Lack of astrocytic glycogen alters synaptic plasticity but not seizure susceptibility

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ABSTRACT

Brain glycogen is mainly stored in astrocytes. However, recent studies both *in vitro* and *in vivo* indicate that glycogen also plays important roles in neurons. By conditional deletion of glycogen synthase (GYS1), we previously developed a mouse model entirely devoid of glycogen in the central nervous system ($\text{GYS1}^{\text{Nestin-KO}}$). These mice displayed altered electrophysiological properties in the hippocampus and increased susceptibility to kainate-induced seizures. To understand which of these functions is related to astrocytic glycogen, in the present study we generated a mouse model in which glycogen synthesis is eliminated specifically in astrocytes ($\text{GYS1}^{\text{Gfap-KO}}$). Electrophysiological recordings of awake behaving mice revealed alterations in input/output curves and impaired long-term potentiation, similar, but to a lesser extent, to those obtained with $\text{GYS1}^{\text{Nestin-KO}}$ mice. Surprisingly, $\text{GYS1}^{\text{Gfap-KO}}$ mice displayed no change in susceptibility to kainate-induced seizures as determined by fEPSP recordings and video monitoring. These results confirm the importance of astrocytic glycogen in synaptic plasticity. (150 words)

Keywords: glycogen, long-term potentiation, plasticity, epilepsy, astrocyte, metabolism

1 INTRODUCTION

Most cell types in the body store glucose in the form of glycogen, a branched macromolecule containing up to 55,000 glucose units. The only enzyme able to form glycogen *in vivo* is glycogen synthase (GYS). There are two isoforms of glycogen synthase in mammals: the muscle isoform, GYS1, which is expressed in all tissues except the liver, and the liver-specific isoform, GYS2. In the brain, glycogen is estimated to comprise about 0.1% of tissue weight [1]. Both astrocytes and neurons express GYS1 and synthesize glycogen, although glycogen levels in astrocytes are much higher than in neurons [2, 3].

In the past decades, numerous studies have demonstrated that brain glycogen plays a role in memory consolidation and synaptic function [reviewed in [4–6]]. In histological studies of the healthy brain, glycogen granules are almost always confined to astrocytic cell bodies and processes [7]. Hence, there is a longstanding belief that the contribution of brain glycogen to cerebral functions is entirely due to its role in astrocytes. However, recent *in vitro* studies suggested an active glycogen metabolism in neurons [2, 8].

To study the role of brain glycogen *in vivo*, we previously developed a transgenic mouse line ($\text{GYS1}^{\text{Nestin-KO}}$) which lacked GYS1 and thus glycogen throughout the whole CNS, while GYS1 expression was normal in other tissues. Paired-pulse recordings at the CA3-CA1 synapse of the hippocampus showed that the $\text{GYS1}^{\text{Nestin-KO}}$ animals displayed increased facilitation, i.e. an increased response to the second pulse [9, 10]. The $\text{GYS1}^{\text{Nestin-KO}}$ animals also exhibited impaired long-term potentiation (LTP) evoked at the CA3-CA1 synapse. LTP is believed to be a primary molecular mechanism underlying long-term memory consolidation [9]. Additionally, the animals were more susceptible to hippocampal seizures induced by kainate or train stimulation [10]. These *in vivo* results demonstrate that brain glycogen plays a role in both short- and long-term synaptic plasticity as well as in the prevention of seizures.

68 Since the $GYS1^{Nestin-KO}$ mice lacked both glial and neuronal glycogen, the differential contribution of each glycogen pool to these results could not be determined with this model. For this reason, we next generated a new model with greater cellular resolution devoid of GYS1 in a subset of glutamatergic neurons, the excitatory Ca^{2+}/calmodulin-dependent protein kinase 2 (Camk2a)-positive neurons of the forebrain [11], including the pyramidal cells of the hippocampal CA3-CA1 synapse. Like the $GYS1^{Nestin-KO}$, these animals presented altered LTP and an associative learning deficiency, although the impairment was not as pronounced. However, unlike the $GYS1^{Nestin-KO}$ mice, $GYS1^{Camk2a-KO}$ mice exhibited no statistically significant change in PPF and no difference in susceptibility to kainate-induced seizures. This study corroborated the presence of an active glycogen metabolism in neurons *in vivo* and illustrated the importance of neuronal glycogen in LTP. However, since only a subset of neurons was affected in this model, it remains unclear whether the differences between the $GYS1^{Nestin-KO}$ and $GYS1^{Camk2a-KO}$ lines are due to the lack of astrocytic glycogen and/or glycogen in another subtype of neuron.

