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Astrocyte glycogen sustains neuronal activity during hypoglycemia: studies with the glycogen phosphorylase inhibitor CP-316,819

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Nonstandard Abbreviations:

CP-316,819, [R-R*,S*]-5-chloro-N-[2-hydroxy-3-(methoxymethylamino)-3-oxo-1-(phenylmethyl)propyl]-1H-indole-2-carboxamide;

DAB, 1,4-dideoxy-1,4-imino-D-arabinitol;

EEG, electroencephalogram;

FBS, fetal bovine serum;

PEG, polyethylene glycol

MEM, minimal essential medium;

MOR-14, N-methyl-1-deoxynojirimycin

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ABSTRACT

Glycogen in brain is localized almost exclusively to astrocytes. The physiological function of this energy store has been difficult to establish because of the difficulty in manipulating brain glycogen concentrations in vivo. Here we used a novel glycogen phosphorylase inhibitor, CP-316,819, that causes glycogen accumulation under normoglycemic conditions but permits glycogen utilization when glucose concentrations are low. Rats treated with CP-316,819 had an 88 ± 3 % increase in brain glycogen content. When subjected to hypoglycemia, these rats maintained brain electrical activity 91 ± 14 minutes longer that rats with normal brain glycogen levels and showed markedly reduced neuronal death. These studies establish a novel approach for manipulating brain glycogen concentration in normal, awake animals and provide in vivo confirmation that astrocyte glycogen supports neuronal function and survival during glucose deprivation. These findings also suggest an approach for forestalling hypoglycemic coma and brain injury in diabetic patients.

Glycogen is the only significant energy store in brain, and it, along with its mobilizing enzyme glycogen phosphorylase, is localized almost exclusively to astrocytes (Phelps, 1972; Koizumi, 1974; Pfeiffer-Guglielmi et al., 2003). Utilization of astrocyte glycogen is accelerated both by neuronal activity (Swanson, 1992; Cruz and Dienel, 2002) and lack of energy substrate (Lewis et al., 1974; Choi et al., 2003). Studies using cell culture and optic nerve preparations demonstrate that elevated astrocyte glycogen can improve neuron survival and axon function during glucose deprivation (Swanson and Choi, 1993; Wender et al., 2000; Brown et al., 2005). In vitro studies have further suggested that lactate or pyruvate derived from astrocyte glycogen can be shuttled to neurons for oxidative metabolism (Dringen et al., 1993; Poitry-Yamate et al., 1995; Pellerin et al., 1998; Brown et al., 2005; Tekkok et al., 2005). Astrocyte glycogen may also serve to fuel energy-demanding functions of astrocytes themselves, such as glutamate uptake, that influence neuronal survival and function (Swanson, 1992; Dienel and Cruz, 2004).

Results of these in vitro studies have been difficult to verify in intact brain, largely because there has not been a useful way of manipulating brain glycogen content that does not affect other relevant aspects of brain metabolism. Astrocyte glycogen content is influenced by several neurotransmitters and neuropeptides, including vasoactive intestinal peptide, insulin, and noradrenalin (Nelson et al., 1968; Cambray-Deakin et al., 1988; Magistretti and Pellerin, 1997); however, these agents have many other actions on both neurons and glia, and most do not readily cross the blood-brain barrier. Brain glycogen can also be increased with barbiturates and other factors that decrease neuronal activity (Nelson et al., 1968; Phelps, 1972; Swanson, 1992), but this approach is similarly unsuitable for selectively targeting glycogen metabolism. Brain glycogen can also be modestly increased with hyperglycemia plus insulin (Nelson et al., 1968),

but it is then difficult to distinguish between the effects of high circulating glucose concentrations and elevated brain glycogen.

In an effort to find a more selective way to increase brain glycogen stores, we used astrocyte cultures to screen several inhibitors of glycogen phosphorylase. One these agents, [R-R*,S*]-5-chloro-N-[2-hydroxy-3-(methoxymethylamino)-3-oxo-1-(phenylmethyl)propyl]-1H-indole-2-carboxamide (CP-316,819), was found to cause astrocyte glycogen accumulation at physiological glucose concentrations, but permit glycogen utilization when glucose levels fall. Using this compound in rats, we found that increased brain glycogen can sustain neuronal activity for long intervals during severe hypoglycemia.

