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Astrogliosis is temporally correlated with enhanced neurogenesis in adult rat hippocampus following a glucoprivic insult

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ABSTRACT

2-Deoxy-D-glucose (2-DG) administration causes transient depletion of glucose derivates and ATP. Hence, it can be used in a model system to study the effects of a mild glycoprivic brain insult mimicking transient hypoglycemia, which often occurs when insulin or oral hypoglycemic agents are administered for diabetes control. In the present study, the effect of a single 2-DG application (500 mg/kg, a clinically applicable dose) on glial reactivity and neurogenesis in adult rat hippocampus was examined, as well as a possible temporal correlation between these two phenomena. Post-insult (PI) glial reactivity time course was assessed by immunoreaction against glial-fibrillary acidic protein (GFAP) during the following 5 consecutive days. A clear increase of GFAP immunoreactivity in hilus was observed from 48 to 96 h PI. Moreover, enhanced labeling of long radial processes in the granule cell layer adjacent to hilus was evidenced. On the other hand, a transient increase of progenitor cell proliferation was detected in the subgranular zone, prominently at 48 h PI, coinciding with the temporal peak of glial activation. This increase resulted in an augment of neuroblasts double labeled with 5-bromo-deoxyuridine (BrdU) and with double cortin (DCX) at day 7 PI. Around half of these cells survived 28 days showing matured neuronal phenotype double labeled by BrdU and a neuronal specific nuclear protein marker (NeuN). These findings suggest that a transient neuroglycoprivic state exerts a short-term effect on glial activation that possibly triggers a long-term effect on neurogenesis in hippocampus.

Transient hypoglycemia occurs frequently when insulin or oral hypoglycemic agents are administered for diabetes control; depending on the severity of this metabolic disorder, temporal and long lasting autonomic, neuro-endocrine and behavioral responses could be induced [22]. Glucose is the most important source of energy for brain metabolism. Reduction of cellular glucose utilization (glucoprivation) increases aspartate and glutamate levels during and after the glycopenic insult inducing a sustained activation of glutamate receptors that results in an excitotoxic neuropathology in which certain neurons are selectively killed [1,2]. Hypoglycemia-induced neuronal death occurs predominantly in the hippocampal formation, superficial layers of the cortex, and striatum [2], probably contributing to neurological sequelae such as cognitive decline. Clinical studies have reported that significant learning and memory deficits correlate with the frequency of hypoglycemia not only in patients with type 1 diabetes but also in the younger group among the population with type 2 diabetes [6]. Despite the high incidence of this common complication for diabetes control, the specific pathophysiological changes derived from glucoprivic brain insult and the mechanisms that contribute to brain repair are still not well defined.

Glial cells have been demonstrated as participating in a vast number of brain transactions, both normal and pathological. They exert a particular prominent role in the repairing processes responding to all forms of central nervous system insults [19]. The astroglial cells become reactive (astrogliosis) after a particular insult by increasing the synthesis of glial-fibrillary acidic protein (GFAP), the main cytoskeletal protein for astrocytes, resulting in hypertrophied somata, thickened and prolonged processes under GFAP immunohistochemical assessment [19,25,26]. There exists substantial information concerning molecules able to induce reactive astrocytosis or about changes in molecular expression during glial activation. Nevertheless, the time course of this process after injury and the specific functions exerted by it are not well defined.

On the other hand, adult neurogenesis has been implicated in processes that lead to neural regeneration following central nervous system disease and injuries. In a rat model of hypoglycemia induced by insulin, Suh et al. [20] reported a transient increase in

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progenitor cell proliferation in the dentate gyrus (DG) subsequent to an extensive neuronal loss, suggesting a remarkable role for ameliorating brain injury by boosting endogenous neural regeneration.