82 In the present study, we generated a new mouse model, lacking GYS1 specifically in astrocytes ($GYS1^{Gfap-KO}$). Our results clarify the specific contribution of astrocytic glycogen to cerebral function, confirming its role in synaptic plasticity and discarding its role in the prevention of epileptic seizures.

86

87 ## 2 METHODS

88

89 ### 2.1 Animals

90 Male and female mice aged 4 ± 1 months were used in this study. All experiments were carried out following European Union (2010/63/EU) and Spanish (BOE 34/11370-421, 2013) regulations for the use of laboratory animals. In addition, all experimental protocols were approved by the Ethics Committee of the Pablo de Olavide University. Animals were kept in collective cages (up to five animals per cage) on a 12-h light/dark cycle with constant temperature (21 ± 1°C) and humidity (50 ± 5%). After electrophysiological studies were initiated, mice were kept in individual cages until the end of the experiments. Animals were allowed access *ad libitum* to commercial mouse chow and water. The Gfap-Cre transgenic line 77.6 used in this study was purchased from Jackson laboratories (Stock #024098) and has been thoroughly characterized [12].

100

101 ### 2.2 Biochemical analysis

102 Mice were euthanized by cervical dislocation and decapitation. For total glycogen measurements, whole brains were dissected and flash frozen in liquid nitrogen. For Western blots, brains were removed, hemisected, and the cerebellum, hippocampus and cortex from each hemisphere were dissected and frozen in liquid nitrogen. All samples were maintained at -80 ºC until use. For glycogen quantifications, whole brains were boiled in 30% KOH for 15 min and glycogen was determined by an amyloglucosidase-based assay as previously described [2]. Tissue lysates for Western blot were prepared as previously described [11] and sample protein content was determined by Bradford assay (BioRad). Lysates were loaded in 10% polyacrylamide gels and transferred to Immobilon membranes (Millipore) for Western blot. The following antibodies were used: anti-glycogen synthase (Cell Signaling cat# 3886) and anti-GFAP (Millipore cat# MAB360). The REVERT total protein

113 stain was used as a loading control and densitometry was performed using Image Studio^{TM}
114 Lite (LI-COR BioSciences).
115
116 **2.3 *In situ* hybridization (ISH) and immunolabeling**

117 Mice were anesthetized by intraperitoneal injection of sodium thiopental (Braun) and
118 perfused transcardially with 4% paraformaldehyde (PFA). Brains were removed, postfixed
119 overnight in 4% PFA, and embedded in paraffin. Paraffin sections (5 µm in thickness) were
120 then cut in a microtome. RNAscope Intro Pack 2.5 HD Reagent Kit Red – Mm (cat#
121 322373, ACD, Biotechne) was used for the detection of the Gys1 (Mus musculus) probe
122 (cat# 546911, ACD, Biotechne) as specified by the manufacturer with minor modifications.
123 Sections were dewaxed and pretreatment was done with RNAScope ® Target Retrieval
124 Reagent for 15 min and with RNAScope Protease Plus for 30 min. Probe incubation was
125 perfomed for 2h at 40ºC. After *Gys1* amplification and signal detection, samples were
126 blocked with 5 % of normal goat serum (16210064, Thermofisher) mixed with 2.5 % BSA
127 diluted in wash buffer for and with Mouse on mouse (MOM) Immunodetection Kit –
128 (BMK-2202, Vector Laboratories). Primary mouse IgG1 anti-GFAP antibody (MAB360,
129 Merck Millipore) was diluted 1:1000 and incubated overnight at 4ºC. Alexa Fluor 488 Goat
130 anti-mouse (405319, Biolegend) was used as the secondary antibody. Samples were stained
131 with DAPI (D9542, Sigma) and mounted with fluorescence mounting medium (S3023
132 Dako).
133 Digital scanned fluorescent images were acquired using a NanoZoomer-2.0 HT C9600
134 scanner (Hamamatsu, Photonics, France) using NDP.scan2.5 software U10074-03
135 (Hamamatsu, Photonics, France).
136
137 **2.4 Animal preparation for the electrophysiological study**

