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論　文　題　目

〈邦　文〉

プレキシン A1 欠損マウスにおける毛繕い行動の亢進とプレパルス抑制障害に関する研究

〈欧　文〉

Study on the excessive self-grooming and impaired prepulse inhibition in PlexinA1-deficient mice

名城大学大学院　薬学研究科　薬学専攻　博士課程

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The dissertation is based on the following report

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Abstract

PlexinA1 (PlxnA1) is a transmembrane receptor for semaphorins (Semas), a large family of proteins that act as axonal guidance cues during nervous system development. The name of Plxn was derived from the plexiform layers of the optic tectum and the neural retina where its expression was primarily observed. Plxns are highly conserved transmembrane receptors throughout the animal kingdom from invertebrates to vertebrates, which suggests crucial roles of Plxns in the homeostatic control and development of various tissues. PlxnA1 is expressed in a broad range of tissues from embryo to adult. In the embryo, PlxnA1 is expressed in cortical interneurons and plays an important role in forebrain development. PlxnA1 is also expressed in the interneurons in the adult mouse cortex, surrounded by perineuronal nets (PNNs) which contain Semaphorin3A (Sema3A), a class 3 Sema as a constituent. Many studies have been conducted on the role of PlxnA1 in the regulation of axon guidance, cardiac morphogenesis, carcinogenesis, bone homeostasis or immune responses. However, the neurobiological role of PlxnA1 in neuronal network development crucial for neurobehavioral performance has not yet been clarified.

In the experiments to see the expression of PlxnA1 in the adult (6-month-old) mouse brain, western blotting analyses performed on protein lysates from different brain regions initially confirmed the expression in the prefrontal cortex, hippocampus, and striatum. To know the cellular localization of *PlxnA1* mRNA, *in situ* hybridization was performed with *PlxnA1* mRNA-specific probes on coronal sections from GAD67-GFP knock-in mice, in which GABAergic neurons are labeled by green fluorescent protein (GFP). *PlxnA1* mRNAs were scattered throughout all layers of the prefrontal cortex (PFC), as well as all subfields of the

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hippocampal region. Also, double labeling revealed that *PlxnA1* mRNAs were expressed in most GFP-positive (GABAergic) neurons in the prefrontal cortex and hippocampus of the adult mouse brain. PlxnA1 knockout (KO) mice under BALB/cAJ background were generated by homologous recombination method. The number of PlxnA1 KO mice born from heterozygous parents was lower than expected by the Mendelian rule at the weaning stage (10.94%) but the number on postnatal day 0.5 (P0.5) was as predicted (23.17%). Thus, it is conceivable that more than half of PlxnA1 KO mice died during the early postnatal period before weaning. Even though neither body nor brain weight differed between wild type (WT) and PlxnA1 KO mice at P0.5, mean body weight and brain weight in PlxnA1 KO mice at 6 months of age were significantly lower than those in WT littermates ($p < 0.0001$; $p < 0.05$, by Student's *t*-test respectively). Thus, PlxnA1 KO mice exhibited notable impairment in postnatal body and brain growth. In addition, about 16% of adult PlxnA1 KO mice showed agenesis of the corpus callosum (AgCC) as confirmed by immunohistochemical staining for myelin basic protein. In contrast, AgCC was more frequently observed at both embryonic day 17.5 (E17.5) and P0.5. Thus, the impairment observed in postnatal body and brain growth along with morphological deficits and early mortality may stem, at least in part, from neurodevelopmental abnormalities occurring during embryogenesis.

Therefore, the present study aims to examine whether the PlxnA1 deficiency leads to abnormal behavioral development in adult mice under BALB/cAJ background. A comprehensive battery of behavioral tests was conducted which includes the wire hang test of muscle strength, motor coordination on the rotarod task, the assessment of spontaneous ambulation, state anxiety, and grooming in the open field test, grooming behavior

in the home cage, information processing as evaluated by prepulse inhibition (PPI), anxiety on the elevated plus maze, social interaction test, spatial working memory on the Y maze spontaneous alternation test, and cued and contextual fear conditioning. PlxnA1 KO mice under BALB/cAJ background displayed excessively repetitive self-grooming, impaired PPI, and lower anxiety level, but the motor performance, working memory, fear memory, and sociability did not differ significantly between WT and PlxnA1 KO mice. The present study consequently suggests a pivotal role of PlxnA1 in the development and function of brain regions controlling self-grooming and PPI. Thus, PlxnA1 KO mice may be a valuable model to explore the repetitive behaviors and information processing deficits characteristic of many neurodevelopmental and psychiatric disorders.

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1. Introduction

During development of the embryonic nervous system, growing axons are navigated toward their targets by multiple extracellular guidance molecules, interacting with specific receptor proteins expressed in the growth cone (1). Various attractive or repulsive axon guidance molecules are expressed in the extracellular environment of the mammalian nervous system, including a large family of semaphorins (Semas) (2). The major class of proteins that act as receptors for Semas is the Plexin (Plxn) family (3, 4, 5, 6). Nine *Plxn* genes have been recognized in the mammalian genome and categorized into four subfamilies, *PlxnA1-4*, *PlxnB1-3*, *PlxnC1*, and *PlxnD1* (7, 8). The extracellular region of Plxn contains a Sema domain comprised of ~500 amino acids, whereas the highly conserved intracellular region contains a GTPase activating protein (GAP) domain for signal transduction (9, 10). Sema family is a diverse group of proteins which divided into 8 major classes. Semas in classes I and IV–VII are membrane-associated, and those in classes II, III, and VIII are secreted (11). Most membrane-bound Semas directly bind to Plxns, whereas class III Semas require neuropilins (Nrps) for the ligand binding as obligate co-receptors of Plxns (11). After binding of Semas to Nrps with very short intracytoplasmic domain, Nrps forms a complex with PlxnA1, a signal transducing receptor to transduce necessary signals intracellularly for axon guidance (Fig.1) (12). In addition to axon guidance, the Sema3A-Nrp1-PlxnA1 signaling pathway promotes the formation of the lymphatic valve (13). The PlxnA1 receptor also interacts with vascular endothelial growth factor receptor 2 (VEGFR2) or tyrosine kinase receptors off-track (OTK) independently of Nrps to regulate cardiac morphogenesis in the chick embryo (3) and with both DNAX-activating protein of 12 kDa (DAP12) and

