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Acute and chronic changes in glycogen phosphorylase in hippocampus and entorhinal cortex after status epilepticus in the adult male rat

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Abstract

Glial cells provide energy substrates to neurons, in part from glycogen metabolism, which is influenced by glycogen phosphorylase (GP). To gain insight into the potential subfield and laminar-specific expression of GP, histochemistry can be used to evaluate active GP (GP_a) or totalGP (GP_a + GP_b). Using this approach, we tested the hypothesis that changes in GP would occur under pathological conditions that are associated with increased energy demand, i.e. severe seizures (status epilepticus or 'status'). We also hypothesized that GP histochemistry would provide insight into changes in the days and weeks after status, particularly in the hippocampus and entorhinal cortex, where there are robust changes in structure and function. One hour after the onset of pilocarpine-induced status, GP_a staining was reduced in most regions of the hippocampus and entorhinal cortex relative to saline-injected controls. One week after status, there was increased GP_a and totalGP, especially in the inner molecular layer, where synaptic reorganization of granule cell mossy fibre axons occurs (mossy fibre sprouting). In addition, patches of dense GP reactivity were evident in many areas. One month after status, levels of GP_a and totalGP remained elevated in some areas, suggesting an ongoing role of GP or other aspects of glycogen metabolism, possibly due to the evolution of intermittent, recurrent seizures at approximately 3–4 weeks after status. Taken together, the results suggest that GP is dynamically regulated during and after status in the adult rat, and may have an important role in the pilocarpine model of epilepsy.

Keywords

astrocytes; epilepsy; glia; mossy fibre sprouting; pilocarpine; seizure

Introduction

Glycogen, located primarily in astrocytes in the central nervous system (Cataldo & Broadwell, 1986; Swanson, 1992; Magistretti *et al.*, 1993), is the principal reservoir of energy substrates in the brain. It is now recognized that the glial energy supply is critical to normal CNS function

(Gruetter, 2003; Magistretti & Pellerin, 1996; Magistretti, 2006). Although the exact role of brain glycogen has not been clarified completely, its relationship to neuronal activity is clear, because glycogen levels in the brain respond rapidly to even the weakest of sensory stimuli (Cruz & Dienel, 2002), and inhibition of brain glycogen metabolism during periods of intense neuronal stimulation have dramatic effects on neural activity (Brown *et al.*, 2005).

In contrast, less is known about the metabolic requirements of the brain under pathological conditions, like seizures and epilepsy. Initially, studies measuring glycogen levels in the brain failed to demonstrate consistent effects of seizures on glycogen levels (Holmes & Holmes, 1926; Kerr & Antaki, 1937; Chance & Yaxley, 1950). However, improvements in biochemical techniques and methods for tissue preservation led to more consistent results, and more recent studies suggest that brain glycogen decreases immediately after generalized activity in whole brain (Bolwig & Rafaelsen, 1972; Hutchins & Rogers, 1970; Duffy *et al.*, 1975), the cerebral cortex (Folbergrova, 2000; Sacktor *et al.*, 1966; Folbergrova *et al.*, 1981; Folbergrova *et al.*, 1985) and the hippocampus (Folbergrova *et al.*, 1985; Folbergrova *et al.*, 1981).

The changes in metabolism after seizures are also of interest because this is a time when neuronal death, glial infiltration to the site of injury, and other changes that require energy occur. The first studies that evaluated changes after the termination of seizures showed that brain glycogen returned to control levels rapidly (Bolwig & Rafaelsen, 1972). However, in a study that examined changes 72 h after seizures, Folbergrova and colleagues (Folbergrova, 1973; Folbergrova *et al.*, 1996) showed that the initial decline was followed by an elevation in glycogen levels, suggesting that glycogen depletion during seizures might be followed by an increase to accommodate energy demands associated with neuronal injury.

To our knowledge no studies have examined changes after status, which may require much more energy because it involves continuous, prolonged seizures. In addition, there has been no report of changes in glycogen metabolism over the days or weeks following status. The changes over days and weeks after status are of interest because at this time, a chronic period of recurrent spontaneous seizures begins. Thus, many attribute the intervening weeks between status and chronic seizures to a time of 'epileptogenesis', when changes in the brain develop that ultimately lead to a permanent state of intermittent, unprovoked seizures. It is of interest to determine how the changes during this period of epileptogenesis may relate to glial glycogen metabolism. Increased metabolic demand might be expected during the period of epileptogenesis due to the diverse changes in structure and function. Increased energy demand may persist as recurrent seizures develop, because each spontaneous generalized event would be expected to place substantial energy demands on the neurons involved in seizure generation.

To test these hypotheses, we examined adult male rats at three times after the onset of status; one hour, a time when status has not yet ceased; one week, a time when changes associated with epileptogenesis occur; and one month after status, a time when animals exhibit spontaneous recurrent seizures. We chose pilocarpine because it is a reliable chemoconvulsant that can induce status, and because pilocarpine-induced status leads to the emergence of recurrent seizures consistently in the rat (Mello *et al.*, 1993).

