

SHORT RESEARCH ARTICLE

Brain glycogen content is increased in the acute and interictal chronic stages of the mouse pilocarpine model of epilepsy

Gi Young Seo¹  | Elliott S. Neal¹  | Felicity Han¹  | Diana Vidovic¹  |
Fathima Nooru-Mohamed¹ | Gerald A. Dienel^{2,3}  | Mitchell A. Sullivan⁴  |
Karin Borges¹ 

¹School of Biomedical Sciences, Faculty of Medicine, The University of Queensland, St Lucia, Queensland, Australia

²Department of Neurology, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA

³Department of Cell Biology and Physiology, University of New Mexico School of Medicine, Albuquerque, New Mexico, USA

⁴Glycation and Diabetes Group, Mater Research Institute, Translational Research Institute, The University of Queensland, Brisbane, Queensland, Australia

Correspondence

Karin Borges, School of Biomedical Sciences, The University of Queensland, St Lucia, QLD 4072, Australia.
Email: k.borges@uq.edu.au

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Abstract

Glucose is the main brain fuel in fed conditions, while astrocytic glycogen is used as supplemental fuel when the brain is stimulated. Brain glycogen levels are decreased shortly after induced seizures in rodents, but little is known about how glycogen levels are affected interictally in chronic models of epilepsy. Reduced glutamine synthetase activity has been suggested to lead to increased brain glycogen levels in humans with chronic epilepsy. Here, we used the mouse pilocarpine model of epilepsy to investigate whether brain glycogen levels are altered, both acutely and in the chronic stage of the model. One day after pilocarpine-induced convulsive status epilepticus (CSE), glycogen levels were higher in the hippocampal formation, cerebral cortex, and cerebellum. Opposite to expected, this was accompanied by elevated glutamine synthetase activity in the hippocampus but not the cortex. Increased interictal glycogen amounts were seen in the hippocampal formation and cerebral cortex in the chronic stage of the model (21 days post-CSE), suggesting long-lasting alterations in glycogen metabolism. Glycogen solubility in the cerebral cortex was unaltered in this epilepsy mouse model. Glycogen synthase kinase 3 beta (Gsk3b) mRNA levels were reduced in the hippocampal formations of mice in the chronic stage, which may underlie the elevated brain glycogen content in this model. This is the first report of elevated interictal glycogen levels in a chronic epilepsy model. Increased glycogen amounts in the brain may influence seizure susceptibility in this model, and this warrants further investigation.

KEYWORDS

glutamine synthetase, Gsk3b, status epilepticus, temporal lobe epilepsy

Gi Young Seo and Elliott S. Neal contributed equally.

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1 | INTRODUCTION

Brain glycogen is used as an energy source during seizures when energy needs are high,¹ and glycogen levels decrease minutes after the onset of seizures induced in rodents by various convulsant chemicals or by maximal electroshock.²⁻⁸ During periods of high neuronal activity compared with resting, glycogen is believed to be the preferred fuel of astrocytes as it quickly yields ATP needed for rapid glutamate uptake⁹ and ion pumping.^{10,11}

Brain glycogen levels are reduced shortly after acute induced seizures in rodents,²⁻⁸ but little is known about glycogen metabolism in chronic epilepsy models. In surgically-excised ictogenic hippocampal foci from humans with chronic epilepsy, glycogen levels are high¹² and glutamine synthetase activity is low.^{13,14} DiNuzzo and colleagues suggested that glycogen levels are high in epilepsy because of reduced glutamine synthetase function.¹⁵ The authors proposed that this glycogen becomes insoluble and unmetabolisable, which may contribute to the high levels.¹⁵

Glycogen metabolism has not been explored in chronic models of epilepsy. Here, we determined whether interictal brain glycogen levels and solubility, glutamine synthetase activity and expression of glycogen metabolism genes were altered in the acute and/or chronic stages of the mouse pilocarpine model of epilepsy.

