Research article

Full title: GluD1 knockout mice with a pure C57BL/6N background show impaired fear memory, social interaction, and enhanced depressive-like behavior

Short title: Behavioral and biochemical analyses of GluD1 knockout mice with a pure C57BL/6N background

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Abstract

The GluD1 gene is associated with susceptibility for schizophrenia, autism, depression, and bipolar disorder. However, the function of GluD1 and how it is involved in these conditions remain elusive. In this study, we generated a GluD1-knockout (GluD1-KO) mouse line with a pure C57BL/6N genetic background and performed several behavioral analyses. Compared to a control group, GluD1-KO mice showed no significant anxiety-related behavioral differences, evaluated using behavior in an open field, elevated plus maze, a light-dark transition test, the resident-intruder test of aggression and sensorimotor gating evaluated by the prepulse inhibition test. However, GluD1-KO mice showed (1) hyper locomotor activity in the open field, (2) decreased sociability and social novelty preference in the three-chambered social interaction test, (3) impaired memory in contextual, but not cued fear conditioning tests, and (4) enhanced depressive-like behavior in a forced swim test. Pharmacological studies revealed that enhanced depressive-like behavior in GluD1-KO mice was restored by the serotonin reuptake inhibitors imipramine and fluoxetine, but not the norepinephrine transporter inhibitor desipramine. In addition, biochemical analysis revealed no significant difference in protein expression levels, such as other glutamate receptors in the synaptosome and postsynaptic densities prepared from the frontal cortex and the hippocampus. These results suggest that GluD1 plays critical roles in fear memory, sociability, and depressive-like behavior.

Introduction

The δ-type ionotropic glutamate receptor consists of GluD1 (GluRδ1) and GluD2 (GluRδ2) [1–3]. Despite having conserved membrane topology and amino acid residues critical for glutamate binding and Ca^{2+} permeability, the δ subfamily members do not function as conventional glutamate-gated receptor channels when expressed alone or in combinations with other ionotropic glutamate receptor subunits [4–6]. Instead, they are components of a tripartite transsynaptic adhesion system, where the extracellular domain of postsynaptic GluD1/2 interacts with that of presynaptic neurexin protein (NRXN) via members of the cerebellin precursor protein (CBLN) family in the synaptic cleft [7–9]. Moreover, slow activity of GluD1/2 ion channels is triggered by activation of group 1 metabotropic glutamate receptors (mGluRs) [10–12].

GluD2 has been intensively studied since it was cloned in 1993 [2,3]. In the rodent cerebellum, GluD2 is exclusively expressed in Purkinje cells and is selectively localized to postsynaptic spines at parallel fiber synapses [13,14]. GluD2 is indispensable during the formation and maintenance of parallel fiber-Purkinje cell synapses by interaction with presynaptic neurexins through its amino-terminal domain [7,8,15,16]. In addition, GluD2 regulates cerebellar synaptic plasticity [15,17] and motor learning [15,18,19] by interaction with the scaffolding proteins through its carboxyl-terminal domain [20–22].

The gene encoding GluD1 in humans (*GRID1*) is associated with a susceptibility for schizophrenia [23–26], major depressive disorder [27], and autism spectrum disorder [28–31]. GluD1 is expressed in various brain regions in rodents, including the cerebral cortex, hippocampus, amygdala, bed nucleus of the stria terminalis, striatum, thalamus, nucleus accumbens, lateral habenular, and the dorsal raphe nucleus [3,32,33]. Similar to

GluD2, postsynaptic GluD1 is required for synapse formation and maintenance *in vitro* via CBLN1/CBLN2 and presynaptic NRXN [7,9,34,35]. In addition, GluD1 regulates group 1 mGluRs-mediated long-term depression in the hippocampus *ex vivo* [36]. Furthermore, activation of group 1 mGluRs trigger the opening of GluD1 channels, which are key determinants of the slow excitatory postsynaptic current *ex vivo* [12].

The GluD1 knockout (GluD1-KO) mice line (*Grid1*^{tm1Jnz}), compared to the relevant control groups, showed abnormal behavioral phenotypes, such as hyper locomotor activity, lower anxiety-like behavior, hyper aggression, higher depression-like behavior, deficits in social interaction [37], enhanced working memory, deficit in contextual and cued fear conditioning [38], and increased stereotyped behavior [39]. However, the *Grid1*^{tm1Jnz} mouse was generated from embryonic stem (ES) cells derived from the 129/SvEv strain, followed by backcrossing to C57BL/6 mice 2–6 times [37,38,40]. It is well known that backcrossing with different mouse strains leads to a change in the basal levels of behaviors, such as anxiety [41–43], aggression [44,45], prepulse inhibition [46], social interaction [47,48], pain sensitivity [49], depressive-like behavior [42,50–52], and learning and memory [41,53–56]. Thus, a concern of using a mixed genetic background is that the resulting phenotype cannot be confidently attributed to either the target gene or closely-linked genes flanking the targeted locus [53,57,58]. In addition, the 129S6/SvEvTac strain used in *Grid1*^{tm1Jnz} lacked the DISC1 gene (disrupted in schizophrenia 1) [59–61], which is a strong candidate gene that contributes to cause schizophrenia and autism spectrum disorder [62,63]. Moreover, the *Grid1*^{tm1Jnz} mouse was generated by knock-in of a selection marker of neomycin

cassette; the promoter of the cassette unexpectedly affected gene expression levels [64–67].

To avoid these issues, we generated GluD1-KO mice with a pure C57BL/6N genetic background and investigated an impact of GluD1 deletion on various behaviors including anxiety, aggression, sensorimotor gating, sociability, learning and memory, and depression.

Methods

Animals

GluD1-KO mice were generated using the C57BL/6N ES cell line, RENKA [65] and maintained in a pure C57BL/6N background [68]. Briefly, exon 4 of the *Grid1* gene and a *Pgk* promoter-driven neomycin-resistance cassette were flanked by loxP sequences (*Grid1*^{flox}). *Grid1*^{flox} mice were crossed with telencephalin-Cre mice [69] to create the null allele (*Grid1*$^{-}$). Mice were fed *ad libitum* with standard laboratory chow and water in standard animal cages in a 12-h light/dark cycle (light on at 8:00 a.m.) at room temperature and relative humidity in the ranges of 22°C–24°C and 30%–70%, respectively. Experimental protocols used throughout the study were approved by an institutional committee at Niigata University (SA00466) and were in accord with Japanese legislation concerning animal experiments.

Behavioral tests were carried out with 8 to 12-week-old male wild-type (WT, *Grid1*$^{+/+}$) (n = 115 in total) and GluD1-KO (*Grid1*$^{-/-}$) (n = 92 in total) litter mates by heterozygous breeding, and were performed during the light phase (between 10:00 a.m.

and 18:00 p.m.). Mice were handled (3 min per day for 3 days) before starting behavioral tests. Behavioral analyses were performed with the experimenter blind to mice genotype. After each trial, the apparatus was cleaned with hypochlorous water to prevent a bias due to olfactory cues. A battery of behavioral tests were performed in the following order: open field, light-dark transition, elevated plus maze, 3-chamber social interaction, and a forced swim.

Open field

Open field tests were carried out using a method similar to that reported previously, with minor modification [70]. Each mouse was placed in the corner of an open field apparatus (50 cm × 50 cm × 40 cm high; O'Hara & Co., Tokyo, Japan) with a chamber illuminated at either 5 or 100 lux. Distance traveled and time spent in the central area (defined as 25% of total area) were recorded and calculated automatically over a 10-min period using Image OFCR software (O'Hara & Co.; see 'Image analysis for behavioral tests').

Elevated plus maze

Elevated plus maze tests were carried out using a method similar to that reported previously, with minor modification [70]. The apparatus consisted of two open arms (25 cm × 5 cm) and two enclosed arms of the same size with transparent walls (height 15 cm). The arms and central square (5 cm × 5 cm) were made of white plastic plates and were elevated 60 cm above the floor (O'Hara & Co.). Arms of the same type were oriented opposite from each other. Each mouse was placed in the central square of the

maze, facing one of the closed arms. The time spent in closed and open arms and the frequency of entry into open arms were observed for 10 min under two different illumination condition (5 and 100 lux). Data acquisition was performed automatically using Image EP software (O'Hara & Co.; see 'Image analysis for behavioral tests').

Light-dark transition test

Light-dark transition tests were carried out using a method similar to that reported previously, with minor modification [70]. The apparatus consisted of a cage (21 cm × 42 cm × 25 cm high) divided into 2 equal chambers by a black partition containing a small opening (5 cm x 3 cm high) (O'Hara & Co.). One chamber was made of white plastic and was brightly illuminated (252 lux), whereas the other chamber was made of black plastic and was dark (no illumination). Mice were placed in the dark chamber and allowed to move freely between the two chambers for 10 min. Time spent in each chamber, total number of transitions and latency to the first transition from dark to light chambers were recorded automatically using Image LD software (O'Hara & Co.; see 'Image analysis for behavioral tests').

