



Behavioral Characterization of GLT1 (+/-) Mice as a Model of Mild Glutamatergic Hyperfunction

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GLT1 is one of the major transporters responsible for maintenance of glutamate homeostasis in the brain. In the present study, glutamate transporter 1-deficient GLT1 homozygous (-/-) and heterozygous (+/-) mice were investigated with the intention that they may provide a model of hyperglutamatergic state resulting in various behavioral alterations. The GLT1 (-/-) mice had lower body and brain weight, mild neuronal loss in CA1 hippocampal region as well as focal gliosis and severe focal neuronal paucity in layer II of the neocortex. The short life-span of GLT1 (-/-) precluded us from systematic behavioral studies in these mice. In contrast, GLT1 (+/-) mice exhibiting a 59% decrease in GLT1 immunoreactivity in their brain tissue, showed no apparent morphological brain abnormalities, and their life-span was not markedly different from controls. Behaviorally, GLT1 (+/-) presented moderate behavioral alterations compared to their wildtype littermates, such as: mild sensorimotor impairment, hyperlocomotion (at 3 month of age only), lower anxiety (at 6 months), better learning of cue-based fear conditioning

but worse context-based fear conditioning. Our results suggest that GLT1 (+/-) mice may serve as a potentially useful model to study neurodegenerative disease conditions with mild hyperglutamatergic activity.

Keywords: GLT1 KO mice; GLT1 (+/-) mice; Glutamate uptake; Histology; Immunocytochemistry; Behavior

INTRODUCTION

The maintenance of glutamate homeostasis is crucial for normal brain physiology since its hypofunction or hyperfunction can result in detrimental consequences. The former has been associated with learning deficits and psychotomimetic activity (Collingridge, 1987; Carlsson and Carlsson, 1990; Danysz *et al.*, 1995b) while the latter, with excitotoxic neuronal death resulting from overactivation of glutamate ionotropic receptors (Rothman and Olney, 1987; Meldrum and Garthwaite, 1990; Danysz and Parsons, 2002). Therefore, very precisely balanced control of glutamate release and

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uptake assures the physiological optimum. There are 5 glutamate transporters, out of which three Na⁺-dependent carriers play a major role in the mouse brain, namely, GLAST - equivalent to EAAT1 (excitatory amino-acid transporter 1) in humans, which is present on glia; GLT1 - equivalent to human EAAT2, present both on neurons and glia, and rEAAC1 - equivalent to EAAT3 in humans, present on neurons (Nicolls and Attwell, 1990; Seal and Amara, 1999; Tanaka, 2000; Danbolt, 2001). Under physiological conditions, GLT1 plays the key role in preventing extrasynaptic glutamate spill-over throughout the brain, except for the cerebellum and retina where GLAST predominates (Tanaka, 2000; Danbolt, 2001). Manipulation of these transporters in animals offers a unique possibility of investigating various disease conditions believed to be connected with either deficit or excess of glutamate.

Pathologies related to excessive levels of glutamate seem to be especially interesting, as they have been implicated in both acute insults, such as either brain ischemia or traumatic brain injury (Benveniste *et al.*, 1984), and chronic neurodegenerative conditions such as epilepsy (Czuczwar and Meldrum, 1982; Meldrum, 1994), Parkinson's disease (Schmidt *et al.*, 1990; Turski *et al.*, 1991), Huntington's disease (Schwarcz and Köhler, 1983), amyotrophic lateral sclerosis (ALS) (Rothstein, 1995), Alzheimer's disease (Greenamyre *et al.*, 1988), and AIDS (HIV infection) (Lipton, 1992) (see Danysz *et al.*, 1995a).

In the above-listed disorders, the general role of glutamate in the pathomechanism of neurodegeneration has been suggested; however, there are indications that a deficit in certain glutamate carriers may be a direct causal factor in some conditions. For example, the most convincing evidence exists for ALS, where a decrease in high affinity Na⁺ dependent glutamate transporter, later defined as GLT1 (Rothstein *et al.*, 1995), has been observed in affected patients (Rothstein *et al.*, 1992) and in SOD1(G93A) mice (Pardo *et al.*, 2006). Similar changes in GLT1 levels have been suggested to occur in Alzheimer's disease (Masliah *et al.*, 1996; Zoia *et al.*, 2004).

It should be stressed that we do not feel that GLT1 could be a model of particular disease, it is rather a model of malfunctioning of glutamate

homeostasis that could be one of the factors contributing to the pathomechanism in several diseases as mentioned above.

As GLT1 plays a major role in glutamate homeostasis (Tanaka, 1993; 2000), its knock-out in mice may serve as a valuable model of glutamatergic disturbances that frequently accompany human brain diseases, as discussed above (Tanaka *et al.*, 1997b). Homozygous GLT1 mice (-/-) lack GLT1 immunoreactivity, and exhibit significant phenotypes after postnatal week 3 (Tanaka *et al.*, 1997a) including decreased weight gain, decreased survival, alterations in EEG indicative of epileptic activity, spontaneous neuronal degeneration occurring in the hippocampal CA1 region, and enhanced susceptibility to injury as evidenced by enhanced edema after cold-induced injury (Tanaka *et al.*, 1997a). In addition, hippocampal slices from GLT1 (-/-) mice show an impairment in LTP induction (Katagiri *et al.*, 2001). These findings indicate that non-contingent, constant elevation of glutamate levels may impair synaptic plasticity phenomena, (see Danysz and Parsons, 2003).

Significantly decreased survival rates in GLT1 (-/-) mice precluded us from performing comprehensive behavioral tests in these mice. Therefore, in the current study we investigated the behavioral alterations in GLT1 heterozygous (+/-) mice which can model relatively mild hyper-glutamatergic conditions thought to underlie several chronic neurodegenerative conditions including Alzheimer's disease.

