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Functional roles of the glial glutamate transporter (GLAST) in emotional and cognitive abnormalities of mice after repeated phencyclidine administration

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Abstract
Alterations of the glutamatergic system components, including *N*-methyl-d-aspartate (NMDA) receptors are relevant to the pathophysiology of schizophrenia. Repeated phencyclidine (PCP) administration induces several schizophrenia-like psychobehavioral abnormalities and decreases extracellular glutamate levels, which are associated with increased levels of glial gluta-

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mate and aspartate transporter (GLAST) in the prefrontal cortex (PFC) of mice. In the present study, we investigated the functional roles of GLAST in the emotional and cognitive abnormalities in mice following repeated PCP administration by using GLAST heterozygous (+/−) mice, since GLAST mutant mice are a useful tool for elucidating the contribution of glutamate dysfunction to the pathophysiology of schizophrenia. PCP-administered GLAST wild-type (+/+) mice showed enhancement of immobility in a forced swimming test, impairments of visual recognition memory in a novel object recognition test, decrease in high potassium (K^+)-induced extracellular glutamate release, and overexpression of GLAST and S100 proteins in the PFC, compared to saline-administered GLAST$^{+/+}$ mice. Such behavioral and neurochemical abnormalities were not observed in PCP-administered GLAST$^{+/-}$ mice. In conclusion, these results clearly suggest that genetic GLAST dysfunction and glial activation play important roles in the development of emotional and cognitive abnormalities in PCP-administered GLAST$^{+/+}$ mice.

1. Introduction

It has been hypothesized that dysfunction of the glutamatergic system is involved in the pathophysiology of psychiatric disorders, such as schizophrenia and mood disorders (Carlsson et al., 1997; Carlsson et al., 1999; Cui et al., 2014; Frankle et al., 2003; Kiselycznyk et al., 2011). Abundant pharmacological evidence has demonstrated that *N*-methyl- d-aspartate (NMDA) receptors are implicated in the pathophysiology of schizophrenia (Carlsson et al., 1999; Noda et al., 2009). Phencyclidine (PCP), a non-competitive NMDA receptor antagonist, has been shown to induce schizophrenia-like psychoses representing positive symptoms, negative symptoms, and cognitive impairments in humans (Javitt and Zukin, 1991). Thus, PCP-administered animals have been utilized as animal models of schizophrenia (Enomoto et al., 2005; Jentsch et al., 1997a, 1997b; Mandillo et al., 2003). These animals exhibit hyperlocomotion as an index of positive symptoms (Noda et al., 2009) and social behavioral impairments in a social interaction test and enhanced immobility in a forced swimming test as indices of negative symptoms (Noda et al., 1995a, 1997). They also show sensorimotor gating deficits and cognitive impairments in several learning and memory tests (Hida et al., 2014b; Mouri et al., 2007; Nagai et al., 2009). Some of the behavioral abnormalities, such as negative-like behaviors and/or cognitive impairments after withdrawal from repeated administration of PCP, appear to be sensitive to second-generation antipsychotics, in agreement with the clinical findings, which improve the negative symptoms and the cognitive impairments in schizophrenia (Nabeshima et al., 2006; Nagai et al., 2009; Noda et al., 1997; Zhang et al., 2013).

Glial glutamate and aspartate transporter (GLAST; excitatory amino-acid transporter 1) and glial glutamate transporter-1 (GLT1; excitatory amino-acid transporter 2) are described as glial glutamate transporters that regulate extracellular glutamate concentration by rapidly clearing glutamate from the extracellular fluid (Eulenburg and Gomeza, 2010; Gomez-Galan et al., 2013; Thomassen et al., 1985). Clinical studies show, an increased incidence of a rare genetic variant in the human gene encoding GLAST (Walsh et al., 2008) and an increased level of GLAST mRNA in the prefrontal cortex (PFC) of schizophrenia (Parkin et al., 2018). Simpson et al. (1998) investigated postmortem tissue of schizophrenics and reported that the number of binding sites and protein levels of glutamate transporters were increased in the PFC. Repeated PCP administration decreases extracellular glutamate levels and high potassium (K^+)-induced glutamate release, which are associated with increased levels of GLAST but not GLT1, and expression and activation of glial cells in the PFC of mice (Murai et al., 2007).

Microinjection of DL-threo-β-benzyloxyaspartate (DL-TBOA), a potent glutamate transporter blocker, enhances extracellular glutamate levels in the PFC and attenuates the psychobehavioral abnormalities induced in PCP-administered mice (Murai et al., 2007). It is possible that DL-TBOA attenuates the psychobehavioral abnormalities associated with activation of the glutamatergic system in the PFC by inhibition of GLT1 because DL-TBOA inhibits not only GLAST but also GLT1 (Shimamoto, 2008), which is the major glutamate transporter present in the PFC (Eulenburg and Gomeza, 2010). However, the functional roles of GLAST in the psychobehavioral abnormalities induced by repeated administration of PCP are not yet clear.

GLAST homozygous (−/−) mice, but not GLAST heterozygous (+/−) mice show a significant increase in the total distance traveled during exposure to a novel open field as well as an increase in cognitive impairment measured by an instrumental visual discrimination task (Karlsson et al., 2009). GLAST mutant mice are a useful tool for elucidating the contribution of glutamate dysfunction to the pathophysiology of schizophrenia (Karlsson et al., 2009; Watase et al., 1998). In the present study, we investigated the functional roles of GLAST in the emotional and cognitive abnormalities induced by repeated PCP administration using GLAST$^{+/-}$ mice, but not GLAST$^{-/-}$ mice, because GLAST$^{+/-}$ mice did not show any baseline abnormalities (Supplemental Fig. S1).

2. Experimental procedures

2.1. Animals

Male and female adult (8-week-old) GLAST heterozygous (+/−) mice and GLAST wild-type (+/+) mice, which were provided by Professor Kohichi Tanaka (Laboratory of Molecular Neuroscience, Medical Research Institute, Tokyo Medical and Dental University: TMDU) (Karlsson et al., 2009; Watase et al., 1998) were used. The mutant mice were obtained by crossing F13

GLAST$^{+/-}$ mice with a 99.99% pure C57BL/6 genetic background. The genotypes of the mice were determined by tail biopsy and PCR. The wild-type allele (214 base pairs, bp) was detected using the forward primer 5′-AAGTGCCTATCCAGTCCAACGA-3′, and the reverse primer 5′-AAGAACTCTCTCAGCGCTTGCC-3′. To detect the mutant allele (362 bp), a neomycin-specific forward primer (5′-AATGGAAGGATTGGAGCTACGG-3′) and a reverse primer (5′-TTCCAGTTGAAGGCTCCTGTGG-3′) were used. PCRs were performed using the Ex Taq DNA polymerase (TaKaRa, Siga, Japan). The reaction profile consisted of a first round at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 61 °C for 1 min, and extension at 72 °C for 2 min, with the final extension reaction carried out at 72 °C for 7 min in a T100TM Thermal Cycler (Bio-Rad Laboratories, Hercules, CA).

