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Hypoxic-Ischemic Brain Injury Exacerbates Neuronal Apoptosis and Precipitates Spontaneous Seizures in Glucose Transporter Isoform 3 Heterozygous Null Mice

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Abstract

We examined the effects of 45 min hypoxia (FiO₂ 0.08, Hx) versus normoxia (FiO₂ 0.21, Nx) on the ipsilateral (Ipsi) and contralateral (Ctrl) sides of the brain in neuronal glucose transporter isoform 3 (Glut3) heterozygous null mice (glut3^{+/-}) and their wild type littermates (WT), undergoing unilateral carotid artery ligation. Glut3^{+/-} mice, under Nx, demonstrated a compensatory increase in blood-brain barrier/glial Glut1 protein concentration, a concomitant increase in neuronal nitric oxide synthase (nNOS) enzyme activity and Bax protein, with a decrease in pro-caspase 3 protein (p<0.05 each). After Hx, re-oxygenation in FiO₂ of 0.21 led to no comparable adaptive up-regulation of the ipsilateral brain Glut3 or Glut1 protein at 4 hr and Glut1 at 24hrs in glut3^{+/-} versus WT. These brain Glut changes in glut3^{+/-} but not WT mice were associated with an increase in pro-apoptotic Bax protein and caspase-3 enzyme activity (p<0.01 each) and a decline in the anti-apoptotic Bcl-2 and procaspase-3 proteins (p<0.05 each). Glut3^{+/-} mice after Hx demonstrated TUNEL positive neurons with nuclear pyknosis in most ipsilateral (hypoxic-ischemia) brain regions. A sub-set (~55%) of glut3^{+/-} mice developed spontaneous seizures after hypoxic-ischemia confirmed by electroencephalography while the WT mice remained seizure-free. Pentylentetrazole testing demonstrated an increased occurrence of longer lasting clinical seizures at a lower threshold in glut3^{+/-} versus WT mice, with no detectable differences in monoamine neurotransmitters. We conclude that hypoxic-ischemic brain injury in glut3^{+/-} mice exacerbates cellular apoptosis, necrosis and precipitates spontaneous seizures.

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Keywords

brain Glut1; brain Glut3; brain mitochondria; brain morphology

Introduction

Glucose, an essential substrate for brain oxidative metabolism is transported across the blood brain barrier into glia and neurons by a family of structurally related membrane spanning glycoproteins called facilitative glucose transporters (glut) (Simpson et al 2007). Of the 14 isoforms cloned to date, multiple isoforms are expressed in brain. While Glut2 (Arluison et al 2004, Young & McKenzie 2004), Glut4 (Sankar et al 2002) and Glut8 (Sankar et al 2002) are noted in specific regions of the brain, Glut1 and Glut3 are ubiquitously expressed in most brain structures (Devaskar et al 1991, Mantych et al 1992). Glut1 is the major isoform that is expressed by the blood-brain barrier and glial cells (Devaskar et al 1991). Disruption of this isoform as noted in the antisense transgenic mouse (Heilig and Saunders et al 2003) or the glut1 haplo-insufficient mouse (Wang et al, 2006) led to structural brain anomalies or electroencephalographic seizures with motor abnormalities respectively. Glut1 deficiency by nature of its strategic location in the blood-brain barrier has a global impact resulting in a small sized brain (Wang et al 2006). This mouse phenotype recreates the autosomal dominant human Glut1 deficiency syndrome where a spastic gait, acquired microcephaly and developmental delays are seen with seizures (DeVivo et al 1991, Klepper et al 1999, Seidner et al 1998).

In contrast to Glut1, Glut3 is more selective being expressed primarily by neuronal cells. Particularly, this isoform is limited to neuronal processes and meets the energy requirements necessary for synaptic neurotransmission (Maher et al 1996). Previously, we observed temporal but no spatial changes during brain development in glut3 expression (Fields et al 1999, Khan et al 1999). Chronic hypoglycemia revealed an increase in neuronal Glut3 protein (Lee et al 2000) and in separate investigations hypoglycemia in rats led to nitric oxide production perhaps mediated by activation of the nitric oxide synthase (NOS) enzyme (Won Suh et al 2008). Thus, neuronal nitric oxide production and neuronal NOS (nNOS) activation serve as surrogate markers for neuronal glucose deficiency (Won Suh et al 2008). Imposition of hypoxic-ischemic brain injury was also associated with an adaptive increase in brain Glut3 expression that maintained the glucose supply and utilization by the brain (Zovein et al 2004). This suggested that Glut3 mediated glucose transport was critical for neuronal glucose mediated energy metabolism (Klepper et al 1999, Zovein et al 2004).

Recently, we created a glut3 null mouse on a C57/BL6 background and observed embryonic lethality in the homozygous mutant (Ganguly et al 2007). However, the heterozygous mutant (glut3^{+/-}) was normal in body and brain size with cognitive abnormalities but demonstrated normal exploratory, sniffing and rearing behaviors and motor ability (Zhao et al 2010). We hypothesized that hypoxic-ischemic brain injury in a glut3^{+/-} mouse would exacerbate brain injury and precipitate an overt clinical phenotype. To test this hypothesis, we subjected glut3^{+/-} and WT mice to unilateral carotid artery ligation with subsequent exposure to hypoxia (FiO₂ = 0.08) or normoxia (FiO₂ = 0.21) for forty-five minutes and

investigated the effects of 4 hr and 24 hr re-oxygenation on brain Glut1 and Glut3 proteins, neuronal nitric oxide synthase protein and enzyme activity, apoptosis and the presenting phenotype. We further characterized this presenting phenotype by pentylene tetrazole (PTZ) testing.

Materials and Methods

Animals

Glut3 heterozygous null mutant C57/BL6 mice ($\text{glut3}^{+/-}$) that lack exons 7 to 10 of the glut3 gene on one allele were employed for these studies. Heterozygous matings generated wild type (WT) and $\text{glut3}^{+/-}$ mice, that were genotyped by Southern blot analysis of EcoR1 restricted tail genomic DNA (Ganguly et al 2007). All mice were allowed ad libitum access to food and water and maintained in a 12-hour light/dark cycle. The care and use of animals were approved by the UCLA Animal Research Committee as per the guidelines established by National Institutes of Health.