One way to probe the complex mechanisms involved in glycogen metabolism is to use a histochemical reaction of the enzyme that catalyses the breakdown of glycogen, glycogen phosphorylase (GP, 4- α -D-glucan, orthophosphate α -D-glucosyl-transferase, EC2.4.1.1). GP occurs in two forms (i) phosphorylated (GP_a; active) or (ii) dephosphorylated (GP_b; inactive). In the central nervous system, immunohistochemical studies localizing the brain or muscle isoforms of the enzyme have shown that GP is primarily localized to astrocytes and ependymal cells (Pfeiffer *et al.*, 1990; Pfeiffer *et al.*, 1992; Pfeiffer-Guglielmi *et al.*, 2003), with very few immunoreactive neurons (Pfeiffer-Guglielmi *et al.*, 2003). At present, there are no antibodies

available that will distinguish the phosphorylated and dephosphorylated isoforms, so histochemistry is a valuable resource.

Here, we provide the first evidence of dynamic changes in GP within the hippocampus and entorhinal cortex after status. The results suggest that GP changes in a subfield and laminar-specific manner, both immediately and weeks after status. The data suggest that more studies of glycogen metabolism in the pilocarpine model could provide greater insight into mechanisms underlying acute and chronic changes that accompany epileptogenesis.

Materials and methods

All animal use and procedures met the guidelines of the National Institutes of Health and New York State Department of Health and were approved by the Institutional Committee on Animal Care and Use at Helen Hayes Hospital. Animals were housed one rat per cage, provided food and water *ad libitum*, and a 12-h light : 12-h dark cycle was used (lights on 07:00 h). Reagents were obtained from Sigma Chemical Company (St Louis, MO) unless otherwise specified.

Pilocarpine treatment

Adult male Sprague–Dawley rats (206–260 g, Taconic Farms, Germantown, NY) were injected with atropine methylbromide (1 mg/kg, s.c.) 30 min prior to pilocarpine HCl (380 mg/kg, i.p.) or physiological saline (equivalent volume) and the onset of status was monitored by behavioural seizures using the Racine scale. The onset of status was defined by the first stage five seizure (Racine, 1972) that did not terminate after 2 min.

One hour after status, animals in the survival groups were treated with diazepam (Wyeth–Ayerst, Collegeville, PA; 5 mg/kg, i.p.) and 2.5 mL of dextrose (5%, s.c.) in Ringers-lactate solution. For 5–7 days after status, the diet was supplemented daily with half an apple, left at the bottom of the cage. This treatment was introduced when it was noted that some rats did not appear to resume normal food and water intake immediately after status. Animals that did not have status, but were administered pilocarpine, or saline-treated rats, received the same treatment.

Histochemistry

General procedures—Histochemical and immunohistochemical procedures were performed on fresh frozen tissue, because fixation denatures GP. Rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.) and decapitated. The brain was frozen within 2 min in cold methylbutane (−50 °C) and stored at −80 °C until use. Brains were sectioned (30 µm) in the horizontal plane using a cryostat and immediately mounted on subbed slides. Slides were stored at −80 °C until they were processed. To optimize comparisons, sections from one hemisphere of a control rat were mounted on the same slide as sections from one hemisphere of a rat that had status. The sections were frozen at the same time, frozen for the same duration of time, and processed concurrently.

GP histochemistry—Histochemistry for GP_a and totalGP (GP_a + GP_b) was conducted as described previously (Walling *et al.*, 2006) using a technique that was adapted from an earlier report (Meijer, 1968). In brief, sections were incubated for 30 min at 37 °C in α -d-glucose-1-phosphate (21 mM), EDTA (6.8 mM), NaF (38 mM), and dextran (FW 40 000; 1 mM) dissolved in 40 mL of sodium acetate buffer (pH 5.6) and brought to 200 mL with distilled H₂O (pH 6.0). For assessment of totalGP activity, the incubation medium also included adenosine 5'-mono-phosphate (2.8 mM). Excess fluid was blotted from the sides of the slide, and sections were allowed to air-dry (20–25 min), then briefly fixed in 40% ethanol (4 min), air-dried again (15–20 min) and then dipped in Lugol's iodine in 11% sucrose for 2–3 min. Sections were

allowed to air-dry overnight and then were coverslipped with Permount (Fisher Scientific, Waltham, MA).

Immunocytochemistry—Sections adjacent to those used for GP histochemistry were immunolabelled using antibodies to NeuN, to visualize neurons (monoclonal, Chemicon; Temecula, CA) and the astrocytic marker GFAP (monoclonal, Boehringer Mannheim; Indianapolis, IN). As for histochemistry, control sections were processed concurrently with sections from rats that had status. In brief, slides with previously cut, frozen sections were brought to room temperature and incubated in 4% paraformaldehyde (in 0.1 m phosphate buffer; pH 7.4) for 20 min. Slides were then washed in 0.1 m trishydroxymethyl-amino methane (Tris buffer; pH 7.6, 3 × 5 min) followed by 30 min in H₂O₂ (1%, made in Tris buffer) to block endogenous peroxidase activity. Following a wash in Tris buffer (5 min) slides were washed in Tris containing 0.1% Triton X-100 (Tris A; 10 min), then Tris A containing 0.005% bovine serum albumin (Tris B; 10 min). Slides were then treated with 10% normal horse serum (Vector Laboratories, Burlingame, CA; 1 h) in Tris B followed by 10-min washes of Tris A and Tris B, respectively. Tissue was then incubated with the antibody to NeuN (1 : 5000) diluted in Tris B, for a minimum of 24 h at 4 °C. Following incubation, slides were washed in Tris A, then Tris B (10 min each) and incubated for 1 h (room temperature) in secondary antibody (anti-mouse IgG made in horse; 1 : 400, Vector Laboratories) in Tris B. Following a 10-min wash in Tris A and a 10-min wash in Tris D (0.5 m Tris containing 0.1% Triton X and 0.005% BSA), slides were incubated for 2 h in avidin-biotin-horseradish peroxidase complex (ABC Elite kit; 1 : 1000; Vector Laboratories) in Tris D. Slides were washed in Tris buffer (3 × 5 min) and visualized using Vectastain SG (Vector Laboratories).