MATERIALS AND METHODS

The studies were performed in accordance with protocols approved by the animal studies committee of the San Francisco Veterans Affairs Medical Center. Reagents were obtained from Sigma-Aldrich , St. Louis, except where noted.

Glycogen phosphorylase inhibitors. [R-R*,S*]-5-chloro-N-[2-hydroxy-3-(methoxymethylamino)-3-oxo-1-(phenylmethyl)propyl]-1H-indole-2-carboxamide (CP-316,819) and 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) were prepared as described (Hoover et al., 1998; Overkleeft et al., 1998). CP-316,819 was purified by chromatography on silica gel to greater than 99% purity, as assessed by HPLC analysis. N-methyl-1-deoxynojirimycin (MOR-14) was a kind gift from H. Fujiwara, Gifu University, Japan. For cell culture studies, all compounds were prepared as 10x concentration stock solutions in water. For in vivo studies, CP-316,819 was dissolved 150 mg / ml or 50 mg / ml in polyethylene glycol (PEG-200), and then diluted 1:5 into physiological saline.

Primary astrocyte cultures. Confluent astrocyte cultures were prepared from forebrain cortices of Swiss-Webster mice as described (Swanson et al., 1989b) and used at 22-25 days in vitro. In brief, forebrain cortices were dissected from anesthetized 1-day-old pups, dissociated by incubation in papain/DNAase, suspended in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS; Hyclone; Ogden, UT) and 2 mM glutamine, and plated in 24 well tissue culture plates at approximately 5 $x10^4$ cells /cm^2. At confluence (12–14 days in vitro), the cells were incubated with 10 μM cytosine arabinoside for 48 hours to prevent the proliferation of other cell types. This medium was replaced with MEM containing 5% FBS and a

total of 5 mM glucose. Medium was exchanged with fresh medium weekly and on the day prior to experiments.

Studies of glycogen content in cultured astrocytes. Incubations with the phosphorylase inhibitors were performed for 24 hours in fresh cell culture medium. Drugs were added in 20 μL volumes of water into 400 μL culture medium, with control wells receiving only the water vehicle. To assess glycogen utilization during substrate deficiency, cultures were placed in glucose-free medium (Earle's balanced salt solution) containing 0.01 μg / ml antimycin A_1 for 60 minutes prior to cell harvest. Glycogen content was measured by the amyloglucosidase method (Swanson et al., 1989b). Duplicate wells from each 24-well plate were combined for each measurement, and 3 measurements from each astrocyte preparation were averaged for each data point ("n").

Rat brain glycogen determination. Male Sprague-Dawley rats (250-300 g) were obtained from Charles River (Wilmington, MA). Non-fasted rats were given intraperitoneal injections of CP-316,819 at an initial dose of 150 mg / kg (5 ml / kg), with subsequent injections of 50 mg / kg (5 ml / kg) 12 and 16 hours later. Controls received 5 ml / kg of vehicle alone at the same dosing schedule. Two hours after the final injection, the rats were used for either brain glycogen determinations or hypoglycemia studies. For brain glycogen determinations the rats were euthanized by high energy (3 kW) focused microwave irradiation (Gerling Applied Engineering, Inc., Modesto, CA) (Swanson et al., 1990). Parietal cortex and hippocampus from both hemispheres was dissected free of underlying white matter and solubilized in 0.1N NaOH / 0.01% SDS for subsequent glycogen determinations by the amyloglucosidase method (Swanson

et al., 1990). In some studies, the rats were decapitated 30 minutes prior to microwave injection in order to evaluate the ability of the increased brain glycogen to be utilized under ischemic, substrate-deficient conditions.

