A mouse model for Glut-1 haploinsufficiency

Dong Wang1, Juan M. Pascual1, Hong Yang1, Kristin Engelstad1, Xia Mao1, Jianfeng Cheng2, Jong Yoo3, Jeffrey L. Noebels3 and Darryl C. De Vivo1,*

1Colleen Giblin Laboratories for Pediatric Neurology Research, Department of Neurology and 2Department of Psychiatry, Division of Biostatistics, New York State Psychiatric Institute, Columbia University, New York, NY, USA and 3Developmental Neurogenetics Laboratory, Department of Neurology, Baylor College of Medicine, Houston, TX, USA

Received December 12, 2005; Revised February 8, 2006; Accepted February 15, 2006

Glut-1 deficiency syndrome (Glut-1 DS, OMIM #606777) is characterized by infantile seizures, developmental delay, acquired microcephaly and hypoglycorrhachia. It is caused by haploinsufficiency of the blood–brain barrier hexose carrier. Heterozygous mutations or hemizygosity of the GLUT-1 gene cause Glut-1 DS. We generated a heterozygous haploinsufficient mouse model by targeted disruption of the promoter and exon 1 regions of the mouse GLUT-1 gene. GLUT-1+/- mice have epileptiform discharges on electroencephalography (EEG), impaired motor activity, incoordination, hypoglycorrhachia, microencephaly, decreased brain glucose uptake as measured by positron emission tomography (PET) scan and decreased brain Glut-1 expression by western blot (66%). The GLUT-1+/- murine phenotype mimics the classical human presentation of Glut-1 DS. This GLUT-1+/- mouse model creates an opportunity to investigate Glut-1 function, to examine the pathophysiology of Glut-1 DS in vivo and to evaluate new treatment strategies.

INTRODUCTION

Glucose is the primary source of energy for the mammalian brain. Glut-1 is the predominant glucose transporter expressed in the blood–brain barrier (BBB), is responsible for glucose entry into the brain (1) and is the first identified member of the facilitated glucose transporter family (SLC2A) (2). The human GLUT-1 gene has been localized to the short arm of chromosome 1 (1p34.2) [UCSC Human Genome Project Working Draft, April 2002 assembly (hg11): http://genome.cse.ucsc.edu/], is 35 kb in length and contains 10 exons encoding a protein of 492 amino acids (2). The protein is highly conserved among different species including human, rat, mouse and pig (3). The mouse GLUT-1 gene is localized to chromosome 4 and has a very similar gene structure to human GLUT-1 (Mouse Genome Informatics). Mouse GLUT-1 cDNA (NM 011400) is >97% identical to that of human GLUT-1 (4).

Glut-1 deficiency syndrome (Glut-1 DS, OMIM 606777) is an autosomal-dominant disorder characterized by infantile seizures, developmental delay, acquired microcephaly, ataxia and spasticity (5–8). It is caused by haploinsufficiency of the BBB hexose carrier. Three clinical phenotypes have emerged since 1991 as more patients have been described (8,9). The first phenotype (classical) is a developmental encephalopathy with seizures affecting the cognitive, behavioral and motor domains. Cognitive impairment ranges from learning disabilities to severe mental retardation. Acquired microcephaly occurs in 50% of patients, but deceleration of head growth is more common. The second phenotype is characterized by mental retardation, dysarthric speech and intermittent ataxia without any clinical seizures (10). The third phenotype is characterized by choreoathetosis and dystonia (11). The molecular basis for this human disease is a wide spectrum of heterozygous mutations, including nonsense, missense, insertion, deletion and splice site mutations, and hemizygosity of the GLUT-1 gene (8,12–15). However, correlations between genotype and phenotype still remain elusive.

Ketone bodies bypass the BBB Glut-1 defect, entering the brain by a monocarboxylic acid transporter (MCT1) and provide an alternative brain fuel (5,16,17). The ketogenic diet is effective in controlling the seizures and other paroxysmal symptoms in Glut-1 DS (11,18), but this treatment has
less effect on the neurobehavioral symptoms. Epigenetic approaches to enhance GLUT-1 expression or Glut-1 transport activity have been attempted with some success in vitro (16). Conversely, pharmacological inhibition of Glut-1 transport should be avoided (19–21).