Slides were subsequently incubated in avidin-biotin blocking solution (Avidin-Biotin Blocking kit; Vector Laboratories), then incubated with the antibody to GFAP (1 : 1000), analogous to the procedure for NeuN, using anti-mouse IgG made in horse (1 : 400; Vector Laboratories). GFAP immunoreactivity was visualized with Vector NovaRed (Vector Laboratories).

Data analysis

Photomicrographs were taken using a brightfield light microscope (Olympus BX51, Olympus America, Center Valley, PA) and digital camera (Optronics, Muskogee, OK). For densitometry, images were converted to grayscale and analysed using MCID image analysis software (Interfocus Ltd; Linton, UK). Three dorsoventral levels were sampled, corresponding to −7.6 to −6.82; −6.82 to −5.32; −5.32 to −3.86 relative to bregma (Paxinos & Watson, 1998) and described previously (Walling *et al.*, 2006). Three sections at each level of the dorsal/ventral plane (total nine sections) were analysed for GP_a and totalGP (GP_a + GP_b) for each animal. There were no detectable differences in either GP_a or totalGP activity between pilocarpine- or saline-injected rats through the septotemporal axis, so the data were pooled. The total number of relative optical density (ROD) sample blocks (250 µm × 250 µm) that were sampled for each region of hippocampus and entorhinal cortex were: CA1 pyramidal cell layer, 189; CA1 stratum oriens, 56; CA1 stratum radiatum, 56; CA1 stratum lacunosum-moleculare, 56; CA3 pyramidal cell layer, 189; CA3 stratum oriens, 135; CA3 stratum radiatum, 56; dentate gyrus granule cell layer, 189; inner molecular layer, 189; outer molecular layer, 54; hilus, 54; entorhinal cortical layer I, 135; Layer II, 135; Layer III-LD (lamina dissecans), 189; Layer V–VI, 135; parasubiculum, 56; and presubiculum, 56. Layers III and LD (lamina dissecans) were pooled because they were difficult to discriminate on the basis of GP histochemistry. Layers V and VI were pooled for the same reason. Furthermore, Layer III and LD appeared to change in a concerted manner, as did Layers V and VI.

Statistics

Statistics are provided in the Results section or tables. Statistical analysis was performed using matched samples (status vs. saline control). A two-way, repeated measures analysis of variance (anova) was performed at each period of survival (one hour, one week or one month) for both GPa and totalGP. *Posthoc* analysis of anova results were performed using the test of least significant difference (LSD), and *P* was set at 0.05.

Results

Behavioural manifestations of pilocarpine-induced status

Pilocarpine administration was followed by behaviours associated with mild seizures and then more severe seizures, which ultimately ended in status. The first behavioural change, occurring within 7 min (range, 2–11 min) of pilocarpine injection, was a low posture. This was followed by behaviours associated with mild seizures (Racine stages 1–2); head bobbing, ear twitching, mastication, and repetitive movement of the vibrissae, and sometimes hypersalivation. Stage 3–5 seizures, characterized by unilateral or bilateral forelimb clonus, rearing and subsequent loss of posture (falling), began 31.8 ± 15.4 min after pilocarpine. The mean latency to status was 57.8 ± 27.5 min (range, 30–114 min) after the injection of pilocarpine. Saline-injected rats did not exhibit any behavioural signs of seizures.

Relative optical density (ROD) measurements of GP histochemistry

Table 1 presents the ROD measurements of GP histochemistry that were averaged from the entire hippocampus and the entorhinal cortex of animals. Animals were examined one hour, one week, or one month after status and compared to saline-injected controls. ROD measurements showed that one hour of status decreased GPa in the hippocampus and entorhinal cortex, but totalGP was not altered. In contrast, levels of both GPa and totalGP were increased at one week and one month (Table 1).

Effects of status on GP – the hippocampus

One hour after the onset of status—Further analyses were conducted in each layer of the hippocampus, because mean ROD levels did not address laminar-specific differences (Fig. 1, hippocampus; Fig. 2, entorhinal cortex). The laminar analysis revealed that GPa was reduced in all layers within hippocampal subfields CA1, CA3, and the dentate gyrus after one hour of status, except for the pyramidal cells layers (Table 2; Fig. 1). In contrast, no changes in totalGP were statistically significant (Table 2; Fig. 1).

One week and one month after status—One week after status, the mean hippocampal GPa activity was increased in all layers (Table 2; see also Fig. 1). In contrast, totalGP was increased only in a few layers; stratum oriens and stratum pyramidale of CA1, stratum oriens of CA3, and the hilus (Table 2). GPa levels remained elevated one month after status in many layers within subfields CA1 and CA3, except for stratum lacunosum-moleculare of CA1 and stratum radiatum and stratum oriens of CA3 (Table 2). In the dentate gyrus, only the hilus remained elevated. TotalGP was elevated at one month only in the CA1 and CA3 pyramidal cell layers (Table 2).

Effects of status on GP – the entorhinal cortex

One hour after the onset of status—Changes in GP in the entorhinal cortex were similar to changes described above for hippocampus, with a few notable exceptions. One hour after the onset of status, the reduction in GPa was restricted to Layer I and II (Fig. 2; Table 3). TotalGP levels at this time were not altered.