138 For electrode implantation, animals were anesthetized with 0.8–3% halothane from a
139 calibrated Fluotec 5 (Fluotec-Ohmeda, Tewksbury, MA, USA) delivered via a homemade
140 mask and vaporizer at a flow rate of 0.8 L/min oxygen. Briefly, animals were implanted
141 with bipolar stimulating electrodes at the right Schaffer collaterals of the dorsal
142 hippocampus and with a recording electrode in the ipsilateral CA1 area using stereotaxic
143 coordinates [13]. Electrodes were made of 50 µm Teflon-coated tungsten wire (Advent
144 Research Materials Ltd., Eynsham, England). The final location of the CA1 recording
145 electrode was determined electrophysiologically, as described by some of us [11, 14].
146 Stimulating, recording and ground wires were soldered to a 6-pin socket. The socket was
147 fixed to the skull with the help of three small screws and dental cement [11, 14].
148
149 **2.5 Input/output curves and LTP procedures**
150
151 Both input/output curves, paired-pulse facilitation (PPF), and LTP were evoked in
152 behaving mice following procedures described elsewhere [11, 14]. For the
153 electrophysiological study, the mouse was located in a small (5 × 5 × 5 cm) box, aimed to
154 avoid over walking. For input/output curves, mice were stimulated at the CA3-CA1
155 synapse with single pulses of increasing intensities (0.02–0.4 mA). PPF was determined by
156 applying double pulses with increasing inter-pulse intervals (10, 20, 40, 100, 200 and 500
157 ms) at a fixed intensity corresponding to ~40% of asymptotic values, as previously
158 described [11]. Evoked field excitatory post-synaptic potentials (fEPSPs) were recorded

with Grass P511 differential amplifiers, across a high impedance probe (2×10^{12} Ω; 10 pF), and with a bandwidth of 0.1 Hz-10 kHz (Grass-Telefactor, West Warwick, RI, USA).

For LTP measurements, baseline fEPSP values evoked at the CA3-CA1 synapse were collected 15 min prior to LTP induction using single 100 µs, square, biphasic pulses. Pulse intensity was set well below the threshold for evoking a population spike (0.15-0.25 mA); i.e., 30–40% of the intensity necessary for evoking a maximum fEPSP response [14, 15]. LTP was evoked with a high-frequency stimulus (HFS) protocol consisting of five 200 Hz, 100-ms trains of pulses at a rate of 1/s, repeated six times, at intervals of 1 min. The stimulus intensity during the HFS protocol was set at the same value as that used for generating baseline recordings to prevent the presentation of electroencephalographic seizures and/or large population spikes. After each HFS session, the same stimuli were presented individually every 20 s for 60 additional min and for 30 min on the following three days [11, 14]. Evoked fEPSPs were recorded as described above.

2.6 Induction of hippocampal seizures with kainate injections in implanted mice

Following procedures described elsewhere [16], we determined the propensity of control and GYS1$^{Gfap\text{-}KO}$ mice to generate convulsive seizures in the hippocampal area. For this, we intraperitoneally (i.p.) administrated the AMPA/kainate receptor agonist kainate (8 mg/kg; Sigma, St. Louis, MO, USA) dissolved in 0.1 M phosphate buffered saline (PBS) pH = 7.4. Local field potentials and electrically evoked fEPSPs were recorded in the hippocampal CA1 area from 5 min before to 60 min after kainate injections.

2.7 Video monitoring of seizures after kainate injections

Animals were placed in individual cages and were administered with three consecutive i.p. injections of kainate (8 mg/kg per dose, 24 mg/kg total) one every 30 min from the onset of the experiment in order to induce convulsive non-lethal seizures. Seizure stages after kainate injections were evaluated as described previously [17–19]. After the first kainate injections, the animals developed hypoactivity and immobility (Stage I–II). After successive injections, hyperactivity (Stage III) and scratching (Stage IV) were often observed. Some animals progressed to a loss of balance control (Stage V) and further chronic whole-body convulsions (Stage VI). Extreme behavioural manifestations such as, uncontrolled hopping activity, or "popcorn behaviour" and continuous seizures (more than 1 minute without body movement control) were included in Stage VI. All behavioural assessments were performed blind to the experimental group (genotype) in situ, as well as recorded and reanalysed blind to the first analysis. Analysis consisted in the record of the time spent until the onset of the first seizure, the number of seizures per animal, the time spent on each grade, as well as the maximum grade reached by each animal.