We have succeeded in creating the first GLUT-1<sup>+/−</sup> mouse model for human Glut-1 DS by targeted disruption. The GLUT-1<sup>+/−</sup> mice display many features that are faithful to the human condition with seizures, hypoglycemia, impaired motor activity, incoordination and learning disturbances, microencephaly, decreased brain glucose uptake by PET scan and decreased brain Glut-1 expression.

**RESULTS**

Generation of GLUT-1<sup>+/−</sup> mice by targeted disruption

The strategy used for targeted disruption of the mouse GLUT-1 gene is shown in Figure 1A. We constructed a targeting vector in which the promoter and exon 1 of the mouse GLUT-1 gene were replaced by the neomycin-resistant gene (Neo<sup>+</sup>), and a diphtheria toxin alpha chain (DT) gene cassette was added adjacent to intron 1 of the GLUT-1 gene. Electroporation of the recombinant construct in embryonic stem (ES) cells was followed by exposure to the neomycin analogue G418. Of the 300 Neo<sup>+</sup> ES clones, 23 showed evidence of recombination of the recombinant construct in embryonic stem (ES) cells and were followed by exposure to the neomycin analogue G418. Of the 300 Neo<sup>+</sup> ES clones, 23 showed evidence of homologous recombination of the targeting construct. Two positive clones were injected into blastocysts and one chimera was mated with 129S6/SvEvTac to transmit the modified GLUT-1 gene to the next (F<sub>1</sub>) generation. The F<sub>1</sub> heterozygous (GLUT-1<sup>+/−</sup>) mice were mated to each other, to generate homozygous knock-out (GLUT-1<sup>/−/−</sup>), heterozygous (GLUT-1<sup>+/−</sup>) and wild-type (GLUT-1<sup>+/+</sup>) animals. Confirmation of gene disruption was obtained by both Southern blot and polymerase chain reaction (PCR)-based analysis (Fig. 1B and C).

Homzygous GLUT-1<sup>/−/−</sup> mice show embryonic lethality

To obtain GLUT-1<sup>/−/−</sup> mice, GLUT-1<sup>+/−</sup> mice were mated. However, in 194 offspring, there were 75 GLUT-1<sup>+/−</sup> mice (39%) and 119 GLUT-1<sup>/−/−</sup> mice (61%). No GLUT-1<sup>/−/−</sup> mice were born, suggesting embryonic lethality. To establish the timing of the embryolethality, we PCR-genotyped 33 embryos collected at different stages of development (from E10.5 to E13.5) and found that the percentages of /−/−, +/− and +/+ genotypes in these embryos were 15% (5), 58% (19) and 27% (9), similar to the ratio expected by Mendelian transmission of the recombinant allele. These findings dated the loss of Glut-1<sup>/−/−</sup> expression to the embryonic window of mature placenta formation (E10 dpc), organogenesis (E12 dpc) and body-mass growth and organ maturation (E13–E14 dpc). As shown in Figure 2A and B, GLUT-1<sup>/−/−</sup> embryos at E13 did show some morphological abnormalities. In addition to their small size, there was the lack of visibly detectable eyes, a comparatively diminutive rostral embryonic pole and overall developmental delay in comparison with their GLUT-1<sup>+/+</sup> and GLUT-1<sup>+/−</sup> littermates.

Heterozygous GLUT-1<sup>+/−</sup> mice show impaired motor performance

Animals were examined periodically to evaluate spontaneous motor activity, reaction to stimuli and the presence of neurological signs. No obvious neurological impairment was detected in the GLUT-1<sup>+/−</sup> population. Seventeen
GLUT-1+/− (five females and 12 males) and 14 GLUT-1+/+ animals (five females and nine males) at the age of 14–22 weeks were tested on the rotation-rod treadmill (Rotarod). The Rotarod test is used to study motor coordination and learning. There were no significant differences between male and female GLUT-1+/− mice. We conducted two-sample t-test between GLUT-1+/− (male and female) and GLUT-1+/+ mice (male and female) and found statistically significant differences (P < 0.0001) at all speeds (25, 30, 35 and 40 rpm) and for all ages (14, 18 and 22 weeks) (Fig. 3A and B). Even after adjusting for multiple comparisons, the statistically significant differences still hold. For GLUT-1+/− mice, there were significant differences in the direction of poorer motor performance between age 14 and 18 weeks at all test speeds (25, 30, 35 and 40 rpm) (P < 0.05) and between age 18 and 22 weeks at 40 rpm (P < 0.05). The abnormal motor performance emerged as early as age 4–5 weeks and worsened with time (22 weeks of age). Beam walking, to evaluate balance and motor coordination, and the footprint test, to evaluate gait, were performed with 10 GLUT-1+/− and 9 GLUT-1+/+ animals (age 20–30 weeks). Significant differences were found for both latency (P < 0.05) and hindpaw slips (P < 0.05) between WT mice (latency, 5.9 ± 2.2 s; hindpaw slips, 0.8 ± 0.3) and GLUT-1+/− mice (latency, 7.9 ± 1.9 s; hindpaw slips, 4.5 ± 0.4) (Fig. 3C and D). The footprint test revealed no statistical difference between stride length (GLUT-1+/−, 7.1 ± 0.8 cm; GLUT-1+/+, 7.0 ± 0.6 cm) and hindpaw base (GLUT-1+/−, 2.8 ± 0.4 cm; GLUT-1+/+, 2.7 ± 0.5 cm).