Experimental Design

Adult WT ($n=40$) and $\text{glut3}^{+/-}$ ($n=40$) mice of both sexes (age range 2–6 months) were used for the studies. The ipsilateral (Ipsi) and contralateral (Ctrl) cerebral hemi-cortices of WT and $\text{glut3}^{+/-}$ mice were examined separately in the two groups of normoxia (Nx) and hypoxia (Hx) (figure 2A). The adult mouse brain injury model was employed for the experimental design (Levine 1960). All animals underwent permanent cauterization (for the 4-hour re-oxygenation studies) or ligation (for the 24-hour re-oxygenation studies) of the left common carotid artery under inhalational isoflurane anesthesia, with an intervening 1–2 hour recovery period during which they had *ad libitum* access to food and water. The modification to common carotid artery ligation was made after a change in the protocol was instituted by our animal research committee's policy on survival surgery. Animals destined for Hx were placed in a flask maintained at 36°C in a heated water bath and exposed for 45 minutes to fractional inspired oxygen (FiO_2) of 0.08 balanced with nitrogen at a flow rate of 1.2 liters/min that was held steady and monitored by a gas flow meter. Animals assigned to Nx underwent similar treatment except for exposure to FiO_2 of 0.21 at a similar flow rate. The mice recovered in their cages for 4 or 24 hours during re-oxygenation following which they were euthanized to harvest their brains. Whole brains were removed from crania, dissected and weighed. The right (Ctrl side representing Nx or Hx exposure alone) and left (Ipsi side representing ischemia or hypoxic-ischemia exposures) cerebral cortices were separated and rapidly frozen.

Western blot analyses of nNOS, Glut1, Glut3, Bax, Bcl-2 and procaspase-3 protein—Brain samples were homogenized (1:5–7 w/v) at 4°C in 1X Laemmli's buffer (50 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% v/v glycerol) supplemented with 2% β -mercaptoethanol, 5 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ pepstatin A, 2 $\mu\text{g/ml}$ leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 200 μM sodium orthovanadate. Homogenates were centrifuged at 3,000 rpm for 10 minutes and the supernatants sonicated on ice and re-centrifuged at 14,000 rpm for 10 minutes at 4°C. These supernatants were subjected to Western blot analysis. Protein concentrations were determined by the Bio-Rad protein assay

(Bio-Rad Laboratories, Inc., Hercules, CA) (Bradford 1976), using bovine serum albumin (BSA) as standards. Twenty μg of samples were separated by 12.5% SDS-PAGE and the proteins subsequently transferred onto nitrocellulose membranes. Immunoblots containing proteins were blocked in 5% non-fat dry milk at room temperature for 1 hour. Membranes were then incubated with a rabbit anti-mouse Glut1 IgG (1:4000 dilution) (Khan et al 1999, Zovein et al 2004) or anti-mouse Glut3 IgG (1:2000 dilution) (Rajakumar et al 2004, Zovein et al 2004) in PBS supplemented with 0.1% Tween-20 in room temperature for 1 hour. Similarly nNOS, Bax, Bcl-2 and procaspase-3 protein concentrations were assessed by subjecting 40–50 μg of brain samples to 14% SDS-PAGE and transferring the separated proteins to nitrocellulose or polyvinylidene difluoride (PVDF) membranes. After blocking in 5% milk, rabbit anti-nNOS (1:100 dilution) (BD Biosciences, San Jose, CA), anti-Bax (1:500 dilution), anti-Bcl-2 antibody (1:400 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-procaspase-3 antibody (1:1000 dilution) (Cell Signaling Technology, Beverly, MA) incubations were carried out at room temperature for 1 hour. Inter-lane loading was standardized by detecting either vinculin (1:3000 dilution) or β -actin (1:4000 dilution) using monoclonal primary antibodies (Sigma Chemical Co., St. Louis, MO). All immunoblots were treated with donkey horseradish peroxidase-linked anti-rabbit or anti-mouse secondary antibody and the antigen-antibody complexes detected using the ECL or ECL Plus detection systems (Amersham Pharmacia Biotech, Piscataway, NJ). The protein bands were semi-quantified by densitometry and expressed as a ratio to the internal control (Zovein et al 2004).

nNOS enzyme activity at 4 and 24 hours of re-oxygenation—Brain homogenates were prepared in 50 mM triethanolamine-HCl (pH 7.4) containing 0.1 mM EGTA, 0.1 mM EDTA, 0.5 mM DTT, 1 mM pepstatin A and 2 mM leupeptin at 4°C. The homogenates were next centrifuged at 20,000g for 60 min at 4°C and the supernatants were assayed for [^3H]-L-arginine to [^3H]-citrulline conversion and calcium-dependent NOS (reflects nNOS) activity was measured as described previously (Singh et al 1997, Singh et al 2000) using the NOSdetect™ assay kit (Stratagene, LaJolla, CA).

Caspase-3 Enzyme Activity at 24 hours of re-oxygenation—Brains were homogenized in 1:2 w/v chilled lysis buffer and incubated on ice for 15 minutes. Samples were centrifuged at 3,000 rpm in 4°C for 20 minutes and the supernatants were centrifuged at 14,000 rpm at 4°C for 15 minutes and at 19,000 rpm at 4°C for 15 minutes. Fifty μl of brain homogenate extracts were added to 50 μl of 2X reaction buffer and 5 μl of caspase-3 specific conjugated substrate (DEVD-pNA) and incubated for 1 hour 15 minutes in a 37°C water bath. Negative control reactions consisted of omission of the conjugated substrate. Subsequently the chromophore p-nitroaniline (pNA) was detected spectrophotometrically (405 nm) after its cleavage by endogenous caspase-3 from the labeled caspase-specific substrate, DEVD-pNA (ApoAlert Caspase Colorimetric Assay Kit; BD Biosciences Clontech, Palo Alto, CA). Caspase-3 enzyme activity was measured (Zovein et al 2004) using a standard curve established with free pNA and calculated using the optical density units (ODU) as follows:

$$\text{Caspase activity (nmol pNA/mg/hr)} = \Delta\text{ODU} \times 1/\text{curve slope/mg protein}/1.25\text{hr (curve slope} = \Delta\text{ODU}/\Delta\text{nmol pNA)}.$$