2 | METHODS

2.1 | Animals

Male CD1 mice (Animal Resource Centre, WA, Australia) were individually housed in open-top cages under a 12/12 light/dark cycle with standard chow and water given ad libitum. Mice weighed 30-40 g when experiments started. Experiments were approved by the University of Queensland's Animal Ethics Committee (SBMS/141/17).

2.2 | Pilocarpine status epilepticus model

Convulsive status epilepticus (CSE) was induced with pilocarpine as described,¹⁶ with some modifications. Mice were acclimatized for 1 week before being injected with methyl-scopolamine (2 mg/kg in 0.9% saline, ip) 15 minutes prior to an injection of pilocarpine (300-350 mg/kg in 0.9% saline, sc). This induced CSE in around 30%-40% of mice and the remainder did not experience CSE or died. For 90 minutes following pilocarpine injection, mice were observed, and seizure behavior

scored every 15 minutes from 0 to 5 according to a modified Racine scale where 0 = no reaction or immobility; 1 = shivering; 2 = light clonic seizure; 3 = whole or partial body continuous clonic seizure while retaining posture; 4 = severe clonic seizure (whole-body severely seizing, mouse close to falling over); and 5 = tonic clonic seizure, loss of balance and/or jumping. After 90 minutes, mice received diazepam (10 mg/kg in 0.9% saline, sc) and were classified as CSE or no CSE. Mice were classified as CSE if they experienced continuous behavioral seizures for a minimum of 45 minutes, mainly consisting of whole-body clonic seizures (stage 3) with occasional severe stage 4 or 5 seizures. Mice that did not display this behavior were classified as no CSE. Some no CSE mice experienced shivering (stage 1) and/or 1-2 infrequent seizures (stage 2-5) over the 90 minute period, but this did not reach a continuous state and occurred usually early after the pilocarpine injection. Please note that in our previous studies, we have referred to mice in this model as being either "SE" or "no SE." Owing to a lack of video-electroencephalography (discussed below), we cannot eliminate the possibility that some mice experienced nonconvulsive SE and thus here refer to mice as CSE or no CSE. Naïve mice were injected with methylscopolamine and diazepam and saline instead of pilocarpine.

A portion of the mice was killed the next day (one day post-CSE, acute stage) by focal microwave fixation of the head³ to assess glycogen content or by cervical dislocation to assess glutamine synthetase activity.

Three days following pilocarpine injection, the remaining CSE mice were injected sc with 1 mL 4% dextrose twice daily, given wetted chow, and handfed peanut butter and jam. If >15% weight loss was observed mice were euthanized, per ethical guidelines. All remaining mice were kept until 21 days post-CSE (chronic stage) and then killed by focal microwave fixation of the head³ to assess glycogen content or by cervical dislocation to determine gene expression. Brain regions were isolated, snap-frozen in liquid nitrogen, and stored at -80°C. No CSE mice were used as "healthy" controls.¹⁶ For day 1 glycogen measurements, data from naïve and no CSE mice were pooled due to the limited availability of microwave-fixed tissue at this timepoint.

Spontaneous seizures were not monitored by video-electroencephalography in this study. However, we and others have previously shown using video-electroencephalography that in our model all CSE mice in the chronic stage develop spontaneous recurrent seizures and on average experience around one seizure per day.^{17,18} All mice that were sacrificed 1 day after pilocarpine injection were not observed to have any behavioral seizures in the 60 minutes prior to being sacrificed. When sacrificing mice in the chronic stage of the model, mice were only sacrificed when no behavioral