The present experiments aimed to determine several aspects of short- and long-term consequences of a single glucoprivic insult. The first goal was to determine the time courses and the interrelationship of reactive astrogliosis and cell proliferation in the DG of hippocampus subsequent to a mild neuroglucoprivic insult. The second goal was to investigate whether the altered cell proliferation has an impact on neuroblast and mature neuron generation. To establish a neuroglucoprivic model system that could induce specifically a glycopenic brain insult restricting both the adrenal medullary release of epinephrine and the cortical release of glucocorticoids as physiological responses to systemic hypoglycemia, we used the antimetabolic glucose analog 2-deoxy-D-glucose (2-DG). 2-DG has the 2-hydroxyl group replaced by hydrogen. Hence, it cannot undergo further glycolysis. Briefly, 2-DG is taken up into the cell through the glucose transporters [16] and it is phosphorylated by glucose hexokinase to produce 2-deoxy-D-glucose-6-phosphate. The fact that this molecule cannot be metabolized via the glycolytic pathway causes its gradual accumulation within the cell, followed by the inhibition of glucose-6-phosphate isomerase and the subsequent blockade of the conversion from glucose-6-phosphate to fructose-6-phosphate, a crucial step in this metabolic pathway [11]. This intrinsic property of 2-DG has been applied in clinical pharmacology for the treatment of two common diseases, cancer and epilepsy, in which elevated metabolic demands are required by the pathophysiological states. Moreover, 2-DG crosses the blood–brain barrier so it can be used experimentally to induce neuroglucoprivation.

Ninety-two young Wistar male rats of 300 ± 10 g were used in this study. All animal procedures were approved by the local Bioethical and Biosecurity committees. Animals were housed on an artificial light/dark cycle (light on at 18:00 and light off at 6:00) with temperatures between 20 and 24 °C, adequate ventilation, food and water *ad libitum* unless specified differently. Fig. 1 depicts the general experimental design of the three experiments.

For glucoprivation induction (GI), experimental subjects were injected (i.p.) a single dose (500 mg/kg) of 2-deoxy-D-glucose (2-DG, Sigma–Aldrich) dissolved in 0.9% saline; control groups were injected with saline. Injection was performed at 10:00, denoted as time 0 (Fig. 1, indicated by an arrow), with 4 h of food restriction before and after the injection.

To characterize the time course of reactive astrogliosis, rats were sacrificed at the following time points post-GI: *T* (*T* denotes a period of 24 h), 2*T*, 3*T*, 4*T* and 5*T*, $n = 6$ for each group (Fig. 1, green group) through transcardial perfusion with 0.9% saline and 4% paraformaldehyde in PB 0.1 M fixative posterior to sodium penthobarbital anesthesia. Coronal sections (50 μm) of dorsal hippocampus were obtained. For immunostaining, sets of one out every six sections from Bregma −3.2 to −3.8 mm were processed. Mouse anti-GFAP (1:1000, Chemicon) and chicken anti-mouse Alexa fluor 594 (1:1000, Molecular Probes) were used as primary and secondary antibodies, respectively through conventional immunohistochemical procedures. Hilus of DG was photographed and optical density was determined for each group using Fovea Pro 4.0 (Reindeer Graphics).

To characterize the time course of cell proliferation due to the GI in the subgranular zone (SGZ), 5-bromo-2-deoxyuridine (BrdU, Sigma, 50 mg/kg/12 h divided in three injections) was prepared, injected and immunoreacted as described elsewhere [27]. In order to get a relatively precise rate of proliferation near the time point, BrdU injections covered only the immediate 12 h before the perfusions at *T*, 2*T*, 3*T*, 4*T*, 5*T*, 7*T* and 14*T* time points (Fig. 1, orange group). Sections were selected with the same criteria of experiment 1. Rat anti-BrdU (1:1000, Accurate Scientific) and goat anti-rat IgG (Alexa Fluor 488, 1:1000, Invitrogen) were used as primary and secondary antibodies, respectively. Immunolabeled nuclei across the section in the SGZ-hilus were counted.