Resident-intruder test

Resident-intruder tests were carried out using a method similar to that reported previously [37]. Resident male WT (27.3 ± 0.2 g) or GluD1-KO mice (24.1 ± 0.4 g) were individually housed for 3-4 weeks before testing. Resident mice were exposed to intruder male WT C57BL/6 mice, which had been group-housed (four to five per cage) and were of lower body-weight than resident mice (0-4 g lighter than intruder mice), for

a duration of 10 min. New intruder mice were used in each test. Latency to attack the intruder and attack frequency were measured manually.

Prepulse inhibition test

Acoustic startle response and prepulse inhibition (PPI) of the acoustic startle response were measured using a startle chamber (SR-Lab Systems; San Diego Instruments, CA, USA)[71]. For acoustic startle responses, a background of white noise was used (70 db). An animal was placed in the Plexiglass cylinder and each test session began after 5 min of acclimatization. Mice were presented with 64 trials. There were eight different sound levels presented: 75, 80, 85, 90, 95, 100, 110, and 120 dB. Each white-noise stimulus was 40 ms and presented 8 times in a pseudorandom order such that each sound level was presented within a block of 8 trials. The intertrial interval was 15 s. Analysis for startle amplitudes was based on the mean of the seven trials (ignoring the first trial) for each trial type.

PPI responses were measured with acoustic stimuli (120 dB) combined with four different prepulse intensities. Each mouse was placed in the startle chamber and initially acclimatized for 5 min with background white noise alone (70 dB**)**. Mice were then presented with 48 trials. Each session consisted of six trial types. One trial type used a sound burst (40 ms, 120 dB) as the startle stimulus (startle trials). There were four different trials consisting of acoustic prepulse and acoustic startle stimuli (prepulse trials). The prepulse stimulus (20 ms) of either 73, 76, 79, or 82 dB was presented 100 ms before the onset of the acoustic startle stimulus. Finally, there were trials where no stimulus was presented (no-stimulus trials). The six trial types were presented in a pseudorandom order such that each trial type was presented once within a block of eight

trials. The intertrial interval was 15 s. Analysis was based on the mean of the seven trials (ignoring the first trial) for each trial type. The percentage PPI of a startle response was calculated using the following equation: 100 − [100 × (startle response on prepulse trials − no stimulus trials)/(startle trials − no stimulus trials)].

Three-chambered social interaction test

The three-chambered social interaction test was performed as previously described, with minor modification [48,70]. The apparatus consisted of a rectangular, illuminated (5 lux) three-chambered box with a lid and an attached infrared video camera (O'Hara & Co.). Each chamber was 20 cm × 40 cm × 22 cm (high) and the dividing walls were made of clear Plexiglas, with small square openings (5 cm wide × 3 cm high) to allow exploration of each chamber. Male mice of the C3H strain, with ages ranging between 8 to 12 weeks, were purchased from Charles River Laboratories (Yokohama, Japan) and used as 'strangers'.

One day before testing, the 'subject mice' were individually placed in the middle chamber and allowed to freely explore the entire apparatus for 5 min. Before testing, subject mice were placed in the middle chamber and allowed to freely explore all three chambers for 10 min (habituation trial). In the sociability test (sociability trial), an unfamiliar C3H male mouse ('stranger 1') that had no prior contact with the subject mouse was placed in one of the side chambers. The stranger mouse was enclosed in a small, round wire cage, which allowed nose contact between the bars but prevented fighting. This cage was 11 cm in height, with a floor diameter of 9 cm and vertical bars 0.5 cm apart. The subject mouse was placed in the middle chamber and presented with stranger 1 in one compartment and an empty cage in another compartment for 10 min.

The amount of time spent around each cage (stranger 1 or empty) was measured. At the end of the 10-min sociability trial, each subject mouse was then tested in a 10-min trial to quantitate social preference for a new stranger (social novelty preference trial). The wire cage enclosing the familiar C3H male mouse (stranger 1) was moved to the opposite side of the chamber that had been empty during the sociability trial. A second, unfamiliar C3H male mouse (stranger 2) was placed in the other side of the chamber in an identical small wire cage. The subject mouse was free to explore the mouse from the previous sociability test (stranger 1), and the novel mouse (stranger 2). The amount of time spent around each cage (stranger 1 or stranger 2) was measured. Data acquisition and analysis were performed automatically using Image CSI software (O'Hara & Co.; see 'Image analysis for behavioral tests').

Contextual and cued fear conditioning test

The contextual and cued fear conditioning test was performed using a method similar to a previous report [70], with minor modifications. Fear conditioning was conducted in a transparent acrylic chamber (33 cm□×□25 cm□×□28 cm high) with a stainless-steel grid floor (0.2 cm-dimeter, spaced 0.5 cm apart; O'Hara & Co.). For the conditioning (conditioning test), each mouse was placed in the chamber and was allowed to explore freely for 3 min. Subsequently, white noise (55 dB) was played through a speaker set on top of the conditioning chamber wall, which served as the conditioning stimulus (CS), was presented for 20 s. During the last 2 s of CS presentation, mice received a footshock (0.7 mA, 2 s), which served as an unconditioned stimulus (US). Two more CS-US pairings were presented with a inter-stimulus interval of 40 s. Animals were returned to their home cages 40 s after the last CS-US paring. Twenty-four hours after

231 conditioning (contextual test), contextual fear memory was tested for 3 min in the same
232 chamber. Forty-eight hours after conditioning (cued test), cued fear memory was tested
233 with an altered context. Each mouse was placed in a triangular chamber (33 cm ×□33
234 cm ×□32 cm high) made of opaque white plastic and allowed to explore freely for 1
235 min. Subsequently, each mouse was given CS presentation for 3 min. In each session,
236 percentage of time spent freezing was calculated automatically using Image FZ software
237 (O'Hara & Co.; see 'Image analysis for behavioral tests').

238 Pain sensitivity was measured as a control experiment using the fear conditioning
239 chamber apparatus in a manner similar to a previous study [38]. Following 2 min of
240 habituation, mice were given footshocks of increasing strength ranging from 0.05 to 0.7
241 mA in a stepwise manner by 0.05 mA, with an intertrial interval of 30 s. We measured
242 current thresholds for three reactions of mice to nociceptive shock: flinch, vocalization,
243 and jump (vertical and horizontal). Scoring indicated the first shock intensity at which
244 each pain reaction was detected.

245

246 Forced swim test with pharmacological manipulation

247 Forced swim tests were performed following Porsolt's method with minor
248 modifications [37,72]. The apparatus consisted of a transparent plastic cylinder (22 cm
249 height; 12 cm diameter) placed in a box (41 cm × 31 cm × 42 cm high; O'Hara & Co).
250 The cylinder was filled with water (22 ± 1°C) up to a height of 10 cm. Each mouse was
251 placed into the cylinder and activity was monitored for 5 min via a CCD camera
252 mounted on the top of the box. The cylinder was refilled with clean water after each test.
253 Image data acquisition and analysis were performed automatically using Image PS
254 software (see 'Image analysis for behavioral tests').

With respect to drugs, saline (0.9% NaCl in H_2O) was used as a vehicle and for control injections. Drug concentration for injections were: 15 mg/kg of imipramine (097-06491; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 10 mg/kg of fluoxetine (F132; Sigma-Aldrich, MO, USA), 30 mg/kg of desipramine (042-33931; FUJIFILM Wako Pure Chemical Corporation). Drug concentrations were chosen on the basis of previous studies for imipramine [52][73][74][75][76], fluoxetine [52][73][77][76], and desipramine[52][73][74][76][78]. Both vehicle and drug solutions were intraperitoneally administered. Sixty min after injection, mice were tested in the open field for 10 min with 5 lux illumination and subsequently subjected to a forced swim test for 5 min.

Image analysis for behavioral tests

The application software used for the behavioral studies (Image OFCR, LD, EP, CSI, PS, and FZ) were based on the public domain NIH Image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/) and ImageJ program (http://rsb.info.nih.gov/ij/), which were modified for each test (available through O'Hara & Co.).

Subcellular fraction and western blot analysis

Subcellular fractions were prepared following Carlin's method [79] with minor modifications. All processes were carried out at 4 °C. Briefly, WT and GluD1-KO mice with a C57BL/6N background (8 to 12 weeks old) were decapitated after cervical dislocation, and the frontal cortex (defined as one third anterior part of the cerebral

cortex) and hippocampus were immediately dissected and removed. Brain tissues were homogenized in homogenization buffer [320 mM sucrose and 5 mM EDTA, containing complete protease inhibitor cocktail tablet (Complete Mini; Roche, Mannheim, Germany)] and centrifuged at 1,000 × g for 10 min. The supernatant was centrifuged at 12,000 × g for 10 min, and the resultant pellet was re-suspended in homogenization buffer as the P2 fraction. The P2 fraction was layered over a 1.2 M/0.8 M sucrose gradient and centrifuged at 90,000 × g for 2 h. The synaptosome fraction was collected from the interface, mixed with equal volume of Triton solution [1% Triton X-100, 0.32 M sucrose, 12 mM Tris-Cl (pH 8.0)] for 15 min, and centrifuged at 200,000 × g for 1 h. The resultant pellet was suspended in 40 mM Tris-Cl (pH 8.0), 1% SDS as the post synaptic density (PSD) fraction. The protein concentration was determined using BCA Protein Assay Reagent (Thermo Fisher Scientific, MA, USA). Equal volume of SDS sample buffer [125 mM Tris-Cl (pH 6.8), 4% SDS, 20% glycerol, 0.002% BPB, 2% 2-mercaptoethanol] was added to the sample fractions and boiled for 5 min at 100 °C.