Immunohistochemical Analyses

Brain Sectioning—Mice (n=6 per genotype) were anesthetized with intraperitoneal pentobarbital (75 µg/gm body weight). Following thoracotomy, the brains were perfused via the left ventricle and the infusate drained via the snipped right atrium. Using a rate-regulated pump, each mouse was perfused with 25–30 ml of 4% paraformaldehyde (PFA) (pH 7.4) at room temperature (RT) for 10 minutes. Whole brains including the cerebella were removed from the cranium, placed in fresh 4% PFA for one hour, and then subsequently fixed overnight in fresh 4% PFA at 4°C. Fixed brains were washed three times in ice-cold 1X PBS for 20 minutes each. Subsequently, the fixed brains were stored in ice-cold 1X PBS supplemented with 0.1% sodium azide until embedded in 2% agar (Bacto-Agar from Difco Laboratories, Detroit, MI). Coronal sections (30 µm) spanning from olfactory bulbs to the superior colliculi were obtained using a Vibratome (Leica VT 1000S, Leica Microsystems Inc., Deerfield, IL). Free floating sections were carefully collected marking the right and left halves of brains (as each half represents the Ipsi and Ctrl sides of the brain).

Glut3 and Glut1 immunofluorescence under normoxia—Brain sections were rinsed in 1X PBS and incubated in fresh 1% sodium borohydride (Sigma, St. Louis, MO) at RT for 10 minutes. Sections were subsequently incubated successively in 0.2% Triton X-100 (Shelton Scientific, Shelton, CT) at RT for 1 hour, 50 mM glycine (Fisher Scientific, Fair Lawn, NJ) at RT for 15 minutes, and 50 mM ammonium chloride (Sigma, St. Louis, MO) at RT for 30 minutes with 1X PBS rinses in between each incubation. Non-specific binding sites were blocked with 0.2% Triton X-100 in 3% normal donkey serum and 1% gelatin at RT for 2 hours. Sections were then incubated with a rabbit anti-mouse Glut3 IgG (1:1000 dilution) or for double staining with a mixture of rabbit anti-Glut3 IgG and guinea pig anti-Glut1 IgG (1:500 dilution) (gift from Dr. Takata, Gunma University Medical School, Japan) (Ganguly et al 2007, Rajakumar et al 2004, Zovein et al 2004) at RT for 1 hour followed by overnight incubation at 4°C. Negative control consisted of absent primary antibody. Sections were washed thoroughly with ice-cold 1X PBS for 30 minutes and then incubated with a mixture of 1:500 dilution of FITC-conjugated donkey anti-guinea pig, Texas-red-conjugated donkey anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA) and 1:1000 DAPI antibody (Sigma, St. Louis, MO) at RT for 1 hour. Following thorough washing with ice-cold 1X PBS for 30 minutes, the brain sections were then mounted on charged slides (ProbeOn Plus, Fisher Scientific, Fair Lawn, NJ) using an anti-bleaching mounting medium and examined under a Nikon E-600 fluorescence microscope (Nikon, Melville, NY) (Shin et al 2004) equipped with a cooled charged-coupled device (CCD) camera (Ganguly et al 2007).

nNOS immunohistochemistry at 4 hours of re-oxygenation—Brain sections were rinsed in 1X PBS to remove sodium azide and incubated in fresh 1% sodium borohydride (Sigma, St. Louis, MO) at room temperature (RT) for 30 minutes. Sections were then incubated with 0.5% hydrogen peroxide (Sigma, St. Louis, MO) at RT for 15 minutes,

followed by 50 mM glycine (Fisher Scientific, Fair Lawn, NJ) at RT for 30 minutes with 1X PBS rinse following all incubations. Non-specific binding sites were blocked with 0.2% Triton X-100 in 3% normal goat serum at RT for an hour. Sections were incubated with a polyclonal rabbit anti-nNOS at 1:100 dilution (BD Biosciences, San Jose, CA) at RT for 1 hour followed by overnight incubation at 4°C. Negative control consisted of absent primary antibody. Sections were washed thoroughly with 1X PBS at RT for 30 minutes and then incubated with a secondary biotinylated goat anti-rabbit antibody at 1:500 dilution in RT for 1 hour. Immunodetection was visualized by incubating sections with ABC reagent (Vectastain ABC System, Vector Laboratories, Burlingame, CA) in RT, followed by diaminobenzidine tetrahydrochloride (DAB) reagent including hydrogen peroxide until optimal staining was detected by light microscopy. Positive nNOS staining was stereologically quantified in the cortex, striatum, amygdala, thalamus, hypothalamus, and hippocampus of WT and glut3^{+/-} animals using Metamorph Imaging series software (Downington, PA) (Sankar et al 2002).

Morphology - Hematoxylin and Eosin (H & E) Staining after 24 hours of re-oxygenation—Perfused and fixed brains were dehydrated in graded ethanol solutions, cleared with xylene, and embedded in paraffin. Brain sections (8 µm) were deparaffinized, rehydrated, and stained with hematoxylin and eosin (all reagents from Fisher Scientific, Santa Clara, CA) prior to examination under light microscopy or under fluorescent light using a fluorescein filter to ascertain injured neurons based on increased eosinophilic staining as previously described (Sankar et al 1997).

TUNEL staining after 24 hours of re-oxygenation—TUNEL labeling was performed using a commercially available kit (NeuroTACSII, Trevigen, Gaithersburg, MD). Brain sections on charged slides were first rehydrated, followed by 35-minute incubation with proteinase K (1:100 dilution) at 37°C. Endogenous peroxidase activity was quenched for 5 min with a solution of 30% hydrogen peroxide in methanol. TUNEL reaction was carried out at 37°C in a humidity chamber for 90 minutes using terminal deoxynucleotidyl transferase to incorporate biotinylated nucleotides at sites of DNA breaks characterizing apoptosis. The reaction product was stained and visualized with streptavidin-HRP according to manufacturer's protocol. Detection of DNA nicks was achieved by nickel enhancement of the DAB reaction. The brain sections were sequentially dehydrated, coverslipped, and examined under light microscopy (Zovein et al, 2004).