One week and one month after status—One week after status, there was increased GPa activity in all areas except Layer I (Table 3; see also Fig. 2). TotalGP was unchanged relative to controls, with the exception of Layers III-LD, where it was decreased (Table 3), possibly related to cell loss in Layer III, which occurs in this animal model (Du *et al.*, 1995).

Analysis one month after status showed that GP levels in the entorhinal cortex, for the most part, returned to the control range. Layers V–VI were the only regions that still demonstrated increased GPa. TotalGP was similar to control values in all layers of the entorhinal cortex (Table 3). Animals that were administered the same dose of pilocarpine as other rats, but did not enter status epilepticus, demonstrated only mild seizures in the hours after pilocarpine administration (stages 1–2). These animals were useful because they could address the possibility that pilocarpine had an influence on GP, rather than status per se. These animals were rare, precluding statistical comparisons. Nevertheless, there was no evidence either qualitatively (data not shown) or by their mean ROD values, that they differed from controls. Total mean ROD values were evaluated for two saline and two pilocarpine controls that were processed concurrently (one saline and one pilocarpine control evaluated at one week after status, another saline and pilocarpine control evaluated at one month after status) and means were similar [saline controls, 0.41 (GPa) and 0.63 (totalGP); pilocarpine controls, 0.43 (GPa) and 0.62 (totalGP)]. In addition, other studies of the pilocarpine model have, to our knowledge, rarely described effects attributed to pilocarpine independent of seizures that are induced by pilocarpine (Turski *et al.*, 1989; Scharfman *et al.*, 2002). It has also been shown that the brain damage, and other sequelae that follow pilocarpine-induced status, is not correlated with the density of muscarinic receptors (Clifford *et al.*, 1987; Scharfman *et al.*, 2000; Scharfman, 2002).

Taken together, it seems likely that the changes in GP in animals with status do not reflect effects of pilocarpine administration itself, but the changes in GP are a reflection of seizures induced by pilocarpine administration.

Changes in GPa relative to totalGP

Table 4 shows the changes in GPa as a function of totalGP to address the hypothesis that the ratio of GPa : totalGP provides additional information about the changes in GP after status. These changes suggest a complex relationship between GPa and totalGP during the first hour of status, because the changes would not be predicted based on the mean values predicted in Tables 2 and 3 alone.

There were laminar-specific changes in the percentage of GPa : totalGP. Analysis of the GPa : totalGP ratio one hour after status demonstrated a decrease in dendritic layers of CA1 and the dentate gyrus, but not CA3. In addition, there was an increase in Layer III-LD. These changes suggest a complex relationship between GPa and totalGP during the first hour of status, because the changes would not be predicted based on the mean values predicted in Tables 2 and 3 alone.

One week after status there was an increase in the ratio of GPa : totalGP in all areas where mean values from Tables 2 and 3 demonstrated an increase in GPa, suggesting that the changes in GPa dominated this time. One month after status, GPa and totalGP ROD levels were higher than normal in some areas (Tables 2 and 3), but the ratio was similar to control conditions (Table 4), suggesting changes had occurred that restored normal relative levels of GPa or totalGP, even if individually the levels of GPa and totalGP were still not completely normal.

Specific changes in GP histochemistry as a result of status

Inner molecular layer—In the inner molecular layer (IML) of the dentate gyrus, a highly reactive band of GPa developed one week after status (Fig. 3). GFAP immunoreactivity

increased in the IML at this time (Fig 3; $n = 5/5$ rats), but did not in any control rats ($n = 0/5$ rats). One month after status, GFAP immunoreactivity in the IML was not distinguishable from control rats (Fig. 3; $n = 0/6$ rats).

GP-intense structures—Small patches, which were intensely GP-reactive, were present one week after status in many regions of the hippocampus and entorhinal cortex (Figs 4 and 5; $n = 5$), but not in control rats, or animals that had pilocarpine but never had status. These areas were highly reactive both for GP_a and totalGP (Figs 4 and 5). They appeared to be cellular because some processes emanated from them (Figs 4 and 5). They were prevalent in cell layers where there normally is gliosis due to neuronal loss (Fig. 5). One month after status, they were detected in the hippocampus in 3/6, suggesting a possible decline after gliosis stabilized (Figs 4 and 5). In entorhinal cortex, GP-intense structures were present in all rats at one week, as shown in Fig. 4. All rats demonstrated such structures at one week ($n = 5$) and one month after status ($n = 6$). Therefore, these structures may not be transient in all cases or in all locations.

Discussion

The results suggest that robust changes in GP occur throughout the hippocampus and entorhinal cortex during status, in the week following status, and there still are differences from control as long as one month after status.

Changes in mean GP reactivity of the hippocampus and entorhinal cortex

When GP values were averaged for the entire hippocampus and entorhinal cortex densitometrically, GP_a declined during status, and increased at one week and one month after status. Interestingly, the reduction in GP_a at one hour after the onset of status was not accompanied by a statistically significant reduction in totalGP. This result suggests that the reduction in GP_a could be due to its conversion to GP_b. It is possible that this is related to the substantial metabolic demands imposed by status. It could also be related to the rise in calcium and cAMP-dependent protein kinase (PKA), which change in response to seizures and excitotoxicity (DeLorenzo & Sun, 2006; McNamara *et al.*, 2006) and regulate phosphorylase kinase (Brushia & Walsh, 1999), the enzyme that catalyses the conversion of GP_b to GP_a.