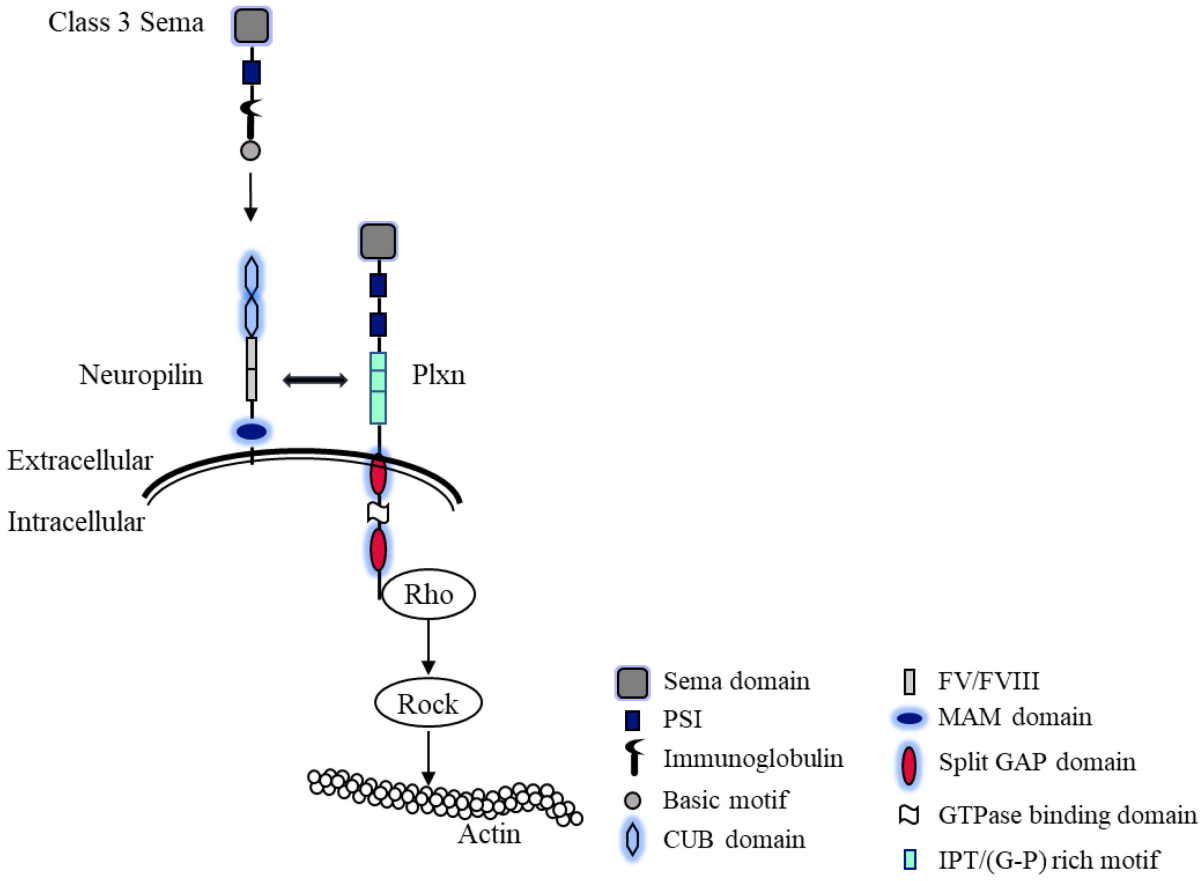


Fig. 1. Semaphorin-Plexin signaling

the triggering receptor expressed on myeloid cells-2 (TREM2) to transduce Sema signals for the regulation of osteoclastogenesis (4). Furthermore, expression of PlxnA1 in dendritic cells induced by major histocompatibility complex class II transactivator (CIITA) is involved in the interactions between T cells and dendritic cells in the immune system (14).

PlxnA1 is widely expressed throughout the developing central and peripheral nervous systems, including the olfactory system, auditory system, neocortex, hippocampus, neural retina, and peripheral ganglia (15, 16). The prominent expression of PlxnA1 in embryos and the early postnatal period suggesting their important

roles in forebrain development (15, 17). Indeed, in a PlxnA1-deficient mouse line on the C57BL/6J background, neuronal progenitor cells of the medial ganglionic eminence, a major source of interneurons, appeared to be poorly anchored to the ventricular surface and showed reduced proliferative ability (18). Thus, the absence of PlxnA1 impairs GABAergic neurogenesis in the developing cortex and also in the formation of striatal projection neurons (18). PlxnA1 also acts as an important receptor for guidance cues for the navigation of callosal axons (19). A PlxnA1 KO mouse line on the BALB/cAJ background exhibited a significantly lower rate of midline crossing compared to wild type (WT) controls at the embryonic stage, indicating the involvement of PlxnA1 in the formation of corpus callosum (CC) (20). Also, mice deficient in synaptic vesicle protein Syb2, which associates with PlxnA1, displayed defasciculation of CC axons during embryonic development (21).

Neuropsychiatric disorders have profoundly intricate and diverse etiologies. Genetic studies have reported that genetic alteration including copy number variations, single nucleotide polymorphism, *de novo* mutations in genes along with environmental interfaces are greatly contributing to underlying pathogenesis (22). In recent years, Sema-Plxn pathway has attracted attention as one of the potential causes of many nervous system disorders including schizophrenia (23, 24, 25), autism (26), Alzheimer's disease (27, 28), and Parkinson's disease (29). One of the direct evidences emerged from the report of an individual with a missense *PlxnA1* mutation (NM_032242.2: c.683G>A, p. Arg228His) presenting with epileptic encephalopathy (30, 31). Another case is an adult human with a missense *PlxnA1*variant (NM_032242, c.4201A>T, p.1401M>L) showing the symptom of schizophrenia (32). The third case was an individual with

a *de novo* heterozygous variant in *PlxnA1* (NM_032242. Chr3: g.126735788, c.3184G>A, p. Gly1062Ser) presenting with a developmental encephalopathy characterized by intractable epilepsy of infantile onset, frequent hyperkinetic movement, stereotypies, autism, and dysmorphic facial features indicative of Dubowitz syndrome (33). Case/control cohort analysis identified *PlxnA1* as a *de novo* schizophrenia-related gene and found an association of disruptive (missense and frameshift) mutation in *PlxnA1* and schizophrenia in the Swedish population (34). Moreover, deregulation in transcript levels of *Sema3A* and *PlxnA1* was reported in the prefrontal cortex of schizophrenic patients (25). Such findings suggested the role of *PlxnA1* allelic variants and mutations to the heterogeneous morphological, neurofunctional, and behavioral abnormalities, consistent with multiple functions in neurodevelopment across brain regions.

The predominant expression of PlxnA1 in the mouse forebrain during development and their associations with several neurodevelopmental and neuropsychiatric disorders prompted my current investigations of PlxnA1 functions in the complex behaviors of adult mice.

2. Material and methods

2.1. Animals

PlxnA1 knockout (KO) mice on the BALB/cAJ genetic background were produced by gene targeting (4). In brief, a gene targeting vector was constructed by replacing the genomic region containing the initiation codon and Sema domain-coding sequence with a neomycin resistance gene. The vector was then transfected into E14.1 embryonic stem (ES) cells by electroporation. G418- and ganciclovir-resistant clones were screened by polymerase chain reaction (PCR) and confirmed by Southern blotting. Mutant ES cells were introduced into mouse blastocysts, and the blastocysts were transferred into uteri of pseudopregnant mice to generate chimeras. F1 heterozygous mice were generated by mating the chimeras with BALB/cAJ mice and then backcrossed with BALB/cAJ mice for 10 generations. Pairs of the resultant heterozygous mice were bred to produce homozygous KO mice and wild type (WT) littermates as controls.

A GAD67-green fluorescent protein (GFP) knock-in mouse (ICR.Cg-Gad1<tm1.1Tama>) was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan (35) and backcrossed with BALB/cAJ mice for 10 generations to establish a BALB/cAJ line with fluorescent marking of GABA neurons.

All mice were raised in the animal center of the Faculty of Pharmacy, Meijo University. WT and PlxnA1 KO littermates were housed in groups of six with ad libitum access to water and food (MM-3, Sankyo Labo Service Corporation, INC., Tokyo, Japan) under a controlled environment with 50.5% humidity, 23.1 °C ambient temperature, 300 lux illuminance, and a 12 h/12 h light/dark cycle (lights on at 8:00). All animal

care and experimental procedures were performed according to the guidelines of the Physiological Society of Japan and the guidelines on animal experimentation of Meijo University. The Animal Ethics Review Committee of Meijo University approved the experimental protocol (authorization number: 2020PE4).

2.2. Genotype analysis

Genotyping of both WT and PlxnA1 KO mice was confirmed by PCR using mouse tail DNA as the template and a *PlxnA1* gene-specific primer set as previously reported (4, 36). Genotyping of the GAD67-GFP knock-in mouse was performed according to the original report (35).

2.3. Western blotting

The dissected brain tissues were homogenized in T-PER Tissue Protein Extraction Reagent containing 25 mM bicine and 150 mM sodium chloride (pH 7.6) (Thermo Fisher Scientific Inc. MA, USA), cOmplete ULTRA Tablets, Mini, *EASYpack* Protease Inhibitor Cocktail (05892970001, Roche Applied Science, Penzberg, Germany), and PhosStop (4906845001, Roche Applied Science). The homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C to obtain the soluble protein fraction. Supernatant protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc.). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and semiquantified by western blotting as previously described (20). Briefly, separated proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes and labeled with primary antibodies against β-Actin (1:10000, 13E5, Cell Signaling Technology, Danvers, MA, USA) and mPlexinA1 (1:5000,

AF4309, R & D Systems, Minneapolis, USA) in Can Get Signal immunoreaction enhancer solution-1 (NKB-101, Toyobo Co. Ltd., Osaka, Japan). The membranes were then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody in Can Get Signal immunoreaction enhancer solution-2 (NKB-101, Toyobo Co. Ltd.) for 1 h at room temperature, followed by visualization using ECL™ prime reagents (GE Healthcare, Piscataway, NJ, USA). Protein band density were analyzed by using Image Quant LAS-4000 (GE Healthcare Biosciences, Sweden).