Continuous video electroencephalogram (EEG) telemetry—Electrodes were implanted into mice 48 hours prior to the introduction of experimental treatment to document baseline electrical activity. Continuous video EEG telemetry monitoring was performed over one week post-treatment. Under isoflurane anesthesia, the scalp was reflected and two burr holes were made on each side of the frontoparietal cranium. The telemetry device (Data Sciences International, Arden Hills, MN) in connection with the electrodes was run subcutaneously along the back of mice. Recording began when mice were placed back in cages and onto a telemetry-recording platform. The Stellate software (Montreal, Quebec, Canada) was used to record and analyze video EEG recordings. Analysis was done manually offline based on amplitude, frequency, and time with

electrographical evidence of seizures to confirm observed clinical seizure activity after hypoxic-ischemic injury (Mazarati et al 2002).

Seizure Threshold—To determine the seizure threshold in WT and glut3^{+/-} mice, pentylenetetrazole (PTZ) testing was performed in 6 to 10 month old mice (body weight ~35g) as previously described (Krall et al 1978). The mice received intraperitoneal PTZ (25, 40 or 60 mg/kg) in incremental order every week allowing for an adequate inter-dosing “wash out” period. Animals were closely observed for 30 min after each dosing and seizures were monitored for latency, duration and assigned a seizure score that was previously developed (Racine et al 1972). Mice that demonstrated seizures lasting 30 min after a PTZ dosing or did not survive the test were coded together.

High Performance Liquid Chromatography (HPLC) for Brain Monoamines—HPLC for monoamines were performed using a modified method as previously described (Haidkind et al 2004, Mazarati et al 2005). The hippocampus, caudate and brainstem were dissected and independently homogenized by an ultrasonic dismembrator (Fisher Scientific, Pittsburgh, PA). Homogenized brain samples (0.09 mol/l perchloric acid; 0.04 mmol/l EDTA and 5 mmol/l sodium bisulfite; 1 ml/100 mg of wet tissue) were centrifuged at 17,000g at 4°C. HPLC was performed using the L-ECD-6A electrochemical detector (Shimadzu, Kyoto, Japan) and an HR-80 column (ESA Biosciences, Chelmsford, MA, USA). Separation step used CAT-APhase II mobile phase (ESA Biosciences). The measurements were done at an electrode potential of +0.7 V. The data was analyzed by the EZ Start software (Shimadzu); where the areas under the peaks were calculated and plotted against areas of peaks generated by known standards. Neurotransmitter concentrations were expressed as pmoles per mg of wet tissue.

Data Analyses

Data are expressed as mean±SEM. Once normality of distribution was established experimental data were analyzed by two-way ANOVA to determine the effect of genotype (glut3^{+/-} versus WT) alone, treatment-exposures (Nx-Ctrl, Nx-Ipsi, Hx-Ctrl, Hx-Ipsi) alone and genotype X treatment-exposure interaction at differing time points (4hr and 24 hr). This was followed by the post-hoc Holm-Sidak test to validate inter-group differences. In specific instances where a genotype X treatment-exposure interaction did not exist, one-way ANOVA was employed within each genotype with the post-hoc Fisher's PLSD test to validate inter-treatment group differences. Seizure occurrence for PTZ testing was evaluated by the Chi square analysis. Median seizure scores were compared by the Wilcoxon rank sum test. The duration and latency of seizures were compared with General estimating equation for multiple testing (GEE) with correction for the PTZ-dose. Neurotransmitter concentrations between the two genotypes were compared by Student's t-test. Statistical significance was assigned at a p<0.05.

Results

Baseline Immunolocalization Investigations

Spatial distribution of Glut3 and Glut1 was similar in WT and glut3^{+/-} brains, with Glut1 noted in endothelial cells of the microvasculature (figure 1A&B) and Glut3 immunolocalization observed mainly in neuronal processes in most structures as previously described (Devaskar et al 1991, Ganguly et al 2007, Mantych et al 1992, Zhao et al 2010), and as presently shown in the sub-fornicular organ (figure 1C), the hypothalamus (figure 1D&E) and the periventricular region (figure 1F). In glut3^{+/-} mice, while similar or increased concentrations of Glut1 immunoreactivity were noted to that seen in WT mice (figure 1I), relatively diminished amounts of Glut3 were observed (figure 1G&H).

Hypoxic-ischemia investigations

The experimental design is presented in figure 2A. Our experimental design includes two mouse genotypes (WT and glut3^{+/-}) that were subjected to unilateral (left) carotid artery ligation, followed by exposure to either Normoxia (Nx, FiO₂ = 0.21) or hypoxia (Hx, FiO₂ = 0.08) for 45 min after a period (1–2 hr) of recovery. The contralateral (Ctrl – represents Nx or Hx alone) and ipsilateral (Ipsi – represents ischemia or hypoxic-ischemia) sides of brains were examined separately after either 4 hr or 24 hr of reoxygenation at FiO₂ of 0.21.

nNOS

4 hours of re-oxygenation—Glut3^{+/-} mice demonstrated no differences in neuronal nitric oxide synthase (nNOS) immunoreactivity in all brain structures examined when compared to WT (figure 2B–E) under the two experimental conditions (figure 2A). In keeping with these findings, no differences in neuronal nitric oxide synthase (nNOS) protein concentrations were noted between the two genotypes (figure 2F) subjected to the two different experimental conditions on both the ipsilateral (Ipsi) and contralateral (Ctrl) sides of the brain. In contrast, increased calcium-dependent nNOS enzyme activity (post-translational nNOS) was evident under Nx in Ctrl (one-way ANOVA, Fisher's PLSD $p < 0.05$) and Ipsi (two-way ANOVA, Holm-Sidak $p = 0.007$) sides of the brain in glut3^{+/-} mouse brains when compared to WT with no inter-genotype differences observed under Hx in the Ctrl and Ipsi sides of the brain (figure 2F). No effect of the treatment condition or exposures on nNOS protein or enzyme activity was observed in either genotype.