Protein samples were separated by 8% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (GE Healthcare, NJ, USA). Both WT and GluD1-KO mice samples were blotted on the same membrane for quantification. Membranes were blocked with 5% skimmed milk in TBS-T [20 mM Tris-Cl (pH 7.6), 137 mM NaCl, 0.1% Tween 20] for 1 h, and incubated with each primary antibody (1 μg/ml) (Table 1) for 3-4 h and horseradish peroxidase-conjugated secondary antibody for 1 h. Between these incubation steps, membranes were washed three times with TBS-T for 30 min. Protein bands were visualized with an enhanced chemiluminescence (ECL) kit (GE Healthcare) using a luminescence image analyzer with an electronically cooled charge-coupled device camera (EZ capture MG; ATTO, Tokyo, Japan). Signal

intensities of immunoreacted bands were determined using CS Analyzer ver.3.0 (ATTO).

Table 1. Primary antibodies used in the present study

	Sequence (NCBI #)	RRID	Host	Specificity	Reference/Source
GluA1	841-907 aa (X57497)	AB_2571752	Rb	KO	FI (GluA1-Rb-Af690)
GluA1	880-907 aa	n/a	Rb	IB	[80]
GluA2	175-430 aa (NM_013540)	AB_2113875	Ms		Millipore (MAB397)
GluN2A	1126-1408 aa	AB_2571605	Rb	KO	[81] FI (GluRe1C-Rb-Af542)
GluN2B	1301-1456 aa (D10651)	AB_2571762	Rb	KO	FI(GluRe2C-Rb-Af300)
GluK2	844-908 aa (P42269)	n/a	Rb	KO	[82] Synaptic systems (180 003)
GluD1	895-932 aa (NM_008166)	AB_2571757	Rb	KO	[32] FI (GluD1C-Rb-Af1390)
GluD2	897-934 aa (D13266)	AB_2571601	Rb	KO	[68]
PSD-95	1-64 aa (D50621)	AB_2571611	Rb	IB	[83] FI (PSD-95-Rb-Af1720)

aa, amino acid residues; FI, Frontier Institute; GluA1, AMPA-type glutamate receptor-1; GluA2, AMPA-type glutamate receptor-2; GluD1, delta-type glutamate receptor-1; GluD2, delta-type glutamate receptor-2; GluK2, kainate-type glutamate receptor-2; GluN2A, N-methyl-D-aspartate glutamate receptor-2A; GluN2B, N-methyl-D-aspartate glutamate receptor-2B; GP, guinea pig polyclonal antibody; KO, lack of

313 immunohistochemical or immunoblot labeling in knockout mice; Ms, mouse monoclonal
314 antibody; Rb, rabbit polyclonal antibody; RRID, Research Resource Identifier.
315

316 Statistical analysis

317 All data are expressed as mean ± SEM. Statistical analyses for behavioral studies were
318 performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama,
319 Japan), which is a graphical user interface for R (The R Foundation for Statistical
320 Computing, Vienna, Austria). Data were analyzed by one-way ANOVA, two-way
321 ANOVA, two-way repeated-measures ANOVA followed by Dunnett's post hoc tests, or
322 Student's t-test with Welch's correction as appropriate to correct for multiple
323 comparisons. Attack latency in the resident-intruder test was analyzed using
324 Kaplan-Meier survival curves followed by Mantel-Cox log-rank tests. All statistical
325 tests were two-tailed. The level of significance set was $p < 0.05$.
326

327 Results

328 Normal anxiety-related behavior in GluD1-KO mice

329 To determine whether GluD1 was involved in anxiety-related behavior, we performed
330 tests in an open field (Fig 1A), an elevated plus maze (Fig 1F), and a light-dark transition
331 test (Fig 1M). It is well established that performance in both the open field and elevated
332 plus maze are influenced by the arena illumination levels [84,85]. We therefore used two
333 different illumination conditions (5 and 100 lux) in these tests.
334

Fig 1. Hyperlocomotor activity but normal anxiety-related behavior in GluD1-KO mice. (A-E) The open field. Schematic representation of the open field test (A). GluD1-KO mice traveled significantly longer than WT in the open field test with 5 lux illumination (WT, n = 19; GluD1-KO, n = 13; $p < 0.05$, unpaired Student's t-test) (B), and 100 lux (WT, n = 33; GluD1-KO, n = 24; $p < 0.05$) (D). No significant difference was observed in the time spent in the central region with 5 lux ($p = 0.46$) (C) or 100 lux ($p = 0.08$) illumination (E). (F-L) The elevated plus maze. Schematic representation of the elevated plus maze (F). There were no significant differences between WT (5 lux, n = 16; 100 lux, n = 21) and GluD1-KO mice (5 lux, n = 10; 100 lux, n = 19) in the time spent in the closed arms [5 lux, $p = 0.87$ (G); 100 lux, $p = 0.25$ (J)] or in the open arms [5 lux, $p = 0.95$ (H); 100 lux, $p = 0.72$ (K)], or in the number of entries into the open arms [5 lux, $p = 0.92$ (I); 100 lux, $p = 0.70$ (L)]. (M-P) The light-dark transition test. Schematic representation of the light-dark transition test (M). There was no significant difference between WT (n = 33) and GluD1-KO mice (n = 23) in the time spent in the dark and light boxes [Dark box, $p = 0.62$; Light box, $p = 0.62$ (N)], in the number of entries into the light box ($p = 0.85$) (O), or in latency to first transition into the light box ($p = 0.17$) (P). *$p < 0.05$, unpaired Student's *t*-test with Welch'correction. All values presented are mean ± SEM.

We performed the open field test for a total duration of 10 min (Fig 1A). The open field test presents a conflict between innate drives to explore a novel environment and personal safety [86]. GluD1-KO mice traveled longer distances compared to WT mice under both illumination conditions (Fig 1B, D). We also calculated the percentage of time spent in the central area of the open field, which is commonly used as an index of anxiety

[41]. There were no significant differences in the percentage of time spent in the central area between WT and GluD1-KO mice under either illumination conditions (Fig 1C, E).

In the elevated plus maze, we did not detect any significant differences in the time spent in the closed or open arms between genotypes, or in the number of entries into the open arm between genotypes under either of the illumination conditions (Fig 1G–L). There was no significant differences between WT and GluD1-KO mice in total distance traveled using 5 lux (WT, 15.6 ± 1.0 meters; GluD1-KO, 18.3 ± 0.96 meters; $p = 0.076$) or 100 lux (WT, 19.5 ± 0.96 meters; GluD1-KO, 21.7 ± 1.1 meters; $p = 0.142$) and no differences in total entries using 5 lux (WT, 26 ± 2.4; GluD1-KO, 30 ± 2.8; $p = 0.256$) or 100 lux (WT, 34 ± 2.0; GluD1-KO, 39 ± 2.4; $p = 0.162$).

Besides, we performed another behavioral assay for studying anxiety in mice, the light-dark transition test (Fig 1M). There was no significant difference between WT and GluD1-KO mice in the time spent in the light and dark portions of the box (Fig 1N), in transition number between the illuminated and dark areas (Fig 1O), or in latency to enter the illuminated area of the box (Fig 1P).

Together, GluD1-KO mice showed hyper-locomotor activity in the open field test; however, GluD1-KO mice did not show any anxiety-related behaviors in the open field, elevated plus maze, or light-dark transition tests.

Normal aggression-like behavior in GluD1-KO mice

GluD1-KO mice were rare to show aggressive-like behavior in their home cage. In accordance with these observations, both latency to attack first and attack frequency in GluD1-KO mice were not significantly different from that of WT mice in the resident-intruder test (Fig 2).

Fig 2. Normal aggression-like behavior in GluD1-KO mice. In the resident-intruder test, there was no significant difference between WT (n = 8) and GluD1-KO (n = 7) mice in attack latency (left; log-rank test, $\chi^2 = 2.451$, $p = 0.117$) or attack frequency (right, unpaired student's *t*-test, $p = 0.304$). All values presented are mean ± SEM.

Normal sensorimotor gating in GluD1-KO mice

Because human mutations of the *Grid1* gene are associated with schizophrenia [23–25], we next performed PPI of the acoustic startle response, which is one of the most promising electrophysiological endophenotypes of both patients and animal models of schizophrenia [87–90]. In the acoustic startle responses, the amplitude of startle responses was dependent on pulse intensity. There was no difference between genotypes (Fig 3A).