Hypoglycemia and brain histology. Non-fasted male Sprague-Dawley rats (250-300 g) were anesthetized with 1- 3% isoflurane in a 75:25 mixture of nitrous oxide and oxygen (Air Liquide America, Houston, TX) and ventilated with a small animal respirator (Harvard Apparatus, South Natick, MA). A femoral artery line was placed for blood sampling and blood pressure monitoring. Hypoglycemia was induced with an intraperitoneal injection of 30 U / kg of insulin (1 ml / kg) (Suh et al., 2003). Blood glucose was measured with an YSI 2700 glucose analyzer (Yellow Spring, OH) at 30 minute intervals, reduced to 15 minute intervals as blood glucose approached 1 mM. Blood gases were measured at 1-hour intervals using an I-STAT machine (I-STAT, Princeton, NJ), and ventilation was adjusted to keep $PaCO_2$ between 35 - 45 mmHg and PaO_2 above 100 mmHg. Blood pressure and electroencephalogram (EEG) were continuously monitored (BIOPAC Systems, Santa Barbara, CA). For EEG monitoring, two burr holes were made in the skull bilaterally over parietal cortex and two monopolar electrodes were placed beneath the dura. A reference needle was placed in neck muscle. EEG isoelectricity was defined as EEG amplitude less than 25 μV for at least 30 seconds. A heating blanket / rectal probe servo-loop was used to maintain core temperature at 36.5 - 37.5 ^{o}C.

For studies of neuron survival, hypoglycemia was terminated with glucose infusion beginning 2 hours after blood glucose had fallen to 1 mM. Glucose infusion was initiated with an injection of 0.2 ml of 50% glucose via the femoral vein, followed by continuous infusion of 1:1 solution of 50% glucose and Krebs-Henseleit buffer (1.5 ml / hour for 3 hours) and

supplemented by 1 ml of 25% glucose by intraperitoneal injection 1 hour after beginning the intravenous glucose infusions. Rats were returned to their home cages after recovery, and brains were harvested 7 days later. Coronal 20 µm sections were prepared with a cryostat, and the sections were stained with hematoxylin and eosin to quantify neuron death in the designated vulnerable brain regions. Five coronal sections were analyzed from each animal, spaced 80 µm apart and spanning the hippocampus. A blinded observer counted the total number of eosinophilic neurons in each structure of interest, in both hemispheres, under a 20x microscope objective. Data from each animal were expressed as the mean number of degenerating neurons per section in the structure of interest.

Statistical analysis. Data were analyzed using ANOVA with the Student-Newman-Keuls test where multiple groups were compared. All data are expressed as means ± s.e.m.

RESULTS

Glycogen phosphorylase inhibitors affect glycogen stores and mobilization

Inhibitors of glycogenolysis can cause glycogen accumulation, but may also limit the ability of glycogen to be utilized when needed. Thus, as an initial step, primary astrocyte cultures were used to evaluate the effects of four compounds, each of which blocks glycogenolysis by a different mechanism. These compounds, CP-316,819 (Hoover et al., 1998; Martin et al., 1998), N-methyl-1-deoxynojirimycin (MOR-14) (Arai et al., 1998), 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) (Fosgerau et al., 2000), and methionine sulfoximine (MSO) (Sellinger and Weiler, 1963), each produced a dose-dependent increase in astrocyte glycogen content when added for 24 hours to standard culture medium containing 5 mM glucose (Fig. 1A). To determine whether the elevated glycogen stores could then be utilized in the presence of these drugs, glycogen content was also measured after subsequent 60 minute incubation in glucose-free medium that contained 10 µg / ml antimycin A to prevent oxidative metabolism of amino acids present in culture medium. The glycogenolysis normally induced by these substrate-free conditions was inhibited by MOR-14 and DAB, but not by CP-316,819 or MSO (Fig 1B).