The increase in GP_a at one week and one month after status was accompanied by increased totalGP, suggesting an up-regulation of GP related to epileptogenesis. More insight into these changes was provided by examining the proportion of GP_a : totalGP, because it suggested that a preferential increase in GP_a occurred one week after status. At one month ROD levels were greater than control for both GP_a and totalGP, but the ratio GP_a : totalGP was similar to controls. One interpretation of this result is that changes in GP_a influence levels of totalGP and vice-versa, reflecting an attempt to maintain constant relative levels under all conditions, i.e. homeostasis.

An increase in energy demand could be related to the changes in GP, although studies of GP by histochemistry alone cannot define glycogen metabolism. Certainly in the weeks after status there are many changes that require energy; neuronal damage due to status is followed by removal of debris and reactive gliosis, neurons that survive status begin to synthesize new proteins and establish new synaptic connections, and neurogenesis is increased in the dentate gyrus (Scharfman, 2002). At one month, animals have recurrent limbic seizures, so activation of the hippocampus and entorhinal cortex would be likely, and would be expected to increase energy utilization.

Laminar changes in GP

When GP was studied with respect to laminar differences, it became apparent that selected layers within the hippocampus and entorhinal cortex were primarily responsible for the changes in mean GP described above.

One hour after the onset of status, GP_a decreased primarily in dendritic layers of hippocampus, suggesting that during status, the primary area of change in GP_a is located at sites of glutamatergic input. This may be due to the fact that reverberatory seizure activity depends on glutamatergic pathways, and astrocytic GP is intense in the area of glutamate release/uptake to support the activity. However, one would expect that somatic action potential generation would be required for the reverberatory excitation, so it is surprising that the cell body layers did not demonstrate a robust change. The lack of GP changes in the cell layers may be due to the fact that relatively few astrocytes are located there.

In the entorhinal cortex, GP_a primarily declined in Layers I and II, which may reflect increased activity of the neurons in Layer II (with cell bodies and dendrites in Layers I and II) that project to the dentate gyrus. Alternatively, astrocytes in Layers I and II may be more sensitive than those in adjacent layers. Regardless, the data were similar to the hippocampus in that GP_a was reduced but not totalGP.

One week after status, GP_a was increased in almost every area of the entorhinal cortex, analogous to the increase in GP_a in the hippocampus that occurred almost in all areas. Interestingly, totalGP was changed in only one area, and that was a reduction, not an increase. This occurred in Layer III-LD, and may reflect that neurons in this region die in the days after status, leading to reduced need for energy afterwards. However, there is striking gliosis in this region in response to neuronal loss, and one would predict that new glia would restore GP. Possibly the new glia in this region have little GP, or there are other regulators of GP in this region of the brain that are unique.

One month after status, there was only one persistent change, and that was an increase in GP_a in Layers V–VI. This change may reflect the fact that recurrent seizures primarily use pathways that are mediated by deep layer neurons, or it may be explained by ongoing changes in the deep layers that involve synaptic reorganization (and hence demand energy). Unlike hippocampus, there was no increase in totalGP after one month in any area of entorhinal cortex. Other than this, changes in entorhinal cortex and hippocampus were similar.

Comparison to previous studies

GP measurements in saline controls were similar to those reported previously in the hippocampus (Harley & Bielajew, 1992; Uecker *et al.*, 1997; Fara-On *et al.*, 2005) and entorhinal cortex (Walling *et al.*, 2006). Changes in GP one hour after status in the present study also were similar to previous studies assessing GP activity using other convulsants, such as methionine sulfoximine. Methionine-sulfoximine-induced seizures in mice reduced the fraction of GP_a in rat cerebral cortex (Folbergrova, 1973). Two days later, totalGP returned to control levels, and it would have been interesting to have known whether GP would have increased after 7 days, because it did so in the present study.

The relationship between GP and seizures

The changes that were observed in the present study suggest that GP levels may be related to changes induced by status. However, a causal relationship was not shown, and the nature of the relationship remains to be defined precisely. Reduction in GP_a during status may exacerbate status-induced damage, because a decline in GP_a would potentially reduce energy supply, and energy depletion may be the reason why neurons die. However, GP is not the only regulator

of energy metabolism. To date there is little direct evidence for a role of GP in seizure-related neuronal damage. However, one study has shown that glycogen levels may play a role in seizure susceptibility. Bernard-Helary *et al.* (2000) investigated the differences in glycogen levels and seizure susceptibility in two strains of mice, using methionine sulfoximine as the convulsant. They found that the strain that was more resistant to seizures had increased levels of glycogen. We suggest that more studies in this area would be useful, so that the role of glycogen metabolism in seizures and epilepsy can be clarified.

GP and mossy fibre sprouting

Mossy fibre sprouting is the growth of axon collaterals from the axons of granule cells into a new target lamina, the inner molecular layer. It occurs in animal models of epilepsy and in humans with temporal lobe epilepsy (Tauck & Nadler, 1985; Sutula *et al.*, 1989), and has been suggested to facilitate seizures by increasing network excitability in the dentate gyrus (Dudek & Spitz, 1997; Sutula *et al.*, 1989). Whether or not it plays a critical role in generating seizures is controversial (Sloviter *et al.*, 2006), but even if it does not, it is a remarkable example of circuit reorganization in epilepsy. Mossy fibre sprouting has been suggested to develop in the weeks following status (Mello *et al.*, 1993). This is consistent with our observations of robust mossy fibre sprouting as early as 3–4 weeks after pilocarpine-induced status, using methods employed in the current study (Scharfman *et al.*, 2002). Therefore, it was interesting that there was increased GP and GFAP-immunoreactivity in the inner molecular layer of the dentate gyrus one week after status. This result suggests a role of astrocytic GP in mossy fibre sprouting. However, such a role appears transient, because GP declined by 4 weeks after status.