2.8 Data collection and statistical analysis

fEPSPs and 1-V rectangular pulses corresponding to brain stimulation were stored digitally on a computer through an analog/digital converter (CED 1401 Plus, CED, Cambridge, England). Data were analyzed off-line for fEPSP recordings with the help of the Spike 2 (CED) program. Five successive fEPSPs were averaged, and the mean value of

203 the amplitude (in mV) was determined. Computed results were processed for statistical
204 analysis using the IBM SPSS Statistics 18.0 (IBM, Armonk, NY, United States). Data are
205 represented as the mean ± SEM. Statistical significance of differences between groups was
206 inferred by Two-way repeated measures ANOVA, followed by the Holm-Sidak method for
207 all pairwise multiple comparison procedures. The Fisher exact test for data collected from
208 kainate experiments. Statistical significance was set at $P < 0.05$. For the biochemical
209 analyses and behavioral assessment of seizure susceptibility after kainate administration,
210 computed results were processed for statistical analysis with PRISM 8.0 (GraphPAD
211 Software, San Diego, USA). Data are represented as the mean ± SEM. Normality of the
212 distributions was checked via the Shapiro–Wilk test; All tests performed were two-sided.
213 Statistical significance of differences between groups was inferred by Student's t-Test or
214 Two-way ANOVA, followed by the Bonferroni post-hoc comparison for all pairwise
215 multiple comparison procedures. Statistical significance was set at $P < 0.05$.

216 **3 RESULTS**

217 **3.1 Generation of GYS1$^{Gfap\text{-}KO}$ mice**

218 GFAP is a cytoskeletal protein found in nearly all astrocytes and a common marker for
219 this cell type. The astrocyte-specific inactivation of *Gys1* was achieved by crossing mice
220 homozygous for the conditional *Gys1* allele [9] with mice expressing Cre recombinase
221 under the control of the *Gfap* promoter [12]. Littermates that were homozygous for the
222 conditional *Gys1* allele and negative for Cre recombinase expression were used as controls.
223 To confirm the inactivation of *Gys1*, we measured GYS1 protein levels in cortex,
224 hippocampus, and cerebellum by Western blot. GYS1 protein was greatly diminished in all
225 regions (Fig. 1a). Quantification by densitometry showed that GYS1 expression was
226 reduced by approximately 80% in the cortex and in the hippocampus, and 60% in the
227 cerebellum (Fig. 1b). Total brain glycogen was decreased by more than 80% (Fig. 1c),
228 which is consistent with the reduction in GYS1 protein. No changes in GFAP levels were
229 observed (Fig. 1a, quantification not shown).
230 To further confirm the specific removal of GYS1 from astrocytes, we visualized *Gys1*
231 mRNA using *in situ* hybridization (ISH) in combination with immunostaining for GFAP. In
232 hippocampus, where astrocytes can be clearly visualized beside neuronal layers, abundant
233 *Gys1* expression was associated with both astrocytes and neurons of the dentate gyrus of
234 control mice (Fig. 1d). In the GYS1$^{Gfap\text{-}KO}$ animals, *Gys1* staining was eliminated from
235 GFAP-positive cells, while neuronal *Gys1* staining was similar to controls. These results
236 confirm the specific elimination of *Gys1* expression from astrocytes, and also illustrate the
237 abundance of *Gys1* expression within neurons of the hippocampus, confirming our previous
238 results [11].
239

240 **3.2 Electrophysiological alterations at the CA3-CA1 synapse in GYS1$^{Gfap\text{-}KO}$ mice**

241 To study the consequences of the lack of astrocytic glycogen on synaptic function, we
242 performed recordings of input/output curves, PPF and LTP evoked at the CA3-CA1
243 synapse of the hippocampus (Fig. 2a). In a first experimental step, we examined the
244 response of CA1 pyramidal neurons to single pulses of increasing intensity (0.02–04 mA)
245 presented to the ipsilateral Schaffer collaterals. Both control and GYS1$^{Gfap\text{-}KO}$ mice
246 presented similar increases in the amplitude of fEPSPs evoked at CA1 pyramidal neurons