**Decreased brain weight and hypoglycorrachia in GLUT-1+/− mice**

The average body weights of GLUT-1+/+ (26.5 ± 3.5 g) and GLUT-1+/− (25.4 ± 4.0 g) mice at age 18–22 weeks were similar, whereas the average brain weights of GLUT-1+/− mice (394 ± 19 mg) were significantly smaller (P < 0.001) than GLUT-1+/+ mice (417 ± 14 mg) (Table 1). There was no significant difference in the growth curves between the GLUT-1+/+ (n = 5) and GLUT-1+/− (n = 5) mice (Fig. 4A and B) even when compared by gender. The blood and cerebrospinal fluid (CSF) glucose values, collected by cisternal puncture within 5 min, were measured after euthanizing with carbon dioxide to avoid anesthetic-related glucose change. There was no significant difference between GLUT-1+/− and GLUT-1+/+ blood glucose values (P = 0.85), but the CSF glucose value in GLUT-1+/− mice (26.3 ± 7.17 mg/dl) was significantly lower (P < 0.001) than GLUT-1+/+ mice (74.6 ± 14.1 mg/dl) (Table 2).

**Spontaneous seizure in GLUT-1+/− mice**

The electrographic features of the GLUT-1 heterozygous disruption were examined by chronic video/electrographic recordings of freely moving mice. The baseline cortical activity of the GLUT-1+/− mice showed relatively normal, low-amplitude desynchronized EEG activity with the frequent (10–20 h−1) appearance of solitary generalized interictal discharges with no obvious behavioral correlate in 3/3 mutants (Fig. 5A and B). Although no seizures could be provoked by startle or photic stimulation, multiple spontaneous cortical seizures were observed in heterozygous GLUT-1+/− mice during the monitoring period. These proved to be of more than one type. First, there were generalized or partial lateralized seizures, beginning with rapid onset of high voltage, rhythmic spiking followed by slower polyspike and wave discharges and terminating abruptly without postictal depression (Fig. 5C). During these electrographic seizures, behavior was uninterrupted, and the mouse continued to engage in either exploratory behavior and grooming or to remain motionless. Second, a bilateral generalized slow (~2–3 s−1) spike and wave pattern was observed, again without interruption of behavior (Fig. 5D). This seizure type occurred less frequently (0–1 per 5-day monitoring session). Finally, in the fasting state, frequent brief (1–4 s) bilateral rhythmic 6 s−1 spike discharges were seen accompanied by periods of behavioral arrest (Fig. 5E). These seizure events were immediately followed by a return to normal EEG activity upon termination of the seizure. Wild-type littermates showed no obvious EEG abnormalities.
Decreased Glut-1 expression and impaired glucose uptake in GLUT-1\(^{-/-}\) mouse brain without obvious histological abnormality

Brain sections from cerebrum, thalamus, hippocampus, brain stem, cerebellum and spinal cord of GLUT-1\(^{-/-}\) mice revealed no obvious abnormalities compared with the GLUT-1\(^{+/+}\) brains (Fig. 6).