METHODS

Subjects

The Animal House in the Polish Academy of Sciences Medical Research Center received 6 GLT1 +/- mice, 3 females and 3 males. They were cross mated. In our study we used mice from the F1 generation, 102 wild-type (WT), 94 GLT1 heterozygotes (+/-) and 22 GLT1 homozygotes (-/-); 104 of them were females and 114 males. The animals used for analysis of body weight, brain weight and pathology were 1-2 months old. All animals, experimentally naive before testing, were housed under a 12-h light/dark cycle, with water and food provided *ad libitum*. Behavioral testing took place during the light cycle. WT ($n=20$), GLT1 (+/-($n=20$) and GLT1 (-/-($n=20$) mice were anesthetized by isoflu-

ranum inhalation (AErrane, Baxter) and the following parameters were estimated: body size, body weight, brain size and fresh brain mass. Afterwards, the brains were subjected to further histological analysis. Approval of all the behavioral procedures preformed, was given by the First Warsaw Animal Ethics Committee.

Western Blot Analysis

For Western blot analysis, plasma membrane-enriched fractions of the whole brains of WT and GLT1(+/-) mice (10 µg) were separated by 5-20% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose and analyzed with the enhanced chemiluminescence (ECL) detection system. An affinity-purified rabbit polyclonal antibody against mouse GLT1 was used (Tanaka *et al.*, 1997a). To quantify changes in expression of each glutamate transporter, densitometry of the immunoblots was used (Ueda *et al.*, 2002). Each blot was within the range of standards of membrane fraction of the normal mouse cerebellum (7.5-60 µg) that were used to generate a standard curve. The linearity of the relationship between optical density and protein concentration was verified on each film. The transferred blotted membrane was stained with Ponceau-S to verify that the lanes were loaded with equivalent amounts of protein.

Immunocytochemistry

The brains were fixed by immersion in 4% paraformaldehyde solution (in 0.1 M phosphate buffered saline (PBS), pH 7.4) for 24 h at 4°C, cryoprotected in 30% sucrose, frozen on dry ice, and sectioned at 40 µm in a cryostat. The sections were collected free-floating in PBS containing 0.1% sodium azide and stored at 4°C until needed. Four markers were used: Nissl staining - to assess the general cytoarchitectonic features; an antibody against NeuN (neuron-specific nuclear protein) - a neuronal marker; an antibody against GFAP (glial fibrillary acidic protein) - an astrocytic marker, and fluorescently labeled isolectin B4 from *Griffonia simplicifolia* (Molecular Probes) - a marker of microglia that recognizes specific carbohydrate moieties on the surface of microglial cells. The Nissl staining was done according to routine procedures. The immunocytochemical reactions were

performed using free-floating sections that were incubated overnight at 4°C with mouse anti-GFAP (1:200; Chemicon) or mouse anti-NeuN (1:200; Chemicon) antibodies or with Alexa-Fluor 488-conjugated isolectin B4 from *Griffonia simplicifolia*. The immunoreactions were detected using appropriate species-specific biotinylated secondary antibodies followed by avidin-peroxidase (Vector) and DAB (diaminobenzidine, Sigma) as a chromogen. The sections were mounted on slides, air dried, and either dehydrated in ethanol solutions and xylene, and coverslipped with Entellan (Merck) or mounted directly in Vectashield antifade medium for fluorescence (Vector). The sections were analyzed and photographed using bright-field optics of the Olympus microscope equipped with a digital camera. Fluorescent confocal images were obtained using a TCS SP2 microscope from Leica.

Behavioral Testing

Two WT and two GLT1 (-/-) mice at an age of about two months were used to assess general motor function in the hindlimb extension reflex. Twenty four young (three months old; 12 WT/12 GLT1(+/-) and 24 older (6 months old; 12 WT /12 GLT1 (+/-)) mice were tested in Sensorimotor tasks, Open Field and Elevated Plus Maze. WT ($n=43$) and GLT1 (+/-) ($n=40$) were submitted to IntelliCage tests, and WT ($n=13$) and GLT1 (+/-) ($n=10$) mice to Fear Conditioning test.

The sequence of behavioral testing was: Neurological and Sensorimotor tests (to assess coordination, climbing, locomotion and orienting reactions), Open Field test (to measure locomotor and exploratory activity), Elevated Plus Maze test (to measure anxiety-like behavior), IntelliCage tests (to measure place preference and place avoidance learning and memory) and Fear Conditioning tests (to measure cue and context learning).

Neurological and Sensorimotor Tests

The Hindlimb Extension Reflex

Hindlimb and forelimb claspings are often observed in transgenic lines with motor dysfunction or degeneration. GLT1 (-/-) mice were analyzed for limb claspings by suspending them by the tail for 1 min. A claspings event was defined as a retraction of either or both hind limbs into the body and toward the midline.

Sensorimotor Tests

A battery of sensorimotor tasks was performed for three successive days. In each test, the time was measured with the criterion of 120 s to complete, and all tasks were given once a day. The mice were subjected to: *1. Walking Initiation.* Mice were placed on a floor where 2 circles were drawn, a smaller (8 cm in diameter) inside a bigger (28 cm in diameter). Mice started walking from the smaller circle and the time from crossing the border of the first circle to crossing the border of the bigger circle by forelimbs was recorded. *2. Bridges.* The flat bridges (50 cm long) were wooden planks of three different widths: 1, 2 and 4 cm. The round bridges were dowels of three different diameters: 0.5, 1 and 3 cm. Each mouse was placed in the middle of a bridge suspended between two platforms, 50 cm above a foam cushion. The latency to reach the platform was measured. In case the mouse fell, its time was taken as 120 s. *3. Wire Suspension.* The mouse was suspended by front limbs on a horizontal wire extended between two platforms, 50 cm above a foam cushion on the floor. The time to fall from the wire was measured. *4. Turning in an Alley.* The time taken by the mouse to turn around in an alley (3 cm wide with walls 13 cm high) was measured. The mouse was placed facing the back wall of an alley and turned to the open end of the alley. *5. Turning on an Inclined Screen.* The mouse was placed facing downward on the center of an inclined screen and the time taken by the mouse to turn to face upward was measured. The screen was a horizontal wire mesh (20 x 20 cm square), placed 50 cm above the table at 45° angle.