247 by the stimuli presented to Shaffer collaterals (Fig. 2b). These two input/output
248 relationships were best fitted by sigmoid curves ($r \geq 0.9$; $P \leq 0.001$; not illustrated),
249 suggesting the normal functioning of the CA3-CA1 synapse in both groups. However, the
250 experimental group reached lower maximal fEPSP amplitudes than their littermate controls.
251 No significant differences [Two-way repeated measures ANOVA; $F_{(19,266)} = 1.222$; $P =$
252 0.239] were observed overall between control and GYS1$^{Gfap\text{-}KO}$ groups. However, fEPSPs
253 evoked by three increasing intensities presented significant differences (All pairwise
254 multiple comparison procedures; $P < 0.05$). We also performed an analysis of PPF at the
255 CA3-CA1 synapse by applying double pulses at a fixed intensity with increasing inter-
256 stimulus intervals (10, 20, 40, 100, 200, 500 ms). Both groups displayed facilitation at 20
257 and 40 ms intervals (Fig. 2c). GYS1$^{Gfap\text{-}KO}$ exhibited no statistical difference in PPF
258 compared to control animals [Two-way repeated measures ANOVA; $F_{(5,95)} = 0.726$; $P =$
259 0.606].
260 In a following experimental step, we evoked LTP at the CA3-CA1 synapse of the two
261 genotypes as an indication of long-term synaptic plasticity. It is well known that the
262 hippocampus is involved in the acquisition of different types of associative [20, 21] and
263 non-associative [22, 23] learning tasks and that the CA3-CA1 synapse is often selected for
264 evoking LTP in behaving mice [14, 24]. For baseline values, animals were stimulated every
265 20 s for ≥ 15 min at the implanted Schaffer collaterals (Fig. 2d). Afterward, they were
266 presented with a high frequency stimulus (HFS) protocol. Immediately after the HFS
267 session, the same single stimulus used to generate baseline records was presented at the
268 initial rate (3/min) for another 60 min. As illustrated in Fig. 2d, recording sessions were
269 repeated for three additional days (30 min each). The control group presented a significant
270 LTP when comparing baseline values with those collected following the HFS session
271 (Holm-Sidak method, all pairwise multiple comparison procedures; $P \leq 0.041$). Although
272 the amplitude of fEPSPs also increased in GYS1$^{Gfap\text{-}KO}$ mice following the HFS session,
273 only a tendency was presented ($P \geq 0.671$) (Fig. 2d). In addition, the amplitude of fEPSPs
274 evoked in the control group was significantly [Two-way repeated measures ANOVA;
275 $F_{(32,512)} = 6.277$; $P < 0.001$] larger and longer lasting than that evoked in the experimental
276 group (Fig. 2d). In summary, GYS1$^{Gfap\text{-}KO}$ mice display no change in PPF but a
277 significantly impaired LTP compared to the littermate controls.
278
279 **3.3 Seizure susceptibility in GYS1$^{Gfap\text{-}KO}$ mice**

280 Kainate is a widely used chemoconvulsant used to study seizure susceptibility in
281 rodents [25]. We previously demonstrated that mice devoid of cerebral glycogen
282 (GYS1$^{Nestin\text{-}KO}$) are more susceptible to kainate-induced seizures [10]. To understand the
283 participation of astrocytic glycogen in seizure susceptibility, we also assessed the response
284 of GYS1$^{Gfap\text{-}KO}$ mice to kainate-induced seizures in the pre-implanted animals. Evoked
285 seizures in control and GYS1$^{Gfap\text{–}KO}$ mice presented similar durations and profiles (Fig. 3a).
286 Both groups presented a noticeable depression in the amplitude of evoked fEPSP recorded
287 following a kainate-dependent seizure (Fig. 3b). Overall, there was no difference in the
288 number of seizures observed per genotype (Fisher exact test; $P = 0.657$) (Fig. 3c).
289 To corroborate these results, we employed an alternative seizure assessment protocol in
290 a new cohort of mice. Mice were given three kainate injections (8 mg/kg, i.p. every 30 min)
291 and video-recorded for 180 minutes to monitor their behavior (i.e. epileptic events)
292 following the first injection. Mice from both genotypes reached similar severity stages (Fig.