2.4. *In situ* hybridization analysis of *PlxnA1*

Coronal sections of the mouse brain were cut at 20 μm on a CryoStar NX70 cryostat (Thermo Scientific, Yokohama, Japan) and mounted on SuperFrost Plus slides (Fisher Scientific #12-550-15, MA, USA). *In situ* hybridization was conducted on the sections using RNAscope Probes (Advanced Cell Diagnostics, CA, USA) targeting the 1091–1984 base pair region of the mouse *PlxnA1* gene (Accession No: NM_008881.2) according to the manufacturer's protocol. Probes used to examine the expression of *PlxnA1* was RNAscope Target Probe Mm-PlexinA1 (cat no. 481388, ACD, Newark, USA). RNAscope Positive Control Probe-DapB (cat No.310043, ACD, Newark, USA) and RNAscope Negative Control Probe-DapB (cat No.310043, ACD, Newark, USA) were used as a positive and negative control to test optimal permeabilization and probe RNA quality. After *in situ* hybridization, sections were incubated with anti-GFP chicken polyclonal antibody (1:1000, ab13970, Abcam, Cambridge, UK) overnight at 4 °C. On the second day, the samples were washed and incubated with Alexa Flour 488 goat anti-chicken secondary antibody (1:1000, A11039, Thermo Fisher

Scientific) for 1 h at room temperature. After incubation, sections were washed again and sealed under glass cover slips (Matsunami Glass Industries Ltd., Osaka, Japan) using Fluoro-KEEPER Antifade Reagent, Non-Hardening type containing DAPI for nuclear counterstaining (cat no. 12745-74, Nacalai Tesque Inc. Kyoto, Japan). The slices were imaged using the All-in-One Fluorescence Microscope (BZ-X710; Keyence, Osaka, Japan) controlled by BZ-X viewer version 1.3.1.1. Images were analyzed with BZ-X Analyzer version 1.4.0.1.

2.5. Behavioral tests

The open field test was evaluated at 2, 4, and 6 months of age, while all other interventional behavioral tasks were conducted once at 6 months of age. The room for behavior tests was maintained at 50.5% humidity, 23.1 °C, and 30 lux illuminance under a 12 h/12 h light/dark cycle (lights on at 8:00). Adult mice in standard mouse cages (27 cm length × 17 cm wide × 13 cm height) were acclimated to the test room for three days before behavioral testing to mitigate the effects of stress due to environmental changes. After every session, each apparatus was cleaned thoroughly with water and 70% alcohol to remove any dirt and odor cues.

2.5.1. Wire hang test

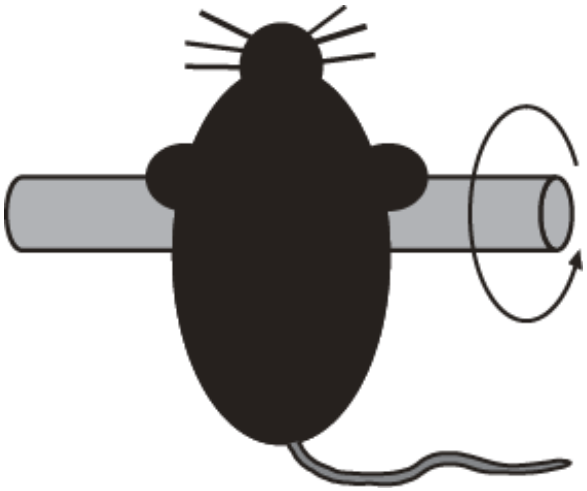
The wire hang test was performed by placing the animal on a wire cage lid, then inverting the lid above a pad for 60 s. The latency of the animal to fall from the cage lid to the pad was evaluated as an index of limb muscle strength. The wire hang test seeks to evaluate the motor function and deficit in rodent models of



CNS disorders. A total of 22 WT and 18 KO mice was used in the experiment.

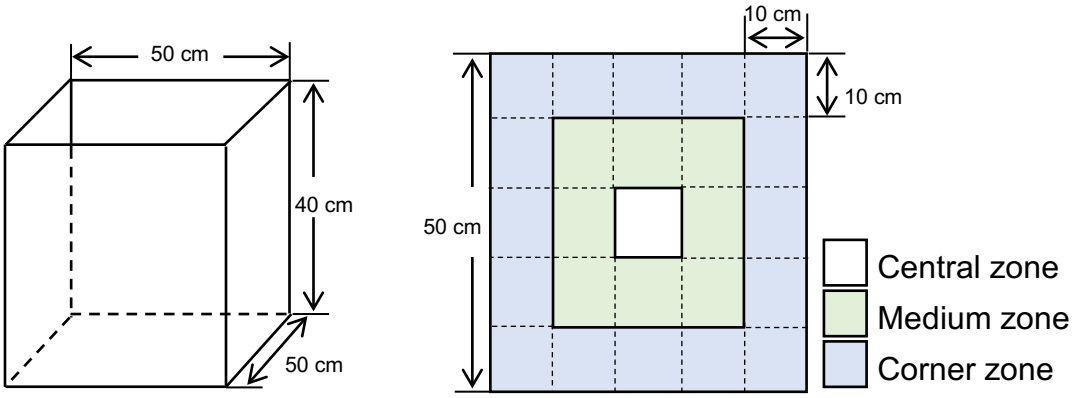
2.5.2. Rotarod test

Motor coordination and learning were evaluated using the standard and accelerating rotarod tests, respectively. In the first test, mice were introduced on a drum (Ugo Basile; Gemonio, Varese, Italy) rotating at 4 rpm. The latency to fall off was recorded as an indicator of motor coordination.



One week later, each mouse was again placed on the drum and rotation speed was gradually increased from 4 to 20 rpm over 5 min. The latency to fall off was recorded once a day for 3 consecutive days to measure motor learning. A total of 32 WT and 15 PlxnA1 KO mice were tested in rotarod tasks.

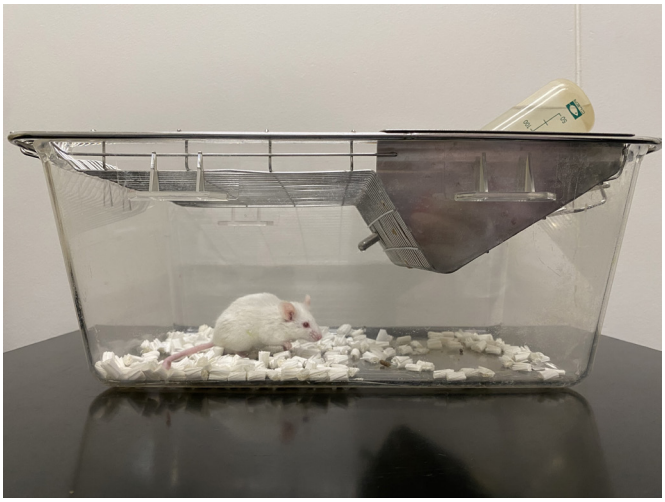
2.5.3. Open field test



The open field test is a well-validated and commonly conducted test for general locomotion and exploratory behavior. At 2, 4, and 6 months of age, each mouse was placed in the center of an open field apparatus (50 × 50 × 40 cm) and allowed for 20 min to explore the environment freely. Total distance traveled, number of intrusions into the central zone (central zone entries), time spent in the central zone, and the number and duration of grooming episodes were recorded and analyzed by ANY-MAZE (Stoelting Co., Wood Dale, IL, USA). A total of 23 WT and 21 PlxnA1 KO mice completed the three successive open field tests.

2.5.4. Grooming in the home cage

In home cage, grooming behavior was observed in a separate cohort of WT and PlxnA1 KO mice. Each mouse was housed individually in a standard mouse cage for behavioral analysis on day 1 and reared in the room for three days. The total durations of grooming and scratching from 20:00 to 8:00 on day 3 were analyzed using ANY-MAZE (Stoelting Co.).



A total of 20 WT and 25 PlxnA1 KO mice were evaluated.