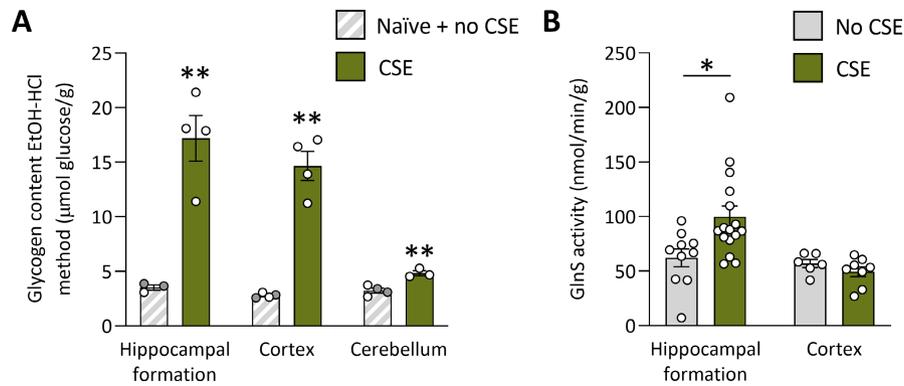


FIGURE 1 Brain glycogen content and glutamine synthetase activity are elevated in the acute stage of the mouse pilocarpine model. (A) Glycogen content (EtOH-HCl method, $n = 2$ naïve mice (gray circle) and $n = 1-2$ no CSE mice (white circle) for pooled control group, $n = 3-4$ CSE mice) and (B) glutamine synthetase (GlnS) activity ($n = 11-16$ /group hippocampal formation, $n = 6-8$ /group cortex) 1 day post-CSE. Data analyzed by unpaired t test (with Welch's correction if required) in A and/or Mann-Whitney U test in B. * $P < .05$, ** $P < .01$. CSE, convulsive status epilepticus

seizures were observed within 60 minutes prior, indicating mice were in the interictal stage.

2.3 | Glycogen levels and solubility

Glycogen was extracted and assayed using the ethanol-HCl-amyloglucosidase procedure.¹⁹ Glycogen was also extracted from matched cortex tissue using a glycogen isolation buffer (GIB) and then KOH to use another method and determine glycogen solubility²⁰ (Appendix S1).

2.4 | Glutamine synthetase activity assay

Cytosolic fractions were isolated from hippocampal formations²¹ (1 day post-pilocarpine) and glutamine synthetase (GlnS) activity was measured via the production of γ -glutamyl-hydroxamate at 540 nm using methods adapted from^{13,22} (Appendix S1).

2.5 | Quantitative PCR

Total RNA from hippocampal formations (21 days post-pilocarpine) was extracted with QIAzol (ThermoFisher Scientific). 1 μ g total RNA was reverse transcribed into cDNA (iScript gDNA Clear cDNA Synthesis Kit; Bio-Rad) and qPCR performed using the QuantiNova SYBR Green PCR Kit (primer details in Table S1). 10 μ L reactions were amplified in duplicate using the QuantStudio 6 Real-Time PCR system (Life Technologies). Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method, compared to the geometric mean of Tbp and Hmbs.

2.6 | Data analysis

Statistical analyses were performed in GRAPHPAD PRISM 9.1.2 (GraphPad Software). All data are mean \pm SEM and were assessed for normality (Shapiro-Wilk normality test) and homogeneity of variance (F test or Brown-Forsythe test). Data with 3 groups were analyzed by one-way ANOVA with the Fisher's LSD post hoc test. All other data were analyzed by unpaired t test (Welch's correction applied if variances are unequal), or Mann-Whitney U test if non-normally distributed. Data points ± 2 SD from the mean were excluded.

3 | RESULTS

Using the EtOH-HCl extraction method, all brain regions assessed showed higher glycogen content in mice 1 day post-CSE compared with a pooled group of naïve and no CSE mice. Glycogen content increased in CSE mice 4.9-fold in the hippocampal formation ($P = .007$), 4.4-fold in the cortex ($P = .003$) and 1.7-fold in the cerebellum ($P = .005$) compared with control mice (Figure 1A). At this timepoint, glutamine synthetase activity increased in the hippocampal formations of CSE mice by 1.6-fold ($P = .010$) but was unchanged in the cortex ($P = .25$) (Figure 1B).

Glycogen content was elevated 21 days post-CSE 2.7-fold in the hippocampal formation and 1.9-fold in the cortex (Fisher's LSD, both $P < .001$) compared with no CSE mice. Cerebellar glycogen content was unchanged (one-way ANOVA, $P = .06$) (Figure 2A).