24 hours of reoxygenation—In WT mice, while the Ctrl brain side exposed to Hx revealed an increase in nNOS protein concentration, no effect of hypoxia was evident either in the Ctrl or Ipsi brain sides in glut3^{+/-} mice when compared to respective WT littermates (Fisher's PLSD $p < 0.03$), or the Nx-Ctrl in WT mice (figure 2G). In keeping with this observation, nNOS enzyme activity was no different between genotypes, under Hx versus Nx conditions in both Ctrl and Ipsi sides of the brain (figure 2G). This finding was unlike that of Nx-Ctrl and Nx-Ipsi observed in glut3^{+/-} mice at 4 hrs post-reoxygenation (figure 2F).

Glut proteins and mitochondrial apoptotic markers

4 hours of reoxygenation—Glut3^{+/-} mice expressed a ~50% decrease in Glut3 protein (Holm Sidak $p<0.001$) with a concomitant compensatory increase in Glut1 protein (Fisher's PLSD $p<0.05$) under baseline Nx condition (figure 3A). During 4 hr of re-oxygenation, in comparison to the Ctrl side under Nx condition, while WT mice demonstrated no change on the Ipsi side, an acute response to Hx on both the Ctrl and Ipsi (hypoxic-ischemia [HI]) sides of the brain revealed an increase in brain Glut3 (Holm Sidak $p<0.001$) and Glut1 protein concentrations (Fisher's PLSD $p<0.05$). In contrast, glut3^{+/-} mice failed to mount a similar acute response of brain Glut3 or Glut1 with Hx on both the Ctrl and Ipsi sides of the brain. Associated with these observed brain Glut protein changes in WT mice, the pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins remained unchanged (Figure 3B). In contrast, glut3^{+/-} mice revealed increased Bax with Hx on the Ipsi (Holm Sidak $p<0.01$) and Ctrl (Holm Sidak $p<0.01$) brain sides and decreased Bcl-2 in the Ipsi brain side alone under Hx (Fisher's PLSD $p<0.05$) (figure 3B).

24 hours of re-oxygenation—In WT mice, with 24 hours of re-oxygenation (Figure 3C), while a decrease in Glut3 protein concentration under Hx in both the Ipsi and Ctrl brain sides (Holm Sidak $p=0.005$) was observed, an associated elevation in brain Glut1 protein concentration under Hx on the Ipsi side (Holm Sidak $p=0.007$) versus Nx on both the Ipsi and Ctrl sides was detected. Glut3^{+/-} mice that started with a baseline reduction in brain Glut3 and elevated Glut1 protein concentration under Nx on the Ctrl side (Holm Sidak $p<0.01$) also revealed no change in either Glut3 or Glut1 protein concentrations on the Ipsi side and under Hx on both sides. In the 24 hr phase of reoxygenation (figure 3D), the WT mice expressed increased Bax under Hx on the Ipsi brain side (Holm Sidak $p<0.006$) with unchanged Bcl-2 concentrations. Glut3^{+/-} mice on the other hand demonstrated decreased Bax with Hx on both the Ipsi and Ctrl brain sides (Holm Sidak $p<0.001$) and decreased Bcl-2 proteins with Hx on the Ipsi brain side (Holm Sidak $p<0.001$) alone. In examining the pro-effector molecule of apoptosis, procaspase-3 (figure 3E), WT mice showed a tendency to decrease but was not significantly different under Hx on both sides versus Nx. Glut3^{+/-} mice, on the other hand, showed exaggerated decreases in procaspase-3 under Nx on the Ipsi brain side, and Hx-Ctrl side (Holm Sidak $p<0.001$), which diminished further on the Hx-Ipsi side (Holm Sidak $p=0.003$). WT mice showed a reciprocal increase in caspase-3 activity with Hx on the Ipsi side of the brain (Holm Sidak $p<0.001$, figure 3F), whereas glut3^{+/-} mice expressed increasing capase-3 activity with Hx (Holm Sidak $p=0.007$) on both the Ctrl and Ipsi sides of the brain (Holm Sidak $p<0.001$).

The morphological integrity of neurons in the hippocampi was preserved in WT mice under Nx on the Ipsi side and those subjected to Hx on both the Ctrl and Ipsi sides of the brain (figure 4A–D). In contrast, glut3^{+/-} mice (figure 4E–H) subjected to Hx, demonstrated extensive pyknotic neuronal nuclei in the hippocampal CA1 region, consistent with neuronal death on the Ipsi side of the brain (figure 4H). Under fluorescence microscopy, the enhanced eosin uptake by these necrotic neurons was seen as green autofluorescence along with irregularly shaped cell bodies (figure 4H), confirming neuronal injury. A similar phenomenon on the Ipsi side of the brain was observed in glut3^{+/-} cortices subsequent to Hx (not shown). TUNEL staining as shown in the motor cortex-cingulum region of the Ipsi

brain in WT mice (figure 5A–D) subjected to Hx resulted in TUNEL positive cells (figure 5D). Glut3^{+/-} mice demonstrated a progressive increase in TUNEL positive cells on the Ipsi side under Nx, Ctrl side under Hx increasing further on the Ipsi side under Hx (figure 5E–H) and superseding that of the WT counterpart. The glut3^{+/-} mouse hippocampus and hypothalamus also revealed similar TUNEL positive cells (not shown).

Phenotypic Expression

Spontaneous and EEG seizures—Glut3^{+/-} mice unlike the WT controls developed clinically evident spontaneous seizure activity subsequent to hypoxic-ischemia and 24 hours of reoxygenation (supplemental materials – video tape recording). This prompted continuous video electroencephalographic telemetry to confirm the observed clinical seizure activity in a sub-set of mice (~30%). The total number of glut3^{+/-} mice that demonstrated clinical seizures only after Hx and unilateral carotid artery ligation were 6 from a total of 11 (6/11; ~55%) (figure 6A&B). None of the glut3^{+/-} mice subjected to Nx had clinical or EEG evidence of seizures (0/11). Similarly, the WT mice demonstrated no clinical or EEG evidence of seizures when subjected to Hx and unilateral carotid artery ligation (0/10) (figure 6C) however 1/11 WT mice subjected to Nx demonstrated EEG evidence of seizures throughout, i.e. before and after the carotid artery ligation which was not accompanied by clinical seizures but rather normal exploratory activity. The rest of these WT mice (10/11; ~91%) were completely seizure free.