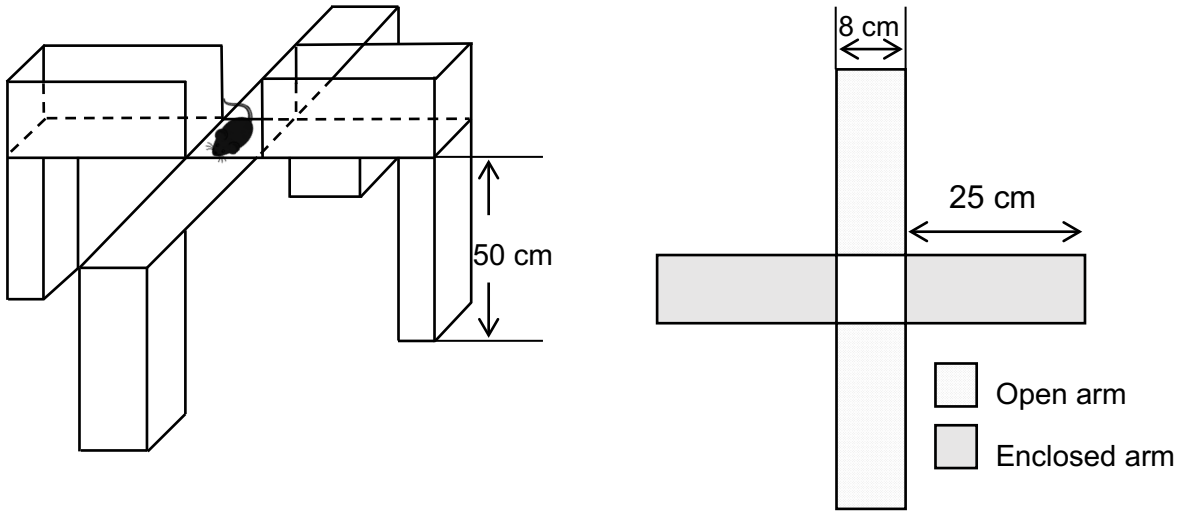
2.5.5. Prepulse inhibition (PPI)



Prepulse inhibition (PPI) is a neurological phenomenon in which a weaker prestimulus (prepulse) inhibits the reaction to a subsequent strong reflex-eliciting stimulus (pulse). PPI was conducted using the SR-Lab System (San Diego Instruments, San Diego, CA, USA). The system includes an acrylic measuring tube (3.8 cm in diameter and 13 cm in length) installed in a soundproof chamber and a vibration sensor connected to a signal amplification sensor. Each session started with 10 min of acclimation under 70-dB background noise, followed by both startle response and PPI trials. Each trial was presented ten times (total 40 times) in a

randomized order. The sound stimulus protocols were (1) pulse alone (120 dB), (2) prepulse (74 dB) + pulse (120 dB), (3) prepulse (78 dB) + pulse (120 dB), and (4) prepulse (86 dB) + pulse (120 dB). The prepulse sound preceded the onset of the 120-dB startle stimulus by 100 ms. Percent (%)PPI was calculated as 100 – [(startle response on prepulse plus pulse trials/startle response on pulse alone trials) × 100]. Auditory startle reflex data were analyzed by Student's *t*-test and %PPI data by two-way ANOVA (factors genotype × prepulse sound level) with repeated measurement. In total, 34 WT and 30 PlxnA1 KO mice completed all trials.

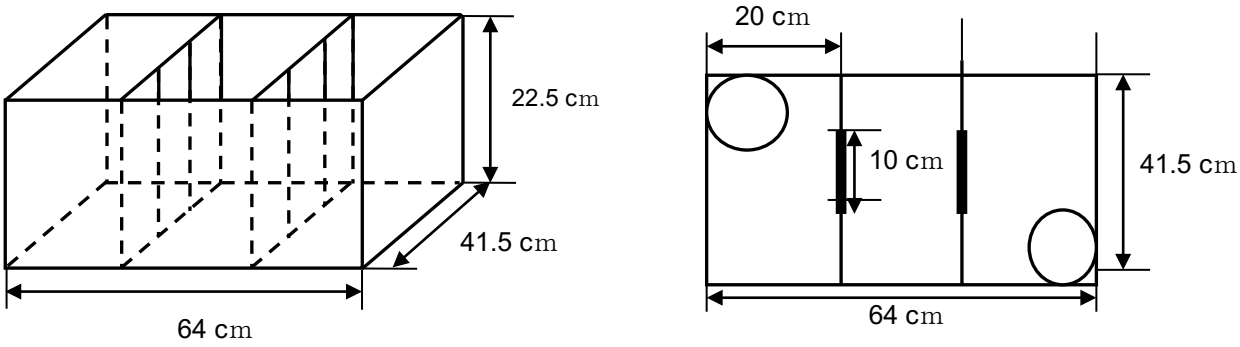
2.5.6. Elevated plus maze test



The elevated plus maze test was performed on 14 WT and 14 PlxnA1 KO mice following previously established method (37). The apparatus consisted of two open arms (25 × 8 cm) and two enclosed arms of the same size, with 20-cm high transparent walls. The arms and central square were made of white plastic plates and were elevated to a height of 50 cm above the floor. Each mouse was placed in the central square

of the maze (8 × 8 cm), facing one of the closed arms. Mouse behavior was recorded during a 10-min test period. The number of entries into, and the time spent in open and enclosed arms were measured using a computer-assisted video tracking system (ANY-MAZE, Stoelting Co.).

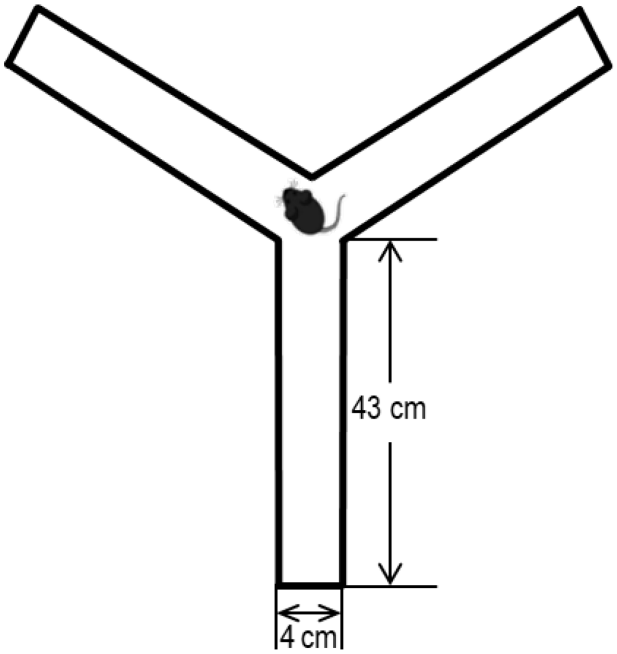
2.5.7. Social interaction test



A three-chamber social interaction test was conducted using a method modified from Silverman et al. (38). The test was composed of three sessions: habituation, sociability and social novelty, which evaluated sociable behavior and recognition of social novelty. Identical columnar wire cages (diameter: 9 cm, height: 13.5 cm) were placed at equivalent positions in the left and right chambers of a three-chamber Plexiglass box (64 × 41.5 × 22.5 cm). On trial one, each mouse was placed in the central chamber and allowed to freely explore all three compartments for 10 min, and the time interacting with each empty cage was measured. The mouse was returned to the home cage, and an unfamiliar sex-matched BALB/cAJ WT mouse (Stranger 1) was put into one of the cages. In trial 2, the test mouse was placed back into the chamber and the time interacting with each cage was again recorded for 10 min (trial 2). After trial 2, the mouse was again returned to the home cage, and a novel mouse (Stranger 2) was introduced into the empty cage. In trial 3, the

interaction times with Stranger mouse 1 and 2 were measured over 10 min. The interaction time with Stranger 1 in trial 2 was used as an index for sociability, while the interaction time with Stranger 2 in trial 3 was measured as an index of social novelty and memory. In total, 14 WT and 14 PlxnA1 KO completed all three trials.