The Open Field Test

The apparatus was constructed of a wooden floor (59.5 x 59.5 cm) and walls (34 cm high) painted grey. The experimental room was illuminated by an evenly focused dim light (40-W bulb) to avoid direct lighting of the open field. The walls of the open field formed a comparatively homogenous environment by blocking the view of the room. The animals' behavior was monitored by a video camera placed above the center of the apparatus to provide a top view of the animal. The mice were put individually in one corner of the open field facing the center and were allowed to explore freely for 5 min. The floor of the apparatus was cleaned with ethanol

after each session to remove odor cues.

To analyze the behavior, the open field apparatus was divided into 3 virtual zones: thigmotaxic (10 cm wide), middle (10 cm wide) and center (19.5 cm wide). Data were analyzed by an EthoVision system (Noldus Information Technology) and the following parameters were counted: the latency to the first occurrence in each zone, the total time spent in each zone, the frequency of passing through each zone, the total distance moved, the maximum distance moved without stopping, the duration of movement and stops.

The Elevated Plus Maze Test

The elevated plus maze apparatus was composed of wood painted grey and placed 63 cm above the floor. The apparatus consisted of four arms of equal size (30 cm x 5.5 cm): two opposing arms were enclosed by 17-cm high walls and the other two arms were surrounded by 0.5 cm high transparent tape. The walls were connected by a common central square platform (5.5 cm). The Elevated Plus Maze test was performed in the same room conditions as the Open Field test. The mice were put individually in the center of the plus maze facing the open arms and were allowed to explore freely for 5 min. The floor of the apparatus was cleaned with ethanol after each session to remove odor cues.

The plus maze apparatus was divided virtually into 3 zones: closed arms, open arms and center. In each of the zones, the behavior was analyzed by EthoVision considering the latency of the first occurrence in each zone, the total time spent and the frequency of passing through each zone.

The IntelliCage Tests

One week before the experiment, mice were exposed to isoflurane anesthesia and injected with glass-covered microtransponder (11.5 mm length, 2.2 mm diameter; Trovan, ID-100) with a unique code. Then, the animals were housed in groups in the experimental room for several days before starting adaptation to the cage. In the main battery of tests, GLT1 (+/-) and WT mice were first subjected to the IntelliCage placed inside a large standard rat cage: 20.5 cm high x 40 cm x 58 cm at the top and 55 cm x 37.5 cm at the base. In each corner, a triangular learning chamber was located with 2 bottles of

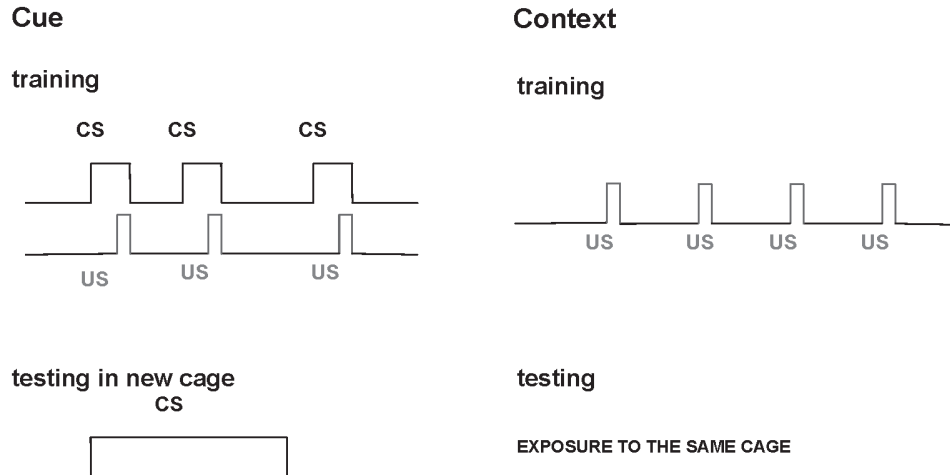


FIGURE 1 Cue and context conditioning scheme. Conditioned stimulus (CS) indicates acoustic stimuli signaling a footshock (US).

water. To drink, only one mouse at the same time could go inside a plastic ring (outer - 50 mm diameter, inner - 30 mm diameter, 20 mm deep into the outer ring) which ended with two 13 mm holes (one on the left, one on the right side) giving access to water-bottle nipples. After 48 h with free access to water in all corners (Simple Adaptation), the holes were closed by small motorized doors and mice learned to open the gates by nosepokes (Nosepoke Adaptation). Then, two learning tests were performed: *Place Preference* and *Place Avoidance*. In the *Place Preference* test, mice received sugar water (10% sucrose) in one of the learning chambers. Mice were supposed to associate the sugar water with a specific corner. The corner with sugar water was the least preferred corner during the Nosepoke Adaptation. In the *Place Avoidance* test the mice received an air-puff (1 bar) when entering the corner that was the most preferred during the *Place Preference* test. Mice were relearning to avoid the corner where they were subjected to the air-puffs.

The learning skills to associate one corner with sugar water (*Place Preference*) and the ability to relearn to avoid one corner after receiving air-puffs (*Place Avoidance*) measured as a percentage of correct nosepokes to all nosepokes were analyzed by IntelliCage software.

