed by glucose infusion; DZP, diazepam; SE, *Status epilepticus*; SEM, standard error of the mean. See also Figures S2 and S3. Fonte: Autor

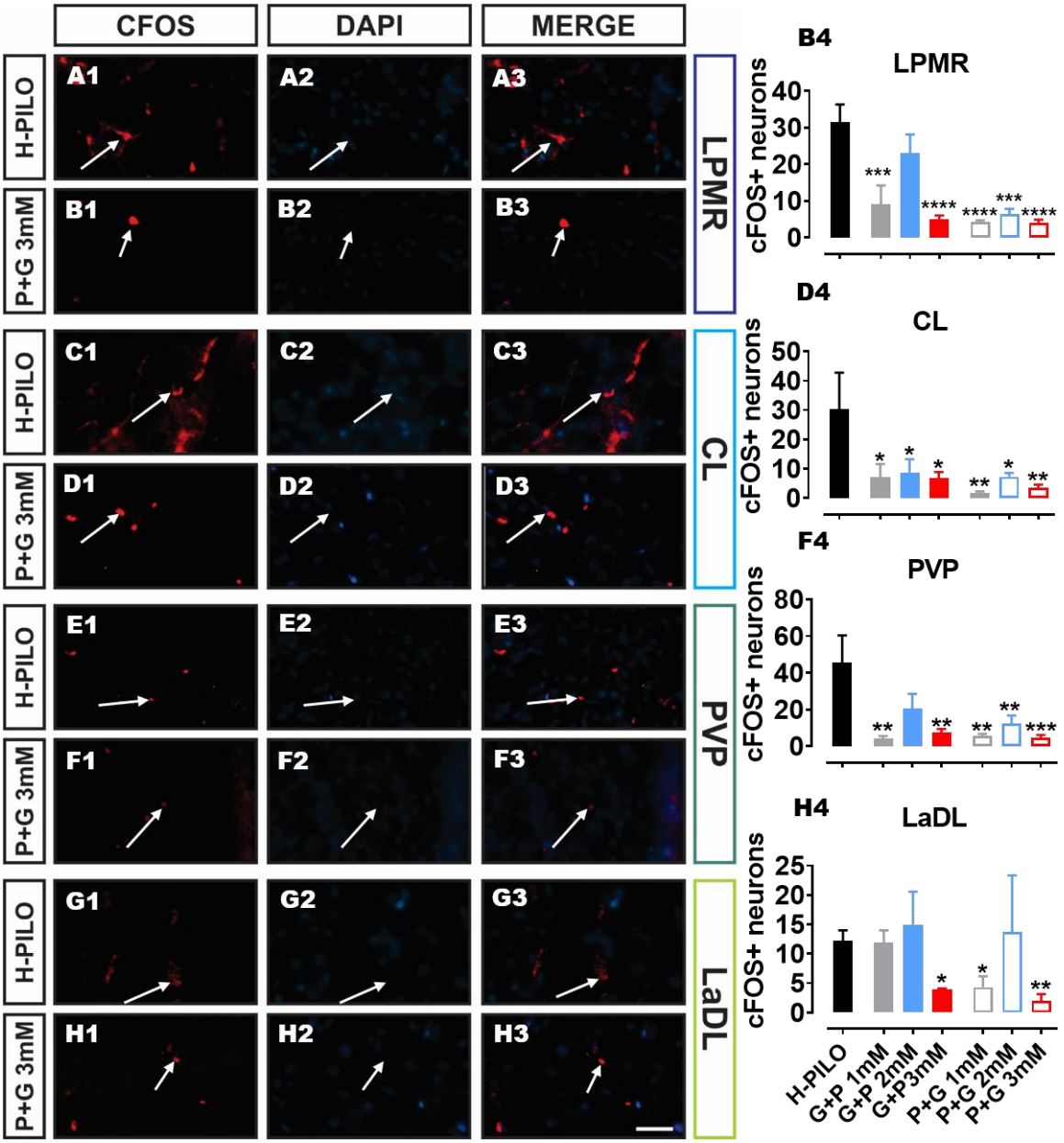
As in neurodegenerative processes, a hippocampal-like result was observed in neuronal activity in other brain areas. Elevated hippocampal glucose supply (3mM) after PILO decreased neuronal activity in retrosplenial (one-way ANOVA, $F (6, 22) = 3.59$, $P = 0.012$), perirhinal (one-way ANOVA, $F (6, 22) = 3.59$, $P = 0.012$), and piriform (one-way ANOVA, $F (6, 16) = 3.70$, $P = 0.017$) cortices in relation to control (Figure S2 A-F 1-4). In addition, the total number of cFOS+ neurons was lower in the subiculum of the group that received the glucose infusion when compared to control (one-way ANOVA, $F (6, 20) = 6.51$, $P = 0.0006$; Figure S2 G-H 1-4). Similarly, intrahippocampal administration of glucose was able to attenuate neuronal activity in thalamic [lateral posterior (one-way ANOVA, $F (6, 22) = 10.94$, $P < 0.0001$), centrolateral (one-way ANOVA, $F (6, 22) = 3.54$, $P = 0.01$) and posterior paraventricular (one-way ANOVA, $F (6, 20) = 5.21$, $P = 0.0023$); Figure S3 A-F 1-4] and amygdaloid (lateral) nuclei (one-way ANOVA, $F (3, 9) = 9.65$, $P = 0.0036$; Figure S3 G-H 1-4).