The GLAST$^{+/+}$pregnant female mice were monitored for their parturition date, and we defined the day of birth as postnatal day 0 (PD 0). The animals were housed in groups of 4–5 per plastic cage without any elements of housing enrichment. The animals were provided with food (CE2, Clea Japan Inc., Tokyo, Japan) and water *ad libitum* under a 12/12 h light/dark cycle (lights on from 9:00 AM to 9:00 PM). Behavioral experiments were carried out in a sound-attenuated and air-regulated experimental room, to which mice were habituated for at least 1 h, during light periods. All experiments were performed in a blind manner and in accordance with the Guidelines for Animal Experiments of Nagoya University School of Medicine (Approved number 29283) and Meijo University Faculty of Pharmacy (Approved number 2017-15). Procedures involving animals and their care conformed to the international guidelines set in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

2.2. Drugs and drug administration

Phencyclidine hydrochloride [1-(1-phenylcyclohexyl) piperidine hydrochloride: PCP] was synthesized by Professor Shinji Kitagaki (Department of Medicinal Chemistry, Faculty of Pharmacy, Meijo University) according to the method described by Maddox et al. (1965) and was checked for purity. PCP was dissolved in 0.9% NaCl solution (saline: SAL).

The mice received SAL (0.1 mL/kg/day, s.c.) or PCP (10 mg/kg/day, s.c.) once a day for 14 consecutive days (Noda et al., 1995b). The GLAST mutant mice were divided into the following four administration groups: SAL-administered GLAST$^{+/+}$ mice, SAL-administered GLAST$^{+/-}$ mice, PCP-administered GLAST$^{+/+}$ mice, and PCP-administered GLAST$^{+/-}$ mice.

2.3. Behavioral experiments

The behavioral test battery was performed during the light periods (in a 12 h light/dark cycle, lights on at 08:00 am) and according to the experimental protocol shown in Fig. 1(A). No sex differences in the behavioral responses were found in our preliminary experiments (data not shown). All experiments were performed independently using different mice.

2.3.1. Spontaneous activity

Spontaneous activity in GLAST mutant mice was measured on the first day after withdrawal of PCP administration (day 15; Fig. 1(A)) in accordance with the protocol described previously (Hida et al., 2014a). The mice were placed individually in a transparent acrylic cage with a black frosted Plexiglas floor (W45 × L26 × H40 cm), and spontaneous activity was measured for 60 min by using digital counters with infrared sensors, SCANET SV-10 LD (Melquest Co. Ltd., Toyama, Japan). The system was equipped with photosensor frames on the side walls. Spontaneous activity was defined as the total number of beam cuts due to horizontal movement, as measured by the photosensors. The acrylic cage was thoroughly sanitized with 70% EtOH solution (in water) after each mouse, and the cage was wiped dry with clean paper towels.

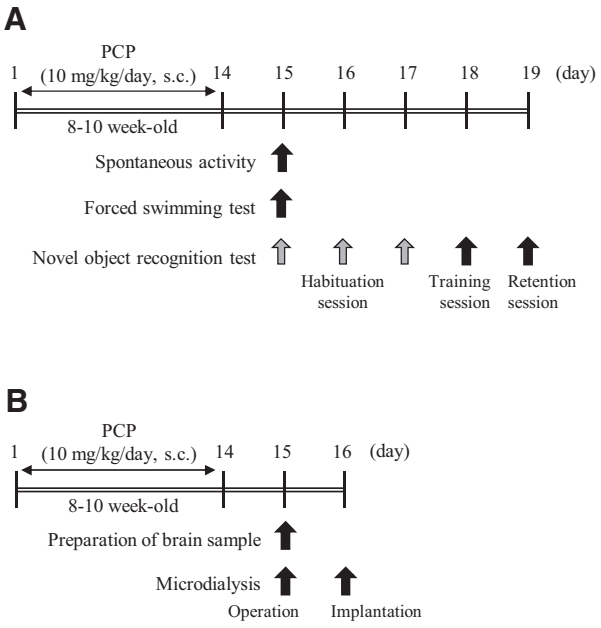

Fig. 1 Schedule of the behavioral experiments (A) and microdialysis experiment or brain sample preparation (B). GLAST mutant mice were divided into the following four administration groups: SAL-administered GLAST$^{+/+}$ mice, SAL-administered GLAST$^{+/-}$ mice, PCP-administered GLAST$^{+/+}$ mice, and PCP-administered GLAST$^{+/-}$ mice. SAL: saline (0.1 mL/kg/day, s.c.), PCP: phencyclidine (10 mg/kg/day, s.c.).

2.3.2. Forced swimming test

The forced swimming test (FST) was carried out 1 day after withdrawal of PCP administration (day 15; Fig. 1(A)) in accordance with the protocol described previously (Noda et al., 1995a) under conditions of 2 klux illumination. Each mouse was placed in a transparent glass cylinder (D15 × H20 cm), which contained water at 22–23 °C to a depth of 15 cm and was forced to swim for 180 s (Noda et al., 1997). The duration of swimming was measured by using a SCANET MV-10 AQ apparatus (Melquest Co. Ltd.). The immobility time was calculated as follows: 180 (s) − swimming time (s) = immobility time (s).

2.3.3. Novel object recognition test

The novel object recognition test (NORT) was carried out on days 1–5 after withdrawal of PCP administration (days 15–19; Fig. 1(A)) in accordance with the protocol described previously (Hida et al., 2014b). The experimental apparatus consisted of a Plexiglas open-field box (W30 × L30 × H35 cm), the floor of which was covered with paper bedding. The apparatus was placed in a sound-isolated room.