293 4a). In the majority of the mice of both genotypes, seizures began approximately 15
294 minutes after the third dose of kainate (*P* = 0.9501, Student's t-test; Fig. 4b). There were no
295 significant differences between groups in the prioritary stage (the behavioral stage in which
296 an animal spends the most time after kainate administration throughout the duration of the
297 experiment) (*P* = 0.5441, Student's t-test; Fig. 4c) nor the maximum stage (the most severe
298 stage reached during the experiment) (*P* = 0.9644, Student's t-test; Fig. 4d). Furthermore,
299 there were no significant differences in the time spent per stage (Two-way ANOVA: Stage
300 factor: *P* = 0.0007, Genotype factor: *P* = 0.2370; Fig. 4e) or in the number of seizures per
301 animal after each injection (Two-way ANOVA: Administration factor: $P < 0.0001$,
302 Genotype factor: *P* = 0.7980; Fig. 4f). These results unequivocally confirmed that
303 $GYS1^{Gfap\text{-}KO}$ animals present a similar seizure susceptibility compared to control
304 littermates.
305
306 ## 4 DISCUSSION
307
308 In this study, we analyze for the first time the physiological consequences of removing
309 glycogen specifically from astrocytes by means of transgenic tools. By comparing this
310 mouse to previous models lacking glycogen in the entire CNS [9, 10] or only in Camk2a-
311 positive excitatory neurons of the forebrain [11], we are able to define the specific
312 physiological roles of glycogen in astrocytes versus neurons.
313 Using Cre/Lox technology, we deleted *Gys1* in GFAP-positive cells to eliminate only
314 astrocytic glycogen synthesis. Western blot analyses confirmed a clear reduction in GYS1
315 protein levels in hippocampus, cortex and cerebellum of $GYS1^{Gfap\text{-}KO}$ mice (Fig. 1, a and
316 b). Total brain glycogen content was also greatly reduced relative to littermate controls
317 (Fig. 1c). These results are consistent with the well-documented observation that the
318 majority of brain glycogen is stored in astrocytes, but also point to a remaining significant
319 fraction of non-astrocytic GYS1 expression and glycogen synthesis, likely in neurons. The
320 analysis of *Gys1* mRNA distribution by ISH validated the astrocyte-specific deletion of
321 GYS1 in the $GYS1^{Gfap\text{-}KO}$ model and also confirmed a significant proportion of *Gys1*
322 expression within neurons, in line with our previous results [11] (Fig. 1d).
323
324 We also studied synaptic function in the $GYS1^{Gfap\text{-}KO}$ model via stimulation of the
325 CA3-CA1 synapse in the hippocampus. Input/output curves showed no overall significant
326 difference between groups, but at some intensities, evoked fEPSPs were statistically higher
327 in the control group compared to the $GYS1^{Gfap\text{-}KO}$ animals, and the latter group reached a
328 lower maximum fEPSP (Fig. 2b). These results suggest that the absence of astrocytic
329 glycogen may reduce basal synaptic strength. A similar situation is obtained with inhibitors
330 of astrocytic glutamate transport, which also reduce basal EPSPs since the accumulation of
331 glutamate causes presynaptic inhibition [26]. Astrocytes take up synaptic glutamate and
332 convert it to glutamine for transfer to neurons, where it is recycled into glutamate and
333 repackaged into synaptic vesicles, a process known as the glutamate/glutamine cycle [27,
334 28]. Since astrocytic glycogen has been shown to play a role in both glutamate uptake and
335 recycling [29, 30], the lack of astrocytic glycogen could cause impaired glutamate uptake
336 leading to presynaptic inhibition and therefore lower synaptic strength. While PPF
337 experiments with increasing inter-pulse intervals showed significantly greater facilitation in
338 $GYS1^{Nestin\text{-}KO}$ mice [10], in the present study, $GYS1^{Gfap\text{-}KO}$ mice only lack astrocytic

339 glycogen displayed only a trend toward increased PPF, with no statistical difference (Fig.
340 2c). PPF is typically attributed to presynaptic mechanisms such increased [Ca^{2+}] in the
341 presynaptic terminal [31]. The altered PPF observed in $GYS1^{Nestin\text{-}KO}$ mice could be
342 exclusively neuronal in origin.