Figure S2: Glucose modulation decreases the cFOS expression in cortical and subiculum areas after pilocarpine-induced SE



The nuclei were labeled with DAPI (blue, middle panels, A-H2). Fluorescent labeling strong cFOS immunoreactivity (red, left panels) in RSGr (A1), Prh (C1), Pir (E1) and DS (G1) in H-PILO rats. Merge of cFOS and DAPI are shown in right panels (A-H3). Quantitative analysis of cFOS+ neurons in H-PILO (black bars), G+P (grey, blue and red bar) and P+G (grey, blue and red bar outline) rats are shown in A4, C4, E4 and G4. Some concentrations of hippocampal glucose (1, 2 or 3mM) before and after PILO attenuated the number of cFOS+ neurons in RSGr (one-way ANOVA, F (6, 22) = 3.59, P = 0.012; B4), Prh (*t-test*, *t5* = 3.79, *P* = 0.013; D4), Pir (one-way ANOVA, F (6, 16) = 3.70, P = 0.017; F4) and DS (one-way ANOVA, F (6, 20) = 6.51, P = 0.0006; H4) areas. The 3mM glucose concentration was used as a representative image. Arrows represent the brain areas. Magnification, 200x; scale bar, 50 μm. Error bars indicate the SEM. Data represent the mean ± S.E.M. of 3-6 rats. *P<0.05, **P<0.01 and ***P<0.001 compared with H-PILO; one-way ANOVA with Dunnett's post-hoc test and unpaired t-test. H-PILO, pilocarpine and saline; G+P, glucose followed by pilocarpine infusion; P+G, pilocarpine followed by glucose infusion; RSGr, retrosplenial; PRh, perirhinal; Pir, piriform; DS, subiculum; SEM, standard error of the mean. Fonte: Autor

Figure S3. Increased glucose supply decreases the cFOS expression in thalamic and amygdaloid areas after pilocarpine-induced SE