The test procedure consisted of three sessions: habituation, training, and retention. All sessions were conducted under conditions of dim illumination (20 lux). On days 15–17, each mouse was individually habituated to the box, with 10 min of exploration in the absence of objects on each of the 3 consecutive days (habituation session). During the training session on day 18, two objects (A: triangular prism and B: triangular pyramid) (Fig. 2) were symmetrically fixed to the floor of the box, 8 cm from the sidewalls. The experimenter used a pair of stopwatches to record the time spent exploring each object. A mouse was then placed in the middle of

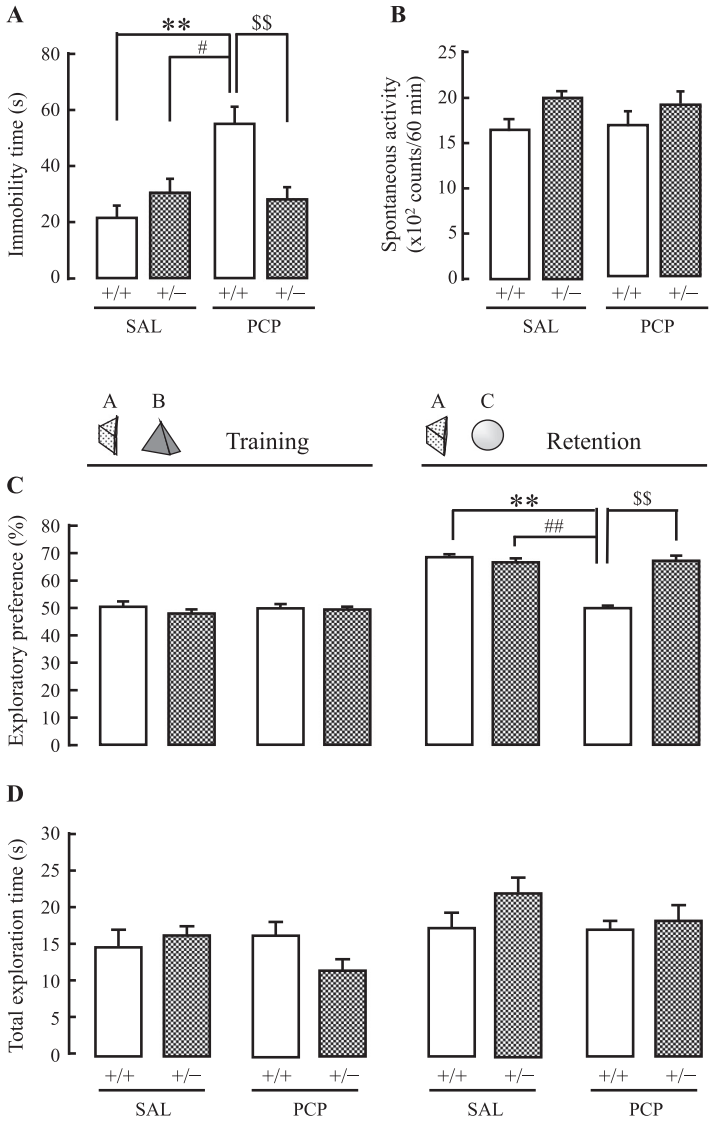

Fig. 2 Performance in the forced swimming test (FST: A), spontaneous activity (B), and novel objective recognition test (NORT: C and D) in GLAST mutant mice. The mice were administered SAL or PCP (10 mg/kg/day, s.c.) once a day for 14 consecutive days. (A) FST in GLAST mutant mice. The immobility time was calculated from the swimming time over 3 min. Values are mean ± S.E.M. (SAL-administered GLAST$^{+/+}$ mice: $n=8$, SAL-administered GLAST$^{+/-}$ mice: $n=11$, PCP-administered GLAST$^{+/+}$ mice: $n=11$, PCP-administered GLAST$^{+/-}$ mice: $n=12$). Two-way ANOVA: $F_{genotype\ (1,\ 40)}=0.17$, $p=0.68$; $F_{drug\ (1,\ 40)}=3.15$, $p=0.08$; $F_{genotype\ \times\ drug\ (1,\ 40)}=9.51$, $p<0.01$. $^{**}p<0.01$ vs. SAL-administered GLAST$^{+/+}$ mice, $^{\#}p<0.05$ vs. SAL-administered GLAST$^{+/-}$ mice, $^{\$\$}p<0.01$ vs. PCP-administered GLAST$^{+/+}$ mice (Bonferroni's test). (B) Spontaneous activity in the GLAST mutant mice. The spontaneous activity was measured over 60 min. Values are mean ± S.E.M. (SAL-administered GLAST$^{+/+}$ mice: $n=6$, SAL-administered GLAST$^{+/-}$ mice: $n=6$, PCP-administered GLAST$^{+/+}$ mice: $n=6$, PCP-administered GLAST$^{+/-}$ mice: $n=6$). Two-way ANOVA: $F_{genotype\ (1,\ 20)}=4.30$, $p=0.05$; $F_{drug\ (1,\ 20)}=0.001$, $p=0.98$; $F_{genotype\ \times\ drug\ (1,\ 20)}=0.18$, $p=0.68$. (C, D) NORT in GLAST mutant mice. (C) Exploratory preference and (D) The total exploration time in the training and retention session were measured for 10 min. (C) In the retention session, the mouse was allowed to explore freely for 10 min, and the time spent exploring each object (B: triangular pyramid as a familiar object and C: golf ball as a novel object) was recorded. A preference index—the ratio of time spent exploring either of the two objects (training session) or the novel object (retention session) over the total amount of time spent exploring both objects was used to assess cognitive function, e.g., A or B/(A + B) × 100 (%) in the training session, and B or C/(B + C) × 100. (D) The total time spent exploring the two objects in the training (A: triangular prism and B: triangular pyramid) and the retention session (B: triangular pyramid and C: golf ball) was recorded for 10 min. Values are mean ± S.E.M. (SAL-administered GLAST$^{+/+}$ mice: $n=10$, SAL-administered GLAST$^{+/-}$ mice: $n=11$, PCP-administered GLAST$^{+/+}$ mice: $n=11$, and PCP-administered GLAST$^{+/-}$ mice: $n=12$). (C) Two-way ANOVA: $F_{genotype\ (1,\ 38)}=1.15$, $p=0.29$; $F_{drug\ (1,\ 38)}=0.04$, $p=0.85$; $F_{genotype\ \times\ drug\ (1,\ 38)}=0.65$, $p=0.43$. (D) Two-way ANOVA: $F_{genotype\ (1,\ 38)}=34.55$, $p<0.01$; $F_{drug\ (1,\ 38)}=46.37$, $p<0.01$; $F_{genotype\ \times\ drug\ (1,\ 38)}=50.61$, $p<0.01$. $^{**}p<0.01$ vs. SAL-administered GLAST$^{+/+}$ mice, $^{\#\#}p<0.01$ vs. SAL-administered GLAST$^{+/-}$ mice, $^{\$\$}p<0.01$ vs. PCP-administered GLAST$^{+/+}$ mice (Bonferroni's test). SAL: saline, PCP: phencyclidine.