343 We also observed an impairment in hippocampal LTP in $GYS1^{Gfap\text{-}KO}$ animals (Fig.
344 2d). However, the LTP impairment in the $GYS1^{Gfap\text{-}KO}$ animals was not as pronounced as
345 was observed in the $GYS1^{Nestin\text{-}KO}$ mice [9]. The present results are reminiscent of those
346 from the $GYS1^{Camk2a\text{-}KO}$ mice, in which LTP was still observed, although it was
347 significantly impaired [11]. Collectively, these three mouse models demonstrate that both
348 astrocytic and neuronal glycogen contribute to LTP. Previous studies using
349 pharmacological agents have shown that astrocytic glycogen is important for long-term, but
350 not short-term, memory formation [32, 33]. The presence of a normal PPF, a measure of
351 short-term plasticity, and the major impairment in LTP that we observed in the $GYS1^{Gfap\text{-}KO}$
352 model are consistent with these observations.

353 The most surprising result regarding the $GYS1^{Gfap\text{-}KO}$ line is its susceptibility to
354 kainate-induced epilepsy. We previously showed that $GYS1^{Nestin\text{-}KO}$ mice were more
355 susceptible to seizures induced by a single convulsive dose of kainate (8mg/kg, i.p.) [10].
356 However, using the same protocol, we detected no statistical difference in the $GYS1^{Gfap\text{-}KO}$
357 line (Fig. 3). Utilizing a second experimental protocol with three consecutive doses of
358 kainate (8mg/kg, i.p., every 30 minutes) we found no statistical differences between the
359 groups in the seizure stages achieved (Fig. 4a), seizure onset (Fig. 4b), prioritary or
360 maximum stage reached (Fig. 4, c and d), time spent per stage (Fig. 4e) or number of
361 seizures per animal (Fig. 4f). These results unequivocally show that $GYS1^{Gfap\text{-}KO}$ animals
362 are not more susceptible to kainate than their littermate controls. Altered glycogen
363 metabolism has been linked to seizures, as reviewed elsewhere [34–37]. A commonly held
364 view is that altered astrocytic glycogen metabolism induces neuronal excitability via
365 impaired glutamate and K^+ uptake. However, herein we show mice lacking astrocytic
366 glycogen do not have more kainate-induced seizures. Since $GYS1^{Camk2\text{-}KO}$ mice also show
367 unaltered kainate susceptibility [11], collectively these mouse models suggest that seizure
368 susceptibility in the $GYS1^{Nestin\text{-}KO}$ line is a consequence of the lack of glycogen in another
369 cell type. Inhibitory neurons play a critical role in suppressing excitability, and their
370 dysfunction is associated with epilepsy in rodent models and humans [38]. Therefore, our
371 results suggest that glycogen in inhibitory neurons might be critical for their regulatory
372 role. This possibility will be addressed in future studies.

373 In summary, the $GYS1^{Gfap\text{-}KO}$ mouse model illustrates the specific contribution of
374 astrocytic glycogen to the physiological roles of glycogen in the brain, further clarifying
375 how brain glycogen is involved in memory and epilepsy. Our results confirm that astrocytic
376 glycogen plays an active role in long-term synaptic plasticity. However, the lack of
377 astrocytic glycogen does not increase susceptibility to kainate-induced seizures in these
378 mice. These data point to a role of neuronal glycogen in cerebral functions, most
379 importantly in the regulation of excitability. A thorough understanding of these processes is
380 essential for better management and treatment of neurological disorders.
381

382 **Figure Legends**

383

384 **Fig. 1** Analysis of GYS1, GFAP and glycogen levels in $GS^{Gfap\text{-}KO}$ mice and controls. (a)
385 Representative Western blot of GYS1 and GFAP protein levels in cortex (Cx),