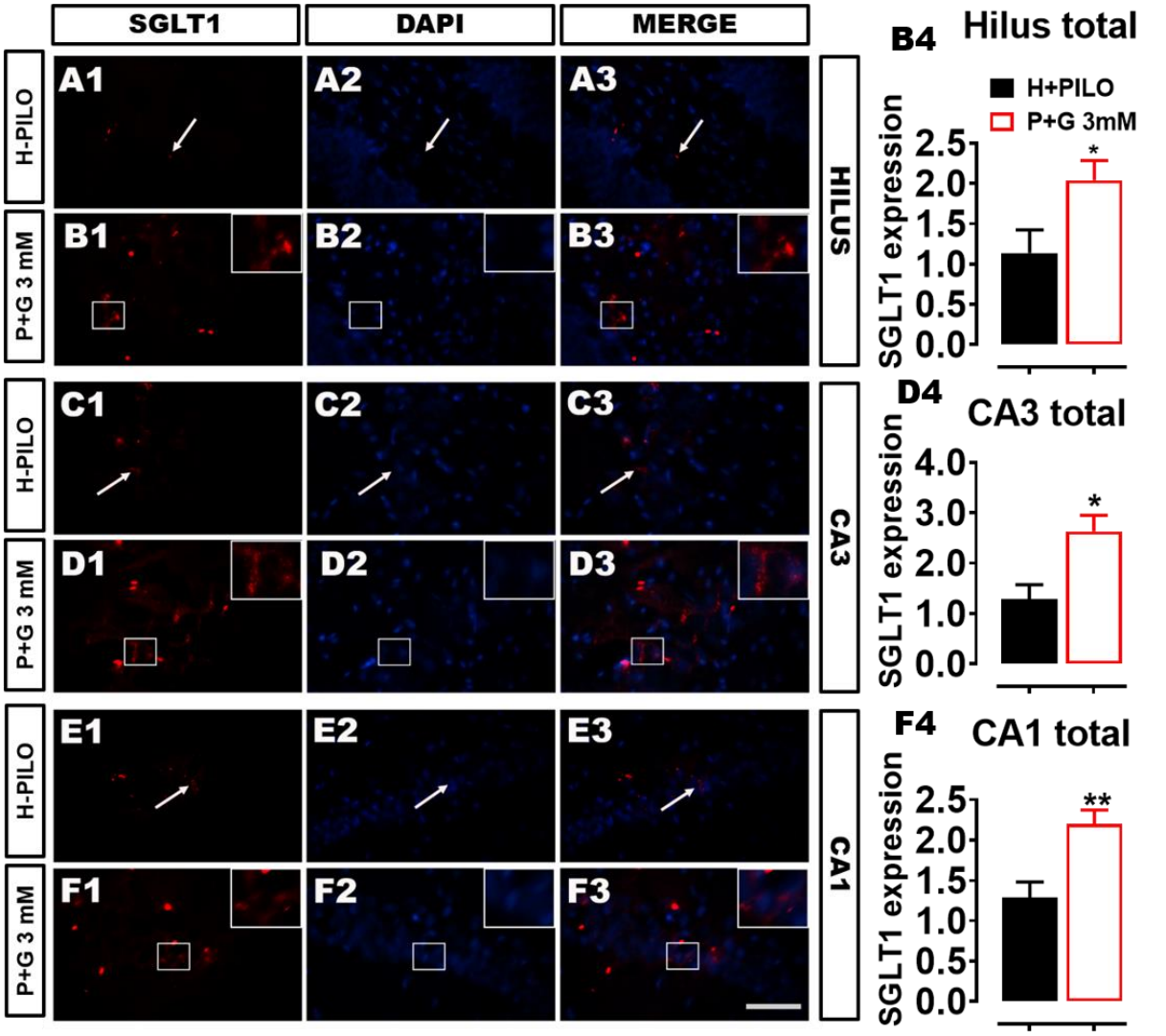


The nuclei were labeled with DAPI (blue, middle panels). Fluorescent labeling of the hippocampus shows strong cFOS immunoreactivity (red, left panels) in LPMR (A1), CL (C1), PVP (E1) and LaDL (G1) in H-PILO rats. Merge of cFOS and DAPI shown in right panels. Quantitative analysis of cFOS+ neurons in H-PILO (black bars), G+P (grey, blue and red bar) and P+G (grey, blue and red bar outline) rats are shown in A4, C4, E4 and G4. Some concentrations of hippocampal glucose (1, 2 or 3mM) before and after PILO decreased the number of cFOS+ neurons in LPMR (one-way ANOVA, $F(6, 22) = 10.94$, $P < 0.0001$; B4), CL (one-way ANOVA, $F(6, 22) = 3.54$, $P = 0.01$; D4), PVP (one-way ANOVA, $F(6, 20) = 5.21$, $P = 0.0023$; F4) and LaDL (one-way ANOVA, $F(3, 9) = 9.65$, $P = 0.0036$; H4) areas. The 3mM glucose concentration was used as a representative image. Arrows represent the brain areas. Magnification, 200x; scale bar, 50 µm. Error bars indicate the SEM. Data represent the mean ± S.E.M. of 5-6 rats. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 compared with H-PILO; one-way ANOVA with Dunnett's post-hoc test. H-PILO, pilocarpine and saline; G+P, glucose followed by pilocarpine infusion; P+G, pilocarpine followed by glucose infusion; PVP, posterior paraventricular th ncl; CL, centrolateral th ncl; LPMR, lateral posterior th ncl; LaDL, lateral amygdaloid ncl; SEM, standard error of the mean. Fonte: Autor

4.2.7 Translocation of the sodium/glucose cotransporter 1 (SGLT1) increases after high hippocampal glucose availability followed by PILO

During a metabolic deficit, such as SE, the uptake of glucose is increased by greater SGLTs function, which are essential during the epileptic seizures (Melo et al., 2016; Poppe et al., 1997; Yu et al., 2010, 2013). We analyzed SGLT1 expression by immunofluorescence to test whether increased glucose availability alters SGLT1 translocation to the neuronal membrane. The higher glucose concentration (3mM) administered after PILO was able to exacerbate the SGLT1 translocation in the DG hilus (*t-test*, $t_7 = 2.407$, $P = 0.0470$), as well as in CA3 (*t-test*, $t_8 = 2.926$, $P = 0.0191$) and CA1 (*t-test*, $t_8 = 3.467$, $P = 0.0085$) subareas of hippocampus compared to the control (Figure 7 A-F 1-4). Thus, modulation of hippocampal glucose plays an anticonvulsive and neuroprotective role possibly involving SGLT1.

Figure 7: Increased glucose supply increases the SGLT1 translocation in hippocampal neurons after pilocarpine-induced SE.



After 24h of SE, the SGLT1 expression was analyzed by immunofluorescence and quantified by densitometry in rats receiving glucose 5 min after PILO. The nuclei were labeled with DAPI (blue, middle panels). Fluorescent labeling of the hippocampus shows strong SGLT1 immunoreactivity (red, left panels) in hilar interneurons (DG hilus, A1) and pyramidal neurons (CA3, C1; and CA1, E1) in P+G rats. Merge of SGLT1 and DAPI shown in right panels. Quantitative analysis of SGLT1 immunofluorescent labeling of pyramidal neurons and interneurons of the H-PILO (black bars) and P+G (red bar outline) rats shown in B4, D4 and F4. Glucose modulation (3mM) after PILO intensified the SGLT1 expression in neurons of DG hilus (*t-test*, t_7 = 2.407, P = 0.0470; B4), CA3 (*t-test*, t_8 = 2.926, P = 0.0191; D4) and CA1 (*t-test*, t_8 = 3.467, P = 0.0085; F4) regions. Representative digital zoom was done on the photomicrographs of the control (B1-3, D1-3 and F1-3; see squares) Arrows represent the DG hilus, CA3 or CA1 regions. Magnification, 400x; scale bar, 25 μm. Error bars indicate the SEM. Data represent the mean ± S.E.M. of 4-6 rats. *P<0.05 and **P<0.01 compared with H-PILO; unpaired t-test. H-PILO, pilocarpine and saline; P+G, pilocarpine followed by glucose infusion; DZP, diazepam; SE, *Status epilepticus*; SEM, standard error of the mean. Fonte: Autor

4.3 DISCUSSION

Glucose consumption is acutely accentuated during epileptic seizures (Poppe et al., 1997; Yamada et al., 2009) and the effects of its modulation are vast and very complex in the epileptic brain. Along the SE, characterized by continuous and self-sustained seizures (Cameron et al., 2019; Meldrum and Brierley, 1973; Meldrum and Horton, 1973; Mohapel et al., 2004; Sánchez and Rincon, 2016), there is a marked increase in cerebral blood flow and oxygen consumption, and consequently, in glucose utilization (Franck et al., 1986). Most patients with SE present an intense secondary hypometabolism, which is capable of compromising several brain areas (Van Bogaert et al., 1994; Farooque et al., 2017; Fernández-Torre et al., 2006; García-García et al., 2017; Kim et al., 2012; Sakakibara et al., 2014). Severe hypometabolism coupled with limited glucose availability (Johansen and Diemer, 1986; Meldrum, 1983; Sapolsky and Stein, 1989) may be directly associated with the typical process of neuronal death in the hippocampus and nearby regions. In view of this, the control of glycemic status can be a determining factor during SE. In the present study, we evaluated the effects of high availability of hippocampal glucose on the severity of epileptic seizures and histological changes following PILO-induced SE. Overall, our main finding was that modulation of the hippocampal glucose index is able to protect the brain from damage resulting from PILO-induced SE.