MSO was used as a positive control in the cell culture studies because it has previously been shown to increase astrocyte glycogen content through an indirect inhibition of glycogenolysis (Swanson et al., 1989b); however, MSO is suboptimal as an agent for evaluating the effect of increased brain glycogen in vivo because it also causes seizures and inhibits glutamine synthetase (Sellinger and Weiler, 1963). CP-316,819 is an indole carboximide inhibitor of glycogen phosphorylase. In hepatocytes, this inhibitory effect is lost at low glucose concentrations (Hoover et al., 1998; Martin et al., 1998) thereby allowing glycogen utilization

when glucose levels are low. As shown in Fig 1B, the inhibitory effect of CP-316,819 in astrocytes also required the presence of glucose, indicating a similar mode of action on the brain-specific isozyme of glycogen phosphorylase. For these reasons we chose to use CP-316,819 to manipulate brain glycogen in vivo.

Similar to the results obtained in culture, CP-316,819 administered to rats at a total of 250 mg / kg in 3 divided doses produced an 88 ± 3% increase in brain glycogen content (Fig. 2). To confirm that this glycogen store can be utilized in the presence of CP-316,819, complete brain ischemia was induced by decapitation 30 minutes prior to microwave brain fixation. The increased brain glycogen store induced by CP-316,819 pretreatment was almost entirely depleted within the 30 minute ischemic interval (Fig. 2).

Elevated brain glycogen prolongs neuron function and improves survival during hypoglycemia

We next examined the effect of increased glycogen stores on neuronal function during severe insulin-induced hypoglycemia, using the cortical EEG as a global measure of cortical neuronal activity. Severe hypoglycemia causes cessation of cortical neuron activity, as evidenced by an isoelectric ("flat-line") EEG. The onset of EEG isoelectricity occurs simultaneously with depletion of brain carbohydrates and fall in brain energy charge (Lewis et al., 1974). If astrocyte glycogen stores can substitute for circulating glucose as an energy substrate, then elevated brain glycogen stores should prolong the interval to isoelectric EEG during severe hypoglycemia. In the rat hypoglycemia model employed for these studies, EEG isoelectricity reproducibly occurs after an interval of 80 - 100 minutes after the blood glucose

levels fall to 1 mM (Suh et al., 2003). This time interval was more than doubled in the group treated with CP-316,819, indicating a prolonged duration of neuronal activity (Fig. 3A).

In a second group of animals, we examined the effect of increased brain glycogen on neuronal survival after hypoglycemia. Rats were pre-treated with CP-316,819 or with saline vehicle. Hypoglycemia was induced with insulin and reversed with glucose 120 minutes after the blood glucose level had fallen to 1 mM. Most rats in the saline-treated group, but none in the CP-316,819-treated group, exhibited isoelectric EEG intervals periods during this 120-minute interval. Blood glucose levels in the two treatment groups were not significantly different at any time point during the hypoglycemic period (Fig. 3B). Rats in the saline-treated group showed substantial neuron death in each of the 4 vulnerable brain regions examined, whereas the CP-316,819-treated group showed negligible injury (Fig. 4), consistent with earlier studies demonstrating a tight link between duration of EEG isoelectricity and extent of neuron death (Auer et al., 1984). CP-316,819 administered less than 2 hours prior to hypoglycemia had no significant effect on brain glycogen content, and likewise had no significant effect on time to EEG isoelectricity or neuronal survival (not shown).

DISCUSSION

The salient findings of this study are that (1) an indole carboximide inhibitor of glycogen phosphorylase, CP-316,819, can be used to elevate brain glycogen in normal, awake rats; and that (2) rats treated with CP-316,819 exhibit prolonged neuronal activity during hypoglycemia and reduced neuronal death following recovery from hypoglycemia.