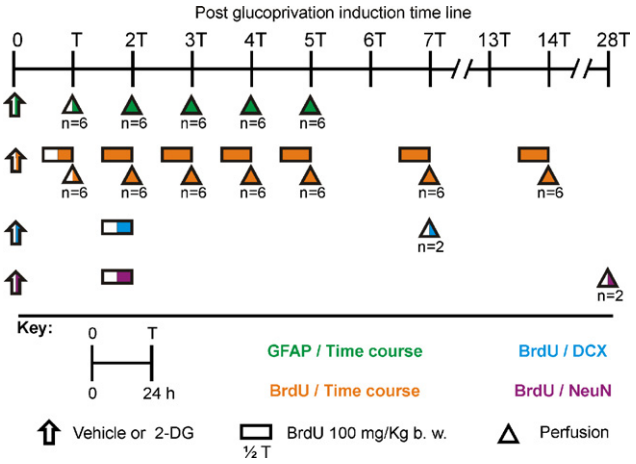

Fig. 1. General experimental design. The time line correlates the experiments performed post-glucoprivation induction (GI). Each interval of time (*T*) represents a period of 24 h. Glucoprivation was induced by an injection of 2-deoxy-D-glucose at time 0, indicated by an open arrow (saline 0.9% for control group). Semi-filled symbols indicate time points where data from control subjects were obtained. Green color indicates the characterization of time course of reactive astrogliosis reflected in GFAP immunoreactivity; orange color indicates the characterization of time course of altered progenitor cell proliferation by GI in SGZ. Blue and purple colors indicate the evaluations of neurogenesis rates reflected in double labeled neuroblast (BrdU/DCX) and matured neuron (BrdU/NeuN) counting, respectively. Triangles indicate perfusion time points and rectangles denote BrdU exposure time (12 h). A total of 92 Wistar male rats were used, the number of animals per group included in each time point is indicated below the point of perfusion. SGZ: subgranular zone; GFAP: glial-fibrillary acidic protein; BrdU: 5-bromo-deoxyuridine (BrdU); DCX: double cortin; NeuN: neuronal specific nuclear protein.

To assess the post-GI neurogenesis rate in the time point where highest cell proliferation was observed, rats from experimental and control conditions ($n = 4$) were injected with BrdU 12 h previous to 2*T*. Half of each group was perfused at 7*T* for BrdU labeled neuroblast detection, using double cortin (DCX) immunolabeling (Fig. 1, blue group), and the other half was perfused at 28*T* for BrdU labeled matured neuron detection, using neuronal specific nuclear protein (NeuN) labeling (Fig. 1, purple group). Perfusion, cutting and section selection were done as described above. Following primary antibodies were used for the corresponding immunoreactions: rat anti-BrdU (1:1000, Accurate Scientific), goat anti-DCX (1:1000, Santa Cruz Biotechnology) and mouse anti-NeuN (1:1000, Chemicon); secondary antibodies: Alexa 488 donkey anti-rat IgG, Alexa 594 rabbit anti-goat IgG and donkey anti-mouse IgG (Molecular Probes). Blind cell counting was performed by two experimentalists directly under fluorescent microscope in the SGZ adjacent to hilus (this region was chosen to set a consistent criterion for all sections). By quickly changing the filters for Alexa 488 and Alexa 594, one can unambiguously identify the double-labeled cell, either BrdU/DCX or BrdU/NeuN.

To evaluate the morphological aspects of the radial glial cell-like processes, we used a modified version of Sholl rings [26]. Briefly, the stereological graticule consists of concentric circles with 10 μm of distance between each. The cellular somata with their visible branches were placed on the center of the graticule and the number of intersections (NoI) of radial glial cell-like processes projections within the graticule was counted.

Quantitative results were expressed as mean ± standard error of mean (SEM). Groups were tested for differences by performing one-way ANOVA followed, when appropriate, by the Dunnett's post hoc test using Prism (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered statistically significant at a value $P < 0.05$.