After intrahippocampal PILO injection, a latency period preceding SE is common in animal models (Castro et al., 2011; De Furtado et al., 2002; Melo et al., 2016). The alteration in latency may be an important factor to be considered for the genesis of epileptic seizures; however, we observed that the latency interval for the first seizure decreased and for SE was not altered after glucose administration. In addition, WDS is typically observed throughout this latency phase prior to kainic acid- or PILO-induced SE (Grimes et al., 1988; Lothman and Collins, 1981; Rodrigues et al., 2005; Shin et al., 2009). Our findings demonstrated that glucose infusion after PILO was able to increase the number of WDS during latency. Previous studies have argued that WDS elevation can act as an endogenous anticonvulsant mechanism (Rodrigues et al., 2005). In other words, the pronounced motor manifestation of the WDS after cerebral glucose supply may be indicative of the reduction in seizures severity, considering that WDS and epileptic seizures may possibly be propagated through different pathways (Grimes et al., 1988; Lee and Hong, 1990).

Classically, epileptic seizures worsen throughout SE (Furtado et al., 2011), starting with milder seizures, such as chewing behavior and head nodding, that intensify for forelimb

myoclonus, rearing, and falling (Castro et al., 2011; Melo et al., 2016). In order to observe the effect of hippocampal glucose control on the severity of seizures, epileptic seizures followed by glucose infusion were evaluated according to Racine's scale (Racine, 1972). We demonstrated that intrahippocampal increased glucose availability after PILO was able to attenuate the number and total time of classes 3-5, indicating the decrease of the severity of the seizures.

Decreased score and duration of seizures have been associated with the anticonvulsant effect of several substances with antiepileptic potential (Citraro et al., 2011; Clinckers et al., 2004). In contrast to our findings, the intraperitoneal infusion of pyruvate, a natural metabolite of glucose, is not able to alter the time or severity of seizures (Kim et al., 2007). On the other hand, it has been previously described that the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) is capable of attenuating susceptibility to seizures (Yang et al., 2013), similarly to our data. The glycolytic inhibitor 2-DG has shown an efficient seizures suppressor effect in several animal models (Gasior et al., 2010; Stafstrom et al., 2009), reducing epileptiform burst, severity and duration of seizures, as well as increasing the latency for PILO-induced seizures in rats (Lian et al., 2007; Stafstrom et al., 2008). Because it is a glucose analog, 2-DG enters the cell via glucose transporters and is converted to 2-DG-6-phosphatase by hexokinase (Bissonnette et al., 1996; Kimmich and Randles, 1976; Sols and Crane, 1954), which impedes its metabolism by glycolysis (Chen and Guéron, 1992). Interestingly, 2-DG attenuates the levels of ATP that activate the non-voltage dependent potassium channel (K_{ATP}) regulated by intracellular ATP/ADP status (Hernandez-Sanchez et al., 2001; Yamada et al., 2001), as well as upregulates the protein and mRNA expression of Kir6.1 and Kir6.2 subunits of this channel (Yang et al., 2013), which together indicate an antiepileptic role (Ma et al., 2007).

Hyperglycemia and hypoglycemia have also influenced seizure susceptibility (Chou et al., 2016; Falip et al., 2014; Moseley et al., 2013; Schauwecker, 2012; Xia et al., 2016). Although no interference was observed in seizures sensitivity of hypoglycemic B6 mice, an interesting study showed that hyperglycemia was able to modulate seizure susceptibility (Schauwecker, 2012). As previous studies (Huang et al., 2009; Stafstrom, 2003), these authors found that streptozotocin (STZ)-induced hyperglycemia (experimental diabetes – chronic or sustained hyperglycemia model) was able to accentuate the duration of seizures during KA-induced SE in B6 mice, indicating a higher susceptibility to seizures. In contrast, using an acute hyperglycemia model, they showed that the severity of seizures was reduced due to the increase in latency time to onset of severe seizures. Based on this, the glycolytic pathway has

been shown to be a determinant factor for the modulation of seizures propagation, but the underlying mechanism for difference in seizures sensitivity in both models is uncertain. Thus, hippocampal glucose control may act as a potential anticonvulsant agent.

Typically, PILO-induced SE causes hyperexcitability neuronal and neuronal death in DG hilus, CA3 and CA1 subfields of the hippocampus (Cameron et al., 2019; Castro et al., 2011; Melo et al., 2016). The role of glucose control in the neurodegeneration process is complex and uncertain. It is known that during the SE there is an exacerbated release of glutamate and excessive intracellular calcium that may lead also to ROS increase (Ambrogini et al., 2019; Coulter and Eid, 2012; During and Spencer, 1993; Vishnoi et al., 2016), promoting cell death by excitotoxicity (Chiu et al., 2015; During and Spencer, 1993; Rossi et al., 2013). In addition, limited energy availability (Johansen and Diemer, 1986; Sapolsky and Stein, 1989), impaired ATP production (Wang et al., 1994), and the release of ROS (Dariani et al., 2013; Mishra et al., 2015; Pestana et al., 2010) may contribute to SE-induced excitotoxic cell death. Therefore, excitotoxic cell death may be sensitive to energy availability.

We report here that the increased infusion of intra-hippocampal glucose decreased neuronal activity and cell loss in areas of the hippocampus. Our findings corroborate previous studies that have shown that infusion of glucose or its metabolites exert a neuroprotective effect against SE-induced neuronal death. A relevant study demonstrated that administration of exogenous glucose (20%, ip) for 3 consecutive days following KA-induced SE significantly reduced cell loss in hilus DG, and CA3 and CA1 subfields (Schauwecker, 2012). Similarly, another study showed that pyruvate (500 mg/kg, ip) was able to attenuate neuronal death caused by KA-induced SE in the same regions of the hippocampus (Kim et al., 2007). These authors have interestingly correlated the lower neuronal loss promoted by pyruvate to the reduced accumulation of zinc in hippocampal neuronal cell bodies, suggesting a putative mechanism of action of the glycolytic pathway that justifies the potential neuroprotective effect. Many studies indicate zinc as an endogenous mediator of KA-induced neuronal death (Lee et al., 2003, 2000; Yi et al., 2003), which has been shown to occur through oxidative necrosis (Kim et al., 1999a, 1999b) associated with mitochondrial damage and energy failure (Jiang et al., 2001; Sensi et al., 2000), as well as NADPH oxidase induction and poly (ADP-ribose) polymerase (PARP) activation (Kim and Koh, 2002; Noh and Koh, 2000). These findings support the idea that controlled infusion of glucose or its metabolites may play a neuroprotective role, reducing neuronal activity and, consequently, the degeneration process caused by SE.