The Context and Cue Fear Conditioning

One week after completing the IntelliCage procedure, the mice were divided into two groups and subjected to Fear Conditioning (FIG. 1), one

group for cue conditioning, the other for contextual conditioning.

Behavioral training was performed in two experimental chambers (25 cm width x 25 cm depth x 25 cm height, Med Associates Inc.), housed in a sound- and light-attenuating box. Foot-shocks (0.7 mA) were delivered by the grid floor (0.3-cm-diameter bars at 1 cm intervals). A 70 dB wide-band noise was emitted from a speaker placed under the grid floor. The chambers were connected with a computer system (located in an adjacent room) monitoring the behavior of the mice. Testing was performed in the same experimental chamber in Context Conditioning or in the new chamber in Cue Conditioning (FIG. 1). The new chamber had a different floor and walls.

Statistics

Results are expressed as mean \pm SEM and analyzed by Student's *t*-test (two groups) or in case of three or more groups, by suitable ANOVA (one way, two way or repetitive measures) followed, if significant, by a *post-hoc* test. The exact procedure is indicated in the respective figure legends.

RESULTS

The Western blot analysis of total brain homogenates prepared from GLT1 (+/-) mice revealed a $59 \pm 5\%$ decrease in GLT1 protein expression compared to the wild-type levels (FIG. 2). Significant differences were also noted for average brain (see below) and body weights between the genotypes

(FIG. 3). GLT1 (-/-) mice had significantly lower, by more than 40%, body weight in comparison to the GLT1 (+/-) and WT mice ($p < 0.001$, One-way ANOVA, $F_{(2,57)}=221.4$, followed by Tukey's test) and their brains were smaller by approximately 40% as well. Brain weight accounted for 2% of body weight independently of the genotype. In particular, for the WT, average body weight was 21.2 g and average brain weight - 0.45 g, for the GLT1 (+/-) - 19.6 g and 0.4 g and for GLT1 (-/-) - 11.6 g and 0.25 g, respectively.

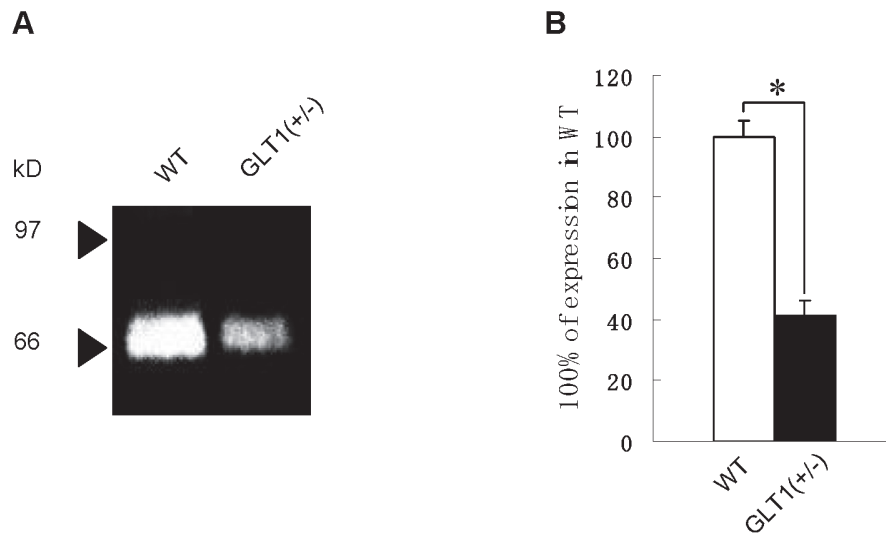


FIGURE 2 Comparison of the expression of GLT1 protein between WT and GLT1 (+/-) mice. (A) Western blot of membrane enriched fraction of brain tissues from WT and GLT1 (+/-) mice using the antibody to GLT1. (B) Densitometric analysis of the immunoblot. Each bar represents the mean (\pm SEM) of the relative density of each band ($n=3$), $*p < 0.005$ (Student *t*-test).

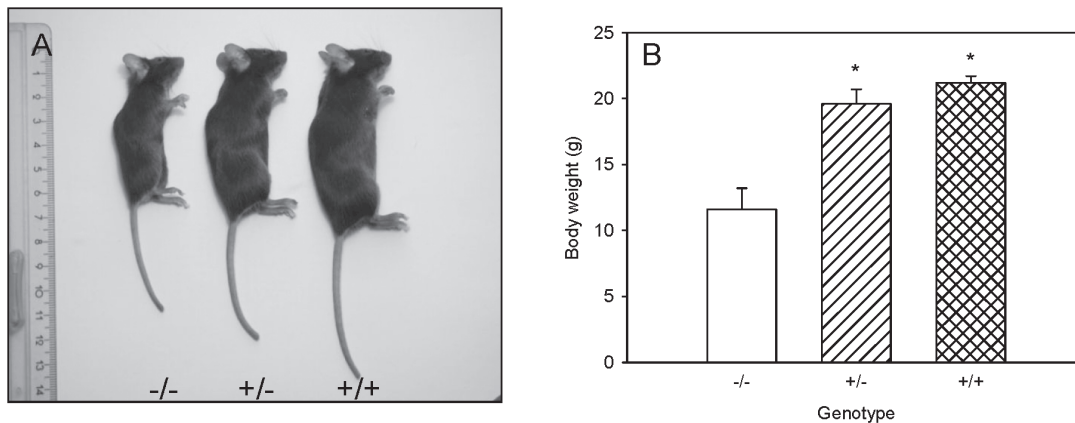


FIGURE 3 A. Comparison of body size between WT, GLT1 (+/-) and GLT1 (-/-) at age about 30 days showed growth retardation of GLT1 (-/-). GLT1 (+/-) did not differ substantially in body and brain weight from WT mice. B. Differences in body weight between genotypes. GLT1 (-/-) mice gained the smallest body weight. Values are means \pm SEM, $*p < 0.001$, One Way ANOVA ($F_{(2,57)}=221.4$) followed by Tukey's test.