In contrast to our results, others have found that GFAP-immunoreactivity is reduced one week after status (Kang *et al.*, 2006). It is possible that the discrepancy is due to the fact that the age of the rats in that study ranged from 9 to 11 weeks postnatal, whereas the rats used in the present study were younger (5–6-weeks old). Animals that are 12-months old have decreased mossy fibre sprouting after status relative to 4-month-old rats (Shetty & Turner, 1999). Therefore, it may be that sprouting is less robust when animals have status at 9–11 weeks.

GP-intense areas after status

To our knowledge, no previous study has reported GP-dense structures in the hippocampus and entorhinal cortex before. In the present study, they were prevalent one week and one month after status. They appeared to be cellular because of fibrous processes emanating from them, but we can not be certain of the cellular origin at the present time, because concurrent labelling with cellular markers were obscured by the dense GP reactivity. However, it seems likely that the GP-dense areas are hypertrophied astrocytes, because they appear similar morphologically to the hypertrophied astrocytes identified by antibodies to GFAP in a previous study of pilocarpine-treated mice (Borges *et al.*, 2003).

Implications

We suggest that robust changes in GP occur during and after status in the rat, and this may be one of the contributing factors in the consequences of status, not only in the hours after status ends, but in the weeks or months that follow. Regulation of energy metabolism may influence epileptogenesis and chronic epilepsy more than has been previously anticipated.

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Abbreviations

GP	glycogen phosphorylase
GPa	active (phosphorylated) GP
GPb	inactive (dephosphorylated) GP
ROD	relative optical density

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Fig. 1.

Glycogen phosphorylase a (GPa) and total glycogen phosphorylase (totalGP) histochemistry in the rat hippocampus after status. Representative examples of GPa (left column, A, C, E and G) and totalGP (right column, B, D, F and H) in saline-treated control rats (A and B), compared to pilocarpine-treated rats that were killed one hour after the onset of status (C and D), one week after status (E and F), or one month after status (G and H). Asterisks in E and F indicate regions of tissue damage. Tissue damage was due to seizure-induced neuronal loss, and in addition, tissue sections were processed without fixation. Therefore, some sections have cracks (e.g. Fig. 3E and F). Fixation was avoided because it is incompatible with GP histochemistry. DG, dentate gyrus. Calibration bar, 500 μ m.

Fig. 2.

GP_a and totalGP in the entorhinal cortex after status. Representative examples of GP_a (left column, A, C, E and G) and totalGP (right column, B, D, F and H) in saline-treated control rats (A and B), compared to pilocarpine-treated rats that were killed one hour after the onset of status (C and D), one week after status (E and F), or one month after status (G and H). I, II, III-LD and V–VI refer to entorhinal cortical layers. LD, lamina dissecans; PreS, presubiculum; PaS, parasubiculum. Scale bar, 500 μ m.

Fig. 3.

Changes in GPa in the inner molecular layer of the dentate gyrus after status. GPa histochemistry (A, C and E) and NeuN/GFAP immunohistochemistry on adjacent fresh tissue sections (B, D and F) in the dentate gyrus in a control rat (A and B) compared to pilocarpine-treated rats that had status one week earlier (C and D) or one month (E and F) earlier. NeuN (blue) and GFAP (brown) illustrate colocalized GPa and GFAP in the inner molecular layer (arrows), just above the granule cell layer. IML, inner molecular layer; GCL, granule cell layer. Scale bar, 100 μ m.

Fig. 4.

Changes in GPα in the dentate gyrus and entorhinal cortex after status. GPα histochemistry is compared for a control rat (A), a pilocarpine-treated rat that was examined one week after status (B) and a pilocarpine-treated rat that was evaluated one month after status (C). Intense GPα reactivity is marked by the arrows. IML, inner molecular layer; GCL, granule cell layer. Note that at 1 month, the hilus appears to have expanded, an observation that has been made previously (McCloskey *et al.*, 2006). (D) Intense GPα reactive structures, similar to the dentate gyrus hilus, were present in the entorhinal cortex (EC) one week after status (arrows). II, III, V refer to entorhinal cortical layers. Scale bar, 100 μm (A–C); 200 μm.

Fig. 5.

GP-dense structures in hippocampal cell layers after status. GPa in CA1 (left column, A–C) and CA3 (right column, D–F) of the hippocampus in saline-injected controls (A and D) and pilocarpine-injected animals one week (B and E) or one month (C and F) after status. Arrows indicate GPa-intense structures found throughout the layers of CA1 (B) and CA3 (E) one week after status and one month after status (C and F). Scale bars, 200 μ m (A–C) and 100 μ m (D–F).