In mouse forebrain, the Glut-1 expression was normalized to \(\beta\)-actin on the plasma membrane fractions. The Glut-1/\(\beta\)-actin ratio was 2.3 \(\pm\) 0.32 (mean \(\pm\) STDEV, \(n = 8\)) in GLUT-1\(^{-/-}\) mice and 3.5 \(\pm\) 0.2 (mean \(\pm\) STDEV, \(n = 8\)) in GLUT-1\(^{+/+}\) mice, which was significantly different (\(P < 0.001\)) (Fig. 7A). The Glut-1 expression in GLUT-1\(^{-/-}\) mice was about 66\% of the GLUT-1\(^{+/+}\) mice (Fig. 7B).

| Table 1. Body and brain weight of wild-type and heterozygous mice (age 18–22 weeks) |
|------------------|-----------------|------------------|
|                  | Brain weight (mg) | Body weight (g)  |
|                  | (mean \(\pm\) SD) | (mean \(\pm\) SD) |
| GLUT-1\(^{-/-}\)  | 394 \(\pm\) 19    | 25.4 \(\pm\) 4.0  |
| GLUT-1\(^{+/+}\)  | 427 \(\pm\) 14    | 26.5 \(\pm\) 3.5  |

Impaired cerebral metabolism associated with Glut-1 deficiency was investigated in the GLUT-1\(^{-/-}\) mice by PET scan. Three pairs of animals showed similar results and representative results for brain are shown in Figure 8. There was a
human molecular genetics, 2006, vol. 15, no. 7 1173

diffuse decrease of brain uptake in GLUT-1+/− mice compared with GLUT-1+/+ mice. As anesthetics affect blood glucose concentration in an unpredictable way, we chose the data only from animals that did not have a significant blood glucose change before and after the scanning process.

DISCUSSION

Glut-1 DS was first described by De Vivo et al. in 1991(5). The predicted defect in glucose transport across the BBB was documented later by the findings of a heterozygous large-scale deletion and a heterozygous nonsense mutation in the GLUT-1 gene of the first two patients (12). These early findings have been confirmed repeatedly since 1998 (7,8,11,13,22–24). The increasing number of mutations and phenotypic variability have made correlation difficult. Now, we report the neurological phenotype of the first GLUT-1+/− mouse model for Glut-1 DS.

The homozygous null mouse (GLUT-1−/−) is embryonic lethal

The homozygous knock-out of GLUT-1 in mice and humans is thought to have a deleterious effect on embryonic or fetal development and is incompatible with life (25). Our observations that no GLUT-1−/− mice were seen in 194 live pups resulting from the mating of GLUT-1+/− mice supports this assumption. We found five GLUT-1−/− (15%), 19 GLUT-1+/− (58%) and nine GLUT-1−/+ (27%) genotypes out of 33 embryos, close to the predicted ratio of Mendelian autosomal transmission of the recombinant allele. GLUT-1−/− embryos at E13 dpc were deformed with lack of visibly detectable eyes, comparatively diminutive rostral embryonic pole and overall developmental delay compared with their GLUT-1−/− or GLUT-1−/+ litters and similar to the size of normal embryos at E9-10 dpc (Fig. 2A and B). These results indicate that the loss of Glut-1−/− embryos started during the period of normal placenta formation (E10 dpc) and organogenesis (E12 dpc). The mouse embryos do not rely on glucose as a major energy source until compaction and blastocyst formation (around E4–E5 dpc) (26). After compaction and polarization, Glut-1 expression is restricted to the basolateral membranes of the outer morula cells and trophoderm. Glut-3 concentrates in the apical trophodermal membranes. Together with the basolateral Glut-1, these transporters deliver glucose to the inner cell mass (26). The mature placenta forms by E10 dpc (27). At this point, transplacental glucose transport in the homozygous GLUT-1−/− embryos would be severely blocked or impaired (as there might be residual Glut-2 expressed on the basolateral membranes). Together with the basolateral Glut-1, these transporters deliver glucose to the inner cell mass (26). The mature placenta forms by E10 dpc (27). At this point, transplacental glucose transport in the homozygous GLUT-1−/− embryos would be severely blocked or impaired (as there might be residual Glut-2 expressed on the basolateral membranes) (26, 28). The lack of glucose severely impedes further development as the early embryo relies almost exclusively on glycolysis for energy metabolism (29). In mouse strains harboring randomly inserted antisense GLUT-1 cDNAs, Heilig et al. (30) also reported findings of smaller embryos (E11.5–18.5 dpc) that exhibited gross somatic malformations after mating pairs of GT1AS heterozygotes.