PTZ testing—During PTZ testing glut3^{+/-} mice at baseline were more likely to have spontaneous seizures, had longer lasting seizures (glut3^{+/-} = 1339s versus WT = 669s; p=0.0087) and presented with higher seizure scores (glut3^{+/-} = 4 versus WT = 3; p=0.04) (figure 7A–C). Glut3^{+/-} mice seized in 50% while WT mice seized in 27% of the total experimental runs (p=0.014). A shorter latency from PTZ dosing to onset of seizures was observed in glut3^{+/-} versus WT mice (glut3^{+/-} = 336s versus WT = 763s; p=0.0085) (figure 7D).

Neurotransmitter concentrations—No differences in neurotransmitter concentrations were observed between the glut3^{+/-} (n=3) and WT (n=3) mice at baseline. In the hippocampus, norepinephrine (glut3^{+/-} = 0.107±0.027 pmol/μg versus WT = 0.099±0.026; p=0.84), dopamine (glut3^{+/-} = 0.003±0.013 versus WT = 0±0.01 pmol/μg; p=0.64) and serotonin (glut3^{+/-} = 0.21±0.072 versus 0.17±0.061 pmol/μg; p=0.69) concentrations did not differ between the two genotypes. In the caudate, norepinephrine (glut3^{+/-} = 0.22±0.069 versus WT = 0.16±0.059 pmol/μg; p=0.55), dopamine (glut3^{+/-} = 0.826±0.634 versus WT = 0.677±0.315 pmol/μg; p=0.82) and serotonin (glut3^{+/-} = 0.399±0.122 pmol/μg versus WT = 0.241±0.098 pmol/μg; p=0.36) concentrations are also no different. Further, even in the brain stem, norepinephrine (glut3^{+/-} = 0.22±0.069 versus WT = 0.183±0.043 pmol/μg; p=0.93), dopamine (glut3^{+/-} = 0.007±0.014 versus WT = 0.001 ± 0.014 pmol/μg; p=0.78) and serotonin (glut3^{+/-} = 0.245±0.082 versus WT = 0.21±0.065 pmol/μg; p=0.75) concentrations were no different among the two genotypes.

Discussion

This is the first report of $\text{glut3}^{+/-}$ mice demonstrating overt spontaneous seizures when subjected to the stress of hypoxic-ischemia. Our observations support a critical role for Glut3 in maintaining neuronal well being in the adult mouse. Glut3 haplo-insufficiency resulted in a compensatory increase in blood-brain barrier/glia Glut1 expression. However, this compensation failed to prevent the resultant increase in nNOS enzyme activity reflective of neuronal glucose deficiency (Won Suh et al 2008), pro-apoptotic protein Bax and decrease in pro-caspase-3 proteins. These baseline changes noted under normoxic conditions were not associated with a visible neurological (seizure) phenotype. Along these lines, there were no baseline differences in hippocampal, caudate or brain stem norepinephrine, dopamine or serotonin neurotransmitter concentrations. While hypoxia (and ischemia) alone led to limited brain cellular changes, hypoxic-ischemia exacerbated brain cell injury. This was related to the absence of the protective Glut3 protein increase that is normally encountered in WT mice subjected to hypoxic-ischemia. Instead in $\text{glut3}^{+/-}$ mice, the absence of an acute increase in neuronal glut3 protein concentration exacerbated neuronal apoptosis and necrosis.

These brain cellular perturbations reflect inadequate fuel supply to meet the needs of neuronal energy metabolism. Since *in-vivo* assessment of neuronal glucose supply separate from the other brain cell types is difficult and *in-vitro* assessment in cultured neurons does not accurately reflect *in-vivo* neuronal events, nNOS activity proved to be an important marker of neuronal glucose deficiency, increasing only acutely at 4 hr after reoxygenation in $\text{glut3}^{+/-}$ versus WT mice under Nx (and ischemia). Superimposition of Hx (and hypoxic-ischemia) however did not further exacerbate the nNOS activity in either the $\text{glut3}^{+/-}$ or WT mice. Similar changes under Nx (and ischemia) in nNOS enzyme activity were absent at 24 hrs in $\text{glut3}^{+/-}$ mice when compared to the respective WT. Superimposition of Hx (and hypoxic-ischemia) did not make any further difference. Thus, unlike neuronal glucose deficiency, hypoxia did not increase nNOS activity.

This inability to meet the glucose needs of neurons compounded with hypoxic-ischemia led to precipitation of overt spontaneous seizures in $\text{glut3}^{+/-}$ mice. However, not all mice subjected to hypoxic-ischemia developed overt seizures on clinical observation. Of the subset that was monitored by video telemetry, all of them demonstrated EEG evidence of seizures regardless of whether clinical seizures occurred. In contrast, none of the WT mice subjected to hypoxic-ischemia experienced overt or EEG seizures. Only one WT mouse exposed to Nx/ischemia demonstrated EEG seizures even before this ischemic exposure, stressing the importance of including strain-specific controls in experiments to establish the incidence of EEG seizures associated with normal behavior within the general population of C57/BL6 mice (D'Ambrosio et al 2005). However, when determining the clinical seizure threshold with dose-responsive PTZ testing under normoxic conditions, the $\text{glut3}^{+/-}$ mice demonstrated a lower threshold seen as a higher proportion of mice with seizures, a higher median seizure score, a longer duration of seizures with a shorter latency period. This observation further supports the precipitation of clinical seizures in $\text{glut3}^{+/-}$ mice under stressful conditions that uncover the existing imbalance between neuronal energy (glucose-dependent) supply and demand.