In agreement with the report of Tanaka and colleagues (1997a) we also found a moderate decrease in the cell density and number in the hippocampal CA1 field of GLT1(-/-) mice, as shown by Nissl-staining (FIG. 4A). In addition, in all GLT1 (-/-) animals the staining for astro- and microglial markers revealed focal gliosis in this area. The thickness and the general cytoarchitectonic organization of the neocortex in GLT1 (-/-) and GLT1 (+/-) mice were similar to that of WT mice. However, both Nissl (not shown) and NeuN (FIG. 4B) staining of

GLT1 (-/-), but not GLT1 (+/-), brains revealed striking, focal neuronal paucity that affected mainly layer II of the neocortex. The foci containing little or no neurons had either a round or oval shape and a diameter up to 0.1 mm (FIG. 4B). By immunoreaction for astrocytes (GFAP), these foci corresponded to the areas of astrogliosis (FIG. 4C). In addition, the staining with isolectin B4 revealed that these areas either contained or were surrounded by the prominent microglial infiltrates (FIG. 5). The foci of gliosis also occurred outside the neocortex (*e.g.*, in the hippocampal formation), however, at those sites, they did not appear to be associated with a pronounced neuronal loss (FIG. 6).

The average life-span of GLT1 (-/-) mice was 23 days precluding their use for systematic behavioral experiments. Neurological examination (the hind limb extension reflex) revealed advanced motor dysfunction of GLT1 (-/-) mice. Wild-type and GLT1 (+/-) mice straightened their legs out while GLT1 (-/-) mice clasped during tail suspension. When held by the tails, GLT1 (-/-) retracted head

and limbs toward the abdomen in a dystonic fashion rather than extending them as did GLT1 (+/-) and WT littermates. The four tested groups (3-months old: WT and GLT1(+/-), 6-months old: WT and GLT1(+/-)) completed all the sensorimotor tasks, although 6-month old GLT1 (+/-) mice achieved worse times in walking initiation than their wild-type siblings, disclosing a slight deterioration in orientation in space and locomotion. The bridge task covering was impaired by age, regardless of the genotype.

Three-month old GLT1 (+/-) mice demonstrated an increased locomotion in a new environment when compared with WT mice. Sensorimotor tests showed no impairment in locomotor activity of 3-month old GLT1 (+/-) mice, however in the open field test, periods of hyperlocomotion were observed that were measured as the maximum distance traveled without stopping (FIG. 7). The animals during the hyperlocomotion appeared aimless and lacking in orientation. Notably, these periods of hyperlocomotion occurred only in young GLT1 (+/-) mice but

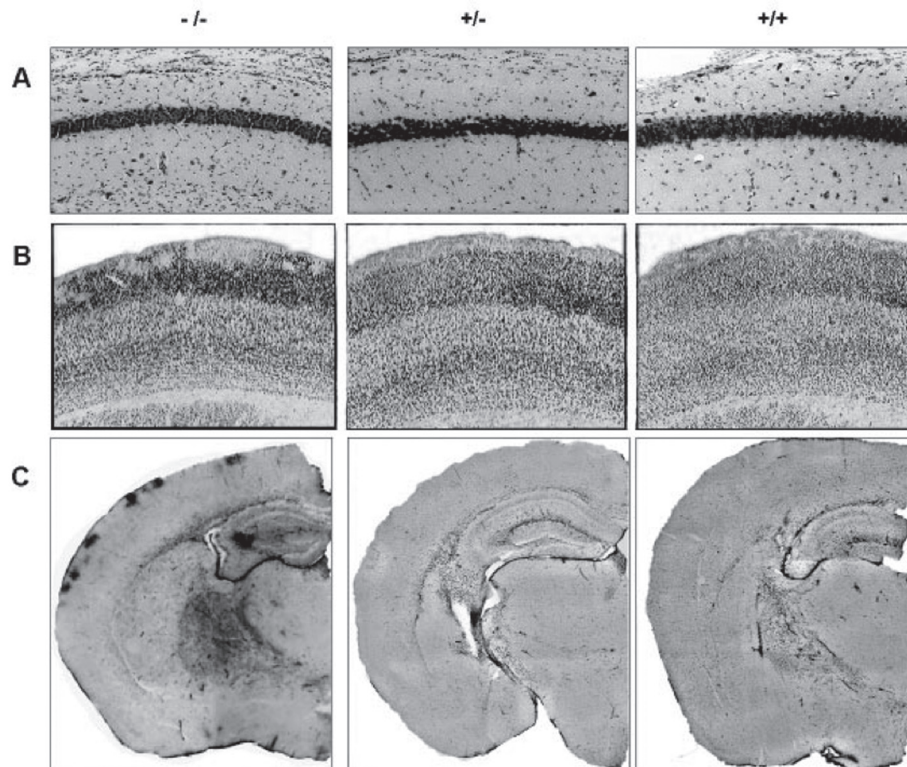


FIGURE 4 Hippocampal CA1 atrophy in GLT1 (-/-) mice (A - Nissl staining). There is no generalized cortical atrophy. In the neocortex of GLT1 (-/-) but not GLT1 (+/-), there is focal neuronal loss (B - NeuN immunoreactivity) that is accompanied by focal gliosis (C - GFAP staining). The gliosis occurs also in other brain structures.