We then examined PPI levels of WT and GluD1-KO mice using four different prepulse intensities. Induction of PPI using 73-, 76-, 79- and 82-dB prepulse in the 120-dB startle condition occurred in both WT and GluD1-KO mice (Fig 3B). There was no significant difference between WT and GluD1-KO mice in PPI levels, suggesting normal sensorimotor gating in GluD1-KO mice.

Fig 3. Normal startle response and prepulse inhibition in GluD1-KO mice. (A) Acoustic startle reflex test: Startle responses amplitudes were dependent on pulse intensity (WT, n = 7; GluD1-KO, n = 9) (two-way ANOVA: $F_{7,112} = 24.2$, $p < 0.001$). There was no difference between genotype ($F_{1,112} = 0.08$, $p = 0.78$), and no interaction between genotype and tone intensity ($F_{7,112} = 0.13$, $p = 1.00$). (B) Prepulse inhibition test:

The PPI levels of WT (n = 11) and GluD1-KO mice (n = 12) were not significantly different with prepulses of 73, 76, 79 and 82 dB (two-way ANOVA: Genotype; $F_{1,84}$ = 2.64, p = 0.11). PPI levels were dependent on prepulse intensity ($F_{3,84}$ =18.09, p < 0.001). There was no significant interaction between genotype and prepulse intensity ($F_{3,84}$ = 0.08, p = 0.97). All values presented are mean ± SEM.

Social deficiency in GluD1-KO mice

We then examined the three-chamber social interaction test, which consists of a sociability test and a social novelty preference test [48] (Fig 4). In the sociability test, a wire cage with a stranger mouse (Stranger 1) was placed in one of the side chambers, and an empty cage was placed in another side chamber (Fig 4A). The preference of the mouse can be quantified based on the time spent around the wire cage with a stranger mouse versus the empty cage. GluD1-KO mice spent a significantly shorter time around the wire cage with the stranger mouse than that of WT mice (Fig 4B) indicating a lack of sociability.

In the social novelty preference test, a second stranger mouse (Stranger 2) was introduced into the empty cage. GluD1-KO mice spent a significantly shorter time around the wire cage with the novel stranger mouse (stranger 2) than that of WT mice (Fig 4C) indicating a lack of preference for social novelty.

Fig 4. Reduced sociability and preference for social novelty in GluD1-KO mice. (A) Schematic representation of the three-chamber social interaction test. Sociability test (middle): a wire cage with a stranger mouse (Stranger 1) was placed in one side chamber and an empty wire cage was placed on the opposite side chamber. Social novelty

preference test (right): a novel stranger mouse (Stranger 2) was placed in a wire cage in one side chamber and a familiar mouse (Stranger 1) was placed in a wire cage on the opposite site. (B) Sociability test: there was a significantly lower time spent near the wire cage with Stranger 1 in GluD1-KO (n = 23) than that of WT mice (n = 25) ($p < 0.01$). (C) Social novelty preference test: there was significantly lower time spent near the wire cage with stranger 2 in GluD1-KO mice than that of WT mice ($p < 0.05$). All values presented are mean ± SEM. *$p < 0.05$; **$p < 0.01$, unpaired Student's t-test with Welch's correction.

Impaired contextual fear memories in GluD1-KO mice

To assess the involvement of GluD1 in fear memory, we performed contextual and cued fear conditioning tests (Fig 5). The freezing responses in the conditioning session did not differ significantly between genotypes (Fig 5A). In the contextual test, GluD1-KO mice exhibited a modest but significant decrease in the freezing response relative to WT mice (Fig 5B).

In contrast, GluD1-KO mice showed no significant difference in freezing response relative to WT mice in the cued test (Fig 5C). There were no significant differences in pain sensitivity (Fig 5D) or hearing ability (Fig 3A) between genotypes, suggesting that ablation of GluD1 caused deficits of contextual, but not cued memories in the fear conditioning tests.

Fig 5. Impaired contextual, but not cued memory in GluD1-KO mice in the fear conditioning test. (A) Schematic representation of the conditioning test (left). Freezing responses on the conditioning test; there was no significant difference between WT (n =

8) and GluD1-KO (n = 9) mice (two-way repeated-measures ANOVA: Genotype; $F_{1,15}$ = 0.0075, p = 0.93, US presentation; $F_{3,45}$ = 14.5, p < 0.0001, Genotype × US presentation; $F_{3,45}$ = 0.523, p = 0.67). (B) Contextual test: freezing responses on the contextual testing 24 h after conditioning. There was significantly lower freezing in GluD1-KO mice during contextual conditioning (p < 0.01, unpaired Student's t-test). (C) Cued test: freezing responses on the cued testing 48 h after conditioning. There was no significant difference between WT and GluD1-KO mice in cued conditioning during pre-tone and tone (pre-tone, p = 0.62; tone, p = 0.14; unpaired Student's t-test). (D) Pain sensitivity test; there were no significant differences between WT (n = 14) and GluD1-KO (n = 11) mice in the footshock test evaluated by flinch (p=0.24), vocalization (p=0.24), vertical jump (p=0.91) and horizontal jump (p=0.56). All values presented are mean ± SEM. **p < 0.01.

Restoring depression-like behavior in GluD1-KO mice using a serotonin transporter blocker

To analyze depression-like behavior, we used the Porsolt forced-swim test [72] (Fig 6). GluD1-KO mice showed significantly increased immobility, indicating enhanced depressive-like behavior (Fig 6A).

Fig 6. Enhanced depressive-like behavior in GluD1-KO mice. (A) Schematic representation of the forced-swim test (left). There was significantly higher percentage immobility in GluD1-KO mice (n = 22) than WT (n = 30) (middle) (two-way repeated measures ANOVA: Genotype, $F_{1,50}$ = 5.66, p < 0.05; Time, $F_{4,200}$ = 25.1, p < 0.001;

Genotype × Time, $F_{4,200} = 1.56$, $p = 0.19$). Average immobility times for minutes 1 to 5 in the forced-swim test (right). There was a significantly higher immobility in GluD1-KO than WT mice ($p < 0.05$, unpaired Student's t-test). (B) Impact of antidepressants on the forced-swim test. Animals were injected intraperitoneally with saline (WT, n = 12; GluD1-KO, n = 8), imipramine (15 mg/kg) (WT, n = 12; GluD1-KO, n = 8), fluoxetine (10 mg/kg) (WT, n = 11; GluD1-KO, n = 9), or desipramine (30 mg/kg) (WT, n = 9; GluD1-KO, n = 9). Mice were subjected to open field test 60 min after injection for 10 min, and subsequently subjected to a forced-swim test for 5 min. Average immobility times for 3 to 5 min: there was a significant genotype and treatment effect between and WT and GluD1-KO mice (two-way ANOVA: Genotype, $F_{1,70} = 29.13$, $p < 0.001$; Drug, $F_{3,70} = 6.16$, $p < 0.001$), but no significant interaction ($F_{3,70} = 2.56$, $p = 0.06$). Within-genotype testing revealed that imipramine and fluoxetine led to a reduction in immobility in GluD1-KO mice (one-way ANOVA with Dunnett's post hoc test: $F_{3,30} = 5.58$, $p = 0.004$; Imipramine, $p = 0.0017$; Fluoxetine, $p = 0.032$; Desipramine, $p = 0.42$). In WT mice, no significant differences in immobility were observed with these antidepressants ($F_{3,40} = 1.78$, $p = 0.17$; Imipramine, $p = 0.85$, Fluoxetine, $p = 0.74$; Desipramine, $p = 0.41$). All values presented are mean ± SEM. *$p < 0.05$; **$p < 0.01$, Dunnett's post hoc test. Desi, desipramine; Flu, fluoxetine; Imi, imipramine.

Next, we tested the impact of representative antidepressants on depression-like behavior in GluD1-KO mice. Imipramine and fluoxetine are inhibitors of the serotonin transporter, while desipramine is an inhibitor of the norepinephrine transporter. [91,92]. These drugs were injected intraperitoneally into WT or GluD1-KO mice 70 min before the forced-swim test (Fig 6B). Before the forced-swim test, an open field test (10 min)

was conducted to confirm that these drugs did not produce false-positive results on restoring depression-like behavior in the forced-swim test due to an increase of locomotor activity.

Injection of imipramine and desipramine, but not fluoxetine led to a reduction in the total distance in WT mice (One-way ANOVA with Dunnett's post hoc test: $F_{3,40}$ = 6.41, p = 0.0012; Saline, 36.9 ± 2.3 meters; Imipramine, 25.5 ± 3.3 meters, p = 0.035; Fluoxetine, 37.0 ± 1.4 meters, p = 1.0; Desipramine, 27.5 ± 1.9 meters, p = 0.032) in the open field test). In contrast, no significant differences were observed in these antidepressants in GluD1-KO mice ($F_{3,30}$ = 3.489, p = 0.028; Saline, 37.6 ± 2.0 meters; Imipramine, 31.7 ± 2.2 meters, p = 0.10; Fluoxetine, 38.1 ± 1.6 meters, p = 1.0; Desipramine, 31.8 ± 1.8 meters, p = 0.093).