Table 1
Mean relative optical density (ROD) measurements of GP in the hippocampus and entorhinal cortex after status using GP histochemistry

	ROD Control	ROD Status	F-value	P-value	(n)
1 hour GPa	0.40 ± 0.08	0.34 ± 0.06↓	$F_{16,160} = 2.03$	$P < 0.0139$	(6)
1 hour GPa + GPb	0.62 ± 0.17	0.54 ± 0.09	$F_{16,160} = 0.71$	NS ($P > 0.05$)	(6)
1 week Gpa	0.33 ± 0.04	0.48 ± 0.03↑	$F_{16,128} = 1.83$	$P < 0.0341$	(5)
1 week GPa + GPb	0.58 ± 0.07	0.62 ± 0.06↑	$F_{16,128} = 2.87$	$P < 0.0005$	(5)
1 month Gpa	0.39 ± 0.04	0.44 ± 0.03↑	$F_{16,160} = 6.07$	$P < 0.00001$	(6)
1 month GPa + GPb	0.61 ± 0.11	0.64 ± 0.60↑	$F_{16,160} = 2.81$	$P < 0.0005$	(6)

Mean ROD measurements ± SD of GPa and totalGP activity in the hippocampus and entorhinal cortex of saline-injected rats (Control) and rats that had pilocarpine-induced status (Status). Animals were examined one hour after the onset of status, after one week, or after one month. Arrows indicate direction of significant change in GP activity compared to control rats (see *p*-values). *n* represents the number of rats per group.

Table 2
ROD measurements of GP histochemistry in the hippocampus after status

Subfield	Control			1 hour status			1 week after status			1 month after status		
	GPa	TotalGP	Gpa	GPa	TotalGP	Gpa	GPa	TotalGP	Gpa	GPa	TotalGP	Gpa
CA1												
PCL	0.194 ± 0.07	0.396 ± 0.11	0.195 ± 0.06	0.324 ± 0.11	0.415 ± 0.12†	0.581 ± 0.20†	0.451 ± 0.08†	0.681 ± 0.13†				
SO	0.369 ± 0.07	0.592 ± 0.13	0.314 ± 0.07↓	0.523 ± 0.10	0.473 ± 0.11†	0.656 ± 0.23†	0.450 ± 0.07†	0.679 ± 0.01				
SR	0.378 ± 0.07	0.600 ± 0.14	0.323 ± 0.07↓	0.539 ± 0.10	0.474 ± 0.11†	0.648 ± 0.23	0.440 ± 0.06†	0.655 ± 0.11				
SL-M	0.406 ± 0.07	0.638 ± 0.15	0.349 ± 0.07↓	0.579 ± 0.10	0.498 ± 0.11†	0.670 ± 0.24	0.463 ± 0.06	0.682 ± 0.01				
CA3												
PCL	0.206 ± 0.07	0.369 ± 0.09	0.201 ± 0.04	0.303 ± 0.09	0.324 ± 0.10†	0.434 ± 0.14	0.313 ± 0.07†	0.488 ± 0.08†				
SR	0.377 ± 0.07	0.590 ± 0.13	0.331 ± 0.06↓	0.546 ± 0.11	0.473 ± 0.11†	0.629 ± 0.21	0.430 ± 0.06	0.641 ± 0.10				
SO	0.456 ± 0.08	0.722 ± 0.20	0.395 ± 0.06↓	0.620 ± 0.08	0.570 ± 0.14†	0.748 ± 0.27†	0.496 ± 0.11	0.753 ± 0.19				
DG												
GCL	0.198 ± 0.06	0.334 ± 0.08	0.129 ± 0.12↓	0.216 ± 0.07	0.223 ± 0.07†	0.318 ± 0.11	0.180 ± 0.02	0.285 ± 0.07				
OML	0.387 ± 0.07	0.601 ± 0.14	0.326 ± 0.10↓	0.538 ± 0.10	0.488 ± 0.11†	0.652 ± 0.23	0.444 ± 0.06	0.653 ± 0.10				
IML	0.344 ± 0.08	0.581 ± 0.13	0.285 ± 0.11↓	0.515 ± 0.07	0.437 ± 0.10†	0.578 ± 0.20	0.331 ± 0.02	0.521 ± 0.09				
Hilus	0.380 ± 0.07	0.596 ± 0.14	0.331 ± 0.06↓	0.535 ± 0.10	0.500 ± 0.12†	0.667 ± 0.23†	0.452 ± 0.06†	0.669 ± 0.11				

ROD measurements (± SD) of GPa and totalGP activity in the hippocampus of saline-injected (Control) rats and rats that had pilocarpine-induced status (Status). Animals that had status were killed immediately after one hour of status, after one week, or after one month survival periods. Control rats were pooled based on the lack of statistical differences in controls killed at different times after status (Table 1). Arrows indicate direction of significant change in GP activity compared to control rat sections processed concurrently ($P < 0.05$). PCL, pyramidal cell layer; SO, stratum oriens; SR, stratum radiatum; SL-M, stratum lacunosum-moleculare; DG, dentate gyrus; GCL, granule cell layer; OML, outer molecular layer; IML, inner molecular layer.

Table 3
ROD measurements of GP histochemistry in the entorhinal cortex after status