Our findings are dissimilar to the previously reported Glut1 antisense transgenic, Glut1 haplo-insufficient (Heilig and Saunders et al 2003) or Glut8 null mutant (Membrez et al 2006, Schmidt and Gawlik et al 2008) mice. This may be related to differing functions of each isoform in brain. Glut1 mediates transport across the blood-brain barrier and into glial cells. Thus more than 50% deficiency as in the transgenic mice led to brain structural abnormalities (Heilig and Saunders et al 2003) which were also seen in the zebra fish (*Danio Rerio*) model (Jensen et al 2006). In contrast, the glut1 haplo-insufficient mice demonstrated smaller brains and electroencephalographic seizures without any provocation (Wang et al 2006) along with motor abnormalities. These characteristics mimic the presenting features of Glut1 deficiency syndrome in human infants (DeVivo et al 1991, Klepper et al 1999, Seidner et al 1998). In contrast, Glut8 null mutant mice demonstrate hyperactivity and features consistent with an anxiety disorder displayed as reduced risk assessment, increased emotional reactivity to a novel environment and enhanced frequency of defecation (Schmidt and Gawlik et al 2008). This may be related to the intracellular location of this isoform in neurons of specific brain structures (Shin et al 2004), namely the hippocampus and substantia nigra (Sankar et al 2002). Whether intracellular neuronal glut8 functionally counteracts the role of plasma membrane glut3 noted in the neuropil (Piroli et al 2004, Reagan et al 2002) remains to be investigated. Glut3^{+/-} mice on the other hand, grossly appear normal (Zhao et al 2010, Schmidt and Richter et al 2008) but when subjected to neurobehavioral testing reveal cognitive deficiencies and electroencephalographically evident seizures not detected clinically (Zhao et al 2010). However, upon provocation by hypoxic-ischemia or PTZ testing develop clinically evident seizures hence precipitating a presenting phenotype. Thus these mice in the presence of adequate oxygen supply overcome any limitations by compensatory mechanisms which primarily include brain Glut1 protein. However, in the presence of hypoxia and hypoxic-ischemia or PTZ, neuronal glut3 deficiency precipitates a clinical phenotype. Whether the associated brain cellular injury is a cause or effect of seizures remains to be determined.

In other cell types, namely the pre-implantation embryo, it has been demonstrated that both glut1 and glut3 are crucial for mediating adequate glucose supply (Moley, Chi and Mueckler 1998, Pantaleon et al 1997). In the presence of diminished glut1 and glut3 as encountered in embryos of a diabetic mother, there is enhanced apoptosis which may underlie congenital malformations (Heilig and Saunders et al 2003, Moley, Chi, Knudson et al 1998, Moley, Chi and Mueckler 1998). Glut1^{+/-} embryonic stem cells when subjected to hypoxia demonstrated apoptosis (Heilig and Brosius et al 2003). In contrast, glut3 absence as in the glut3^{-/-} embryos led to exacerbation of apoptosis in the inner cell mass which ultimately resulted in embryonic demise (Ganguly et al 2007). However, our present study is the first to look at the effect of glut3 deficiency on brain cellular apoptosis. As in pre-implantation embryos, brain cells too demonstrate increased cellular apoptosis and necrosis. These cellular changes are a result of reperfusion after oxygen deprivation that is superimposed on existing neuronal glucose deficiency reflected by the acutely increased nNOS activity (Won Suh et al 2008) evident in normoxic and ischemic (which also served as a surgical sham control) glut3^{+/-} mouse brains. Despite enhanced cellular apoptosis and necrosis, gross brain structure was preserved with no major congenital malformations observed in normoxic or hypoxic-ischemic glut3^{+/-} mice. Since, the effect of complete glut3 absence on brain

development could not be examined in the face of early embryonic lethality (Ganguly et al 2007), we cannot predict the impact of such a condition on development of brain structural anomalies due to generalized neuronal apoptosis and necrosis. Instead in the $\text{glut3}^{+/-}$ mouse, we observed functional changes in the form of seizures when stressed with hypoxic-ischemia.

While there continues a controversy regarding the role of alternate fuels in protecting neuronal energy metabolism, our present observations clearly support a crucial role for Glut3 mediated glucose supply to neurons. We have not particularly examined brain metabolism in this study, however Pellerin and Magistretti (Pellerin & Magistretti 2004) have offered the astrocyte-neuron shuttle hypothesis suggesting that lactate released from the astrocyte in *in-vitro experiments* can exclusively fuel neuronal oxidative metabolism in the complete absence of glucose (Pellerin 2005). In contradiction, Simpson et al based on mathematical modeling refuted this hypothesis, stating that glucose was critically important for neuronal energy metabolism and lactate could not adequately substitute glucose (Simpson et al 2007). The creation of the $\text{glut3}^{+/-}$ mouse and previous demonstration of enhanced brain lactate uptake from the blood may suggest some role for lactate in neuronal energy metabolism (Zhao et al 2010). However, our present investigation supports a need for glucose to fuel neurons and protect these cells against energy failure as evidenced by clinical seizures, neuronal apoptosis and necrosis. If lactate released from astrocytes could adequately overcome the relative lack of glucose as a fuel for neuronal activity, the $\text{glut3}^{+/-}$ mice may have remained asymptomatic in response to hypoxic-ischemia. However, previous *in-vitro* investigations in $\text{glut3}^{+/-}$ astrocytes failed to reveal enhanced lactate release by these cells (Schmidt and Richter et al 2008).

In summary, hypoxic-ischemic brain injury superimposed on glut3 deficiency exacerbates brain cellular apoptosis/necrosis and precipitates overt clinically apparent seizures, thereby unmasking an acute neurological (seizure) phenotype. These novel observations provide support to the role glucose deficiency may play in inducing seizures in a sub-set of hypoxic-ischemic injuries. More importantly when clinical seizures are encountered with hypoxic-ischemia, it becomes imperative to stay vigilant against associated glucose metabolic derangements in the form of hypoglycemia and/or neuroglycopenia. Future studies in the human may uncover a Glut3 related genetic basis for certain seizure disorders.

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Figure 1. Brain Glut1 and Glut3 distribution

(WT = A–G, I panels and $glut3^{+/-}$ = H panel) Coronal brain sections demonstrate Glut1 antibody negative control (A) with only DAPI nuclear stain seen, Glut1 immunoreactivity seen as green FITC stain in the endothelium (arrowheads) (B) coupled with the DAPI stain. Similar Glut1 immunolocalization was observed in the endothelium of both WT and $glut3^{+/-}$ mice. Glut3 immunoreactivity seen as the Texas red stain in neuronal processes (arrows) is seen in the sub-fornicular organ (SFO) (C), Glut3 antibody negative control (D) and Glut3 immunoreactivity in the hypothalamus (E) and the periventricular region (F) in WT mice. Focusing on the SFO a comparison between WT (G) and $glut3^{+/-}$ (H) is shown along with double staining for both Glut3 (red; arrows) and Glut1 (green; arrowheads) along with the blue nuclear DAPI stain (I). Scale bar = 50 μ m.