2.5.8. Y maze spontaneous alternation test

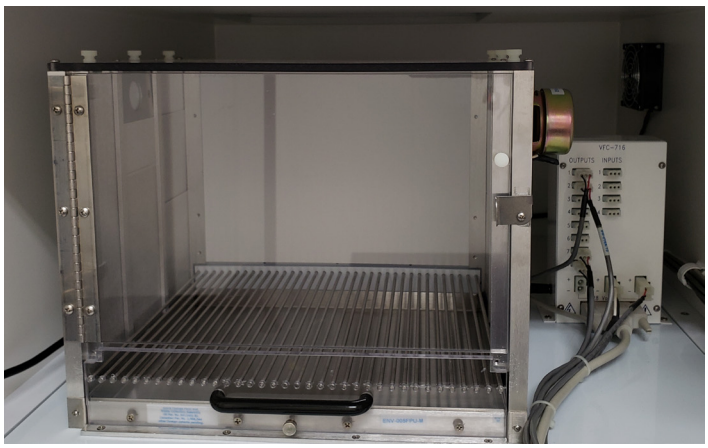


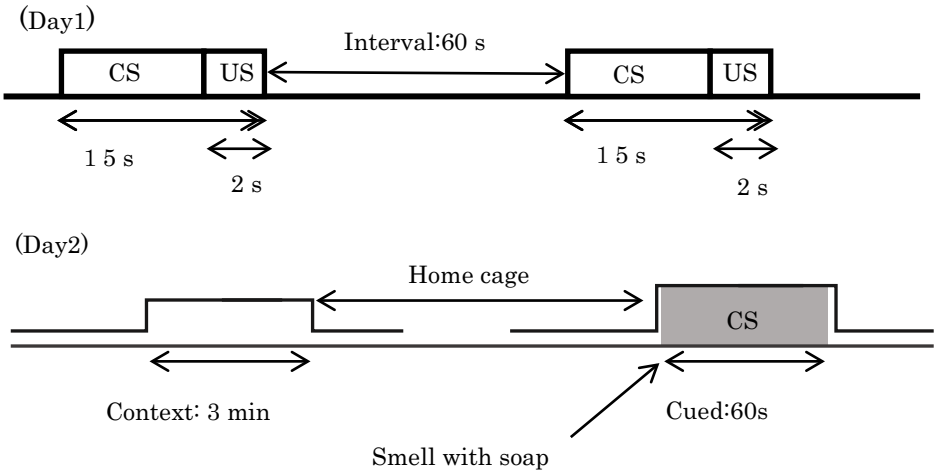
The Y maze spontaneous alternation test was evaluated following the method of Yamada et al. (39). The Y maze used in this study is composed of three arms (43 cm long, 25 cm high, and 4 cm wide) projecting from a central triangular area. Mice were placed in the central area and allowed to explore freely for 5 min. The observer recorded an arm entry when the hind paws were completely within the arm. Spontaneous alternation was defined as successive entries into the three different arms (i.e., without returning to any arm). The percentage alternation was calculated as the ratio of actual to possible alternations (the total number of

arm entries – 2) × 100. In total, 14 WT and 14 PlxnA1 KO mice were observed in the 5-min trial.

2.5.9. Fear conditioning

On the training day, each mouse was putted in a conditioning chamber (25.5 × 32 × 25.5 cm) equipped with a speaker and stainless steel grid floor connected to a shock source.





Six pairings of a conditioned stimulus (CS: 80 dB, 15 s) followed by a mild foot shock (0.4 mA, 2 s) as an unconditioned stimulus (US) were delivered at 60-s intervals. A contextual fear conditioning test was conducted 24 hours after training in the same chamber for 3 min, during which the percentage time freezing (absence of all movement except from respiration) was measured. Mice were returned to home cage for 30

min. Each mouse was then placed in the chamber with a novel odor (soap) and exposed to the 80-dB cue alone for 60 s. Freezing time was evaluated during tone delivery. Freezing was recorded and analyzed automatically using VIDEO FREEZE SOFTWARE (MED Associates Inc, Brain Science-idea.co., Osaka, Japan). A total of 14 WT and 14 PlxnA1 KO mice were examined under both contextual and cued conditions.

2.6. Assessment of agenesis of the corpus callosum by immunohistochemistry

One day after the final behavior tests at 6 months of age, each mouse was transcardially perfused with ice-cold PBS followed by ice-cold 4% paraformaldehyde (PFA) in PBS. The brain was quickly removed, post-fixed in 4% PFA solution overnight and cryoprotected by sequential immersion in 10%, 20%, and 30% sucrose solution. The brain was then embedded in O.C.T. Compound, Tissue-Tek (Sakura Finetek Japan Co. Ltd., Tokyo, Japan), frozen on dry ice, and preserved at – 80 °C. Coronal slices of 10 μm thickness were prepared using a CryoStar NX70 cryostat (Thermo Scientific, Yokohama, Japan), mounted on glass slides (MAS-05, Matsunami Glass Industries Ltd., Osaka, Japan), and air-dried for 1 h. Sections were then washed with PBS plus Tween 20 (PBST) for 5 min, washed three times in PBS (5 min/wash), incubated with 0.3% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity, blocked by incubation with 5% goat serum (codeX0907, Dako, Denmark) in PBS for 1 h, and then immunostained with a primary antibody against myelin basic protein (1:1000) for 1 h at room temperature to label white matter (including the CC). After three washings with PBS, labeled sections were incubated with secondary antibody (Envision + System-HRP labeled polymer, anti-rabbit, Dako) for 1 h, washed three times with PBS, and treated with

Histofine DAB substrate kit (Nichirei Bioscience, Tokyo, Japan) as coloring reagent. The status of the CC was examined under an All-in-One Microscope (BZ-X710; Keyence, Osaka, Japan).

2.7. Statistics

All values are represented as mean ± SEM. Statistical analyses were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Unless otherwise noted, group means were compared by ANOVA with the indicated post hoc test. A $p < 0.05$ (two-tailed) was considered significant for all tests.

3. Results

3.1. Expression of PlxnA1 in the adult mouse brain

In adult brain, PlxnA1 under BALB/cAJ mice expressed in prefrontal cortex, hippocampus, and striatum, as well as GFP positive GABAergic neurons. To confirm the localization, expression analyses of PlxnA1 was performed using western blotting and *in situ* hybridization followed by immunohistochemistry. Western blot was conducted on protein lysates from several brain regions of the adult (6-month-old) mouse brain. As shown in Fig. 2A, PlxnA1 protein was widely expressed in WT mouse brain, especially in the prefrontal cortex, hippocampus, and striatum, but was undetectable in all regions of PlxnA1 KO mouse brain. To visualize the cellular localization of *PlxnA1* mRNA in WT adult mouse brain, *in situ* hybridization was performed with *PlxnA1* mRNA-specific probes on coronal sections from GAD67-GFP knock-in mice, in which GABAergic neurons are labeled by GFP (35, 18). Most GFP-positive (GABAergic) neurons in the medial prefrontal cortex and hippocampus of the adult showed detectable *PlxnA1* mRNA expression (Fig. 2B, C). Thus, PlxnA1 is expressed in adult as well as developing mouse brain.