Using the GIB-KOH extraction method, CSE mice had 1.4-fold greater cortical glycogen content compared with no CSE mice 21 days after pilocarpine injection ($P = .003$,

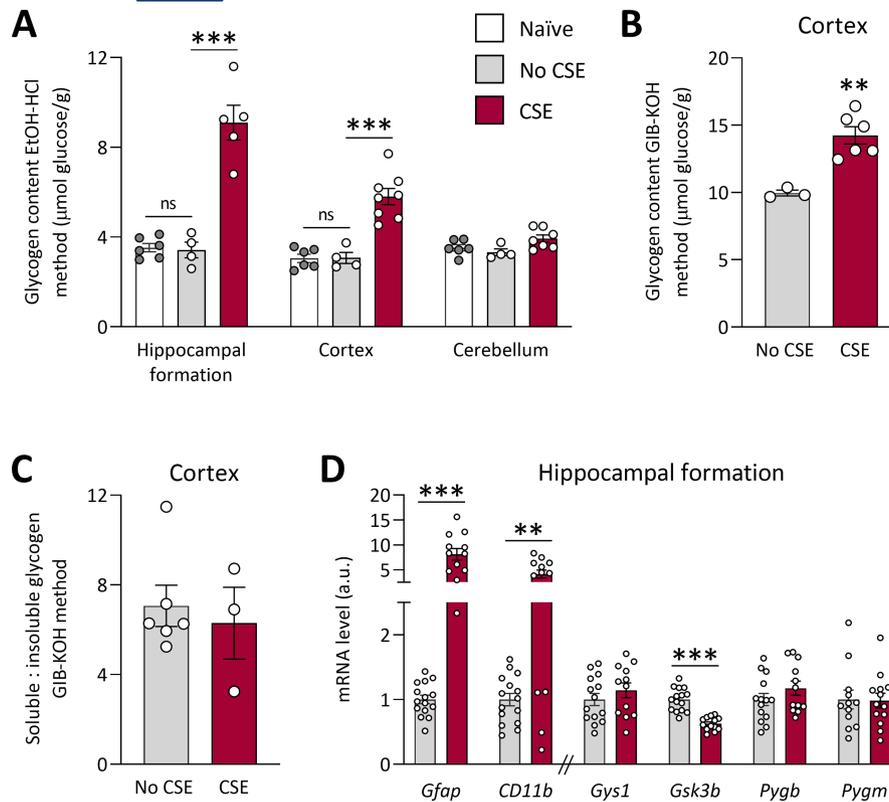


FIGURE 2 Glycogen content is elevated in the chronic stage of the mouse pilocarpine model. (A) Interictal glycogen content 21 days post-CSE (EtOH-HCl method, $n = 4-8/\text{group}$). (B) Interictal glycogen content and (C) ratio of soluble to insoluble glycogen in cortex 21 days post-CSE using GIB-KOH method ($n = 3-6/\text{group}$). (D) Expression of CSE-associated markers and glycogen metabolism genes in hippocampal formations ($n = 12-15/\text{group}$). Data analyzed by one-way ANOVA with Fisher's LSD post hoc test in A, unpaired t test (with Welch's correction if required) in B, D, and Mann-Whitney U test in C. ** $P < .01$, *** $P < .001$. CSE, convulsive status epilepticus; a.u., arbitrary units; Gfap, glial fibrillary acidic protein; CD11b, integrin subunit alpha M; Gys1, glycogen synthase 1; Gsk3b, glycogen synthase kinase 3 beta; Pygb, brain glycogen phosphorylase; Pygm, muscle glycogen phosphorylase; ns, not significant

Figure 2B). At this timepoint, cortical glycogen solubility in GIB was unchanged ($P = .66$, Figure 2C). Note that the GIB-KOH extraction method yielded 9.2-fold higher glycogen levels than the EtOH-HCl method for the microwave-fixed cerebral cortical tissue (Figure S1).

mRNA levels of markers associated with CSE, glial fibrillary acidic protein (Gfap, 8.1-fold, $P < .001$), and integrin subunit alpha M (CD11b, 4.1-fold, $P = .003$), were higher in the hippocampal formation 21 days post-CSE as reported previously. mRNA levels of glycogen synthase kinase 3 beta (Gsk3b) were reduced by 37% 21 days post-CSE ($P < .001$). Glycogen synthase 1 (Gys1, $P = .34$), brain glycogen phosphorylase (Pygb, $P = .23$) and muscle glycogen phosphorylase (Pygm, $P = .93$) mRNA levels were unchanged (Figure 2D).