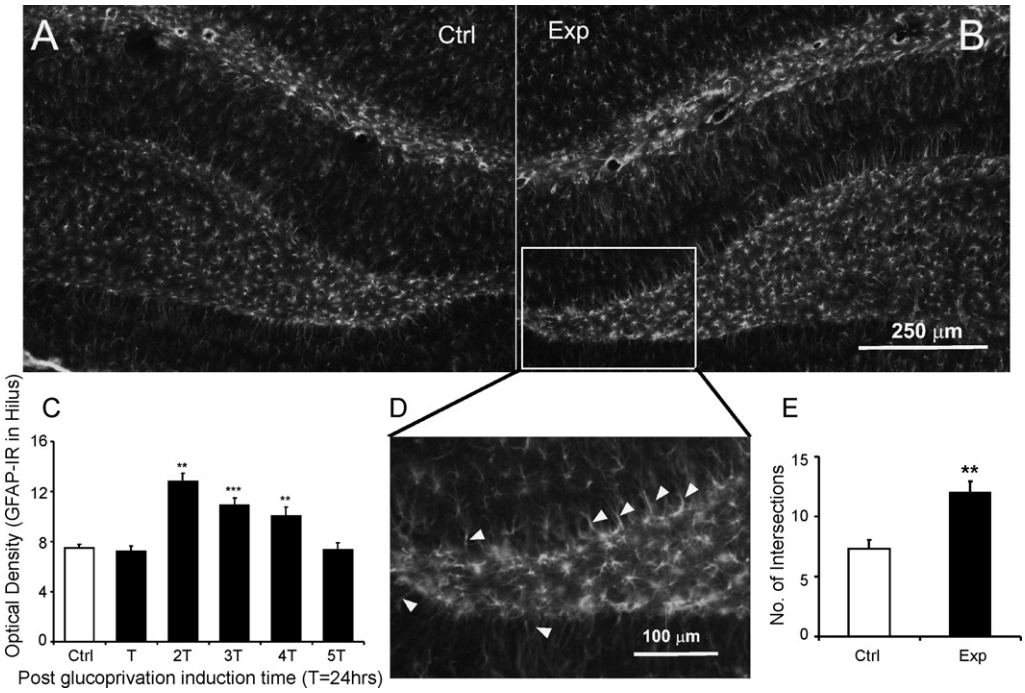

Fig. 2. Representative photomicrographs of GFAP immunohistochemistry in the DG from control (A) and experimental (B) subjects perfused 48 h post-glucoprivation induction (PGI) are shown. Insert (D) shows the hilar region adjacent to the merge region of dorsal and ventral granule cell layers (GCL) with higher magnification. Some radial-like GFAP-IR processes extending into the GCL are indicated by arrowheads. (C) Histogram showing time course of astrocytic reactivity measured by optical density analysis in the hippocampus PGI. $T = 24$ h. (E) Quantification of the number of intersections of radial glial cell-like cytoskeletal processes with the modified Sholl rings at 2*T*. $^{**}P < 0.01$, $^{***}P < 0.001$.

Reactive astrogliosis after a single glucoprivic induction peaked at 48 h post-insult (PI) and remained significantly different to control for 3 days: 1 day after the 2-DG administration, astroglial cells appeared normal under general observation; measurements performed at hilus of hippocampus revealed no difference compared with control. However, optical density at 2*T* showed a sheer augment of GFAP-IR of almost twofolds, it remained elevated in the following 2 days and decreased to the control level at 5*T* (Fig. 2C, optical density at Ctrl = 7.50 ± 0.28; $T = 7.24 \pm 0.40$; $2T = 12.78 \pm 0.71$; $3T = 10.89 \pm 0.59$; $4T = 10.07 \pm 0.69$; $5T\,7.34 \pm 0.57$). During morphological analysis at 2*T*, an increase of length (larger number of intersections) of radial glial cell-like processes was observed. These processes arise from GFAP-IR somata located on the border between hilus and granule cell layer (GCL) and extend into GCL more distally than control (Fig. 2E).

A single glucoprivic insult induced by 2-DG altered SGZ progenitor cell proliferation in a time dependent manner with its peak value observed at 48 h PI: immunolabeling revealed an elevation of BrdU+ nuclei at 2*T* (26.7 ± 3.5), 3*T* (19.2 ± 0.8) and 4*T* (21.9 ± 1.9) with the highest value at 2*T* PI, coinciding with the peak of GFAP-IR. From 5*T* to 14*T*, no significant difference of BrdU+ counts between experimental and control (15.5 ± 1.7) was observed (Fig. 3).