SE-induced seizures preferentially affect one or more extrahipocampal areas, including thalamus, amygdala, substance nigra and neocortical regions, increasing glucose utilization, excitability and neuronal death (Jung et al., 2009; Loss et al., 2012; Sakurai et al., 2015; Sanabria et al., 2002; Scholl et al., 2013; Zenki et al., 2018). Thalamus is a strategically located region that communicates through afferent and efferent pathways with motor areas of the cerebral cortex, amygdaloid nuclei, and motor-related subcortical structures, such as the basal ganglia (Bosch-Bouju et al., 2013; Young and Sonne, 2019). Since the basal ganglia-thalamo-cortical loops are responsible for controlling voluntary movement, involving events such as muscular contraction, motor planning and execution (Castro et al., 2011; Hooks, 2017; Iseki and Hanakawa, 2010), its dysfunction is associated with the development of generalized motor seizures (Lothman and Collins, 1981), neuronal damage and cognitive deficit (Jung et al., 2009; Loss et al., 2012; Ma et al., 2016). We demonstrated that intrahippocampal glucose supply was able to reduce neuronal activity and death following SE-induced PILO in the amygdaloid and thalamic nuclei, subiculum, substantia nigra and cortical areas. Similar to our data, as in the hippocampus, pyruvate protected the cortex and thalamus from neuronal death, which was correlated with decreased zinc in these regions (Kim et al., 2007). Since there is mitochondrial dysfunction, tricarboxylic acid cycle (TCA) damage and oxidative stress in hippocampal formation and other brain areas, such as the cortex and thalamus (Folbergrová et al., 2016; McDonald et al., 2017; Smeland et al., 2013), cerebral glucose control may be a putative therapeutic approach to protect against neuronal damage caused by SE.

Hippocampal and extrahipocampal regions are responsible for memory consolidation and learning processes. PILO-induced SE can cause damage to the hippocampus and several adjacent brain areas, triggering neuronal death and, consequently, learning deficits and memory dysfunction (Gröticke et al., 2007; Khalil et al., 2017; Lenck-Santini and Holmes, 2008; Long et al., 2017; Ma et al., 2016; Peixoto-Santos et al., 2015; Shetty, 2014). In order to evaluate the effect of hippocampal glucose modulation on memory consolidation, cognitive deficit followed by PILO-induced SE was analyzed by the inhibitory avoidance test. Our findings showed that glucose supply after PILO was not able to prevent memory dysfunction. According to our findings, it has been established that STZ-induced diabetic hyperglycemia worsens the memory and learning performances followed by PILO-induced SE (Huang et al., 2009). These authors demonstrated that chronic and progressive glycemic exacerbation plays an excitotoxic role that leads to neuronal death, which justifies cognitive damage. Although our data showed that controlled glucose increase in hippocampal region can protect from

neuronal loss, the deficit in memory consolidation and learning processes remained unchanged.

Additionally, PILO-induced SE is able to raise ROS generation by mitochondria, which are their major target (Santos et al., 2008; dos Santos et al., 2011; Shakeel et al., 2017; Xue et al., 2011). Mitochondrial dysfunction includes deficits in mitochondrial oxygen consumption ratios to form ATP (Pearson et al., 2015), decrease of respiratory chain complex I activity from 20h of SE (Folbergrová et al., 2016, 2018; McDonald et al., 2017), mitochondrial ultrastructural damage and reduction of cytochrome oxidase III (complex IV of respiratory chain) mRNA and protein levels in chronic epileptic rat tissue (Gao et al., 2014). Although attenuated neurodegeneration, our results showed that increased glucose availability did not interfere with oxidative stress followed 24h after SE, both in MDA (product of lipid peroxidation) levels and in CAT and SOD oxidant enzyme activity. The effect of hippocampal glucose modulation on oxidative stress in pilocarpine-induced SE is poorly described, but a study indicated that the use of a synthetic antioxidant prevented oxidative stress, deficits in mitochondrial oxygen consumption rates, hippocampal neuronal death and cognitive damage (Pearson et al., 2015). Therefore, the neuroprotective effect of hippocampal glucose control is not associated with oxidative stress.

During SE, a metabolic deficit, it has been established that SGLTs play a crucial compensatory role in glucose uptake by hippocampal neurons (Poppe et al., 1997; Yu et al., 2010, 2013). Previously, we demonstrated that nonspecific inhibition of SGLTs with phlorizin was able to enhance severity seizures and neuronal death, indicating the importance of their expression during SE (Melo et al., 2016). Here, we also showed for the first time, that intrahippocampal infusion of glucose possibly *via* glucose sensors T1R2/T1R3 (Ren et al., 2009) increased SGLT1 expression in hippocampal subfields, that have been associated with neuroprotection process similar to presented in our results.