	Control		1 hour status		1 week after status		1 month after status	
	GPa	TotalGP	GPa	TotalGP	GPa	TotalGP	GPa	TotalGP
Entorhinal cortex								
Layer I	0.674 ± 0.10	0.837 ± 0.17	0.574 ± 0.09↓	0.851 ± 0.19	0.701 ± 0.20	0.826 ± 0.30	0.646 ± 0.12	0.805 ± 0.12
Layer II	0.438 ± 0.08	0.706 ± 0.13	0.377 ± 0.12↓	0.546 ± 0.11↓	0.522 ± 0.11↑	0.655 ± 0.23	0.526 ± 0.10	0.699 ± 0.07
Layer III-LD	0.521 ± 0.08	0.850 ± 0.19	0.511 ± 0.11	0.716 ± 0.16	0.570 ± 0.11↑	0.705 ± 0.26↓	0.566 ± 0.10	0.757 ± 0.14
Layer V-VI	0.267 ± 0.08	0.437 ± 0.09	0.284 ± 0.06	0.413 ± 0.11	0.317 ± 0.07↑	0.424 ± 0.15	0.348 ± 0.06↑	0.460 ± 0.06
Parasubiculum	0.332 ± 0.08	0.587 ± 0.12	0.356 ± 0.09	0.570 ± 0.14	0.457 ± 0.10↑	0.626 ± 0.22	0.404 ± 0.09	0.599 ± 0.07
Presubiculum	0.527 ± 0.09	0.876 ± 0.23	0.520 ± 0.12	0.819 ± 0.23	0.648 ± 0.16↑	0.803 ± 0.30	0.582 ± 0.12	0.833 ± 0.18

ROD measurements (± SD) of GPa and totalGP activity in the entorhinal cortex of saline-injected (Control) rats and pilocarpine-injected rats that had status, immediately after one hour of status, or after one week and one month survival periods. Arrows indicate direction of significant change in GP activity compared to control rats ($P < 0.05$). Levels of GP activity of control rats were similar regardless of time after saline injection when they were examined (one hour, one week, one month), so control data were pooled. PCL, pyramidal cell layer; SO, stratum oriens; SR, stratum radiatum; SL-M, stratum lacunosum-moleculare; DG, dentate gyrus; GCL, granule cell layer; OML, outer molecular layer; IML, inner molecular layer; LD, lamina densicans.

Table 4
GPa : total GP ratio in the hippocampus and entorhinal cortex

	1 hour		1 week		1 month	
	Control	Status	Control	Status	Control	Status
Hippocampus						
CA1						
PCL	57.31 ± 11.69	62.55 ± 12.06	39.02 ± 15.44	72.74 ± 13.08↑	50.84 ± 12.19	67.07 ± 10.11
SO	64.10 ± 14.40	61.88 ± 13.43	53.88 ± 11.89	68.40 ± 12.10↑	58.23 ± 11.82	63.00 ± 9.89
SR	68.86 ± 13.06	59.08 ± 10.60↓	51.52 ± 13.60	68.54 ± 10.03↑	61.11 ± 11.76	65.41 ± 10.75
SL-M	72.98 ± 15.86	59.60 ± 13.46↓	56.08 ± 14.50	71.24 ± 8.94↑	63.43 ± 10.39	69.83 ± 10.18
CA3						
PCL	62.75 ± 7.96	68.24 ± 10.99	45.60 ± 13.64	74.15 ± 11.88↑	56.51 ± 10.70	64.67 ± 11.26
SR/SL-M	63.94 ± 10.96	61.48 ± 10.07	59.66 ± 11.03	73.92 ± 13.07↑	62.59 ± 7.87	64.25 ± 11.79
SO	66.66 ± 13.82	64.26 ± 7.80	62.88 ± 11.17	77.08 ± 6.28↑	65.75 ± 13.18	67.08 ± 10.61
DG						
GCL	66.51 ± 10.20	60.42 ± 9.05	46.64 ± 15.35	70.54 ± 9.40↑	64.18 ± 10.68	64.72 ± 9.39
OML	71.62 ± 9.95	56.21 ± 10.26↓	60.99 ± 12.53	74.24 ± 8.26↑	68.16 ± 11.15	70.44 ± 10.04
IML	66.20 ± 12.54	55.39 ± 9.95↓	52.06 ± 15.53	75.87 ± 5.39↑	61.99 ± 9.73	65.20 ± 12.19
Hilus	68.38 ± 12.48	68.16 ± 854	60.81 ± 15.18	80.24 ± 4.25↑	74.98 ± 22.95	69.30 ± 9.45
Entorhinal cortex						
Layer I	80.38 ± 11.27	69.84 ± 13.88↓	82.8 ± 5.00	86.82 ± 1.86	83.72 ± 11.53	81.49 ± 8.11
Layer II	64.60 ± 11.45	69.95 ± 10.08	57.69 ± 9.09	81.91 ± 3.44↑	67.71 ± 14.71	77.18 ± 10.88
Layer III-LD	64.66 ± 13.10	72.77 ± 7.29↑	55.99 ± 11.76	81.31 ± 2.46↑	69.16 ± 13.68	76.32 ± 10.89
Layer V-VI	66.49 ± 14.73	70.32 ± 6.79	49.36 ± 7.62	76.57 ± 4.88↑	68.11 ± 14.61	76.14 ± 8.73
Parasubiculum	59.45 ± 6.49	64.21 ± 10.18	51.11 ± 8.89	73.96 ± 5.87↑	62.93 ± 14.02	69.52 ± 10.83
Presubiculum	61.49 ± 8.71	65.98 ± 8.62	58.85 ± 9.88	80.88 ± 2.36↑	68.88 ± 13.79	71.78 ± 11.37

Ratio of GPa to total GP in the hippocampus and entorhinal cortex of saline- injected (control) and pilocarpine-injected rats that had status. Animals that had status were examined after one hour of status, one week after status, and one month after status. Arrows indicate direction of significant change in GP activity compared to control rats ($P < 0.05$). PCL, pyramidal cell layer; SO, stratum oriens; SR, stratum radiatum; SL-M, stratum lacunosum-moleculare; DG, dentate gyrus; GCL, granule cell layer; OML, outer molecular layer; IML, inner molecular layer; LD, lamina densicans.