the box, and the total time spent exploring the two objects was recorded for 10 min. Exploration of an object was defined as directing the nose to the object at a distance of <2 cm and/or touching it with the nose. After the training session, the mouse was immediately returned to its home cages. During the retention session on day 19, the mouse was returned to the same box 24 h after the training session, with one of the familiar objects (e.g., A) used during the training session replaced by a novel object, C: golf ball. The mouse was allowed to explore freely for 10 min, and the time spent exploring each object was recorded as before. Throughout the experiments, the different objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index—the ratio of time spent exploring either of the two objects (training session) or the novel object (retention session) over the total amount of time spent exploring both objects was used to assess cognitive function, e.g., A or B/(A + B) × 100 (%) in the training session, and B or C/(B + C) × 100 in the retention session.

2.4. Microdialysis

Microdialysis procedures were performed according to the procedure described previously with a minor modification (Murai et al., 2007). On the first day after withdrawal of PCP administration (day 15; Fig. 1(B)), the mice were anesthetized with pentobarbital Na (50 mg/kg i.p.) and fixed in a stereotaxic apparatus (David Kopf Instruments, CA, USA). A guide cannula (AG-6, EICOM, Kyoto, Japan) was implanted into the PFC [AP: +1.7, ML: −0.3 from bregma, DV: −1.5 mm from the skull] according to the atlas (Paxinos and Franklin, 1997). On day 16 (24 h after implantation of the guide cannula and 2 days after PCP withdrawal), a dialysis probe (A-I-6-01; membrane length 1 mm, EICOM) was implanted into the PFC, and ringer solution (147 mM, NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) was perfused at a flow rate of 1.0 μL/min. The dialysate was collected every 10 min and the amount of glutamate in the dialysate was determined using an HPLC system (HTEC-500, EICOM) with electrochemical detection (ECD). Three samples were taken to establish baseline levels of extracellular glutamate. For depolarization stimulation, 100 mM KCl-containing ringer solution was delivered through the dialysis probe for 10 min in order to induce the high K^+-evoked release of glutamate. Then, dialysate was collected for 60 min with ringer solution.

2.5. Western blotting

Western blot analyses were performed as described previously, with a minor modification (Enomoto et al., 2005). On the first day after withdrawal of PCP administration (day 15; Fig. 1(B)), the mice were sacrificed by decapitation, and the brains were immediately removed. The PFC containing the cingulate and prelimbic area (Bregma +2.96 to +1.34), defined according to a mouse brain atlas by Franklin and Paxinos (Paxinos and Franklin, 1997), was rapidly dissected, frozen, and stored at −80 °C until used. To prepare the tissue extracts, the dissected brain tissue was homogenized by sonication or a tissue grinder in ice-cold lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 50 mM NaF, 2 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1% NP-40, 1 mM sodium orthovanadate] supplemented with a mixture of proteinase inhibitors (CompleteTM, Roche Diagnostics, Mannheim, Germany). The homogenate was centrifuged at 16,000 g for 20 min and the supernatant was used. The protein concentration was determined using a DC Protein Assay Kit (Bio-rad, Richmond, CA, USA). Samples (10–100 μg of protein) were boiled in sample buffer (125 mM Tris-HCl pH 6.8, 10% 2-mercaptoethanol or 0.2 M dithiothreitol, 4% SDS, 10% sucrose, and 0.004% bromophenol blue), separated on a polyacrylamide gel, and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, MA, USA). The membranes were blocked with a Detector Block Kit (Kirkegaard and Perry Laboratories, MD, USA) and probed with primary antibodies. The membranes were washed with a pH 7.4 washing buffer (50 mM Tris-HCl, 0.05% Tween 20, and 150 mM NaCl) and subsequently incubated with a horseradish peroxidase-conjugated secondary antibody. The immune complexes were detected by EZ capture MG (ATTO, Tokyo, Japan) or ChemiDoc XRS Plus system (Bio-rad) based on chemiluminescence (ECL kit, GE Healthcare, Buckinghamshire, UK). The band intensities were analyzed by densitometry using the ATTO Densitograph Software Library Lane Analyzer (ATTO) or using Image Lab Software (Bio-rad). To confirm equal loading of each protein, membranes were stripped with a pH 6.7 stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl) at 50 °C for 30 min, and β-actin protein expression was detected as described above. The primary antibodies were guinea pig anti-glutamate/aspartate transporter (GLAST) (1:1000; Frontier Institute, Hokkaido, Japan), rabbit anti-calcium binding protein (S100) (1:1000; Dako, CA, USA), and mouse anti-GFAP (1:1000; Sigma-Aldrich, St. Louis, MO). The secondary antibodies, used at a dilution of 1:2000, were horseradish peroxidase-linked anti-guinea pig, anti-rabbit, anti-goat or anti-mouse IgG (Kirkegaard and Perry Laboratories).

2.6. Preparation of the brain slices and staining

Histological procedures were performed as described previously, with a minor modification (Murai et al., 2007). On the first day after withdrawal of PCP administration (day 15; Fig. 1(B), the mice were lightly anesthetized with urethane (150 mg/kg i.p.) and euthanized by transcardial perfusion with ice-cold phosphate-buffered SAL (PBS), followed by 4% paraformaldehyde in PBS. The brains were removed, postfixed in the same fixative for 24 h, and then soaked in 30% (w/v) sucrose in PBS. Coronal sections 20 μm thick were cut with a cryostat (CM 1850, Leica, Nussloch Germany). The sections were incubated with 4% paraformaldehyde for 20 min, followed by incubation in 0.4% Triton X-100 in PBS for 15 min, 50 mM glycine in PBS for 15 min, and blocking buffer consisting of 3% normal goat serum (Sigma-Aldrich), and 5% bovine serum albumin (Wako, Tokyo, Japan) in PBS for 60 min. After incubation in primary antibody against guinea pig anti-GLAST (1:300; Frontier Institute, Hokkaido, Japan), rabbit anti-calcium binding protein (S100) (1:500; Dako) antibody at 4 °C for 24 h, sections were incubated in a fluorescently conjugated secondary antibody (Alexa 488, 546; Molecular Probes, OR, USA) for chromogen detection at room temperature for 2 h. The relative intensity of GLAST and S100 per section was measured in the prelimbic area using a computer-based image analysis system (Image J, MD, USA). Images were acquired with a light microscope (Axioskop2 plus; Carl Zeiss, Germany). An optical threshold was set above the background level to identify positively stained structures. Relative intensity values used for comparison were calculated by multiplying the mean optical density in the areas of interest and then subtracting the background staining (mean optical density *positive area - value of background). For each mouse, 4–6 sections of the prelimbic area were randomly selected, and the prelimbic area was defined according to a mouse brain atlas by Franklin and Paxinos (1997).