In the forced-swim test, no significant differences were observed in these antidepressants in WT mice, possibly due to a ceiling effect. Of note, differences in percentage immobility for the saline-injected versus naïve WT mice in the forced-swim test may have arisen due to injection stress. Injected of GluD1-KO mice with imipramine and fluoxetine, inhibitors of the serotonin transporter, led to a reduction in percentage immobility in the forced-swim test (Fig 6B). In contrast, a reduction in percentage immobility was not observed with GluD1-KO mice with desipramine, an inhibitor of the norepinephrine transporter (Fig 6B). These results suggest that inhibition of the serotonin transporter, but not norepinephrine transporter, restored depression-like behavior in GluD1-KO mice.

Normal glutamate receptor levels and expression of PSD-95 protein in the frontal cortex and hippocampus of GluD1-KO mice

Finally, we measured levels of synaptic protein expression in GluD1-KO mice. We analyzed the excitatory synaptic proteins, such as AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type (GluA1 and GluA2), NMDA (N-methyl-D-aspartate)-type (GluN2A and GluN2B), Kainite-type (GluK2), and δ-type (GluD2) glutamate receptors and PSD-95 in the synaptosome and PSD fractions prepared from the frontal cortex and the hippocampus. Inconsistent with previous reports [38,39], we did not observe significant alterations of protein expression levels in any of these proteins in both synaptosome (Fig 7A) and PSD fractions (Fig 7B) in GluD1-KO mice. What did, however, find small but significant increases in expression of GluD2 in the PSD fractions of both the frontal cortex and the hippocampus of GluD1-KO mice (Fig 7B).

Fig 7. Normal protein expression in synaptosome and PSD fractions prepared from the frontal cortex and the hippocampus of GluD1-KO mice. (A) Protein expression in synaptosome fractions prepared from the frontal cortex and hippocampus. The protein loaded in lanes for GluA1, GluA2, GluN2A, GluN2B, GluK2, and PSD-95 were 20 μg, and 30 μg for GluD2. There was no significant difference between WT and GluD1-KO mice in the protein expression prepared from the synaptosome fractions of the frontal cortex (GluA1, $p = 0.59$; GluA2, $p = 0.89$; GluN2A, $p = 0.34$; GluN2B, $p = 0.33$; GluK2, $p = 0.73$; GluD2, $p = 0.27$; PSD-95, $p = 0.71$) and the hippocampus

(GluA1, p = 0.59; GluA2, p = 0.60; GluN2A, p = 0.96; GluN2B, p = 0.46; GluK2, p = 0.86; GluD2, p = 0.22; PSD-95, p = 0.50). (B) Protein expression in PSD fractions prepared from the frontal cortex and hippocampus. The protein loaded in lanes for GluA1, GluA2, GluN2A, GluN2B, GluK2, and PSD-95 were 10 μg, and 20 μg for GluD2. Except for GluD2, there was no significant difference between WT and GluD1-KO mice in protein expression prepared from the PSD fractions of the frontal cortex (GluA1, p = 0.50; GluA2, p = 0.99; GluN2A, p = 0.70; GluN2B, p = 0.43; GluK2, p = 0.71; GluD2, p = 0.01; PSD-95, p = 0.68) or the hippocampus (GluA1, p = 0.27; GluA2, p = 0.76; GluN2A, p = 0.51; GluN2B, p = 0.49; GluK2, p = 0.50; GluD2, p = 0.03; PSD-95, p = 0.89). All values presented are mean ± SEM from 3-4 experiments. *p < 0.05, unpaired Student's t-test with Welch's correction. FC, the frontal cortex; HPC, the hippocampus.

Discussion

To avoid the variability inherent in mixed genetic backgrounds and effects of closely linked genes flanking the targeted locus, we generated GluD1-KO mice with a pure C57BL/6N background and performed behavior analysis to assess GluD1 functions *in vivo*. Our GluD1-KO mice showed hyperlocomotor activity, abnormal social behavior, a deficit in contextual (but not cued) fear memory, and enhancement of depressive-like behavior, that is partially consistent with the previous studies using *Grid1*^{tm1Jnz} mice [37,38].

We did not observe significant differences in either aggressive behavior in the resident-intruder test, anxiety-related behavior evaluated by the open field test or the light-dark transition test, or the elevated plus maze in our GluD1-KO mice compared to WT mice, whereas robust aggression and lower anxiety-related behavior were observed in *Grid1*^{tm1Jnz} mice [37]. There are three conceivable possibilities that may explain the different behavioral phenotypes between our GluD1-KO and the *Grid1*^{tm1Jnz} mice. The first possibility is the strain difference and flanking-genes effect. It is known that there are mouse-strain difference in basal levels of both aggression [44,45] and anxiety [41–43]. Moreover, it remains a concern that flanking alleles of the target locus generated during the backcrossing of ES cell-derived knockout mice to appropriate mice strains may influence phenotype [53,57,58]. The *Grid1*^{tm1Jnz} mouse was generated using the 129S6/SvEvTac ES cell line [40] followed by backcrossing to C57BL/6 strain 2–6 times [37–39], so a phenotype difference may have arisen due to this. The second possibility is deleterious effects of selection maker gene in the target locus on the neighboring genes expression. The *Grid1*^{tm1Jnz} mouse harbors the neomycin phosphotransferase cassette that allowed the selection of homologous recombinants in the targeted allele [40]. However, such marker genes can interfere with the transcription and splicing of the neighboring genes, thereby resulting in ambiguous genotype-phenotype relationships [64–67]. The gene encoding microRNA (miRNA) *miR-346* that regulates the translation of mRNAs via interaction with their 3' untranslated regions, is located in intron 2 of the GluD1 gene [94]. In contrast, the neomycin phosphotransferase cassette in our GluD1-KO mice deleted via the Cre/loxP system, so it is more appropriate for analyses of GluD1 function. The third possibility is a gene-environment interaction where

591 differences in laboratory environments becomes an additional contributing factor that
592 modulates the behavioral outcome of genetically modified animal models in psychiatry
593 [95,96].

594 We found that there was no significant alteration of postsynaptic protein
595 expression, including AMPA-, kainite-, and NMDA-type glutamate receptor subunits,
596 and PSD-95, in the frontal cortex or the hippocampus of GluD1-KO mice, which is
597 inconsistent with the previous studies [37–39]. In the prefrontal cortex, a significantly
598 lower expression of GluA1 and GluA2 was observed in *Grid1*^{tm1Jnz} mice [37]. In
599 addition, there was a significantly lower expression level of GluA1, GluA2, and GluK2,
600 and a significantly higher expression level of GluN2B and PSD-95 in the hippocampus of
601 *Grid1*^{tm1Jnz} mice [38]. In that study, the authors used a quantification method for
602 synaptosome fraction in which the optical density of each protein of interest was
603 normalized to β-actin [37,38]. In this case, however, normalization of synaptosome
604 fraction by β-actin might be an inappropriate method for two reasons. Firstly, β-actin is a
605 cytoplasmic protein and synaptosomal preparation may increase the variability of the
606 amount of β-actin proteins in the synaptosome fractions. Secondly, Gupta and colleagues
607 suggested that morphological abnormality in the hippocampus and the medial prefrontal
608 cortex in *Grid1*^{tm1Jnz} mice was probably due to an alteration of actin dynamics [39]. If
609 this was the case, then β-actin is not an appropriate protein for normalization. In contrast,

610 we investigated expression levels of synaptic proteins in synaptosome and PSD fractions
611 between WT and GluD1-KO mice using western blot without normalization by β-actin
612 (see Method). In addition, we confirmed that results of western blot for synaptosome and
613 PSD fractions without normalization were consistent with those on semi-quantitative
614 analysis using immunofluorescence [32,97–99].