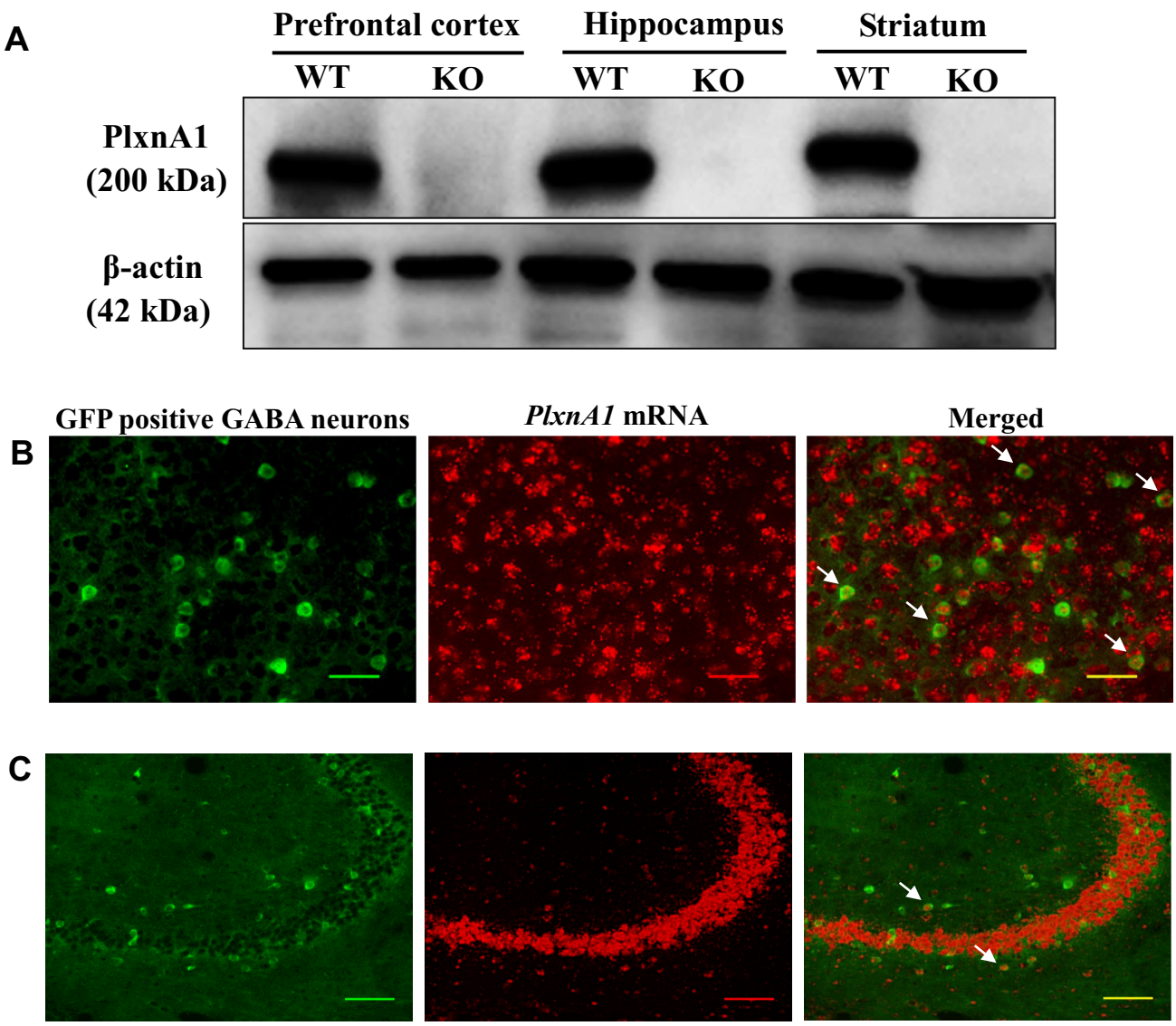


Fig. 2. Expression of PlxnA1 in the adult mouse brain.

(A) Western blotting analysis demonstrating the expression of PlxnA1 protein in prefrontal cortex, hippocampus, and striatum of adult WT BALB/cAJ mice (the background for PlxnA1 KO mice). (B) *In situ* hybridization with a *PlxnA1*-specific probe detected *PlxnA1* mRNA in GFP-expressing GABAergic neurons of the medial prefrontal cortex and (C) hippocampus of GAD67-GFP knock-in mice.

WT: wild type, KO: PlxnA1 knockout mice, GFP: green fluorescent protein, GAD67: glutamic acid decarboxylase 67, Arrows: merged cells of GAD67-GFP and *PlxnA1* mRNA. Scale bars: 50 μm.

3.2. Reduced postnatal survival, body weight, and brain weight of PlxnA1 KO mice

PlxnA1 knockout (KO) mice born from heterozygous parents was lower in numbers than expected by the Mendelian rule at the weaning stage (Total: 192; WT: 59, 30.73%; Heterozygous: 112, 58.33%; KO: 21, 10.94%) but was as expected on postnatal day 0.5 (WT: 22, 26.83%; Heterozygous: 41, 50.00%; KO: 19, 23.17%) (20). Thus, more than half of PlxnA1 KO mice died during the early postnatal period. At 6 months of age, mean body weight of PlxnA1 KO mice was significantly lower than that of WT littermates (25 ± 1.274 g vs. 34.16 ± 0.5911 g, $p < 0.0001$ by Student's *t*-test). Similarly, brain weight at 6 months was significantly lower compared to WT controls (0.4433 ± 0.01 g vs. 0.4756 ± 0.0058 g, $p = 0.0132$ by Student's *t*-test). Alternatively, neither body nor head weight differed at P0.5 (body weight: 124.00 ± 2.11 mg vs. 125.45 ± 2.52 mg; head weight: 22.79 ± 0.43 mg vs. 22.86 ± 0.49 mg; in both $p > 0.05$ by Student's *t*-test). Thus, PlxnA1 KO mice demonstrate substantial impairment in postnatal body and brain growth.

About 13 out of 80 (16%) adult PlxnA1 KO mice at 6 months of age, exhibited AgCC. To confirm the agenesis, immunohistochemistry with Anti myelin basic protein was conducted on anterior and posterior locations of coronal brain slices as shown in Fig. 3. Whereas, in case of both embryonic day 17.5 (E17.5) and P0.5, the agenesis of corpus callosum (AgCC) in PlxnA1 KO mice was observed frequently (20) than adult. Neurodevelopmental abnormalities during early embryonic condition in PlxnA1 KO mice might contribute to these postnatal morphological deficits and premature death to some extent.

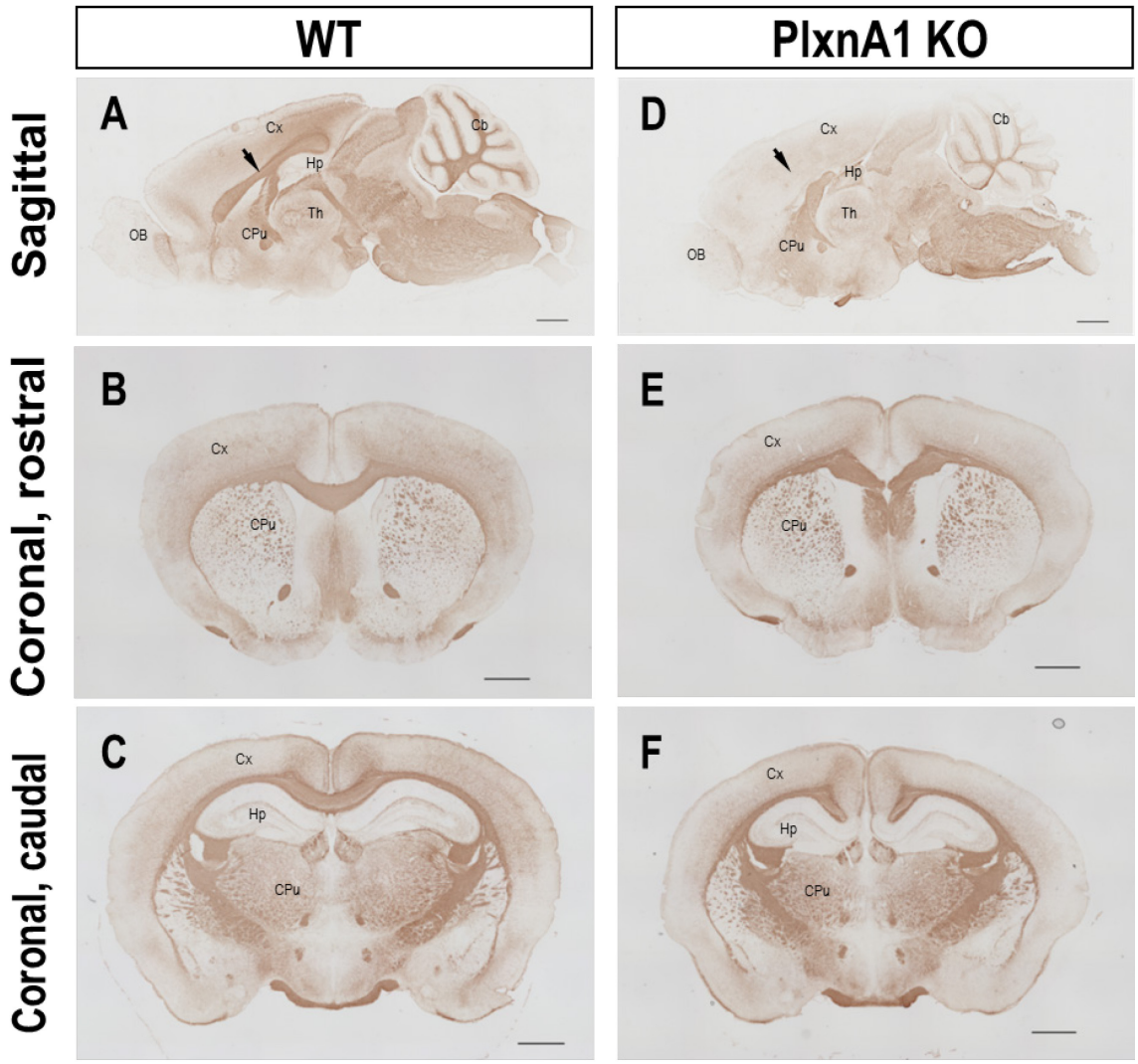


Fig. 3. Comparison of corpus callosum formation in WT and PlxnA1 KO mice.