Long-term modification on neurogenesis rate was revealed by higher counts of newly formed cells becoming neuroblasts at 7*T* and matured neurons at 28*T* in 2-DG rats: the number of BrdU+/DCX+ cells was significantly higher in the 2-DG-injected group (13.63 ± 1.4) than in the control (5.5 ± 1.1; Fig. 4). These cells were commonly observed in clusters along the SGZ. To study whether this increased rate of neuroblast formation reaches its final differentiation and become mature neurons, perfusion of animals at 28*T* PI and immunostained sections with BrdU and NeuN was performed (Fig. 1, purple group). A twofold increase in the number of BrdU+/NeuN+ nuclei was observed in the 2-DG group, compared to the control (5.0 ± 0.5 vs 2.5 ± 0.2, respectively; Fig. 4).

Hypoglycemia is a major risk factor of insulin and oral hypoglycemic agent therapy for diabetes. Clinical studies revealed that diabetes patients have a greater rate of decline in cognitive function [5]. The mechanisms behind this dysfunction are not well known.

In the present work, experimental rats were treated with the synthetic non-metabolizable glucose analog 2-DG, a molecule used extensively as an inhibitor of glycolysis from bacteria to humans. Experimental rats received a single dose of 500 mg/kg of 2-DG. This single pharmacological dose used in our study specifically blocks brain glucose metabolism in a transient fashion, without producing behavioral effects, coma or convulsions [3]. The brain, in contrast to other tissues, is particularly vulnerable to hypoglycemia due to its critical dependence on glucose for adequate performance. It has

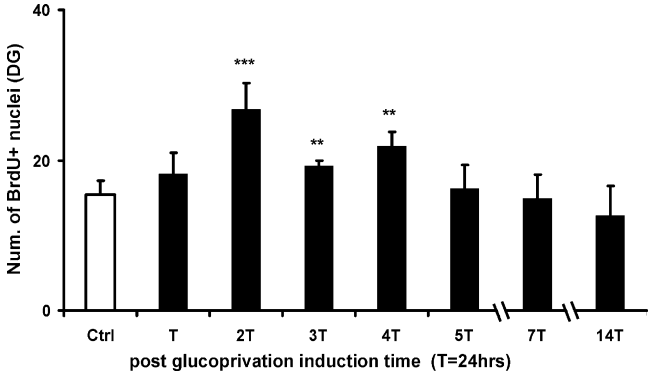

Fig. 3. Histogram showing time course of cell proliferation in the DG post-glucoprivation induction. The temporal effect of a single dose of 2-DG (500 mg/kg) on the number of BrdU nuclei was observed during the first 5 days. Note that the peak in BrdU nuclei temporally coincides with the peak in GFAP expression in the hilus at 2*T* (Fig. 2). $T = 24$ h. $^{**}P < 0.01$, $^{***}P < 0.001$. Counts were obtained from the total numbers of BrdU+ nuclei per section's hilar subgranular zone.