386 hippocampus (Hp) and cerebellum (Cb). REVERT protein stain (Li-COR BioSciences) was
387 used as a loading control. (b) Quantification of GYS1 and GFAP protein levels by region
388 normalized to total protein determined by REVERT. (c) Total brain glycogen in control
389 versus $GS^{Gfap\text{-}KO}$ animals. All data are expressed as average ±SEM (n = 4-6 per group).
390 Significant differences were calculated using student's t-test (*, $P < 0.05$; ***, $P < 0.001$;
391 ****, $P < 0.0001$). (d) GYS1 *in situ* hybridization (ISH). *Gys1* mRNA expression was
392 visualized via ISH (orange), GFAP protein by immunostaining (green), and nuclei using
393 DAPI (blue). The dentate gyrus of the hippocampus from control and $GYS1^{Gfap\text{-}KO}$ are
394 shown. Scale bar = 50 µm.
395
396 **Fig. 2** Electrophysiological properties of hippocampal synapses in behaving control and
397 $GYS1^{Gfap\text{-}KO}$ mice. (A) Animals were chronically implanted with bipolar stimulating (St.)
398 electrodes in the right CA3 Schaffer collaterals and with a recording (Rec.) electrode in the
399 ipsilateral CA1 area. DG, dentate gyrus; Sub., subiculum. (B) Input/output curves of
400 fEPSPs evoked at the CA3-CA1 synapse via single pulses of increasing intensities (0.02–
401 0.4 mA) in control and $GYS1^{Gfap\text{-}KO}$ mice. Although no significant differences [Two-way
402 repeated measures ANOVA; $F_{(19,266)} = 1.222$; $P = 0.239$] were observed between groups,
403 fEPSPs evoked by three different intensities presented significant differences (All pairwise
404 multiple comparison procedures; $P < 0.05$). (C) Paired-pulse facilitation in control and
405 $GYS1^{Gfap\text{-}KO}$ animals with increasing inter-stimulus intervals. No significant differences
406 between the two groups were observed [Two-way repeated measures ANOVA; $F_{(5,95)} =$
407 1.222; $P = 0.606$]. (D) LTP evoked at the CA3-CA1 synapse of control and $GYS1^{Gfap\text{-}KO}$
408 mice following the HFS session. The HFS was presented after 15 min of baseline
409 recordings, at the time marked by the dashed line. LTP evolution was followed for four
410 days. At the right are illustrated representative examples of fEPSPs collected from control
411 and $GYS1^{Gfap\text{-}KO}$ mice at the times indicated in the bottom graph. fEPSP amplitudes are
412 given as a percentage of values measured from baseline recordings, and statistical
413 differences between control and $GYS1^{Gfap\text{-}KO}$ from two-way repeated measures ANOVA
414 are shown (*, $P \leq 0.01$). All data are expressed as average ± SEM (n= 7-9 mice/group).
415
416 **Fig. 3** Kainate susceptibility of $GYS1^{Gfap\text{-}KO}$ mice compared to controls. (a) Representative
417 examples of hippocampal seizures evoked in control and $GYS1^{Gfap\text{-}KO}$ mice following the
418 administration of 8 mg/kg i.p. of kainate. (b) Representative examples of fEPSPs evoked
419 before and immediately after a kainate-evoked seizure. (c) Percentage of control (n = 14)
420 and $GYS1^{Gfap\text{-}KO}$ (n = 9) mice presenting spontaneous seizures at the CA1 area during the
421 recording period (60 min). No significant differences between groups (Fisher exact test; P
422 = 0.657) were observed.
423
424 **Fig. 4** Comparison of kainate-induced seizure profile in control and $GYS1^{Gfap\text{-}KO}$. 3-4
425 months old mice were subjected to three kainate injections (8 mg/kg every 30 min) and
426 epileptic responses were analyzed for 180 minutes after the first injection. (a) Percentage of
427 mice reaching seizure stages I to VI and kainate-induced mortality. (b) Onset of the
428 epileptic activity. Student's t-test ($P = 0.9501$). (c) Prioritary stage displayed by each
429 animal during the course of the experiment. Student's t-test ($P = 0.5441$). (d) Maximum
430 stage reached by each animal during the course of the experiment. Student's t-test ($P =$
431 0.9644). (e) Percentage of time spent on each stage during the course of the experiment.
432 Two-way ANOVA (Stage factor: $P = 0.0007$; Genotype factor: $P = 0.2370$). (f) Number of

433 seizures experimented per animal divided on time segments after the first, second and third
434 kainate administrations. Two-way ANOVA (Administration factor: $P < 0.0001$; Genotype
435 factor: $P = 0.7980$). All data are expressed as average ± SEM (n = 6-7 mice/group).
436
437 **AUTHOR CONTRIBUTIONS**
438
439 JD and JJG conceived the study. JD generated and maintained the $GYS1^{Gfap\text{-}Cre}$ line. JD and
440 MKB collected brain tissues and performed biochemical and histological analyses. AG and
441 JMD-G performed electrophysiological studies before and after single kainate injections.
442 AH and JAR performed seizure video-monitoring with multiple kainate injections. All
443 authors analyzed data and contributed to the writing of the manuscript.
444
445 **CONFLICT OF INTEREST**
446
447 All authors declare they have no conflicts of interest.
448
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