615 In the fear conditioning test, GluD1-KO mice showed significantly lower freezing
616 times in contextual tests, but not in the cued tests. It is well known that the hippocampus
617 and amygdala are critical regions underlying contextual fear conditioning, whereas the
618 amygdala underlies cued conditioning [8]. The deficit of GluD1-KO mice in the
619 contextual test might suggest that GluD1 is more functionally important in the
620 hippocampus. Interestingly, forebrain-specific knockout mice of Cbln1, a partner
621 molecule of GluD1, showed a deficit in contextual and cued memory in the fear
622 conditioning test [100]. The GluD1-CBLN2-NRXN transsynaptic adhesion system
623 requires the formation and maintenance of synapses in the hippocampus *in vitro* and *ex*
624 *vivo* [1,2]. Moreover, Cbln1/2 double knockout mice have decreased synapse density in
625 the hippocampus of 6-month-olds but not 1- or 2-month-olds [3]. Of note, we observed
626 increased GluD2 expression in the PSD fraction of the hippocampus of GluD1-KO mice.
627 In contrast, increased GluD1 expression has been reported in the cerebellum of
628 GluD2-KO mice [9]. These results imply that compensatory regulation for GluD
629 subunits expression exists in brain regions, including the hippocampus. Further analysis
630 needs to clarify the specific role of the GluD-CBLN-NRXN transsynaptic adhesion
631 system in the hippocampus. In addition, there are reports that GluD1 also has unique
632 functions, such as (1) a regulator for group 5 mGluR-mediated AMPA-type glutamate

receptor trafficking [4], and (2) the ion channel activity via group 1 mGluR signaling [5–7]. So, there are other possibilities that may explain how dysfunction of GluD1 affects hippocampal-dependent contextual fear memory. To determine details of the molecular function of GluD1, the hippocampus is a suitable brain area for further analyses.

Human GluD1 gene (*GRID1*) is a gene associated with susceptibility to schizophrenia, autism spectrum disorder, and depression [23–28,94,101]. However, how *GRID1* affects the pathophysiology of these conditions largely remains elusive. Our GluD1-KO mice showed significantly lower sociability in the three-chambered social interaction test, consistent with a previous report [37]. In contrast, our GluD1-KO mice showed lower social novelty preference that is inconsistent with a previous report [37]. Further analysis, such as novel object recognition, may clarify whether this phenotype is derived from either impairment of memory function, sociability, or both. Interestingly, lower *GRID1* mRNA expression is observed in the cerebral cortex of patients with schizophrenia [94] and autism spectrum disorder [102]. Downregulation of *GRID1* mRNA expression is also observed in iPS (induced pluripotent stem) cells derived from Rett syndrome patients, which is a condition associated with autism spectrum disorder [103]. In addition, downregulation of *Cbln1* mRNA was observed in mice carrying a triple dose of Ube3a, a model mouse for autism spectrum disorder [104]. Deletion of *Cbln1* in the glutamatergic neurons of the ventral tegmental area led to lower sociability by weakening excitatory synaptic transmission [104]. This behavioral abnormality might support the hypothesis that GluD1-CBLN-NRXN-dependent synapse formation and maintenance, in particular brain regions, is related to sociability.

Our GluD1-KO mice showed enhanced depressive-like behavior assessed by the forced-swim test. Pharmacological studies further revealed that imipramine and fluoxetine, but not desipramine, significantly restored the enhanced depressive-like behavior in GluD1-KO mice. Because imipramine and fluoxetine are more effective in inhibiting serotonin transporters than desipramine [91,92], increased serotonin concentration in the brain regions related to depressive-like behavior may account for the abnormal behavior of GluD1-KO mice; in other words, the serotonin signaling pathway might be altered in GluD1-KO mice. Candidate regions, in which GluD1 is expressed, related to depression are the lateral habenula and dorsal raphe nucleus [32,33,100]. Increased neuronal activity in the lateral habenula is observed in patients with depression and in animal models of depression [105,106] and lesions of the lateral habenula alters extracellular serotonin concentration in the dorsal raphe nucleus when receiving uncontrollable stress [107]. Furthermore, GluD1 mRNA is downregulated in the frontal cortex of anhedonic rats as an animal model of depression [108], and this phenotype is completely reversed by intraperitoneal injection of the antipsychotic quetiapine [109]. Further analysis is required to identify regions involved in the enhanced depressive-like behavior mediated by the serotonergic system in GluD1-KO mice. As to the effect of antidepressants in the forced-swim test, selective serotonin reuptake inhibitors (SSRIs) tend to lead to more swimming behavior, whereas serotonin and norepinephrine reuptake inhibitors (SNRIs) tend to lead to more climbing behavior [110,111]. Thus, evaluating climbing and swimming behavior with more specific SSRIs (e.g. citalopram or escitalopram) [91,112] and SNRIs (e.g. reboxetine or atomoxetine) [113] will allow us to more precisely discriminate the pathway underlying enhanced depressive-like behavior in GluD1-KO mice.

Behavioral analyses under a pure C57BL/6N genetic background suggest that GluD1 plays critical roles in contextual fear memory, sociability, and depressive-like behavior. We originally developed the *Grid1*$^{+/flox}$ mouse in which exon 4 of *Grid1* gene was flanked by loxP sequences [68]. *Grid1* $^{flox/flox}$ mice under a C57BL/6N genetic background allow us to delete the *Grid1* gene in a brain region-specific manner using region-specific Cre mice or Cre-expressing virus injections. This brain region-specific GluD1-KO mouse could be a useful tool to clarify the neuronal circuits and molecular mechanisms involved in contextual fear memory, sociability, and depressive-like behavior.

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Author contributions

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2. Formal analysis: CN, TT.
3. Funding acquisition: TT, KS.

4. Investigation: CN.
5. Methodology: CN, MK, MA, KS.
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7. Resources: EN, RN, MW.
8. Validation: CN, KT, TT.
9. Visualization: CN, TT.
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All authors discussed the manuscript.

Conflict of interest

The authors declare no competing financial interests.

Data Availability Statement

All the raw data of behavioral tests (doi: 10.6084/m9.figshare.10052663) and western blot images (doi: 10.6084/m9.figshare.10053092) in this study are disclosed in the figshare.

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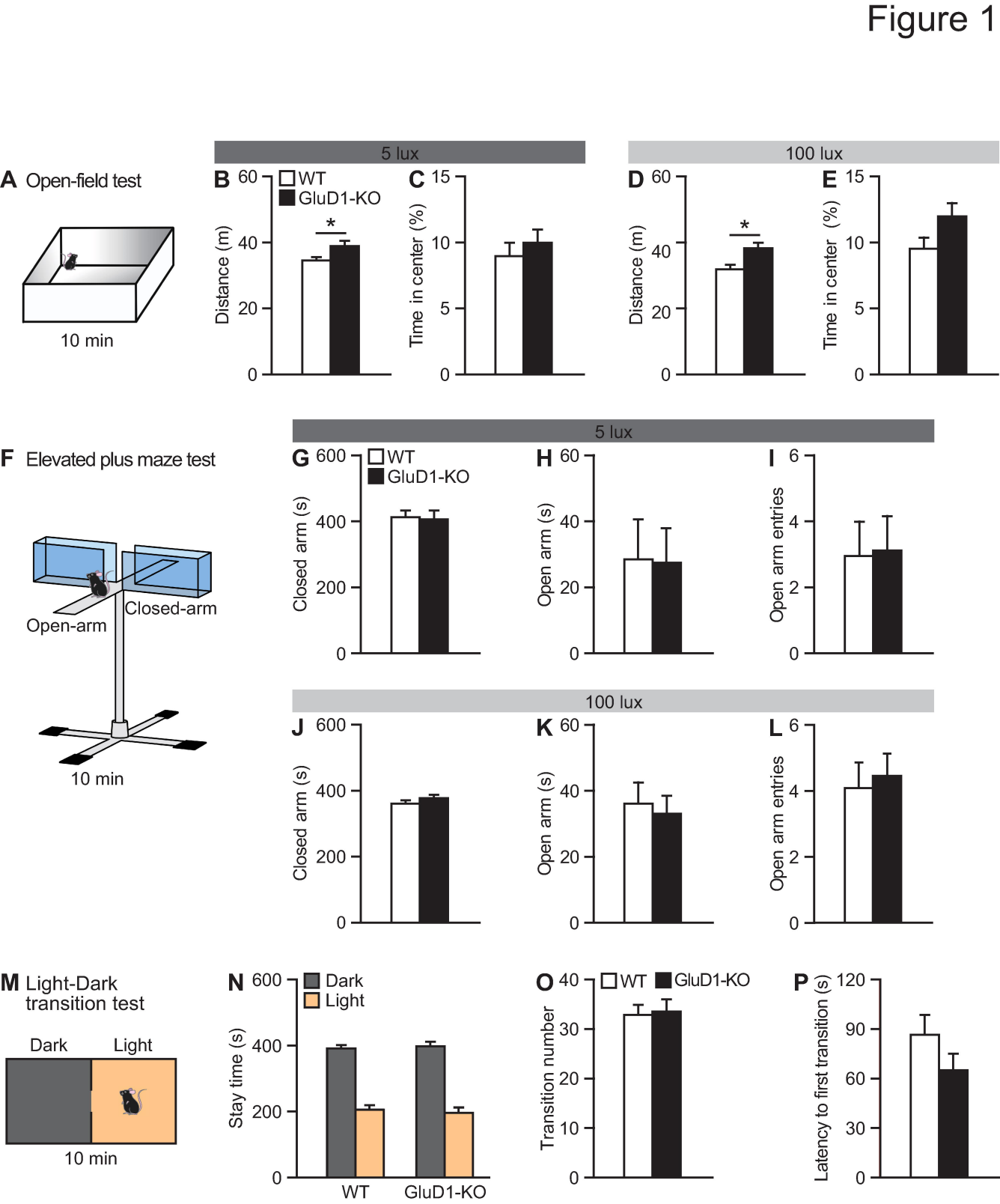
Figure 1