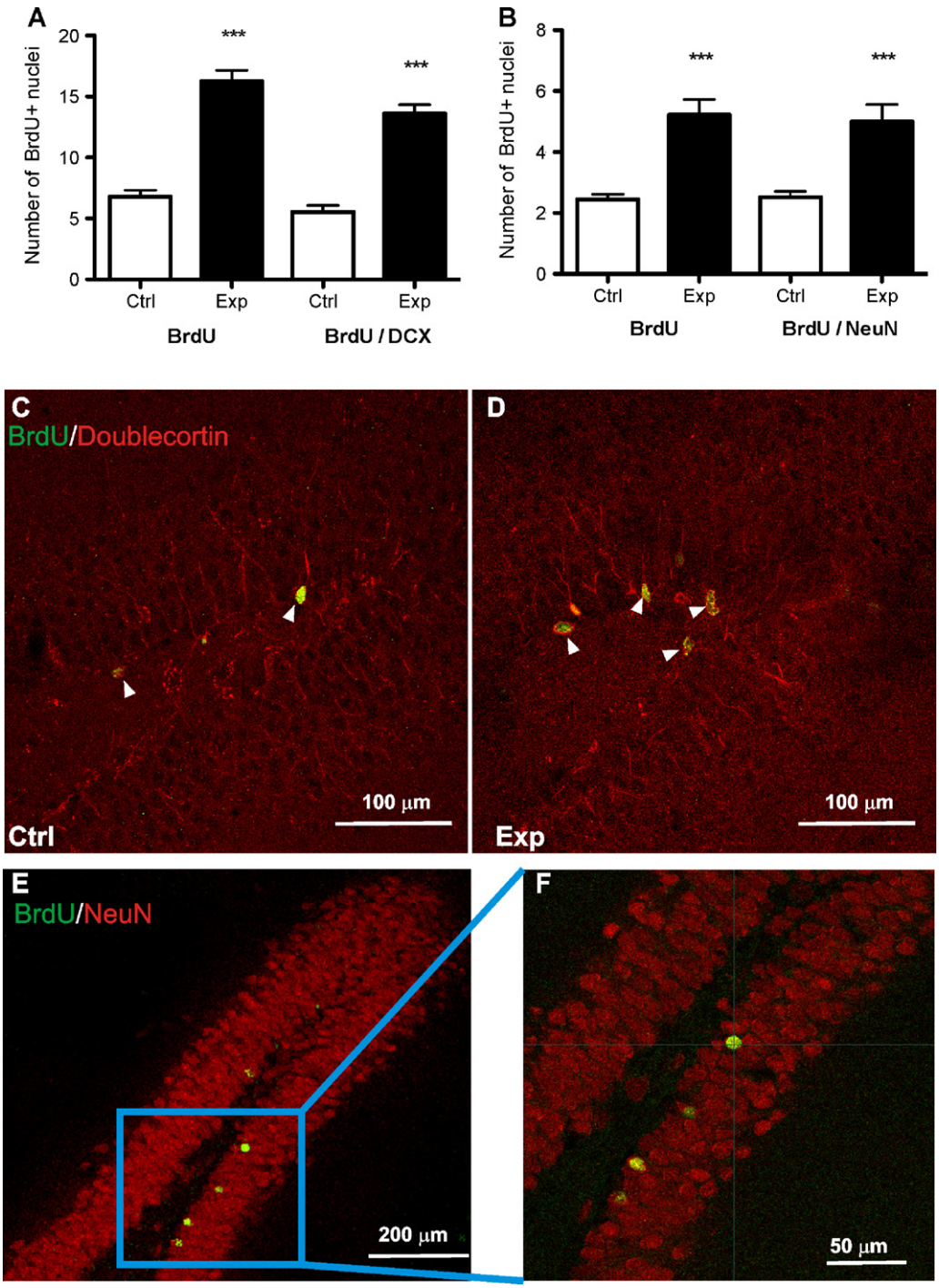

Fig. 4. Effect of transient glucoprivation on neurogenesis in hilar granule cell layer. (A) Histogram showing the number of BrdU+ nuclei and the number of cells BrdU+/DCX+ in control (Ctrl) and experimental (Exp) subjects. (B) Histogram showing the number of BrdU+ nuclei and the number of cells BrdU+/NeuN+ in control (Ctrl) and experimental (Exp) subjects. To analyze the nature of the newly formed neurons induced by 2-DG, BrdU was injected 2 days after the 2-DG dose, taking into account the temporal peak of cell proliferation showed in Fig. 3. (C and D) Representative photomicrographs of double immunohistochemistry for BrdU/DCX (arrowheads) from control (left) and experimental (right) groups. (E) Representative photomicrograph of double immunohistochemistry for BrdU/NeuN. (F) Insert of E. ***$P < 0.001$. Counts were obtained from the total number of double-labeled cells (A) and nuclei (B) per section's hilar subgranular zone.

been shown that neuronal damage and cell death may occur when insulin or other hypoglycemic drugs are administered [4,28]. Studies in human cases and animal models of hypoglycemic brain injury revealed that the dentate gyrus of the hippocampus is preferentially damaged, whereas glial cells are generally spared [1]. Evidence suggests that neuronal death stimulated by hypoglycemia involves a process of excitotoxicity. Hypoglycemia induces neuronal depolarization and therefore raises extracellular glutamate and aspartate concentrations, leading to sustained activation of glutamatergic receptors. Consequently, there is an increase of Ca^{2+} influx that generates mitochondrial calcium deregulation and excessive production of free radicals, inducing mitochondrial dysfunction and DNA damage, finally conducting to cell death [9,15]. The increase in cell proliferation after 2-DG injection observed in the present study is in accordance with other reports, showing that diverse brain injuries stimulate cell proliferation in the SVZ of the DG [12,23],

probably contributing to the replacement and repair of damaged tissue.