2.7. Statistical analysis

All results were expressed as the mean ± S.E.M. for each group. No statistical methods were used to predetermine sample sizes, but the sample sizes were similar to those reported previously (Murai et al., 2007). For all experimental data, the homogeneity of variance was analyzed using Levene's test for equality of variances. For data with a homogenous variance, statistical significance was determined using two-way analysis of variance (ANOVA), followed

by the Bonferroni test in multiple sets of data and using Student's t-test in two sets of data. For non-homogenous data, statistical significance was determined using Bonferroni's test in multiple sets of data. Data were analyzed with SPSS 24 software (IBM, Chicago, IL, USA). *P* values <0.05 were taken to indicate statistically significant differences.

3. Results

3.1. Characteristics of conventional behaviors, NMDA receptor subunits, GABA transporter, GLAST, and glial cell expression in GLAST mutant mice

Anxiety-related and social behaviors in GLAST$^{+/-}$ mice: There were no differences in the amount of ambulation (Supplemental Fig. S1A), ambulation in the center of the open field apparatus (Supplemental Fig. S1B), rearing behavior (Supplemental Fig. S1C) or grooming behavior (Supplemental Fig. S1D) in the open field test, and in the time spent in the open arm (Supplemental Fig. S1E) or open arm entries (Supplemental Fig. S1F) in the elevated plus maze test between the both groups. Further, there were no differences in the total duration of contacts in the social interaction test (Supplemental Fig. S1G) and in the immobility time in the forced swimming test (Supplemental Fig. S1H) between both groups.

Associative learning in the conditioned fear learning test: GLAST$^{+/-}$ mice hardly exhibited a freezing response in the training phase. There were no differences in the basal levels of the freezing response between both groups (Supplemental Fig. S1I and S1J). GLAST$^{+/-}$ mice showed marked cued and contextual freezing responses 24 h after fear conditioning (Supplemental Fig. S1I and S1J). There were no differences in the minimal current required to elicit vocalization/jumping, indicating no alterations of the nociceptive response between both groups (data not shown).

Sensitivity to a non-competitive NMDA receptor antagonist: There was no significant difference in the spontaneous activity of pre-administration during 30 min (−25-0 min) used as baseline between both groups. When PCP (3 mg/kg) was acutely administered into GLAST$^{+/-}$ mice, they showed a significant increase in spontaneous activity (Supplemental Fig. S1K).

Expression of NMDA receptor subunits and GABA transporters in the PFC of GLAST$^{+/-}$ mice: There were no differences in the ratios of phosphorylated GluN1 at Ser^{897}, GluN2A at Tyr^{1325}, and GluN2B at Tyr^{1472}, which exhibit receptor activity, in the PFC between both groups (Supplemental Fig. S2A, S2C, and S2E). The total protein levels of GluN1, GluN2A or GluN2B (Supplemental Fig. S2B, S2D, and S2F), and GABA transporters (mGAT1 and mGAT4) (Supplemental Fig. S2G and S2H) were not different in the PFC of both groups.

3.2. Volition and visual recognition memory in PCP-administered GLAST mutant mice

To examine whether PCP-induced emotional and cognitive impairments involve GLAST, we employed GLAST mutant mice to test the effect of PCP on emotional and cognitive functions.

In the FST, PCP-administered GLAST$^{+/+}$ mice showed a significantly prolonged immobility time, compared to SAL-administered GLAST$^{+/+}$ mice, indicating enhancement of immobility ($p < 0.01$) (Fig. 2(A)). When PCP was administered to GLAST$^{+/-}$ mice, the effects of PCP on immobility were significantly prevented ($p < 0.01$) (Fig. 2(A)). There was no significant difference in immobility time between SAL-administered GLAST$^{+/+}$ and GLAST$^{+/-}$ mice ($p = 1.00$) (Fig. 2(A)) or in the spontaneous activity across all groups ($p = 0.25$) (Fig. 2(B)).

In the training session of NORT, there was no difference in the exploratory preference for the objects across all groups ($p = 0.63$) (Fig. 2(C)). In the retention session, PCP-administered GLAST$^{+/+}$ mice showed a significantly reduced exploratory preference for a novel object, compared to SAL-administered GLAST$^{+/+}$ mice, indicating impairments of visual recognition memory ($p < 0.01$) (Fig. 2(C)). Such impairments were not observed in PCP-administered GLAST$^{+/-}$ mice ($p < 0.01$) (Fig. 2(C)). The number of seconds engaged in the known and novel objects were indicated in Supplementary Fig. S3. The total exploration time in either the training or retention session did not differ across all groups ($p = 0.10$, $p = 0.20$, respectively) (Fig. 2(D)).

3.3. Ability to release high K$^+$-induced glutamate in the PFC of PCP-administered GLAST mutant mice

We have previously demonstrated that repeated PCP administration disables glutamatergic neurotransmission in the PFC of mice, a region where such transmission is necessary for volition and visual recognition memory (Mouri et al., 2007; Murai et al., 2007; Nagai et al., 2009). We asked whether the impairment of glutamine release in the PFC of GLAST$^{+/+}$ mice administered PCP is protected against in the GLAST$^{+/-}$ mice showing no behavioral abnormalities following repeated administration of PCP.

The stable extracellular levels of glutamate were obtained about 12 h after dialysis probe insertion. After a steady state, basal release of glutamate was monitored during 30 min of dialysis. There were no differences in the basal levels of extracellular glutamate in the PFC of any groups in the present study (Supplemental Fig. S4). We investigated the ability to release glutamate of high K$^+$ (% of baseline) in the PFC of SAL- and PCP-administered GLAST mutant mice. There was no significant difference in the ability to release glutamate due high K$^+$ (% of baseline) in the PFC between SAL-administered GLAST$^{+/+}$ and GLAST$^{+/-}$ mice ($p = 1.00$) (Fig. 3). The amount of glutamate released in the PFC of PCP-administered GLAST$^{+/+}$ mice was significantly less than that released in SAL-administered GLAST$^{+/+}$ mice ($p < 0.05$) (Fig. 3). However, such a decrease was not observed in PCP-administered GLAST$^{+/-}$ mice ($p < 0.05$) (Fig. 3).