The GLUT-1+/− mouse phenotype mimics the major features of classic Glut-1 DS

A significant, consistent reduction in the motor performance of the GLUT-1+/− mice was detected by Rotarod, and beam walking tests (Fig. 3A–D), which started as early as age 4–5 weeks and worsened with age. The findings resemble deficits in motor activity, balance and coordination seen in Glut-1 DS patients. There was no significant difference in the footprint test, which is different from the Glut-1 DS patients who display a spastic ataxic gait. We have not observed any generalized tonic-clonic seizures in the mutant mice. However, several patterns of electrographic seizures were observed in the heterozygous GLUT-1+/− mice during the monitoring
period (Fig. 5B–E). Seizure types included: (1) generalized or partial seizures; (2) a bilateral generalized slow (2–3 s<sup>−1</sup>) spike and wave pattern without interruption of behavior; (3) frequent brief (1–4 s) bilateral rhythmic 6 s<sup>−1</sup> spike discharges accompanied by periods of behavioral arrest in the fasting state (Fig. 5E).

Figure 5. Representative electroencephalographic activity recorded from awake wild-type GLUT-1<sup>+/+</sup> (A) and heterozygous GLUT-1<sup>+/−</sup> mice (B). Traces from left and right hemispheres (alternating from anterior to posterior electrodes). The baseline cortical activity was a relatively normal, low-amplitude desynchronized EEG activity with frequent appearance of solitary generalized interictal discharges with no behavioral correlate. A spontaneous seizure in an adult GLUT-1<sup>−/−</sup> mouse is shown (C). This electrographic seizure showed a lateralized discharge arising in the right hemisphere with largest amplitude over the parieto-occipital neocortex, and with a decremental EEG pattern over the contralateral hemisphere. Another electrographic seizure pattern, featuring a slow (<3 Hz), high-amplitude, bilateral spontaneous cortical spike-wave synchronous discharge accompanied by normal behavior is shown (D). A spontaneous 6 s<sup>−1</sup> rhythmic spiking seizure accompanied by behavioral arrest is shown (E). In all seizures, the EEG activity reverts to normal low-amplitude, high-frequency patterns immediately following the end of the discharge. Calibrations: (A–C, E) 200 mV, 1 s; (D) 200 mV, 1.5 s.

Figure 6. Cresyl violet-Nissl stains of (top row) GLUT-1<sup>+/+</sup> and (bottom row) GLUT-1<sup>+/−</sup> mice showing normal regional histology: (A, F) cerebellar cortex, 200×; (B, G) spinal cord, 40×; insets: anterior horn motor neurons, 200×; (C, H) hippocampus, 40×; (D, I) hippocampal cortex, 100×; (E, J) temporal cortex, 100×.

Owing to the high fat content in maternal milk, ketonemia is present in neonatal rats (31–33) and humans (34). During the same postnatal period, circulating glucose levels are almost as high in rat pups as in adults (35). The brain of the suckling rat uses glucose and ketone bodies as substrates for energy metabolism and biosynthesis (36–39). Active cerebral glucose
utilization is initially low, correlating with the limited transport capacity of glucose across the BBB of the suckling rat, approximating 20% of the adult rate during the first two postnatal weeks (39–43). In contrast, the permeability of the BBB to β-hydroxybutyrate is very high at this age (40, 41, 44). By the end of the suckling period, the rate of glucose transport across the BBB increases 3-fold, whereas the diffusion of β-hydroxybutyrate decreases by 62% (41). These observations may explain the relative mitigation of symptoms in the rodent glut-1 DS compared with the human and also may imply an increased benefit from early diagnosis and treatment of the condition with the Ketogenic diet.

We did not recognize any brain histological abnormalities despite the smaller brain size (Fig. 6). This is consistent with the normal CT and MRI findings in Glut-1 DS patients (23). We found brain glucose uptake to be diffusely decreased with the normal CT and MRI findings in Glut-1 DS patients (45) and create an opportunity to investigate developmental vulnerability and regional growth arrest of the brain.

Brain Glut-1 expression was significantly decreased in the GLUT-1+/− mice compared with the GLUT-1+/+ mice. These results are consistent with the complete knock-out of one GLUT-1 allele and a compensatory upregulation of the wild-type allele. The hypoglycorrhachia with decreased CSF glucose values (Table 1) and the brain hypometabolism evidenced by PET scan (Fig. 8) are appropriate surrogate biomarkers of the Glut-1 haploinsufficiency.