A Open-field test
10 min
5 lux
100 lux
B
C
D
E
WT
GluD1-KO
Distance (m)
Time in center (%)
F Elevated plus maze test
Open-arm
Closed-arm
10 min
G
H
I
J
K
L
Closed arm (s)
Open arm (s)
Open arm entries
M Light-Dark transition test
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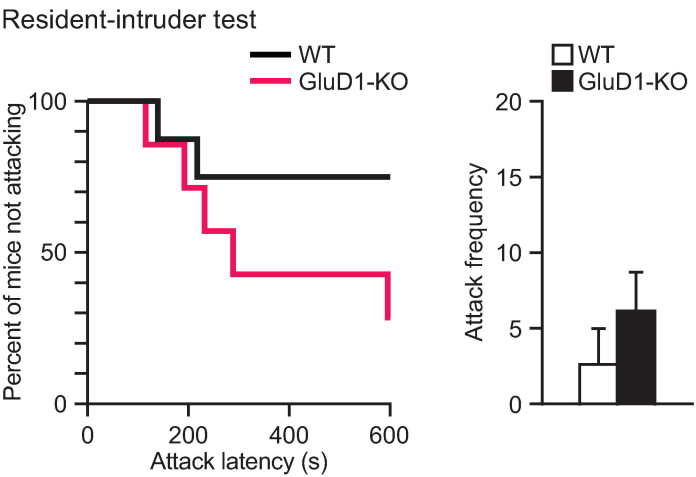
Stay time (s)
Transition number
Latency to first transition (s)
WT
GluD1-KO

Figure 2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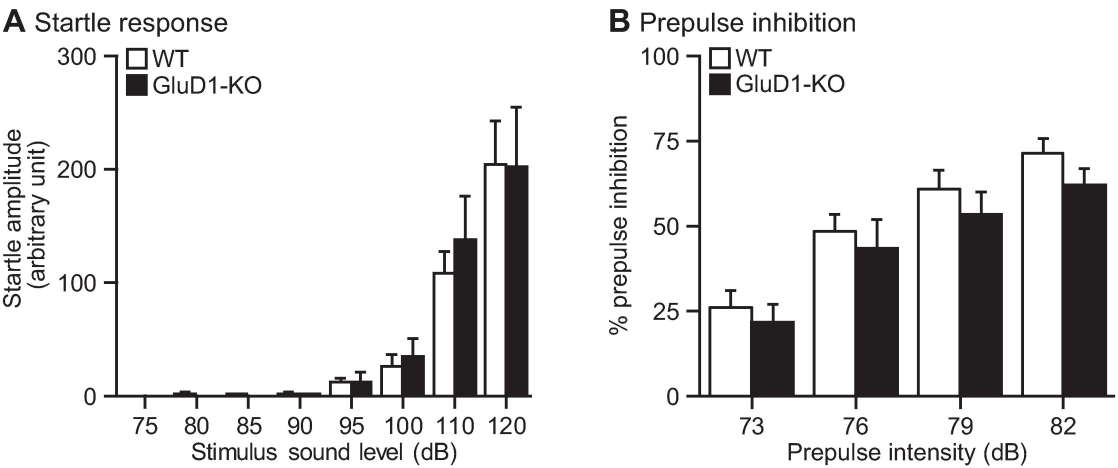
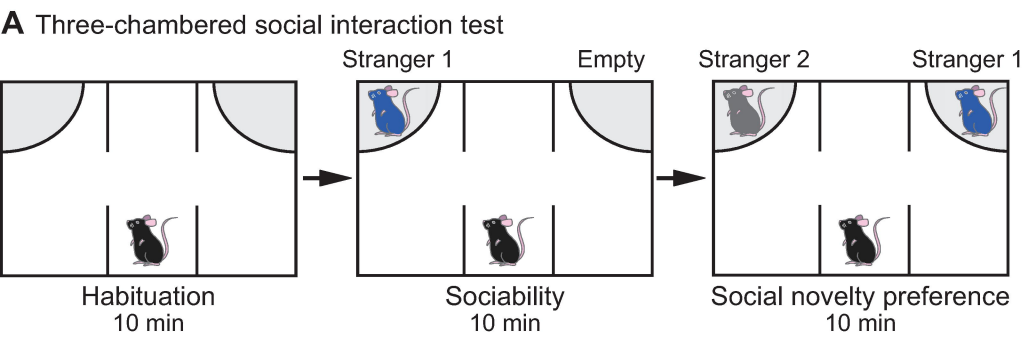
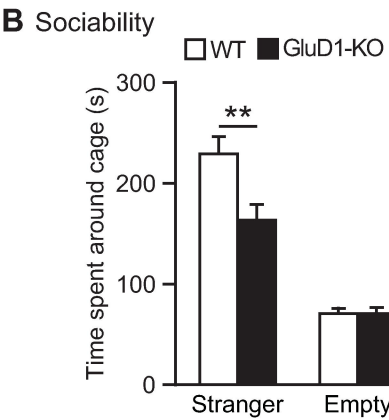
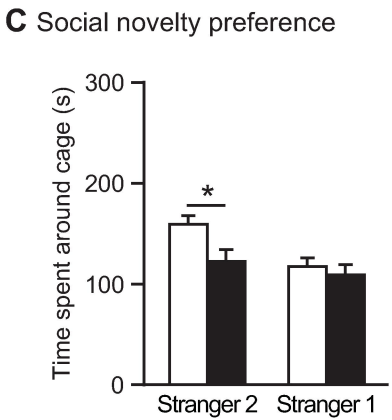
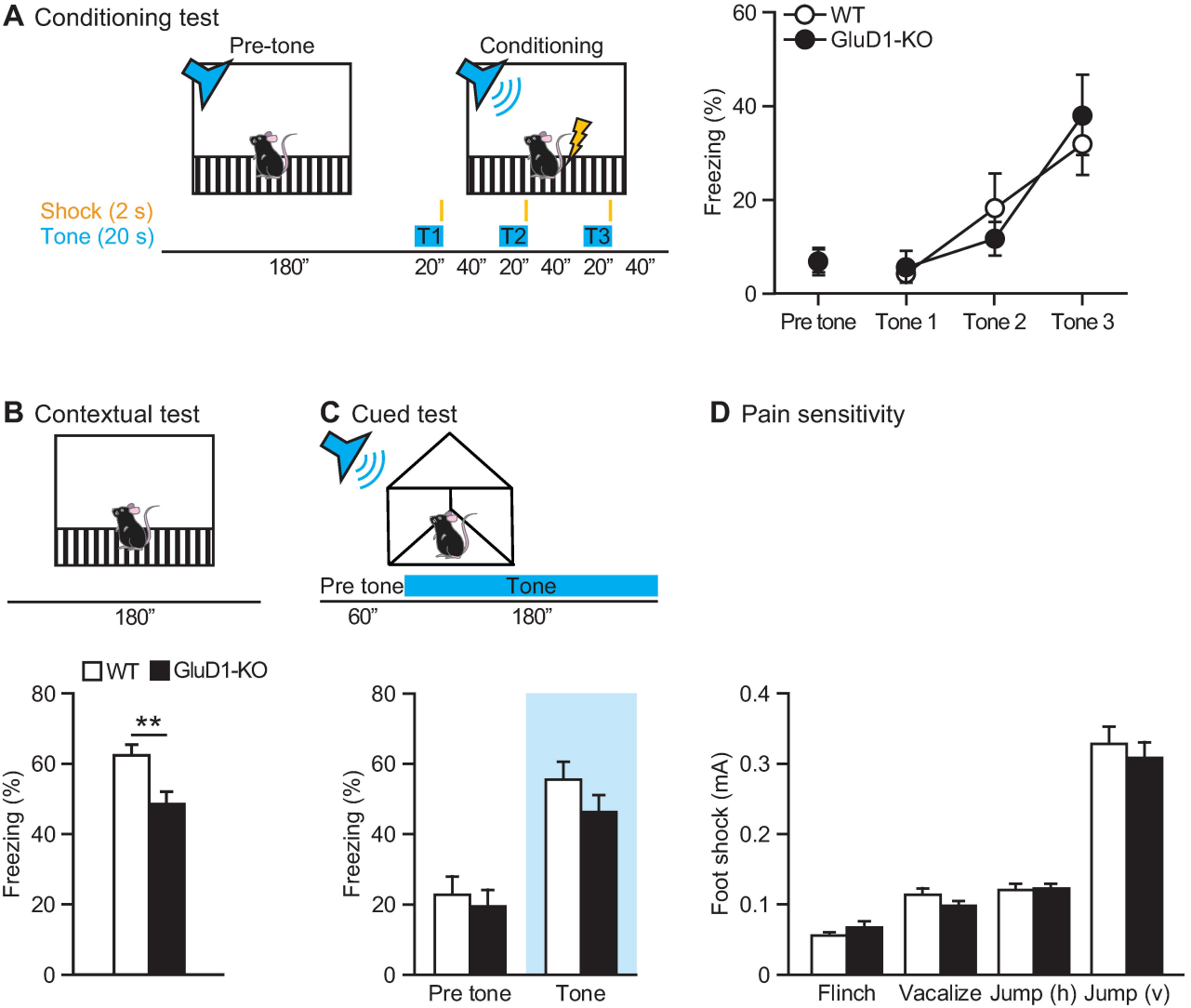
GluD1-KO
Percent of mice not attacking
100
50
0
0
200
400
600
Attack latency (s)
WT
GluD1-KO
Attack frequency
20
15
10
5
0