(A-C) The corpus callosum (CC) was detected by immunostaining for myelin basic protein (brown color) at mid-sagittal plane of WT brain (arrow in A), and at coronal planes in both the rostral (B) and caudal brain (C) of WT mice. (D-F) The CC was not detected at the mid-sagittal plane (arrow in D), rostral coronal plane (E), or caudal coronal plane (F) in 5 of 21 PlxnA1 KO mice (termed PlxnA1 KO-AgCC mice). WT (n = 23); PlxnA1 KO (n = 21); PlxnA1 KO-AgCC (n = 5). WT: wild type, KO: PlxnA1 knockout mice, PlxnA1 KO-AgCC: PlxnA1 KO mice with agenesis of the corpus callosum. OB: olfactory bulb, Cx: cortex, Hp: hippocampus, Th: thalamus, CPu: caudate putamen, Cb: cerebellum. Scale bars: 1000 μm

3.3. Intact limb muscle strength and motor coordination in PlxnA1 KO mice

PlxnA1 KO showed normal limb muscle strength compared to WTs when assessed by the wire hang test (Fig. 4A; 58.89 ± 1.11 s vs. 58.18 ± 1.82 s, p = 0.7421 by Welch's *t*-test). The latency to fall from wire was similar in both WT and PlxnA1 KO mice. In the accelerating rotarod test of motor learning that was observed 3 consecutive days, PlxnA1 KO mice showed significantly longer latencies to fall from accelerating rotarod compared to WT mice on all three daily trials (Fig. 4B; main effect of genotype: F (1, 45) = 9.328, p = 0.0038; p = 0.0163 on trial 1, p = 0.0368 on trial 2, p = 0.0334 on trial 3 by Bonferroni's multiple comparisons test).

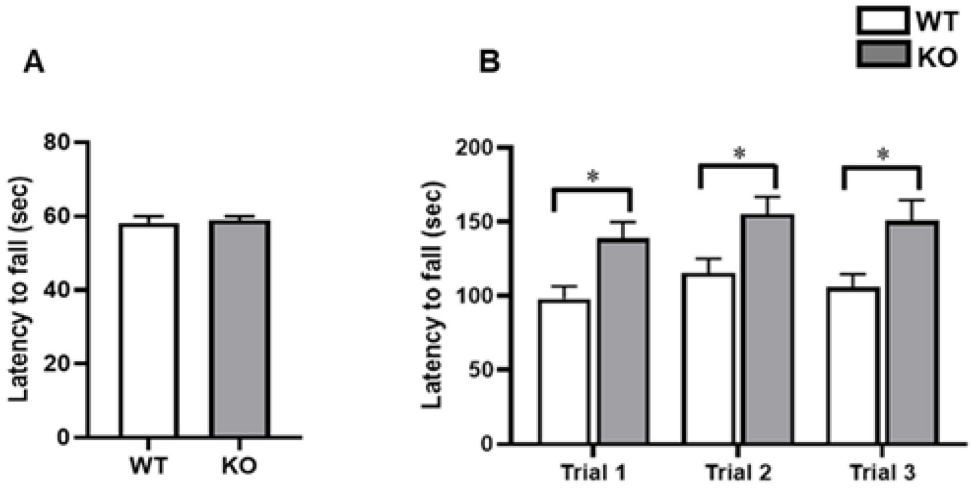


Fig. 4. Limb strength and motor coordination were not affected in PlxnA1 KO mice.

(A) No difference in wire hang time was observed between PlxnA1 KO and WT mice (A). WT (n = 22); PlxnA1 KO (n = 18). (B) PlxnA1 KO mice exhibited significantly longer latencies to fall during the rotarod test than WT mice. WT (n = 32); PlxnA1 KO (n = 15), WT: wild type, KO: PlxnA1-deficient mice. (Data presented as mean ± SEM; *p < 0.05).

3.4. Excessive grooming by PlxnA1 KO mice in the open field

PlxnA1 expressed in GABAergic neurons in prefrontal cortex and hippocampus of adult mice at 6 months suggests the involvement of PlxnA1 in the postnatal brain (Fig. 2). Thus, deficiency of PlxnA1 in mice may result in the abnormal behaviors long after maturation. To examine if the absence of PlxnA1 causes the development of abnormal behaviors with age, the open field test was conducted at 2, 4, and 6 months of age. During open field exploration, the effect of genotype and AgCC on total distance traveled, central area entries as a measure of state anxiety, and on grooming (Table 1, Fig. 5) was measured. There was a significant main effect of genotype ($F (1, 42) = 4.33$, $p = 0.0436$) and age ($F (2, 84) = 21.76$, $p < 0.0001$) on the total distance traveled but no age × genotype interaction ($F (2, 84) = 0.2196$, $p = 0.8033$). Despite the main effect of genotype, there was no significant difference in distance traveled between genotypes at 2, 4, and 6 months (Table 1, all $p > 0.05$ by Bonferroni test; Fig. 5A). To examine the effect of AgCC on total distance traveled (Fig. 5B), PlxnA1 KO mice were retrospectively divided into those with an intact CC (PlxnA1 KO-CC group) and those with substantial AgCC (PlxnA1 KO-AgCC group). While there was a significant main effect of age (Table 1, $F (2,82) = 17.82$, $p < 0.0001$), there was no main effect of group (Table 1, PlxnA1 KO-CC, PlxnA1 KO-AgCC, and WT; $F (2, 41) = 2.282$, $p = 0.1149$) or age × group interaction ($F (4, 82) = 0.3762$, $p = 0.8250$).

Two-way ANOVA revealed a significant main effect of genotype on both the number of entries into the central zone (Fig. 5C; $F (1, 43) = 7.100$, $p = 0.0108$) and time spent in the central zone (Fig. 5E; $F (1, 43) = 4.505$, $p = 0.0396$), and 4-month-old PlxnA1 KO mice entered the central zone significantly more frequently

than age-matched WTs (Table 1, Fig. 5C; $p < 0.05$ by Bonferroni test). In contrast, there were no significant genotype differences in central zone time at any age (Table 1, all $p > 0.05$ by Bonferroni test). There were significant main effects of AgCC group and age on the number of entries into the central zone (Fig. 5D; $F(2, 42) = 4.476$, $p = 0.0173$; $F(2, 84) = 9.300$, $p = 0.0002$). At 2 and 4 months of age, PlxnA1 KO-CC mice made significantly more entries into the central zone than age-matched WTs (Table 1, Fig. 5D; $p < 0.05$ by Bonferroni test). There was also a significant main effect of AgCC group on time spent in the central zone (Fig. 5F; $F(2, 42) = 3.605$, $p = 0.0359$), but PlxnA1 KO-AgCC and PlxnA1 KO-CC mice did not differ significantly in number of entries into the central zone or time spent in the central zone at any age (Table 1, Fig. 5D and 4F; both $p > 0.05$ by Bonferroni test).