3.4. The changes in GLAST and glial cells in the PFC of PCP-administered GLAST mutant mice

In order to confirm whether the decrease in high K$^+$-induced glutamate release is due to changes in GLAST and/or glial

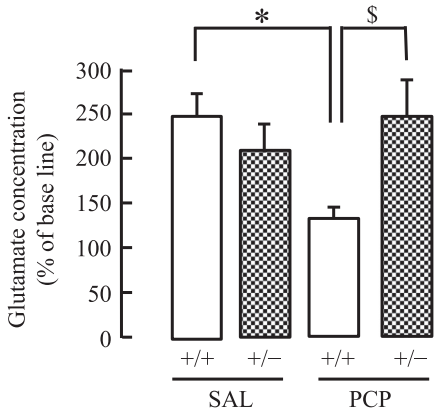

Fig. 3 Ability to release high K^+-induced glutamate in the PFC of PCP-administered GLAST mutant mice. Mice were administered SAL or PCP (10 mg/kg/day, s.c.) once a day for 14 consecutive days. The ability to release high K^+-induced (100 mM) glutamate in the PFC was determined by the microdialysis method. Fractions were collected for 60 min. Values are mean ± S.E.M. (SAL-administered GLAST$^{+/+}$ mice: $n = 12$, SAL-administered GLAST$^{+/-}$ mice: $n = 11$, PCP-administered GLAST$^{+/+}$ mice: $n = 11$, and PCP-administered GLAST$^{+/-}$ mice: $n = 10$). Two-way ANOVA: $F_{genotype\ (1,\ 40)} = 1.91$, $p = 0.18$; $F_{drug\ (1,\ 40)} = 1.86$, $p = 0.18$; $F_{genotype\ \times\ drug\ (1,\ 40)} = 7.06$, $p < 0.05$. $^*p < 0.05$ vs. SAL-administered GLAST$^{+/+}$ mice, $^{\$}p < 0.05$ vs. PCP-administered GLAST$^{+/+}$ mice (Bonferroni's test). SAL: saline, PCP: phencyclidine.

cells, we investigated the protein levels of GLAST and S100 in the PFC of PCP-administered GLAST mutant mice by immunoblotting.

The basal protein levels of GLAST, but not S100 were significantly decreased in the PFC of SAL-administered GLAST$^{+/-}$ mice, compared to those in SAL-administered GLAST$^{+/+}$ mice ($p < 0.05$) (Supplemental Fig. S2I and S2J). When PCP was administered to GLAST$^{+/+}$ mice, the protein levels of GLAST were significantly increased in the PFC, compared to those in SAL-administered GLAST$^{+/+}$ mice ($p < 0.05$) (Fig.4(A)). An increase in the protein levels was not observed in the PFC of PCP-administered GLAST$^{+/-}$ mice, being significantly lower than that in PCP-administered GLAST$^{+/+}$ mice ($p < 0.05$) (Fig. 4(A)). The protein levels of S100, a marker of astroglial injury, in PCP-administered GLAST$^{+/+}$ mice were about 2.1-fold higher than those of SAL-administered GLAST$^{+/+}$ mice, whereas such an increase was not observed in PCP-administered GLAST$^{+/-}$ mice ($p = 0.22$) (Fig. 4(B)). There was no significant difference in the protein levels of GFAP, used as a marker of astrocytes, in the PFC across all groups (data not shown).

Since immunoblot data showed increased protein levels of GLAST and S100, the localization of GLAST in the PFC of PCP-administered GLAST mutant mice was analyzed by immunohistochemical methods. As shown in Fig. 4(C), GLAST was localized to the processes of cells positive for S100, and merged with S100 in SAL- or PCP-administered GLAST mutant mice as previously reported (Murai et al., 2007).

The integrated optical densities of GLAST, but not S100 immunoreactivities were decreased in the PFC of SAL-administered GLAST$^{+/-}$ mice, compared to those in SAL-administered GLAST$^{+/+}$ mice ($p < 0.01$) (Supplemental Fig. S2K and S2L). The integrated optical densities of GLAST and S100 immunoreactivities were increased in the PFC of PCP-administered GLAST$^{+/+}$ mice, compared to those in SAL-administered GLAST$^{+/+}$ mice ($p < 0.05$) (Fig. 4(D) and 4(E)). Increase in the integrated optical densities of GLAST and S100 immunoreactivities were not observed in the PFC of PCP-administered GLAST$^{+/-}$ mice ($p < 0.01$) (Fig. 4(D) and 4(E)).

4. Discussion

Our and other studies indicated that repeated administration of PCP induced schizophrenia-like psychobehavioral abnormalities in rodents (Mandillo et al., 2003; Noda et al., 2009) and led to alteration of glutamatergic functions via activation of glial cells with overexpression of GLAST in the PFC (Murai et al., 2007). The microinjection of DL-TBOA, a potent glutamate transporter blocker, enhanced extracellular glutamate levels in the PFC and attenuated the psychobehavioral abnormalities in adult (Murai et al., 2007) and prenatal (Lu et al., 2011) mice administered PCP repeatedly. Based on these findings, there is the possibility that overexpression of GLAST involving presynaptic dysfunction induces psychobehavioral abnormalities in PCP-administered mice. To confirm the functional roles of GLAST in the emotional and cognitive abnormalities induced by repeated administration of PCP, we investigated it using GLAST$^{+/-}$ mice.