4.4 CONCLUSION

In summary, we showed that increased glucose availability increases the amount of WDS, as an anticonvulsant attempt, and reduces the severity of seizures during PILO-induced SE. In addition, the ectopic intrahippocampal supply of glucose attenuates neuronal activity and the process of neurodegeneration in hippocampal and extrahippocampal regions, without preventing memory deficit and oxidative stress. Finally, we observed that these findings can be sustained by increased SGLT1 expression. Although hypo and hyperglycemia have been

reported as the reason for increased seizure susceptibility and neuronal damage following SE, our results support the hypothesis that local glucose control exerts a neuroprotective profile *via* T1R2/T1R3 induced-SGLT translocation, during the acute phase of epileptogenesis, indicating a putative therapeutic strategy, especially in patients with diabetes and others epileptic comorbidities.

4.5 METHODS

4.5.1 Animals

This study was conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the Brazilian Society of Laboratory Animals Science (SBCAL). All experimental procedures were approved by the Ethical Committee of the Federal University of Alagoas (Protocol # 04/2016), according to Ethical Principles adopted by the Brazilian College of Animal Experimentation (COBEA). Animal studies are reported in compliance with the approved guidelines. Experiments were conducted in male Wistar rats (*Rattus norvegicus* [n= 81, 240-340g, 2-3 months]) from the main breeding stock of the Federal University of Alagoas. Animals were maintained on a 12h/12h light/dark cycle at 21 ± 2°C, with lights on at 07:00 AM and lights off at 07:00 PM. They were individually housed in plastic cages with food and water *ad libitum*. All experiments were designed to minimize animal suffering and to limit the number of animals used.

All animals were monitored by research staff at least 2 times per day, in order to observe signs of illness or impairment by observing the general body condition, respiration rate, dehydration, posture, immobility, social interaction and response to manipulation. For the animals submitted to SE, monitoring the health was carried out for 2 hours/day until the complete post-ictal recovery (about 2 days after SE; note that H-PILO model allows rapid recovery and a high rate of survival (Castro et al., 2011; Melo et al., 2016). During this period, animals were treated with electrolyte and nutrient replacement (i.p. injection of saline 0.9%; and by feeding animals with pasty food). None of the animals presented clinical/behavior signal of pain or unexpected distress, used as humane endpoint criteria for euthanasia.

4.5.2 Surgical procedure

Animals were anesthetized with ketamine (100 mg/kg, ip), and xylazine (10 mg/kg, ip), received 0.1 mL/100g veterinary pentabiotic (Fort Dodge®, subcutaneous) before the surgery. After fixing on stereotaxic, animals received local anesthetic (lidocaine with epinephrine, subcutaneous [Astra®]). Posteriorly, a cannula was implanted stereotaxically in the hilus of the dentate gyrus (DG) of the left hippocampus, according to following coordinates: - 6.30 mm anterior-posterior (AP, reference: bregma); 4.50 mm medial-lateral (ML, reference: sagittal sinus); - 4,50 mm dorsal-ventral (DV, reference: dura mater) (Castro et al., 2011; De Furtado et al., 2002; Melo et al., 2016; 2007). After the surgery, animals were seven days in recovery.

4.5.3 Intrahippocampal microinjections

Animals received either glucose (G), pilocarpine (H-PILO) or its vehicle (VEH), intrahippocampally. The rats were divided into 8 experimental groups: VEH (n=10), H-PILO (n=10), G+P (1mM, n=10), G+P (2mM, n=10), G+P (3mM, n=10), P+G (1mM, n=10), P+G (2mM, n=10) and P+G (3mM, n=11).

Animals were gently immobilized and the drugs microinjection was performed. Animals H-PILO and G+P received microinjections of VEH (saline 0.9%) or G (1, 2 or 3mM/μL [diluted in saline]) in the left hilus of the dentate gyrus (DG) of hippocampus followed 30 minutes later by PILO (1.2mg/μL) to evoke limbic seizures (H-PILO or G+P). In addition, P+G received glucose (1, 2 or 3mM/μL) after 5 minutes of PILO. VEH group received only 1μL of intrahippocampal saline. We used a 5μL syringe (Hamilton Company, Reno, NV, USA) connected to a microinjection pump (Harvard Apparatus PHD 2000, Holliston, MA, USA) at a speed of 0.5 μl/min.

All animals that develop SE were rescued with diazepam (5 mg/kg; i.p.) after 90 minutes of SE onset. Furthermore, animals that did not develop SE received the injection of diazepam under the same conditions.

4.5.4 Behavioral analysis

4.5.4.1 SE seizures

After microinjection of PILO, behavioral activity was recorded by video camera (Full HD Digital Camcorder Sony DCR-PJ6) for a period of 90 minutes, which is enough time to observe neurodegeneration (Castro et al., 2011; Melo et al., 2016). Racine's scale (1972) (Racine, 1972) was used to categorize the behavioral analysis into the following classes were observed: (0) immobility; (1) facial movements; (2) head nodding; (3) forelimb clonus; (4) rearing; (5) rearing and falling.

Furthermore, the latency period for the first seizures (class 2) and for the SE were analyzed. Number of wet dog shake (WDS) was quantified before and along SE. During the SE, the 90 minutes observation time was split into 18 windows of 5 minutes and the most severe seizure with more frequency in each interval was used to represent the window (Castro et al., 2011). In addition, the number and total time of classes 3-5 seizures were analyzed to better understand the severity and evolution of seizures along the SE among different experimental groups. Finally, to determine the severity of seizures the representative scales of each window were summed and the result was divided by the total number of windows.

4.5.4.2 Inhibitory Avoidance Test (IAT)

In the inhibitory avoidance test (IAT), all animals were placed in an automatically operated box (40x25x25 cm) with a wall glass front, being the floor constituted by a steel grid coupled to an energy generating box. The test was divided in: (1) learning/training session, before SE, animal was kept on the platform and received a shock (2.0s of 0.2 mA) after getting off with its four paws on the grid; (2) test session, 24 hours after training and SE, animal was placed in the same apparatus, under the same environmental conditions, without the aversive stimulus.