Glycogen exists in brain, as in liver, as a highly branched polymer. Brain glycogen is localized almost exclusively to astrocytes, with only negligible amounts found in endothelia, ependymal cells, and certain specialized sensory cells of the brainstem (Phelps, 1972; Koizumi, 1974; Pfeiffer-Guglielmi et al., 2003). There is normally an ongoing utilization and re-synthesis of brain glycogen (Watanabe and Passonneau, 1973), which is thought to provide a rapid coupling or buffering between local energy supply and demand during brain activity (Swanson, 1992; Shulman et al., 2001; Dienel and Cruz, 2004). Breakdown of glycogen is performed by glycogen phosphorylase in conjunction with glycogen debranching enzyme. Inhibitors that bind to the catalytic sites of these enzymes, such as the compounds DAB and MOR-14 used in the present study, cause glycogen accumulation and do not allow glycogen breakdown. A novel feature of CP-316,819 is that it acts at the indole inhibitor site of glycogen utilization in a manner that requires millimolar concentrations of glucose for optimal inhibitory activity (Martin et al., 1998). As a result, glycogen utilization is not inhibited during hypoglycemia. CP-316,819 and related compounds have been extensively evaluated as anti-hyperglycemic agents for type-2 diabetes because of their potential to limit hepatic production of glucose from glycogen without inducing hypoglycemia (Treadway et al., 2001). These studies have shown the drugs to be highly selective for glycogen phosphorylase.

In the present studies, rats treated with CP-316,819 showed an 88 ± 3% increase in brain glycogen, and maintained neuronal activity for more than 90 minutes longer than untreated rats. The absolute increase in brain glycogen content was roughly 3 mM. This amount of glycogen would be expected to fuel brain metabolism for less than 15 minutes if it were the only energy supply available in brain (Sokoloff et al., 1977). However, the flux of glucose from blood to brain is reduced, but not zero, during hypoglycemia, such that glycogen is not the sole energy source. There is, in addition, a compensatory reduction in the rate of glucose utilization and synaptic activity under hypoglycemic conditions (Suda et al., 1990). As a result, brain glycogen is consumed very gradually during hypoglycemia (Ratcheson et al., 1981; Choi et al., 2003). A quantitative analysis suggests that normal levels of astrocyte glycogen should be able to support brain metabolism for about 100 minutes of hypoglycemia (Gruetter, 2003). Our observation that near-doubling of brain glycogen content increases the time to EEG isoelectricity by 90 minutes is in good agreement with this estimate.

The findings of this study also suggest that pharmacological elevation of brain glycogen stores could be useful in forestalling hypoglycemic coma and brain injury in diabetic patients. Indole carboximides similar to CP-316,819 are currently under development as anti-hyperglycemic agents by virtue of their inhibitory effect on liver glycogenolysis (Treadway et al., 2001), although it remains to be established whether brain glycogen stores are affected at the doses currently used to target hepatic glycogen phosphorylase. Whether increased glycogen might similarly provide a buffer against energy failure in cerebral ischemia is less clear. Only glycolytic ATP production can occur under the anoxic conditions of ischemia, and thus much less ATP is generated per glycogen glucose moiety. Consequently, and in contrast to hypoglycemia, brain glycogen is consumed very rapidly (within 4 minutes) during complete

ischemia (Lowry et al., 1964; Swanson et al., 1989a). Moreover, glycogen is metabolized to lactic acid under ischemic conditions, and the resulting acidosis may have deleterious effects that outweigh the beneficial effects of increased energy reserve (Yip et al., 1991).