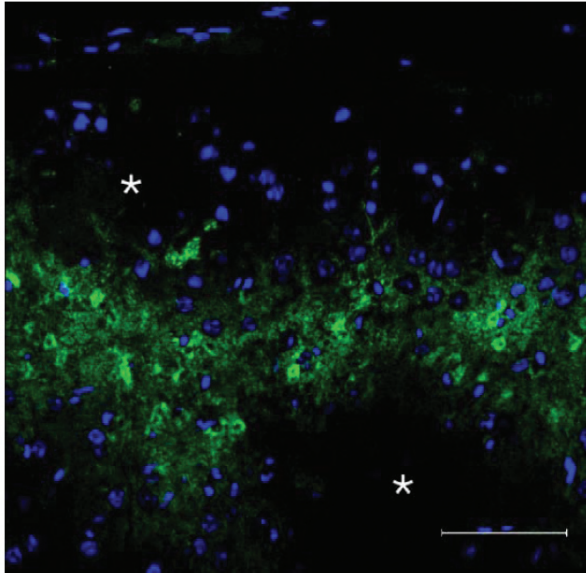


FIGURE 5 Isolectin B4 staining in GLT1 (-/-) neocortex. Microglial cells (green) form dense infiltrate surrounding the area of the apparent neuronal paucity (asterisks). Cell nuclei are stained with DAPI (blue). Bar, 75 μ m.

not in 6-month old GLT1 (+/-) mice.

In the Elevated Plus Maze test, mice typically explore aversive open arms only after becoming familiar with the new environment. As the test revealed, 6-month old GLT1 (+/-) mice entered open arms after very short latency from beginning the test when compared with wild-type mice (FIG. 8).

In the Place Preference paradigm, learning skills were measured as a percentage of nose-pokes in the corner with sugar water (correct nose-pokes) to the total number of nose-pokes in all corners. During the first 24 h, WT and GLT1 (+/-) mice achieved a similar percentage of correct nose-pokes (FIG. 9). In the Place Avoidance task, the ability to relearn to avoid one corner was measured as a percentage of nose-pokes performed in the corner with a punishment, to the total number of nose-pokes in all corners of the cage. The percentage of incorrect nose-pokes was not different between wild-type and GLT1 (+/-) mice during first 24 h of the Place Avoidance test (FIG. 9).

Both WT and GLT1 (+/-) groups acquired the

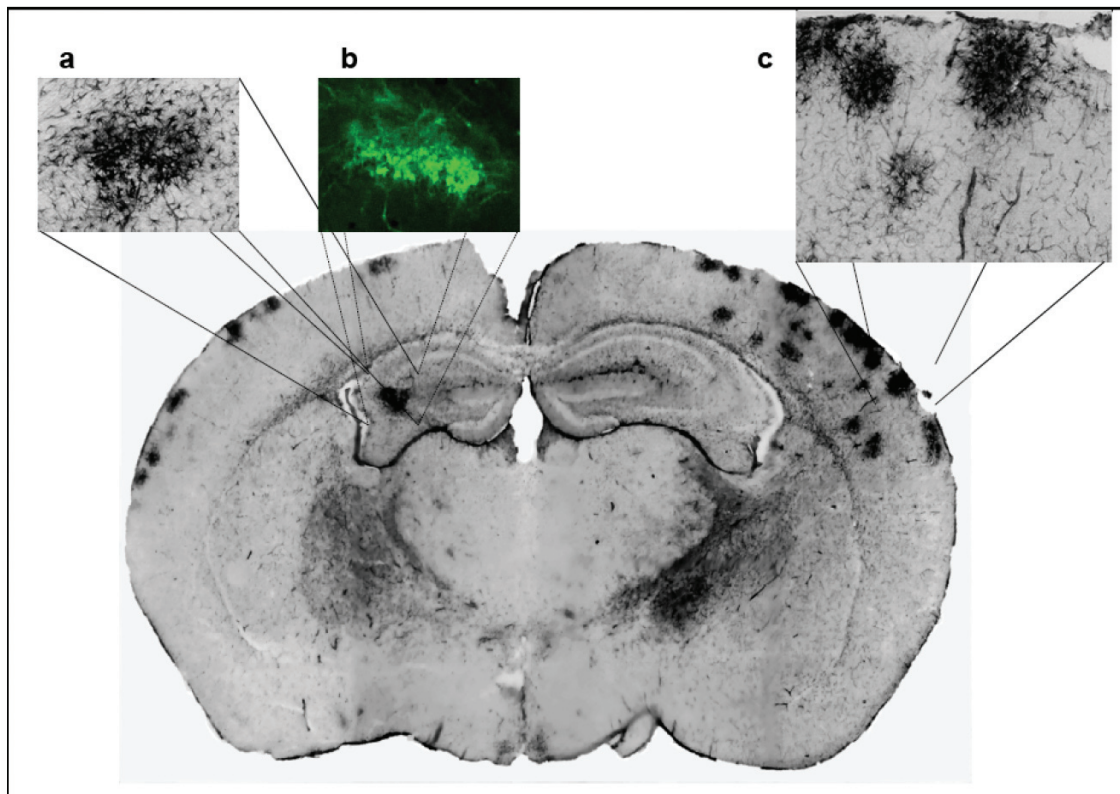


FIGURE 6 The foci of gliosis in hippocampus (a, GFAP staining; b, Isolectin B4 staining) and in the cortex (c) of GLT1 (-/-) mice.

cue- and context-associated fear. In cue conditioning, during the day of adaptation, all tested mice had a similar low level of freezing in the first minute of noise. At 24 h after the training, the level of freezing was checked during the first minute of noise. The rates of freezing were greater in GLT1 (+/-) mice as compared to WT mice (FIG. 10A). One-way ANOVA with repeated measures followed by a Duncan *post-hoc* test showed interaction between genotype and the level of response to the