4 | DISCUSSION

Important findings in the acute stage of the pilocarpine model were that brain glycogen content and hippocampal glutamine synthetase activity were increased in mice

1 day post-CSE. Major findings in the chronic stage of the pilocarpine model were that (1) interictal glycogen content increased in the hippocampal formation and cerebral cortex, (2) in the cerebral cortex, this finding was reproduced with a second extraction method, (3) glycogen solubility in GIB in the cerebral cortex was unchanged, and (4) Gsk3b mRNA levels were reduced in the hippocampal formation.

Increases in brain glycogen content were most pronounced 1 day post-CSE. This is similar to another study that found higher cortex glycogen content 24 hours after acute homocysteic acid-induced seizures in rats.²³ Increased glycogen content was also found in the cortex and cerebellum following acute seizures induced by administration of the glutamine synthetase inhibitor methionine sulfoximine (MSO) in mice, but these increases in glycogen began prior to seizures and are thought to be a direct effect of MSO (reviewed in²⁴). In contrast, we found increased glutamine synthetase activity in the hippocampal formation on day 1, similar to previous findings 3 days after SE in a rat lithium-pilocarpine model.²⁵ Due to higher glutamine synthetase activity, and known depletion of

brain glycogen levels after stress^{26,27} and seizure onset,²⁻⁸ we expected reduced brain glycogen levels 1 day post-CSE. We propose that high glycogen levels 1 day post-CSE are due to inhibition of GSK3 β , an enzyme that can phosphorylate glycogen synthase to inactivate it. Inhibition of GSK3 β has been found in hippocampal extracts from mouse and rat pilocarpine models, appeared most pronounced early after pilocarpine-induced SE, and is seen as increased inhibitory phosphorylation of GSK3 β .^{28,29} Similar changes in GSK3 β phosphorylation were found 8 hours following kainate-induced SE in mice.³⁰

GSK3 β inhibition is expected to enhance GYS activity, promoting glycogen accumulation. Although Gsk3b deletion in the kidney³¹ and skeletal muscle³² increased glycogen levels in these tissues in mice, no study has used brain-specific Gsk3b-deficient models to determine whether Gsk3b ablation increases brain glycogen content.

Brain glycogen content was also high interictally in the chronic stage of the pilocarpine model, suggesting long-lasting interictal changes in glycogen metabolism. Importantly, two extraction methods both showed that cortex glycogen amounts were higher in chronic CSE mice (methods compared in Appendix S1). High interictal glycogen levels may be an adaptive mechanism to reduce seizure generation. Higher glycogen amounts will increase reserves of quickly accessible fuel, which can be used when energy needs are high to fuel the sodium-potassium ATPase to reset membrane potentials, which can avert the generation of a seizure. Cloix and Hevor proposed that increased brain glycogen levels may contribute to the high latency to seizure (minimum 4-6 hours³³) after MSO administration in mice.²⁴ Consistent with this, GYS1^{Nestin-KO} mice were more susceptible to kainate-induced seizures.³⁴ However, mice with GYS1 deleted from astrocytes³⁵ or forebrain excitatory neurons³⁶ did not show this altered susceptibility, which suggested that greater seizure susceptibility in GYS1^{Nestin-KO} mice may be driven by a loss of GYS1 in another cell type.³⁵ Conversely, once a seizure has developed, high levels of glycogen may also fuel seizures.