4.5.5 Biochemical Assessments

Animals (n = 14) that survived within 24h after pilocarpine-induced SE were guillotined and the brains were directly placed on an ice-plate and dissected in order to remove the hippocampus. Hippocampi were immediately frozen in liquid nitrogen for further future use and stored at -80ºC.

4.5.5.1 Total thiol content (Sulfhydryl groups)

Sulfhydryl content was determined from reaction with compound DTNB (5,5′-Dithiobis(2-nitrobenzoic acid). Aliquot of homogenate (100 µg protein) was incubated in the dark with 25 µL of DTNB (20 mM) and the final volume of 1 mL with extraction buffer was completed. Absorbance reading was taken on spectrophotometer (AJX-6100PC) at 412 nm (Ellman, 1959). Results were expressed in mmol/mg protein.

4.5.5.2 Lipid peroxidation

The colorimetric technique was used for the determination of thiobarbituric acid reactive substances (TBARS) (Buege and Aust, 1978). A total of 0.3 mg/mL of hippocampi homogenate were added to 200 µL of 30% (*w/v*) trichloroacetic acid and stirred for 1 min. Then, 200 µL of 10 mM TRIS HCl, pH 7.4 was added to the material, stirred for 1 min and then centrifuged at 2500 rpm for 10 minutes at 4 ° C. The collected supernatant (450 µl) was mixed with 0.73% (*w/v*) thiobarbituric acid (450 µL) which reacted with the lipoperoxidation products to form a pink colored compound. The mixture was incubated for 15 minutes at 100°C and then cooled. The absorbance reading was performed in a spectrofluorimeter (Flex Station 3, Molecular Devices) at 535nm. The results were expressed as mmol/mg protein.

4.5.5.3 Superoxide dismutase (SOD) activity

Determination of SOD activity was carried out accordingly to Misra and Fridovich (1972). The hippocampi homogenate (100 µg protein) was incubated in sodium carbonate buffer (50 mM, pH 10.2, + 0.1 mM EDTA) in a water bath at 37 ° C. The reaction was initiated by the addition of 30 µL of epinephrine (150 mM) in acetic acid (0.05%) in a final volume of 1mL. The absorbance was read at 480nm for 1 min on the spectrophotometer (AJX-6100PC). One unit of SOD was defined as the amount of protein required to inhibit the autoxidation of 1 µmol of epinephrine per minute. The results were expressed in U/mg protein.

4.5.5.4 Catalase activity (CAT)

Catalase activity was monitored accordingly to Aebi (1984). The test is based on the determination of the decomposition of H_2O_2. 80 µg of proteins were added to a 50 mM phosphate buffer (sodium phosphate monobasic monohydrate + dibasic sodium phosphate), pH 7.0, 35 °C. The reaction was then started with 0.3 mM H_2O_2 in a final volume of 1 mL. The decrease in absorbance was monitored at 240 nm on a spectrophotometer (AJX-6100PC) for 1 min. One unit of CAT was defined as the amount of protein required to convert 1 µmol de H_2O_2 per minute to H_2O. The results were expressed in U/mg protein.

4.5.6 Histological processing

In order to perform the histological procedures, animals were injected with an overdose of xylazine and ketamine at 24 hours after SE induction, and were transcardially perfused with 0.1 M phosphate-buffered saline, pH 7.4 (PBS), followed paraformaldehyde solution (4%, diluted in PBS). Afterwards, the brains were removed, cryoprotected with sucrose 20%, frozen at -20 ºC for 3 hours and stored at −80°C. Sections were then cut (30 µm thickness) using a cryostat (Leica CM 1850) at a temperature ranging from -18 to -22°C and were processed for FJ-C staining and immunofluorescence techniques.

4.5.6.1 FJ-C staining procedure

Brain sections were placed onto slides and then subjected to successive washes of 100% ethanol for 3 minutes, 70% ethanol for 1 minute, distilled water for 1 minute. Afterwards, slides were transferred to a solution of 0.06% potassium permanganate for 15 minutes on a rotating platform. Slides were rinsed three times for 1 minute in distilled water and then transferred to the FJ staining solution (0.0001%) for 30 minutes. After, slides were rinsed three times for 1 minute in distilled water (Schmued et al., 1997). Finally, slides were coverslipped using *fluoromount* (EMS). The sections were examined and images captured using a fluorescence microscope (Nikon DS RI1).

4.5.6.2 SGLT1 and c-Fos immunofluorescences

We used an antibody that binds to SGLT1 or nuclear protein c-fos. After PILO-induced SE there is an increased glucose uptake via SGLT and c-Fos overexpression, which indicates a neuronal hyperexcitability. Immunofluorescence was used to analyze the expression of both proteins primarily in the hippocampus and other areas of the brain.