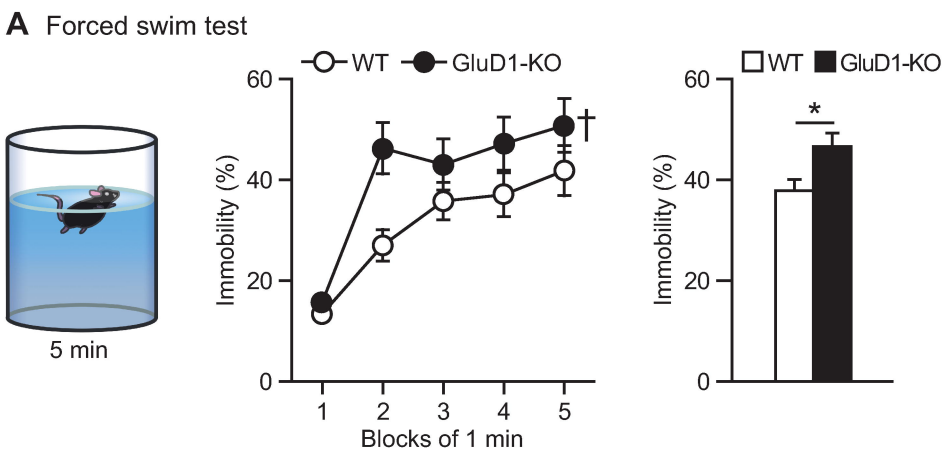

A Startle response
WT
GluD1-KO
Startle amplitude (arbitrary unit)
300
200
100
0
75
80
85
90
95
100
110
120
Stimulus sound level (dB)
B Prepulse inhibition
WT
GluD1-KO
% prepulse inhibition
100
75
50
25
0
73
76
79
82
Prepulse intensity (dB)

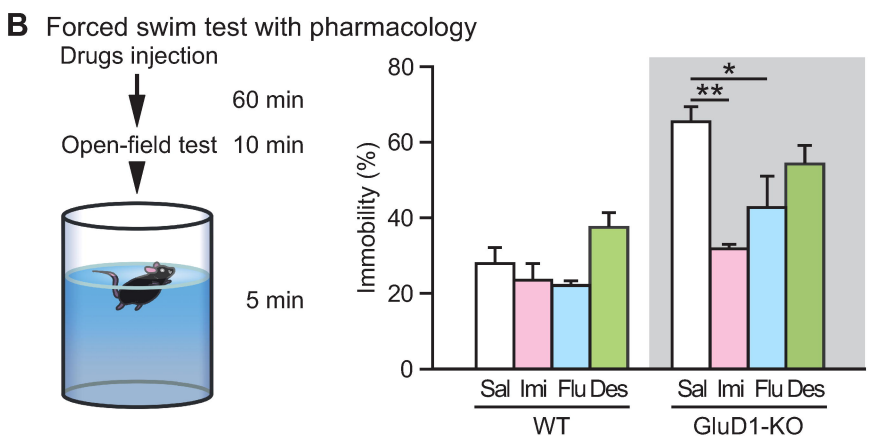

A Three-chambered social interaction test
Stranger 1
Empty
Stranger 2
Stranger 1
Habituation
10 min
Sociability
10 min
Social novelty preference
10 min

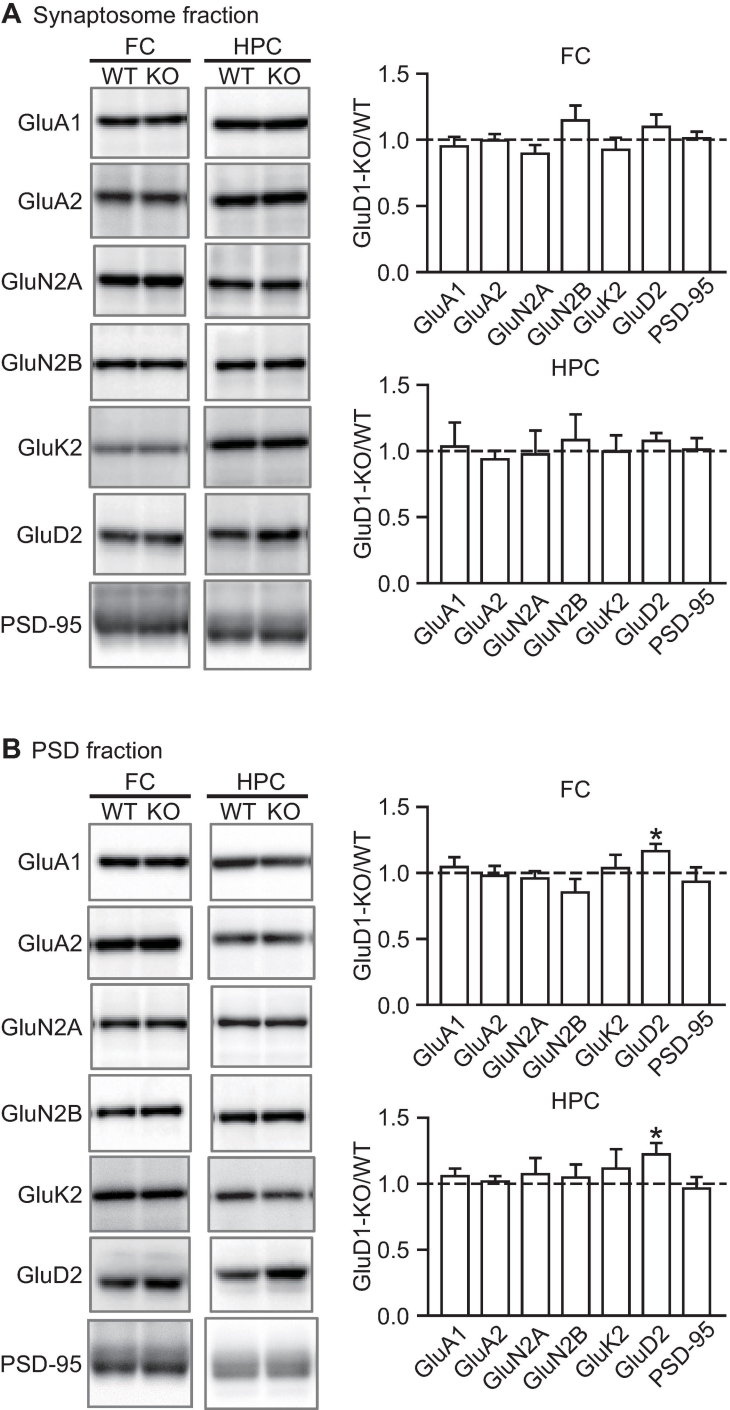

B Sociability
WT
GluD1-KO
Time spent around cage (s)
300
200
100
0
**
Stranger
Empty


C Social novelty preference
Time spent around cage (s)
300
200
100
0
*
Stranger 2
Stranger 1


A Conditioning test
Pre-tone
Conditioning
Shock (2 s)
Tone (20 s)
T1
T2
T3
180”
20” 40” 20” 40” 20” 40”
WT
GluD1-KO
Freezing (%)
60
40
20
0
Pre tone
Tone 1
Tone 2
Tone 3
B Contextual test
180”
WT
GluD1-KO
**
Freezing (%)
80
60
40
20
0
C Cued test
Pre tone
Tone
60”
180”
Freezing (%)
80
60
40
20
0
Pre tone
Tone
D Pain sensitivity
Foot shock (mA)
0.4
0.3
0.2
0.1
0
Flinch
Vacalize
Jump (h)
Jump (v)


A Forced swim test
WT
GluD1-KO
5 min
Immobility (%)
Blocks of 1 min
†
*


B Forced swim test with pharmacology
Drugs injection
60 min
Open-field test
10 min
5 min
Immobility (%)
*
**
Sal Imi Flu Des
WT
Sal Imi Flu Des
GluD1-KO


A Synaptosome fraction
FC
HPC
WT KO
WT KO
GluA1
GluA2
GluN2A
GluN2B
GluK2
GluD2
PSD-95
FC
GluD1-KO/WT
1.5
1.0
0.5
0.0
GluA1
GluA2
GluN2A
GluN2B
GluK2
GluD2
PSD-95
HPC
B PSD fraction
*