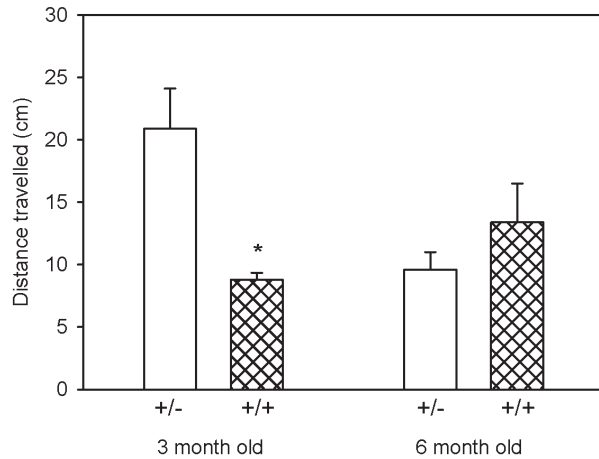


FIGURE 7 The Open Field test. 3-month old GLT1 (+/-) mice moved significantly longer distance without stopping than their wild-type siblings. * $p=0.002$, One Way ANOVA ($F_{(1,21)}=12.80$). Values are means \pm SEM, $n=12$.

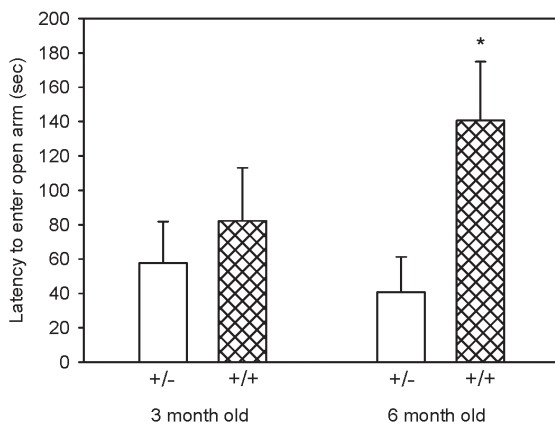


FIGURE 8 The Elevated Plus Maze. 6-month old GLT1 (+/-) mice had three times shorter latency to the first entrance to open arms than wild-type mice. * $p=0.021$, One Way ANOVA ($F_{(1,20)}=6.27$). Values are means \pm SEM, $n=12$.

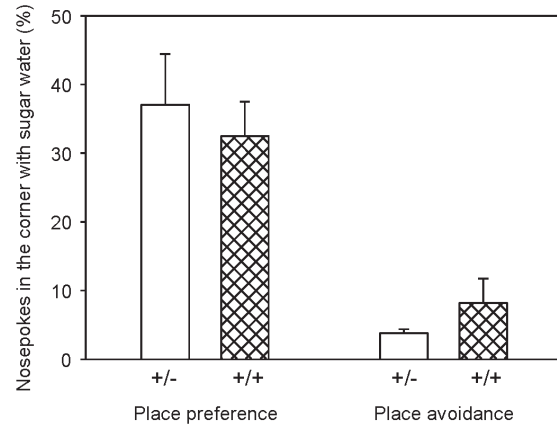


FIGURE 9 The IntelliCage tests. No significant difference between GLT1 (+/-) and wild-type mice in learning (Place Preference) and relearning tests (Place Avoidance). Place Preference, $p=0.6$, One Way ANOVA ($F_{(1,10)}=0.26$), Place Avoidance, $p=0.2$, One Way ANOVA ($F_{(1,10)}=1.55$). Values are means \pm SEM, $n=6$.

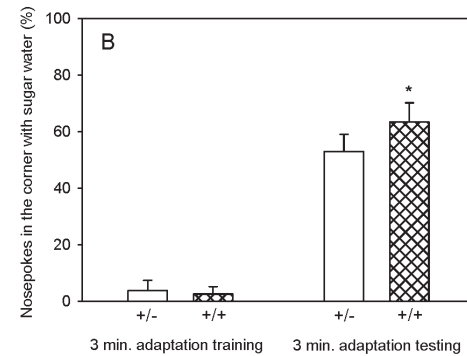
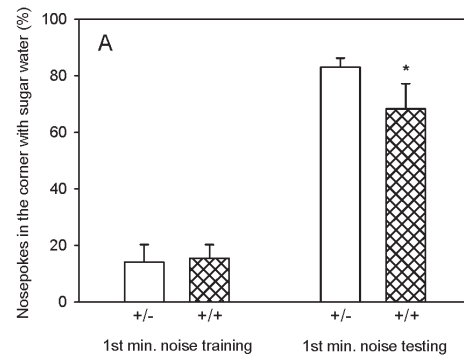


FIGURE 10 Both WT and GLT1 (+/-) groups acquired the cue and context associated fear. However, in the first minute of testing, GLT1 (+/-) mice displayed a slightly stronger freezing response to the acoustic stimulus (A) and slightly lower to the context (B) in comparison to the WT mice. Values are means \pm SEM., * $p < 0.05$ (One Way ANOVA ($F_{(1,9)}=6.43$) with repeated measures followed by a Duncan test).

acoustic stimulus during the first minute of testing: $F_{(1,10)}=4.88$; $p = 0.05$. In the context conditioning test carried out 24 h after the training, the freezing response in GLT1 (+/-) was less frequent as compared to WT mice (FIG. 10B). One-way ANOVA with repeated measures followed by a Duncan test revealed interaction between genotype and the level of response to the context during first 3 minutes of testing: $F_{(1,9)}=6.44$; $p = 0.03$.