Interictal increases in glycogen levels have been suggested to be due to the formation of insoluble glycogen.¹⁵ However, in the present study, cortex glycogen solubility in GIB was unchanged in chronic CSE mice. In both CSE and no CSE groups, the quantity of soluble glycogen was approximately six to seven times that of the insoluble form. Although there was close to no insoluble glycogen in control mouse skeletal muscle,²⁰ we observed moderate levels of insoluble glycogen even in microwave-fixed “healthy” mouse brain. This may be attributable to differences in tissue type, fixation, and extraction procedure. Similar to day 1, glycogen accumulation may be due to reduced GSK3 β function. Increases in inhibitory phosphorylation of GSK3 β are reported in chronic mouse and rat

pilocarpine models.^{37,38} In our chronic model, we demonstrated reduced Gsk3b mRNA levels in the hippocampal formation. Although not measured, like in previous studies,^{37,38} we expect lower GSK3 β activity and inhibition of GSK3 β at the phosphorylation level. GSK3 β controls neuronal excitability by regulating the expression and activity of voltage-gated sodium, potassium, and calcium channels, and glutamatergic and GABAergic receptors (reviewed in³⁹). However, there is no consensus about the extent and how GSK3 β function alters seizure susceptibility. A study using rat pilocarpine and mouse 6 Hz models found pharmacological inhibition of GSK3 β lowered seizure susceptibility.⁴⁰ In contrast, a genetic mouse model with low GSK3 α/β activity displayed greater seizure severity during kainate-induced SE³⁰ and constitutive GSK3 β activation in mice reduced susceptibility to kainate-induced seizures.⁴¹

Gys1 mRNA levels were unaffected in the hippocampal formations of chronic CSE mice. If translated, lowered Gsk3b mRNA levels are expected to influence GYS1 only via post-translational modification. Evidence of increased GYS activity has been seen in other tissues in Gsk3b-deficient mice.^{31,32} Similarly, brain and muscle glycogen phosphorylase mRNA levels were unchanged in hippocampal formations from chronic CSE mice. We note that in the rat pilocarpine model, glycogen phosphorylase activity was increased one month after SE in CA1 and CA3 pyramidal layers of the hippocampus.⁴²

Overall, we suggest that in our chronic mouse pilocarpine model of epilepsy there is upregulated synthesis of glycogen by GYS1. This may be due to reduced Gsk3b mRNA, which in other tissues is reported to increase glycogen synthase activation/activity and promote glycogen accumulation.

4.1 | Limitations

We acknowledge that without electroencephalogram recordings, we cannot eliminate the possibility that some no CSE mice experienced nonconvulsive SE and/or developed spontaneous recurrent seizures. This may have reduced our power to detect differences between no CSE and CSE mice. We are aware that not all analyses were repeated in all brain regions at both timepoints and understand that this is a limitation of our study. Finally, it is possible that exposure to pilocarpine altered baseline levels of GlnS activity compared with naive mice. Since we did not measure GlnS activity in naive mice, the magnitude of the difference we found in hippocampal GlnS activity between no CSE and CSE mice may be underestimated. If basal GlnS activity increased in the no CSE mice, changes in cortex GlnS activity could have been masked.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

AUTHOR CONTRIBUTIONS

KB and GD conceptualized the study. KB, FH, GS, ESN, and DV generated mice and collected tissues. FH and FN (Figures 1A and 2A) and GS (Figure 2B-C and Figure S1) measured glycogen amounts and solubility, ESN measured gene expression (Figure 2D) and DV measured glutamine synthetase activity (Figure 1B). All authors contributed to the analysis and interpretation of data. GS, ESN, and KB made the figures and wrote the manuscript. KB obtained funding.

ORCID

Gi Young Seo  <https://orcid.org/0000-0001-5955-1868>
 Elliott S. Neal  <https://orcid.org/0000-0002-1632-9615>
 Felicity Han  <https://orcid.org/0000-0001-7260-5832>
 Diana Vidovic  <https://orcid.org/0000-0002-9460-4926>
 Gerald A. Dienel  <https://orcid.org/0000-0001-8887-3057>
 Mitchell A. Sullivan  <https://orcid.org/0000-0001-9913-4030>
 Karin Borges  <https://orcid.org/0000-0001-7448-0770>

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SUPPORTING INFORMATION

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