It remains to be established, at the cellular level, how increased astrocyte glycogen stores preserve neuronal function during hypoglycemia. Several lines of evidence suggest that glycogenolysis in astrocytes can serve to produce and supply neighboring neurons with metabolic intermediates, notably lactate (Dringen et al., 1993; Swanson and Choi, 1993; Poitry-Yamate et al., 1995; Pellerin et al., 1998; Wender et al., 2000; Brown et al., 2005; Tekkok et al., 2005). Moreover, studies in astrocyte cultures suggest that lactate derived from glycogen is preferentially exported from the cells (Sickmann et al., 2005). Together, these observations suggest that lactate generated from astrocyte glycogen can be shuttled to neurons as an energy substrate to supplement the reduced supply of glucose available to brain during hypoglycemia. It is also possible, however, that glycogenolysis in astrocytes serves to fuel astrocyte functions that more indirectly support brain activity (Dienel and Cruz, 2004). One important and energetically demanding astrocyte function is the uptake of neurotransmitter glutamate (Anderson and Swanson, 2000), and failure of glutamate uptake leads to neuronal depolarization, EEG isoelectricity, and neuronal death in hypoglycemia and other conditions. Thus, glycogen support of astrocyte metabolism, neuronal metabolism, or both could be important in prolonging neuronal activity and limiting neuronal death during hypoglycemia. Manipulation of brain glycogen content by the methods presented here may provide an approach for discriminating between these potential effects of astrocyte glycogen on neuronal and astrocyte metabolism in vivo.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Glycogen phosphorylase inhibitors affect glycogen stores and utilization. **A**. Glycogen in astrocyte cultures was significantly increased by 24-hour incubations with each of the inhibitors. Control glycogen content was 29.5 ± 1.4 nmol / mg protein. n = 4 – 8 for each data point. **B**. Inhibitor concentrations were chosen to produce comparable elevations in astrocyte glycogen relative to controls ($p < 0.01$). The cultures were subsequently placed in glucose-free medium to determine whether the elevated glycogen store could be utilized in the presence of the drugs. ** $p<0.01$ vs. the no glucose-free incubation; n = 4 - 6.

Fig. 2. CP-316,819 increases brain glycogen content, but does not prevent glycogen utilization during 30 minutes of complete brain ischemia. ** $p<0.01$; n = 5.

Fig. 3. Treatment with CP-316,819 prolongs neuronal function during hypoglycemia. **A**. Interval between the time that blood glucose fell to 1 mM and the onset of isoelectric EEG in rats treated with CP-316,819 or saline vehicle. Insert shows representative normoglycemic and isoelectric EEG traces. ** $p < 0.01$, n = 7. **B**. Blood glucose levels after insulin administration to rats treated with CP-316,819 or saline.

Fig. 4. Treatment with CP-316,819 improves neuronal survival after hypoglycemia. **A.** Photomicrographs show representative hematoxylin and eosin stained sections from brains harvested 7 days after hypoglycemia. Rats had been treated with either CP316,819 or saline (Cont) prior to hypoglycemia. Sections are from the CA1, subiculum, and dentate gyrus (DG) of the hippocampus, and from piriform cortex (Ctx). Degenerating neurons are shrunken and

eosinophilic. **B.** Quantification of neuron death in these regions. * $p < 0.05$, $n = 6$-8. Scale bar = 50 μm.

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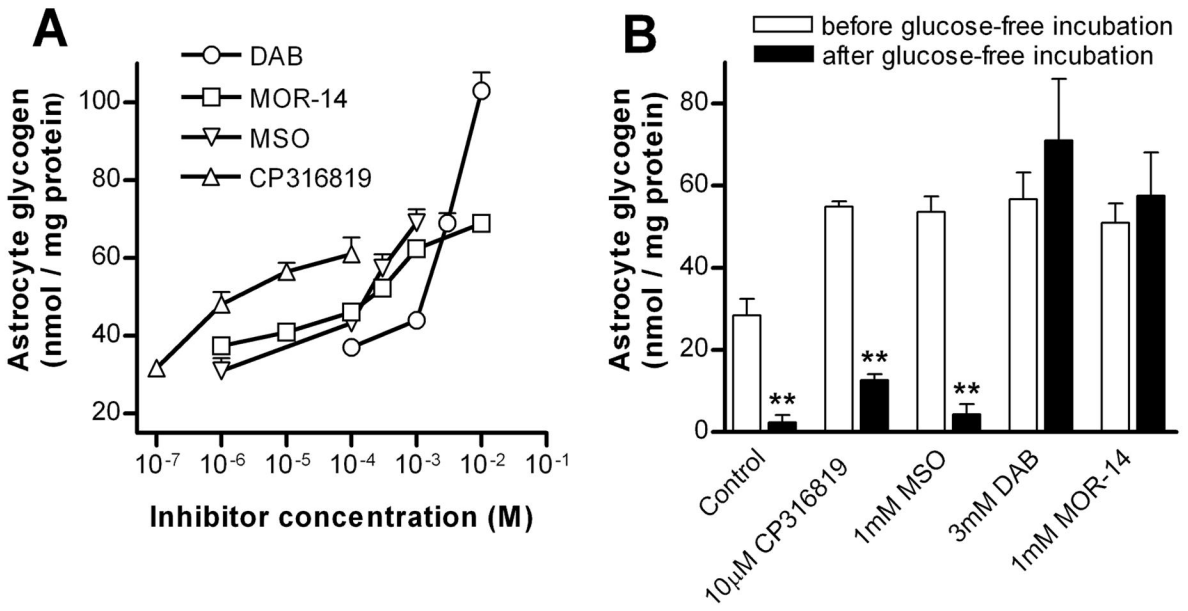