We reconfirmed that PCP-administered GLAST$^{+/+}$ mice showed enhanced immobility in the FST, an impairment of visual recognition memory in the NORT, and overexpression of GLAST in the PFC, consistent with previous reports (Mouri et al., 2007; Nagai et al., 2009; Noda et al., 1995a). These changes were not observed in PCP-administered GLAST$^{+/-}$ mice. GLAST$^{-/-}$ mice, but not GLAST$^{+/-}$ mice showed emotional and cognitive impairments in a novel open field test and in an instrumental visual discrimination task, respectively (Karlsson et al., 2009). In the present study, there were no significant differences in spontaneous activity in a novel environment, immobility in FST, or object recognition memory in a NORT between SAL-administered GLAST$^{+/+}$ and GLAST$^{+/-}$ mice. Therefore, it is unlikely that the performance seen in PCP-administered GLAST$^{+/-}$ mice is due to essential changes in motivation and/or neophobia. There was no significant difference in the total or phosphorylated protein levels of the NMDA receptor subunits (GluN1, GluN2A, and GluN2B), in the expression levels of GABA transporters (mGAT1 and mGAT4), and in the ability to release high K^+-induced glutamate in the PFC between SAL-administered GLAST$^{+/+}$ and GLAST$^{+/-}$ mice. There was no significant difference in the NMDA receptor sensitivity to PCP in GLAST$^{+/-}$ mice. Thus, these results suggest that there are no compensatory changes in GABA transporter and pre- or postsynaptic glutamatergic functions in GLAST$^{+/-}$ mice. Our results using GLAST$^{+/-}$ mice demonstrate that genetic GLAST dysfunction prevents the development of emotional and cognitive ab-

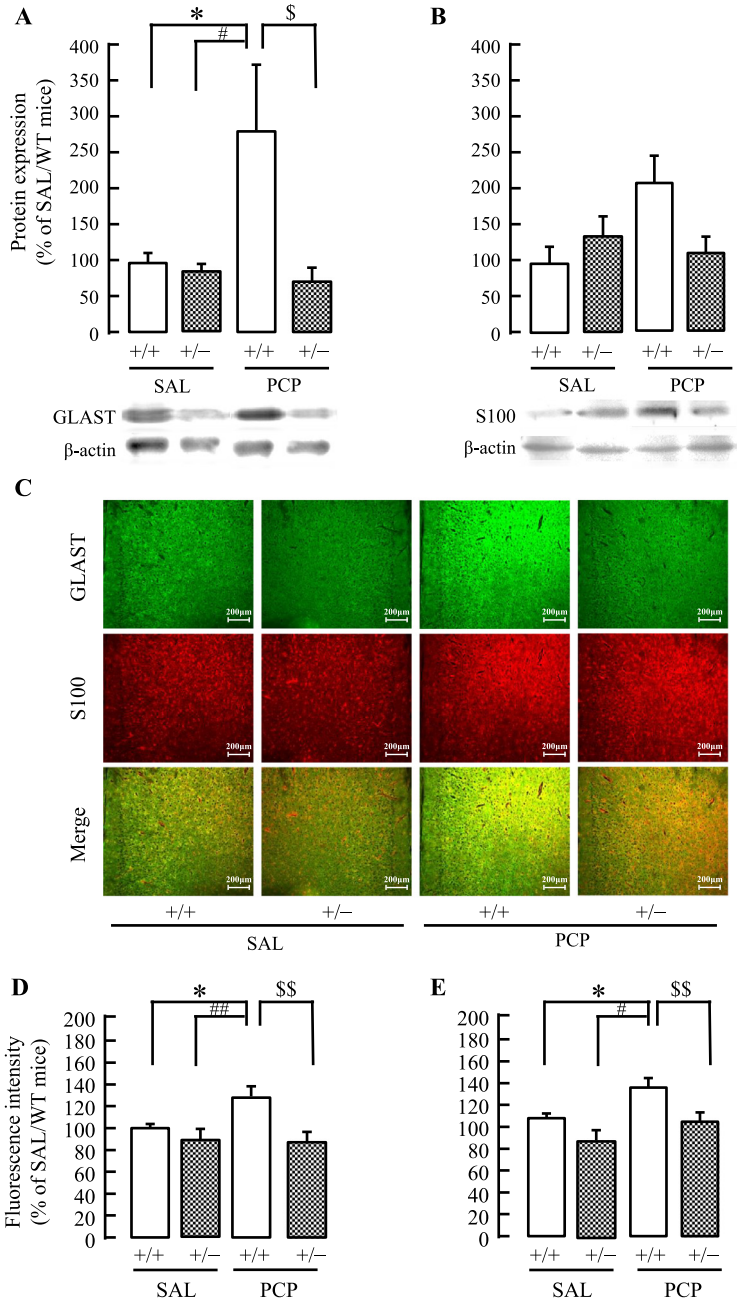

Fig. 4 Changes in GLAST and glial cell expression in the PFC of PCP-administered GLAST mutant mice. The mice were administered SAL or PCP (10 mg/kg/day, s.c.) once a day for 14 consecutive days. Representative western blot bands for the expression of GLAST and S100 proteins (A, B). The amount of protein loaded was normalized using an antibody against β-actin. Results are represented as the protein levels of GLAST (A) and S100 (B) in the PFC. Values are mean ± S.E.M. (SAL-administered GLAST$^{+/+}$ mice: $n = 12$, SAL-administered GLAST$^{+/-}$ mice: $n = 11$, PCP-administered GLAST$^{+/+}$ mice: $n = 11$, and PCP-administered GLAST$^{+/-}$ mice: $n = 10$). (A) Two-way ANOVA: $F_{genotype\ (1,\ 28)} = 5.92$, $p < 0.05$; $F_{drug\ (1,\ 28)} = 3.45$, $p = 0.07$; $F_{genotype\ \times\ drug\ (1,\ 28)} = 4.78$, $p < 0.05$. (B) Two-way ANOVA: $F_{genotype\ (1,\ 28)} = 0.82$, $p = 0.37$; $F_{drug\ (1,\ 28)} = 1.97$, $p = 0.17$; $F_{genotype\ \times\ drug\ (1,\ 28)} = 4.84$, $p < 0.05$. $^{*}p < 0.05$ vs. SAL-administered GLAST$^{+/+}$ mice, $^{\#}p < 0.05$ vs. SAL-administered GLAST$^{+/-}$ mice, $^{\$}p < 0.05$ vs. PCP-administered GLAST$^{+/+}$ mice (Bonferroni's test). (C) Representative image of GLAST (green) and S100 (red) immunostained sections from mice administered SAL or PCP in the PFC. Scale bar: 200 μm. Stereological analysis of the integrated optical density of GLAST (D) and S100 (E) immunoreactivities in the PFC. Values are mean ± S.E.M. (SAL-administered GLAST$^{+/+}$ mice: $n = 8$, SAL-administered GLAST$^{+/-}$ mice: $n = 8$, PCP-administered GLAST$^{+/+}$ mice: $n = 8$, and PCP-administered GLAST$^{+/-}$ mice: $n = 8$). (D) Two-way ANOVA: $F_{genotype\ (1,\ 28)} = 10.59$, $p < 0.01$; $F_{drug\ (1,\ 28)} = 3.78$, $p = 0.06$; $F_{genotype \times drug\ (1,\ 28)} = 6.27$, $p < 0.05$. (E) Two-way ANOVA: $F_{genotype\ (1,\ 28)} = 6.25$, $p < 0.05$; $F_{drug\ (1,\ 28)} = 5.34$, $p < 0.05$; $F_{genotype \times drug\ (1,\ 28)} = 4.41$, $p < 0.05$. $^{*}p < 0.05$ vs. SAL-administered GLAST$^{+/+}$ mice, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. SAL-administered GLAST$^{+/-}$ mice, $^{\$\$}p < 0.01$ vs. PCP-administered GLAST$^{+/+}$ mice (Bonferroni's test). SAL: saline, PCP: phencyclidine, SAL/WT mice: SAL-administered GLAST$^{+/+}$ mice.