**Figure 7.** Brain Glut-1 expression of GLUT-1+/+ and GLUT-1+/− mice. (A) The purified plasma membrane samples from GLUT-1+/+ and GLUT-1−/− mice forebrains were prepared and subjected to western blot analysis as described in Materials and methods. Twenty-five micrograms of prepared membrane protein was loaded in each lane. This figure is representative of the western blot analyses for eight GLUT-1+/+ and eight GLUT-1−/− mice. The two Glut-1 isoforms (55 kDa Glut-1 in the brain endothelial cells and 45 kDa Glut-1 in the astrocytes) and β-actin (42 kDa) are indicated by arrow heads. M indicates the molecular weight marker. Lanes 1–3: GLUT-1+/+ lanes 4–7: GLUT-1−/−. (B) The Glut-1 and β-actin signals were quantitated digitally using a densitometer. The densities of Glut-1 signals were normalized to β-actin signals. The averaged Glut-1/β-actin ratio in eight GLUT-1+/+ and eight GLUT-1−/− mice are presented. The relative Glut-1 expression in GLUT-1−/− mice is about 66% of GLUT-1+/+ mice. *P < 0.001.

**Figure 8.** FDG-PET scans in GLUT-1+/+ and GLUT-1+/− mice. Representative PET images of a pair of GLUT-1+/+ and GLUT-1+/− mice are shown. The upper and lower panels represent the comparable coronal and axial sections, respectively.

### Clues into the mechanism of human Glut-1 deficiency

Recent experimental evidence suggests that neurons preferentially consume lactate rather than glucose, as had been traditionally held (46, 47). Why, then, is there clinical and electrophysiological evidence of neuronal dysfunction in Glut-1 DS, such as epilepsy? We believe that this apparent paradox can be explained by tracing the flow of glucose from blood into brain. Blood glucose traverses the brain endothelial cell to the extracellular space before entering brain cells. Most of the astrocytic lactate derives from the anaerobic metabolism of glucose (48), with a small contribution from glycogenolysis (49). In order for a glucose molecule to reach the astrocyte, a minimum of three Glut-1 transporters arranged in series must be available; one each in the luminal and abluminal sides of the capillary endothelium and a third in the astrocytic plasma membrane. The glucose gradient between the blood and the intracellular compartment of the astrocyte must be large under normal conditions. Glut-1 haploinsufficiency should increase this gradient substantially thereby compromising the availability of glucose for the astrocyte, diminishing the intracellular stores of glycogen and decreasing the production of lactate (50). The neuron would suffer as a consequence with the decreased availability of lactate. The low CSF lactate values recorded in humans with Glut-1 DS is consistent with these pathogenetic speculations (5). Also, the predictably low brain extracellular concentration of glucose in this...
condition limits the alternative neuronal usage of this fuel, transported by Glut-3. In brief, the neuron is energy-deprived because of decreased availability of astrocyte-derived lactate and decreased extracellular concentration of glucose. All these metabolic speculations can be tested in the GLUT-1\(^{+/−}\) mouse model.

In summary, we have successfully created the first haplo-insufficient GLUT-1\(^{+/−}\) mouse model, which mimics the major features of the classical phenotype of human Glut-1 DS. The physical robustness of the GLUT-1\(^{+/−}\) model should facilitate studies focusing on pathophysiological disturbances and therapeutics development that are relevant to the human condition.

**MATERIALS AND METHODS**

All animal studies have been approved by the Institutional Review Board of Columbia University.