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Fig. 2. Suh et al.

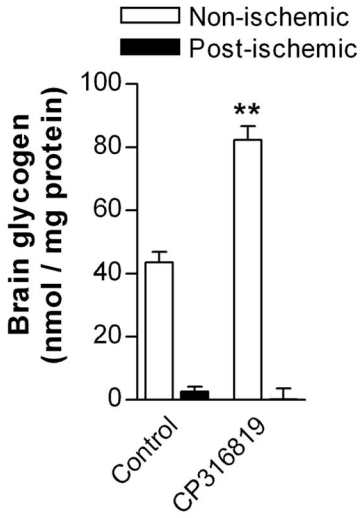

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Fig. 3. Suh et al.

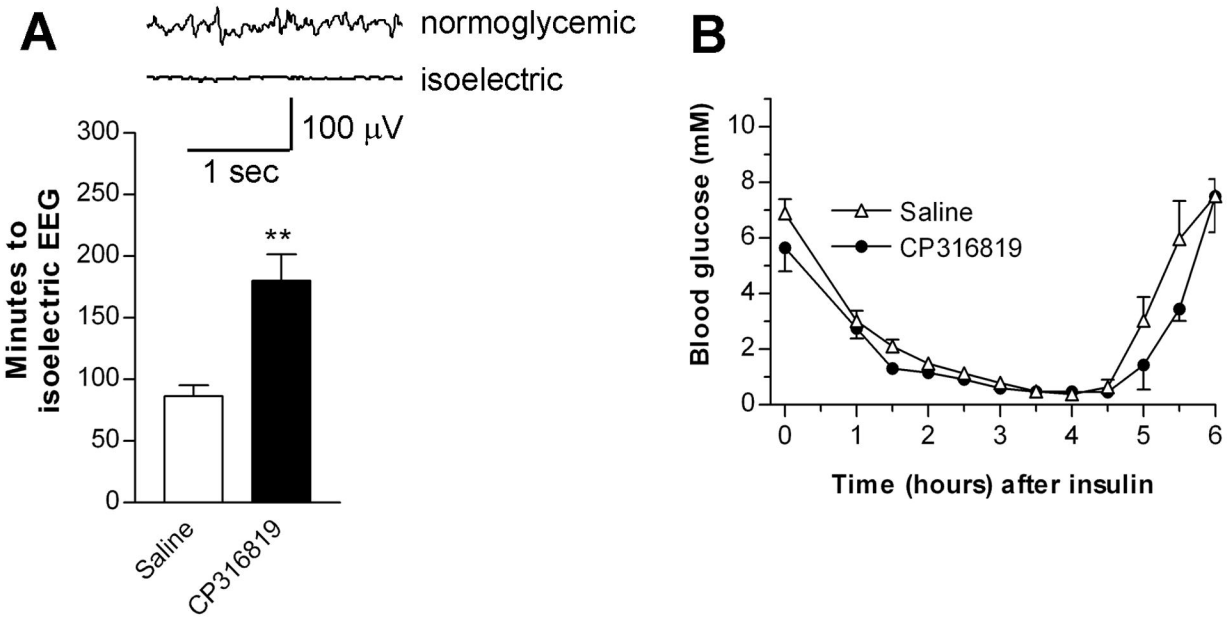

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Fig. 4. Suh et al.