normalities induced by repeated administration of PCP. GLAST plays an important role in the development of glutamatergic dysfunction in the PFC of mice administered PCP repeatedly.

The functional facilitation of glutamate transporters including GLAST in mice receiving PCP repeatedly causes glutamatergic hypofunction by decreasing extracellular glutamate release (Murai et al., 2007). Acute administration of PCP increases the levels of extracellular glutamate, which contributes to excitotoxicity and leads to neuronal damage in the PFC (Adams and Moghaddam, 1998). To prevent excitotoxic neuronal damage, glutamate transporters are up regulated to remove the excess extracellular glutamate (Eulenburg and Gomeza, 2010; Gomez-Galan et al., 2013; Thomassen et al., 1985). Repeated administration of PCP leads to the overexpression of GLAST in the PFC of GLAST$^{+/+}$ mice, and then elicits long-lasting glutamatergic dysfunction even after PCP withdrawal (Nabeshima et al., 2006). Like in a previous report (Murai et al., 2007), PCP-administered GLAST$^{+/+}$ mice showed decreased high K^+-induced extracellular glutamate release, because of the increased levels of GLAST. Unlike GLAST$^{+/+}$ mice, the glutamatergic functions in GLAST$^{+/-}$ mice showing decreased basal protein levels of GLAST were not exacerbated following repeated administration of PCP, because repeated administration of PCP did not lead to excessive expression of GLAST in the PFC. Thus, PCP-administered GLAST$^{+/-}$ mice, as well as SAL-administered GLAST$^{+/+}$ mice, showed facilitation of the extracellular glutamate release evoked by high K^+.

Astrocytes play important roles in glutamate uptake to prevent from excitotoxic neuronal damage (Anderson and Swanson, 2000). S100, a calcium-binding protein, is expressed in astrocytes at high levels in brain lesions and is used as a marker for identifying astrocytes especially in the excitotoxic-damaged CNS (Cerutti and Chadi, 2000; Ridet et al., 1997). Repeated administration of PCP influences neuronal dysfunction by decreasing cell size in the PFC (Murai et al., 2007). Usually, reactive gliosis occurs after injury to the CNS (Ridet et al., 1997). In the present study, increased levels of S100 were not detected in the PFC of PCP-administered GLAST$^{+/-}$ mice compared to PCP-administered GLAST$^{+/+}$ mice. There was no significant difference in the levels of GFAP, an astrocytic marker, in the PFC across all groups. Up-regulation in the synthesis of GFAP, an intermediate filament protein of the astroglial cytoskeleton, accompanies the activation of astrocytes which consists of an enlargement of the glial cell body and an increased number and size of astroglial processes (Cerutti and Chadi, 2000). Although astrocytes may be activated in this case, their number and size remained unchanged. These findings suggest that GLAST plays an important role in the regulation of astrocyte activation induced by neuronal dysfunction in the PFC of mice administered PCP repeatedly. The sensitivity to the development of astroglial abnormalities induced by repeated administration of PCP is decreased in GLAST$^{+/-}$ mice. Thus, PCP-administered GLAST$^{+/-}$ mice could not prevent the neuronal damage and glutamatergic dysfunction after drug withdrawal by suppressing the overexpression of GLAST in the PFC. However, the mechanism of activation of glial cells following the up-regulation of GLAST expression and neuronal dysfunction in the PFC of PCP-administered GLAST$^{+/-}$ mice remains unclear. Further experiments will be needed.

5. Conclusions

These results clearly suggest that overexpression of GLAST and activation of astrocytes play important roles in the development of psychobehavioral abnormalities in mice following repeated administration of PCP. GLAST$^{-/-}$ mice, which show a decreased expression of GLAST, have been reported to show psychobehavioral abnormalities including cognitive impairment (Karlsson et al., 2009). It is therefore necessary to strictly regulate the expression of GLAST to maintain normal brain function. Some antipsychotics inhibit or down-regulate the function of glutamate transporters (Melone et al., 2001; Vallejo-Illarramendi et al., 2005). Treatment of co-cultures with high concentrations of either NMDA or glutamate resulted in neuronal death and caused GLAST levels to increase (Schlag et al., 1998). Studies targeting GLAST may lead to the development of medications for emotional (negative symptoms) and cognitive impairments in schizophrenia.

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Conflict of interest

Dr. Noda has received research support or speakers' honoraria from Sumitomo Dainippon Pharma, Janssen Pharmaceuticals, Otsuka Pharmaceutical, and Kyorin Pharmaceutical. Dr. N. Ozaki has received research support or speakers' honoraria from, or has served as a consultant to Astellas, Sumitomo Dainippon, Eisai, Eli Lilly, Janssen, Meiji Seika Pharma, Mochida, MSD, Nihon Medi-Physics, Novartis, Ono, Kyowa Hakko Kirin, Novartis, Otsuka, Pfizer, Takeda, Taisho, Mitsubishi Tanabe,Tsumura, and KAITEKI.

CRediT authorship contribution statement

Mizuki Uchida: Conceptualization, Formal analysis, Writing - review & editing. **Hirotake Hida:** Conceptualization, Formal analysis, Writing - review & editing. **Kentaro Mori:** Conceptualization, Formal analysis. **Akira Yoshimi:** Formal analysis. **Shinji Kitagaki:** Conceptualization. **Kiyofumi Yamada:** Formal analysis. **Yuichi Hiraoka:** Conceptualization.

Tomomi Aida: Conceptualization. **Kohichi Tanaka:** Conceptualization. **Norio Ozaki:** Formal analysis. **Yukihiro Noda:** Conceptualization, Formal analysis, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2019.06.005.

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