DISCUSSION

Present results confirm previous data indicating severe disturbances in mice lacking GLT1 glutamate transporter (Tanaka *et al.*, 1997a; Tanaka, 2000; Katagiri *et al.*, 2001; Mitani and Tanaka, 2003). GLT1 (-/-) mice showed growth retardation and reduced body weight gain compared to GLT1 (+/-) and WT. The life span of GLT1 (-/-) mice was significantly shorter than WT mice. There was apparent hippocampal CA1 atrophy in GLT1 (-/-) mice, as observed in the previous findings (Tanaka *et al.* 1997a), but no generalized cortical atrophy. In the GLT1 (-/-), but not GLT1 (+/-) neocortex, there was focal neuronal loss accompanied by gliosis, which was also observed in other brain structures.

Sensorimotor tests revealed that GLT1 (-/-) mice displayed feet-clasping, while the performance of all sensorimotor tests did not differ between 3-month old GLT1 (+/-) and wild-type mice. In other behavioral tests only GLT1 (+/-) but not GLT1 (-/-) mice were tested, since the lifespan of GLT1 (-/-) mice did not allow for the accumulation of a sufficient number of surviving subjects. The IntelliCage test failed to reveal any difference between WT and GLT1 (+/-) mice in reaction to novelty, general activity, exploration rate, circadian activity, ability of learning and relearning. However, the results presented herein showed some differences in selected aspects of behavior. In the fear-conditioning test, both GLT1 (+/-) and wild-type mice acquired the cue and context associated fear, but in the first minute of testing, GLT1 (+/-) mice displayed a greater freezing response to the acoustic stimulus. However, the contextual freezing response in GLT1 (+/-) mice was smaller than in wild-type mice. These differences may appear surprising at

first glance, however, it should be kept in mind that these two types of learning cue versus context have different substrates and involve different brain regions. For example, cue-based learning is generally regarded as hippocampus independent (Phillips and LeDoux, 1992) and both types may involve different neurotransmitter receptors (Gravius *et al.*, 2006).

GLT1 (+/-) mice also showed hyperlocomotion in a new environment compared to WT. As was demonstrated by others (Eilam *et al.*, 2003) spatial navigation requires an animal to integrate its path of movement with visual and olfactory cues. In wild-type mice this is expressed as stops in different places of the field in a changeable sequence (Eilam *et al.*, 2003). In the present study we found that 3-month old GLT1 (+/-) mice had significantly longer episodes of movement than the older GLT1(+/-) and wild-type mice.

Behavior of GLT1 (+/-) mice also differed from that of WT siblings in the elevated plus maze test measuring the general level of anxiety. For the wild-type mice, exploration of aversive open arms comes after risk assessment, which involves examining the open arms from the closed arms or the centre without moving. The 6-month old GLT1 (+/-) mice had a significantly shorter latency to the first entrance to the open arms of elevated plus maze than wild-type mice. These mice showed a reduced level of anxiety and a lack of risk assessment. Interestingly, similar changes to that observed in GLT1 (+/-) mice can be pharmacologically induced in wild-type animals through hyperlocomotion-induced by drugs like amphetamine or phencyclidine (PCP) (Fradley *et al.*, 2005).

It is possible, however, that GLT1 (+/-) mice are not hyperactive per se, but show hyperactivity in a new environment due to a reduced level of anxiety and/or habituation. Interestingly, mice lacking the D₃ dopamine receptor show a phenotype similar to GLT1 (+/-) mice. They are characterized by hyperactivity (Accili *et al.*, 1996), and a reduced level of anxiety (Steiner *et al.*, 1997).

The main objective of the present study was to characterize GLT1 (+/-) as a possible model of mild glutamatergic hyperactivity that may take place in chronic neurodegenerative diseases, for example, Alzheimer's disease (Greenamyre,

1991). It is therefore interesting to note that transgenic mice overexpressing mutant amyloid precursor protein appear to have reduced GLT1 and GLAST protein levels (Masliah *et al.*, 2000). Such direct change in GLT1 or its inhibition by A β or oxidative stress could be one of the factors contributing to glutamatergic hyperactivity, resulting in short term plasticity impairment (Katagiri *et al.*, 2001), and in long term neuronal cell loss (Harris *et al.*, 1996; Noda *et al.*, 1999; Danysz and Parsons, 2003). However, changes in GLT1 (+/-) mice reported here, indeed mild, may indicate the presence of an efficient compensatory mechanism even in conditions of over 50% decrease in density of GLT1 transporters.

Whether glutamate transporters actually play a role in termination of excitatory postsynaptic potentials (EPSPs) is questionable. Since such transporters are located on neurons and glia outside the synapse, they may prevent glutamate spill-over to metabotropic autoreceptors or extrasynaptic ionotropic receptors. In fact, it has been calculated that after release, glutamate levels within synaptic cleft falls to low levels in 500 ms (Danbolt, 2001). It is, therefore, not clear what is the mechanism of behavioral alterations in GLT1 (+/-). For example, secondary decreases in glutamate release could be due to stronger activation of inhibitory mGluR autoreceptors; or activation of, *e.g.*, extrasynaptic NR2B-containing NMDA receptors (Li *et al.*, 1998). A rather minor role of GLT1 has been also recently confirmed in cerebellum, studying kinetics of EPSPs in climbing fibers using GLT1 and GLAST KOs (Takatsuru *et al.*, 2007). Nevertheless, whatever the specific mechanisms of behavioral disturbances of GLT1 (+/-) mice are, the present study may suggest indirectly the role of extrasynaptic receptors in modulation of behavior.

In conclusion, given the mild behavioral alterations in GLT1 (+/-) mice, they may offer an excellent model for studying how other factors such as A β , oxidative stress, inflammation, tau phosphorylation etc. work in concert with modest impairment of glutamate homeostasis to trigger neurodegenerative changes. One of the ways may be to cross GLT1 (+/-) with transgenic mice served to model such neurodegenerative diseases as Alzheimer disease, Parkinson's disease, or ALS.

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