Study Design

Figure 2. Study Design and Neuronal Nitric Oxide Synthase

A. A schematic representation of the study design is shown resulting in two major groups of mice. The Nx (FiO₂ = 0.21) group consists of the contralateral (Ctrl) side exposed to Nx alone and the ipsilateral (Ipsi) side exposed to ischemia. The Hx (FiO₂ = 0.08) group consists of the Ctrl side that is exposed to Hx alone and the Ipsi side exposed to hypoxic-ischemia (HI). B–E. One time period at 4hr after reoxygenation was assessed for nNOS immunoreactivity. Striatum (B,D) and hippocampus (C,E) from WT (B,C) and *glut3*^{+/-} (D,E) mice were immunostained for nNOS seen as dark staining neuronal cells (arrows). The central inset reveals a higher magnification of nNOS positive neurons. F–G. Two time periods at 4 hr (F) and 24 hr (G) after reoxygenation were assessed by Western blots which demonstrate nNOS protein (upper panels) and β -actin or vinculin (lower panels) as controls in the inset. Quantification of nNOS protein and calcium-dependent enzyme activity are depicted below. nNOS served as a marker for acute oxidative stress secondary to neuroglycopenia. N=7 (F) or n=6 (G) per genotype per experimental group which includes Ctrl and Ipsi sides of brain for all measurements. 2-way ANOVA reveals an effect of genotype (F value=10.89, p=0.002) but not that of treatment-exposure or genotype X treatment-exposure. Significant difference by Holm-Sidak test is shown by *p=0.007, and by Fisher's PLSD test $^{\circ}$ p<0.05 (F) or p<0.03 (G).

Figure 3. Brain Glut and Apoptotic Proteins

A 4 hr reoxygenation response is seen as changes in brain Glut3 (G3) and Glut1 (G1) noted as an effect of genotype ($F=231.17$, $p<0.001$), treatment-exposure ($F=8.24$, $p<0.001$) and genotype X treatment-exposure ($F=5.20$, $p=0.003$) on Glut3 (A). A genotype effect in Nx-Ctrl and a treatment-exposure effect of Hx-Ctrl and Hx-Ipsi is observed on Glut1 (Fisher's PLSD $^Op<0.05$) (A). Mitochondrial apoptotic proteins, Bax and Bcl2 (Fisher's PLSD $^Op<0.05$) demonstrate an effect of genotype ($F=16.35$ or 25.76 , $p<0.001$) but not that of treatment-exposure or genotype X treatment-exposure (B). A 24 hr response of brain Glut3 (G3) is seen as genotype ($F=192.25$, $p<0.001$) and treatment-exposure ($F=4.99$, $p=0.004$) effects alone and Glut1 (G1) is also seen as genotype ($F=10.84$, $p=0.002$) and treatment-exposure ($F=3.61$, $p=0.02$) effects alone (C). Bax (genotype – $F=8.10$, $p=0.007$; genotype X treatment-exposure – $F=9.93$, $p<0.001$) and Bcl2 (genotype X treatment-exposure – $F=2.98$, $p=0.04$) (D), pro-caspase-3 (genotype – $F=67.52$, $p<0.001$; treatment-exposure – $F=8.26$, $p<0.001$) (E) and caspase-3 enzyme activity (genotype – $F=13.22$, $p<0.001$; treatment-exposure – $F=18.04$, $p<0.001$) (F) in Nx-Ctrl, Nx-Ipsi, Hx-Ctrl and Hx-Ipsi in WT and glut3^{+/-} mice are seen. In all panels representative Western blots are shown in the inset with vinculin (vin) or β -actin as internal controls and the quantification depicted below. $N=7$ per genotype per experimental group (Hx vs Nx) and per exposure (ischemic vs non-ischemic side) for all measurements. † or *Holm-Sidak test following two-way ANOVA or OF Fisher's PLSD test following one-way ANOVA demonstrates inter-group differences.

Figure 4. Brain morphology

Hematoxylin and eosin staining of brain coronal sections from WT (A–D) and *glut3*^{+/-} (E–H) mice subjected to the two experimental conditions, Nx-Ctrl (A,E), Nx-Ipsi (B,F), Hx-Ctrl (C,G) and Hx-Ipsi (D,H) with re-oxygenation of 24 hr. Arrows demonstrate pyknotic neuronal nuclei supporting hypoxic-ischemia induced necrosis in the CA1 region of *glut3*^{+/-} hippocampus (H) which are highlighted by their autofluorescence (H). The inset demonstrates three neuronal cells with autofluorescence. In contrast, WT Hx-Ipsi hippocampus appears normal with no cellular or nuclear change (D) and absent autofluorescence (D).

Figure 5. Brain Cellular Apoptosis

TUNEL staining of the motor cortex-cingulum region of WT (A–D) and *glut3*^{+/-} (E–H) mice subjected to Nx-Ctrl (A,E), Nx-Ipsi (B,F), Hx-Ctrl (C,G) and Hx-Ipsi (D,H) with re-oxygenation for 24 hours is shown. Positive TUNEL is seen as dark condensed neuronal nuclei (arrows).

Figure 6. Continuous video EEG recording

during 24 hours of re-oxygenation following hypoxic-ischemic injury. Abnormal spike waves were noted in *glut3^{+/-}* mice prior to hypoxic-ischemia. After hypoxic-ischemia, *glut3^{+/-}* mice demonstrated running seizures (A) and/or non-motor seizures (B). The insets demonstrate stills from simultaneous video recording of mice. WT after hypoxic-ischemia demonstrated background EEG (C) with no seizures.

Figure 7. PTZ testing at baseline

Clinical seizures following PTZ dosing (25, 40 and 60 mg/kg) in WT (n=9) and glut3^{+/-} (n=11) mice were characterized as the proportion of mice with seizures represented as a percent, *p=0.014 by GEE (A), their seizure scores on a scale of 0 to 6 with the 1st and 3rd quartile demonstrated along with the minimum and maximum scores. The median scores were higher in glut3^{+/-} vs WT, p=0.04 by Wilcoxon rank sum test (B). The duration in seconds (s) of seizures, *p=0.0087 by GEE (C) and latency in seconds from PTZ dosing to initiation of seizures, *p=0.0085 by GEE (D).