**Construction of the targeting vector and generation of the chimera**

A short fragment (1890 bp) and a long fragment (3193 bp) were amplified by PCR using mouse genomic DNA (129 S6/SvEvTac) with two primer sets SF–SR and LF–LR (SF: AC ACTATCCCTAGGCTTTGTAATCAGCTTGTGACTATCCTAAGGCTTTGTATCTCGAG; LF: CTAATCAGCTTGTGACTTCAGGGAGTGACTATCCTAAGGCTTTGTATCTCGAGGGCTTTCTTTGG; SR: CCTCTCGGGTTATGAGAATTACTCGGATTTAGTTTG; LR: CTAATCAGCTTGTGACTTCAGGGAGTGACTATCCTAAGGCTTTGTATCTCGAGGGCTTTCTTTGG). The PCR was carried out in 50 \(\mu\)l volume containing 100 ng genomic DNA, 0.5 \(\mu\m\) each primer and 0.2 \(\mu\m\) each dNTP. After an initiation denaturation at 95°C for 2 min, the PCR condition was 95°C for 30 s, 62°C for 30 s, 68°C for 2 min, for 35 cycles, followed by a final extension at 6°\(C\) for 8 min. The long fragment was cut with KpnI and EcoRI and purified by with QIA quick PCR purification kit (Qiagen, Valencia, CA, USA). The purified fragment was then cloned into the KpnI and EcoRI sites of the targeting vector containing Neo\(^r\) gene and DT gene (a kind gift from Victor Lin, Columbia University). The purified short fragment, cut with XhoI, was cloned into the above targeting vector. The orientation of the two fragments, the Neo\(^r\) gene and the DT alpha chain, were verified by direct sequencing before electroporation into the ES cells. The ES cell line was derived from the 129 S6/SvEvTac mouse substrate. Culture of ES cells, electroporation of NotI linearized vector and selection procedure were performed as described (51). Genomic DNA from clones surviving G418 selection were isolated, digested with HindIII and analyzed with the 3’ probes on Southern blots. Subsequent hybridizations of the same blots with internal probes from the Neo\(^r\) gene confirmed the homologous recombination in the clones.

ES cells that were heterozygous for the targeted GLUT-1 mutation were microinjected into C57BL/6 blastocysts and implanted into pseudopregnant females. The resulting male chimeras were mated with C57BL/6j to check for germline transmission and then mated with female 129 S6/SvEvTac mice. Germ-line transmission of the injected ES cells was confirmed by inheritance of the agouti coat color in the F\(_1\) mice and the agouti offspring were tested for the presence of the mutated GLUT-1 allele by Southern blot analysis or PCR of mouse tail DNA. Three PCR primers were used (WF: CCATAAAGTCA GAAATGGAGGGATTGGTGTT, WR: GCGAGACCG GAACCAGCGCTGTAACTA and MR: CTACCGGTTG ATGTGAAATGTTGCGAGGC). Mice used in this study had a pure background from the 129 strain mice.

**Body weight, brain weight, blood glucose and CSF glucose measurement**

Mice were fasted overnight and their weight was measured before they were euthanized with carbon dioxide. Ten microliters of blood was collected by cutting the tail tip to measure the glucose level using Ascensia Elite XL glucose meter (Bayer Corporation, Mishawaka, IN, USA). CSF was isolated from the cisterna magna as described previously with modification (52,53) within 5 min. Briefly, an incision from the top of the skull to the dorsal thorax was made and the musculature from the base of the skull to the first vertebrae was removed to expose the meninges overlying the cisterna magna. The tissue above the cisterna magna was excised with care not to puncture the translucent meninges. The surrounding area was gently cleaned with the use of cotton swabs to remove any residual blood or other interstitial fluid. A micropipette with a 6–7-mm 30-gauge needle (Hamilton Company, RENO, NV, USA) was used to puncture the arachnoid membrane covering the cistern and collect CSF. The CSF, which was under positive pressure as a result of blood pressure, respiration and positioning of the animal, flowed into the plastic tip. Usually, 5–15 \(\mu\l\) of clear CSF was obtained. The CSF glucose level was measured immediately with Ascensia Elite XL glucose meter. The brain was then dissected and weighted.

**Rotarod test**

Motor coordination was measured by fixed speed Rotarod testing (Rota-Rod Treadmill for Mice Cat#7650, UGO Basile Biological research apparatus, Comerio, Italy). Mice were trained by being placed individually on a rotating drum in acceleration mode from 4 to 40 rpm (over a maximum of 5 min) four times daily for three continuous days. On the fourth day, all mice were tested three times at each speed (25, 30, 35 and 40 rpm). The maximum score was set for 1000 s. The time when the mouse fell off the drum was recorded. The room was illuminated with standard fluorescent lights. Mice were returned to their home cage between tests.

**Beam walking**

Beam walking test measured the motor coordination and balance of mice (54). A strip of wood with a 12-mm\(^2\) cross-section was placed horizontally, 30 cm above the bench surface, with one end mounted on a narrow support and the other end attached to an enclosed box. Two anglepoise lights were positioned above. Mice were trained over four consecutive days with four trials each day before they were tested for five trials. Mice were allowed 60 s to traverse the beam. The time latency to traverse the beam and the number of hind limb slips from the beam were recorded for each trial.
The average latency and hindpaw slips were used for the analysis.