Astrocytes are involved in normal brain functioning through intense interactions with neurons. Astroglial cells also play a pivotal role in brain homeostasis regulation and repairing process after brain injury. The assessment of reactive astrogliosis in the neurogenic regions is of great importance for understanding the mechanisms underlying the integrative response to brain injury. The observed glial reactivity early after 2-DG treatment suggests a change in astrocyte-neuron dynamics, which may include modifications on blood–brain barrier, extrasynaptic space diffusion, neurotrophic factors secretion, neurotransmitter removal [21]. Although the consequences of astrocytic reactivity are a controversial issue [7], there is strong evidence suggesting that altered neuron–glia interactions may be beneficial [8]. Astrocytes have been implicated in reducing neuronal vulnerability to excitotoxicity, since they exert a key role for glutamate uptake within the CNS [18]. Furthermore, due to their high-energy demands and restricted energy stores, neurons are acutely and critically dependent on a stretched regulation of energy metabolism and supply. This metabolic energy regulation is mainly sustained in the brain by astrocytes, as they directly participate in matching glucose supply to neuronal demands [24]. There exists evidence suggesting a core glucose-sensing role in the CNS to the glucose transporter type 2, which is expressed in glial cells, implicating astrocytes as hypoglycemic sensors [13]. Experimental evidence suggests that glucose could be first metabolized by astrocytes, generating extracellular lactate, which is shuttled to the neurons as a substrate for neuronal pyruvate synthesis [17]. Other molecules, like fatty acids and ketone bodies, may also be used as energy sources in the brain under certain circumstances. Astrocytes are the only identified cell type to perform β-oxidation of fatty acids in the brain and they also use fatty acids as precursors for synthesis of ketone bodies that are exchanged with neurons [10].

We have observed a phase-matching relationship between the time courses of both astrogliosis in the hilus of DG, where resides the SGZ – the germinal layer for adult hippocampal neurogenesis – and the cell proliferation rate variation of the same layer post-insult. This observation suggests an intrinsic interrelationship between the two phenomena. Besides, cytoskeletal hypertrophy of radial-glia-like astrocytes towards the deep level of granule cell layer (GCL) was clearly observed. The phenotype, biological properties and the fate of the glial-fibrillary acidic protein-expressing cells in the SGZ are currently of great interest, regarding both normal function and the capacity for ameliorating brain injury. Do all of these GFAP-expressing astrocytes in the SGZ have multipotent neurogenic potential? To answer this question satisfactorily, more detailed knowledge is needed. However, several feasible explanations can be given for this phase-matching condition above mentioned. For instance, it is well documented that astrocytes participate importantly in the creation of the microenvironment—or "niche" that stimulates neurogenesis [29]. In the 2-DG rat SGZ, there were consistent increases of both GFAP immunoreactivity and BrdU+/DCX+ cell counting in the niche-like region where the dorsal and ventral GCL merged (Figs. 2 and 4 photomicrographs). This phenomenon suggests that astrogliosis in this region has an active role in neurogenesis. On the other hand, Muller et al. [14] showed that ciliary neurotrophic factor (CNTF) is necessary for regulating the extent of newly formed neurons in the adult SGZ. In unlesioned brain, CNTF is expressed by astrocytes at low concentrations, whereas its expression increases after a lesion. Thus, the injury-induced neurogenesis can be promoted by a transient increase in CNTF levels by astrocytes. Our finding of similar temporalities of both GFAP immunoreactivity and DG cell proliferation supports this hypothesis.

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