The immunohistochemistry assays were done in histological slide and the protocol used for detection of SGLT1 and c-Fos antigens was the same, with only alteration of primary antibodies. The following primary antibodies were used: rabbit polyclonal IgG to SGLT1 (Catalog Number - Orb11364, Biorbyt®, 1:100) and rabbit polyclonal IgG to c-Fos (Lot # C1010, Santa Cruz Biotechnology®, 1:50). Secondary antibody was used: Alexa Fluor 594 Donkey secondary antibody (anti-rabbit IgG, Biolegend®, San Diego, CA; 1: 2000). Briefly, the immunohistochemistry protocol begins with brain tissue slices submerged in methanol (10 min), followed by two baths of 10 min in PBS 1x. An antigenic rescue was then performed with citrate buffer (pH 6) for 10 min (output 6) and, after a cooling period (30 min, room temperature), immersed in the same solution. Sections were then incubated in an autofluorescence blocking solution with PBS/glycine 3% (1h, room temperature), followed by a second blocking solution for nonspecific sites using fish skin gelatin in 0.05% in PBS 1X and equine serum 1.5% (1h, room temperature). Shortly thereafter, it was incubated with the anti-SGLT1 or anti-cfos primary antibody diluted in fish skin gelatin in 0.05% in PBS 1X (overnight, 4°C). In the second stage, the slices were washed with PBS 1x (2 times in 10 min), followed by incubation with Alexa 594 diluted in fish skin gelatin 0.05% in PBS 1X (1h, room temperature). Sections were washed with PBS 1x (two baths of 5 minutes) and DNA was counterstained with fluorescent dye 4',6-diamidino-2-phenylindole (DAPI, ab104139, Abcam®, USA; 1:1000, diluted in PBS 1x, 15 min, room temperature). Finally, the sections were washed (PBS 1x, five baths of 2 min) and used as mounting medium PBS/glycerol. To control for binding specificity, sections were subjected to the same protocol with omission of anti-SGLT1 and anti-cfos primary antibodies. Sections were examined and images captured using a fluorescence microscope (Nikon DS RI1).

4.5.6.3 Cell counting and densitometry

SGLT1 expression was quantified by densitometry, while fluoro-Jade positive (FJ+) and c-Fos positive (c-Fos+) cells were quantified by using the ImageJ software (Wayne Rasband; Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA). In order to quantify the FJ+ and c-Fos+ neurons in the hippocampus and extrahippocampal areas, different coordinates were used (2007). All cells were counted on the contralateral side because animals that received microinjection of PILO developed a scar around the microinjection site (Castro et al., 2011).

In hippocampus, we sampled in three different coordinates: CA1, CA3 and hilus of dentate gyrus, (AP −2.56 mm; AP −3.30 mm and AP −6.30 mm), as showed by Castro et al. (2011). These regions were selected because of the high sensitivity to the neurodegenerative process. In addition, for the mapping of cortical areas we used three different coordinates: motor primary and secondary (M1 and 2), somatosensory primary (S1), retrosplenial granular (RSGc), agranular insular (AIP), ectorhinal (Ect), perirhinal (PRh), and piriform (Pir) (AP -2.64 mm; AP -3.36 mm; AP -4.80 mm). Additionally, the mapping of the dorsal *subiculum* (DS) and the substantia nigra (reticular part, SNR) were made based on three other coordinates: DS (AP -4.92 mm; AP -5.04 mm; AP -5.20 mm) and SNR (AP -4.80 mm; AP -4.92 mm; AP -5.04 mm). Finally, the thalamic (dorsal lateral geniculate [DLG]; lateral posterior, mediorostral part [LPMR]; centrolateral [CL]; and paraventricular, posterior part [PVP] and amygdaloid [lateral, dorsolateral part [LaDL]) nuclei were mapped according to the following coordinates: thalamus (AP -2.92 mm; AP -3.36 mm; AP -3.72 mm) and amygdala (AP -2.92 mm; AP -3.48 mm; AP -3.84 mm).

4.5.7 Statistical analysis

All experimental values are presented as mean ± SEM and a significance level of 5% (described as $p < 0.05$) was adopted for all statistical tests. Comparisons of most of the results were performed by unpaired t test or one-way analysis of variance (ANOVA), followed by Dunnett's post-test (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA). Only in the inhibitory avoidance test, the data were expressed as median with interquartile range and compared by the Kruskal-Wallis test. The number of animals is cited in the figure legends.

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AUTHOR CONTRIBUTIONS

Conceptualization, I.S.M., R.S.S. and O.W.C.; Methodology, I.S.M., Y.M.O.S., A.L.D.P., M.A.C., V.O.S., J.F.S., C.M.B.C., R.C.S.F, A.C.R.L., R.S.S. and O.W.C.; Investigation, I.S.M., A.C.R.L., A.U.B., R.S.S. and O.W.C.; Formal Analysis, I.S.M., R.C.S.F, A.C.R.L., R.S.S. and O.W.C.; Supervision and Fund Acquisition, O.W.C.; Writing – Review & Editing, I.S.M., D.G.L.G., M.D., A.U.B., R.S.S. and O.W.C.; Resources, M.D., A.U.B., R.S.S. and O.W.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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ANEXO

Anexo 1. Comitê de Ética em Experimentação Animal

UNIVERSIDADE FEDERAL DE ALAGOAS
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CEUA

CERTIFICADO

Certificamos que a proposta intitulada "Papel da glicose e de peptídeos miméticos de receptores de peptídeos N-formil (FPR) em cotransportadores de sódio/glicose (SGLTs) após *Status Epilepticus* induzido por microinjeção intra-hipocampal de pilocarpina", registrada com o nº **04/2016**, sob a responsabilidade de **Olagide Wagner de Castro,** que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Federal de Alagoas - CEUA/UFAL, em reunião de 08/04/2016.

Finalidade	( ) Ensino (X) Pesquisa científica
Vigência da autorização	21.06.2016 a 21.06.2020
Espécie/linhagem/raça	Rato heterogênico / Wistar
Nº de animais	164
Peso/idade	150g-300g
Sexo	Machos
Origem/Local de manutenção	Biotério Central da Universidade Federal de Alagoas – UFAL / Biotério do Laboratório de Neurofarmacologia e Fisiologia Integrativa do Instituto de Ciências Biológicas e da Saúde (UFAL)

Maceió, 13 de abril de 2016.

Profª. Drª. Silvana Ayres Martins
Coordenadora CEUA/UFAL

Profa. Dra. Silvana Ayres Martins
Coordenadora da Comissão de
Ética no uso de Animais
SIAPE 1120858