**Footprint test**

The footprint was used to test the gait of the GLUT-1+/− mice (54). The mice were trained three times to walk along a 50-cm long, 10-cm wide runway (with 10-cm high walls) into an enclosed box. Before the test, a sheet of white paper was put on the floor of the runway and the hindpaws were painted with black ink. The foot pattern was measured for two parameters—stride length and hindlimb-base width. The mean value of each set of three values was used in the analysis.

**Electroencephalographic recordings**

Silver wire electrodes (0.005 in. diameter) soldered to a microminiature connector were implanted bilaterally into the subdural space over the frontal, parietal and occipital cortices of mice under Avertin anesthesia several days prior to recording. Simultaneous cortical activity and behavioral video EEG monitoring were performed using a digital electroencephalograph (Stellate Systems, Montreal, Canada) with heterozygous mutants and unaffected +/+ controls. The mice were allowed to move freely in the test cage for prolonged periods, including sleep. Some mutant mice were also studied following 24 h of fasting with free access to water. Seizure behavior was observed directly and annotated on all recordings.

**PET scan**

A small animal PET scanner with a full-width at half-maximum resolution of 2 mm was used. The mice were littermates and bred together. After 4 h of fasting, one heterozygous GLUT-1+/− and one wild-type GLUT-1+/+ mouse were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) 30 min prior to the scan. Immediately after anesthesia, their tail veins were catheterized. Following positioning in the scan, an attenuation data set was obtained; then the animals were simultaneously injected with 35 μCi of isotope and maintained together in the scanner for 1 h. They were then euthanized by cervical dislocation. Blood glucose was measured to ensure similarity of values between the GLUT-1+/+ and GLUT-1+/− mice, before and after the study. Only mice pairs with similar blood glucose values were selected for analysis.

**Brain tissue preparation and histological analysis**

The mouse brain was fixed by infusion of 4% paraformaldehyde fixative. The brains were divided manually and processed automatically using a series of graded alcohol baths for dehydration and penetration by xylene prior to embedding in hot paraffin. The brains were stored and later softened to fit in molds from which paraffin blocks were obtained. Brains were sectioned in 5 μm coronal and sagittal slices and mounted onto glass slides. The slides were deparaffinized and rehydrated using xylene, ethanol and water. The sections were then stained using a cresyl violet-Nissl method, magnified 4−10× and digitally photographed.

**Western blot analysis of Glut-1 expression in mouse forebrain**

Western blot analysis was performed as described previously (7). Briefly, mouse forebrain was collected after anesthesia, quickly immersed in liquid nitrogen and kept at −80°C. Half of the forebrain was homogenized and centrifuged at 1000g for 10 min at 4°C three times and the supernatant was saved. The combined supernatant was centrifuged at 10 000g for 20 min to obtain the plasma membrane fraction. The pellet was suspended in homogenization buffer and stored at −80°C until used. Twenty-five micrograms of the purified plasma membrane samples from GLUT-1+/+ and GLUT-1−/− mice was loaded. Primary antibodies (rabbit anti-Glut-1) from FabGennix (Frisco, TX, USA) and β-actin from Sigma (A-5441) were diluted 1:5000. The horseradish peroxidase-conjugated antibodies against rabbit (for Glut-1, donkey anti-rabbit IgG-HRP from Santa Cruz Biotechnology) and mouse (for β-actin, sheep anti-mouse IgG-HRP from Amersham) immunoglobulins were diluted 1:5000. The Glut-1 and β-actin signals were quantified digitally using a densitometer equipped with ImageQuant software (Molecular Dynamics, CA, USA). The densities of Glut-1 signals were normalized to β-actin signals.

**Statistics**

Student’s t-test was used for comparisons between two groups. The significance level for statistical comparison was $P < 0.05$, and also was adjusted for multiple comparison.

**ACKNOWLEDGEMENTS**

This work was supported by USPHS grants NS01698 (J.M.P. and D.W.), NS37949 and RR00645 (D.C.D.), the Will and the Colleen Glidden Foundations (D.C.D.), NS29709 (J.L.N.) and HD24064 (EEG Core of BCM-MRRC). Ronald L. Van Heertum, Chitra M. Saxena and John H. Kim facilitated the PET studies. Umrao Monani provided valuable discussion and advice regarding embryo studies. Funding to pay the Open Access publication charges for this article was provided by The Will Foundation.

**Conflict of Interest statement.** None declared.

**REFERENCES**