However, significantly enhanced self-grooming behavior was observed in PlxnA1 KO mice in the open field test. There were significant main effects of age (Fig. 6A; $F(2, 86) = 7.868$, $p = 0.0007$) and genotype (Fig. 5A; $F(1, 43) = 9.953$, $p = 0.0029$) on number of grooming episodes in the open field as well as a significant age × genotype interaction ($F(2, 86) = 5.097$, $p = 0.0081$). Moreover, Bonferroni's multiple comparison tests revealed a significant increase in the number of grooming episodes by PlxnA1 KO mice compared to WTs at 6 months of age (Table 1, Fig. 6A; $p < 0.0001$). There was also a significant main effect of age (Fig. 6C; $F(2, 86) = 6.288$, $p = 0.0028$) and genotype (Fig. 6C; $F(1, 43) = 11.17$, $p = 0.0017$) on the duration of grooming in the open field test but no age × genotype interaction ($F(2, 86) = 2.399$ $p = 0.0969$). Bonferroni's multiple comparison tests confirmed a significant increase in grooming duration by PlxnA1 KO mice compared to WT mice at 6 months old (Table 1, Fig. 6C; $p < 0.0004$). Finally, there was a significant

main effect of age (Fig. 5B; $F(2, 84) = 18.38$, $p < 0.0001$) and AgCC group (Fig. 6B; $F(2, 42) = 6.412$, $p = 0.0037$), and a significant age × group interaction ($F(4, 84) = 6.241$, $p = 0.0002$) on number of grooming episodes, with PlxnA1 KO-AgCC mice showing a significantly greater number than WT and PlxnA1 KO-CC mice at 6 months of age (Table 1, Fig. 6B; $p < 0.0001$ vs. WT and $p = 0.0017$ vs. PlxnA1 KO-CC by Bonferroni test). Also at 6 months of age, PlxnA1 KO-CC mice demonstrated a significantly greater number of grooming episodes compared to WT mice (Table 1, Fig. 6B; $p = 0.0122$ by Bonferroni test). In addition, there was a significant main effect of age (Fig. 6D; $F(2, 94) = 11.08$, $p < 0.0001$) and AgCC group (Fig. 6D; $F(2, 47) = 11.99$, $p < 0.0001$) on grooming duration and a significant age × group interaction ($F(4, 94) = 3.201$, $p = 0.0164$). At 2 and 6 months of age, grooming duration was significantly longer among PlxnA1 KO-AgCC mice compared to WT mice (Table 1, Fig. 6D; $p = 0.0210$ at 2 months and $p < 0.0001$ at 6 months by Bonferroni test). At 6 months of age, PlxnA1 KO-AgCC mice demonstrated significantly longer grooming times than PlxnA1 KO-CC mice (Table 1, Fig. 6D; $p = 0.0026$ by Bonferroni test), while PlxnA1 KO-CC mice exhibited significantly longer grooming times than WT mice (Table 1, Fig. 6D; $p = 0.0430$ by Bonferroni test). Thus, enhanced self-grooming behavior of PlxnA1 KO mice may be exacerbated by AgCC.

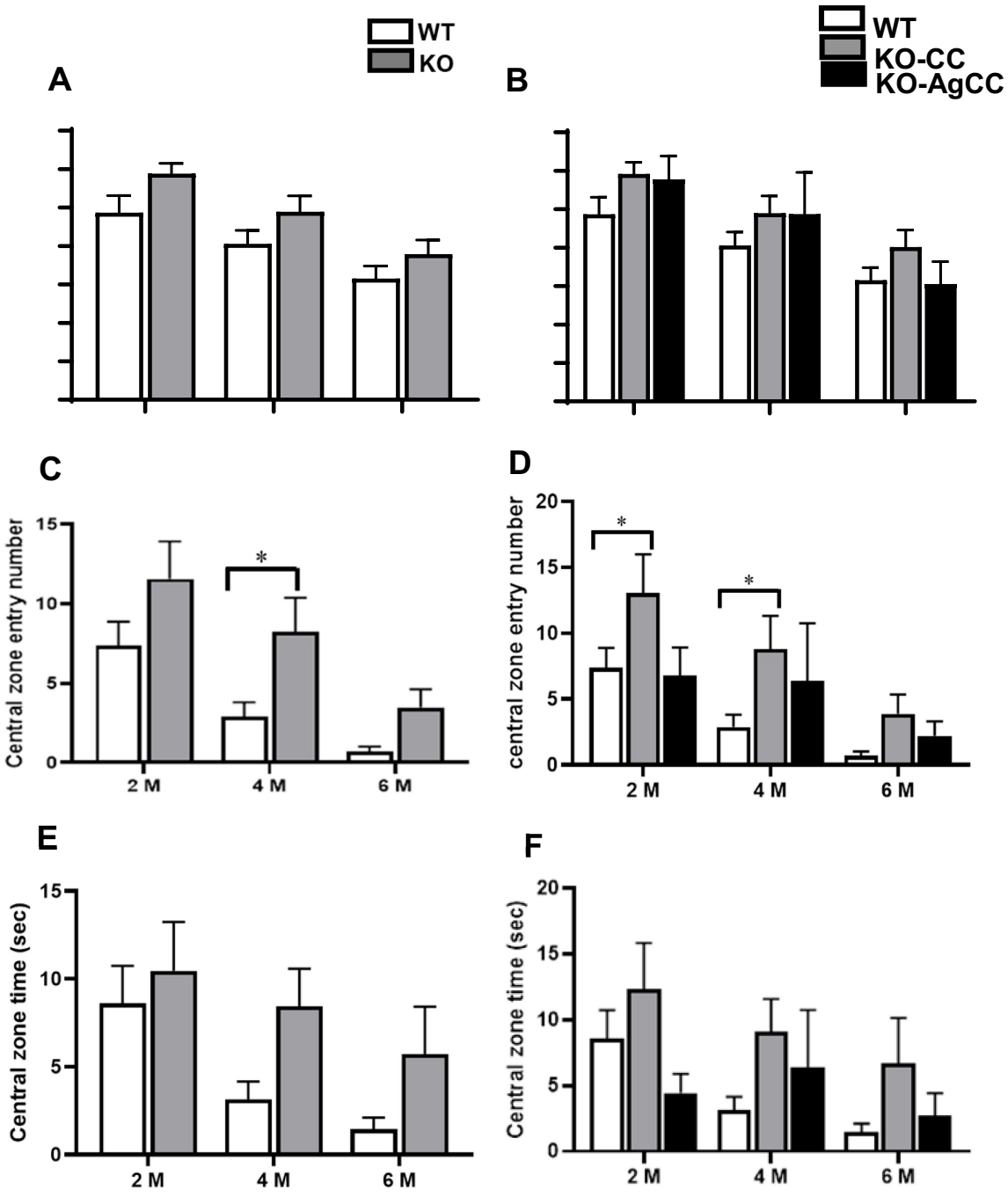


Fig. 5. Locomotor activity in the open field did not differ between PlxnA1 KO and WT mice.

(A) Distance traveled at 2, 4, and 6 months of age did not differ between age-matched PlxnA1 KO and WT mice. (B) There were no significant differences in distance traveled among WT, PlxnA1 KO-CC, and PlxnA1 KO-AgCC mice. WT (n = 23); PlxnA1 KO (n = 21); PlxnA1 KO-CC (n = 16); PlxnA1 KO-AgCC (n = 5).

(C) The number of entries into the central zone was significantly higher in the PlxnA1 KO mouse group compared to the WT group at 4 months of age. (D) The number of central zone entries was significantly higher in the PlxnA1 KO-CC group at 2 and 4 months of age compared to age-matched WT mice. (E) Time spent in the central zone did not differ among groups at any age. (F) PlxnA1 KO-AgCC do not show any significant differences in central zone time compared to age-matched WT and PlxnA1 KO-CC mice. WT (n = 23); PlxnA1 KO (n = 21); PlxnA1 KO-CC (n = 16); PlxnA1 KO-AgCC (n = 5). WT: wild type, KO: PlxnA1 knockout mice, PlxnA1 KO-CC: PlxnA1 KO mice with corpus callosum, PlxnA1 KO-AgCC: PlxnA1 KO mice with agenesis of corpus callosum. 2M: 2 months old, 4M: 4 months old, 6M: 6 months old. Central zone entry number: number of entries into the central zone, Central zone time: Time spent in the central zone. (Data presented as mean ± SEM; *$p < 0.05$).

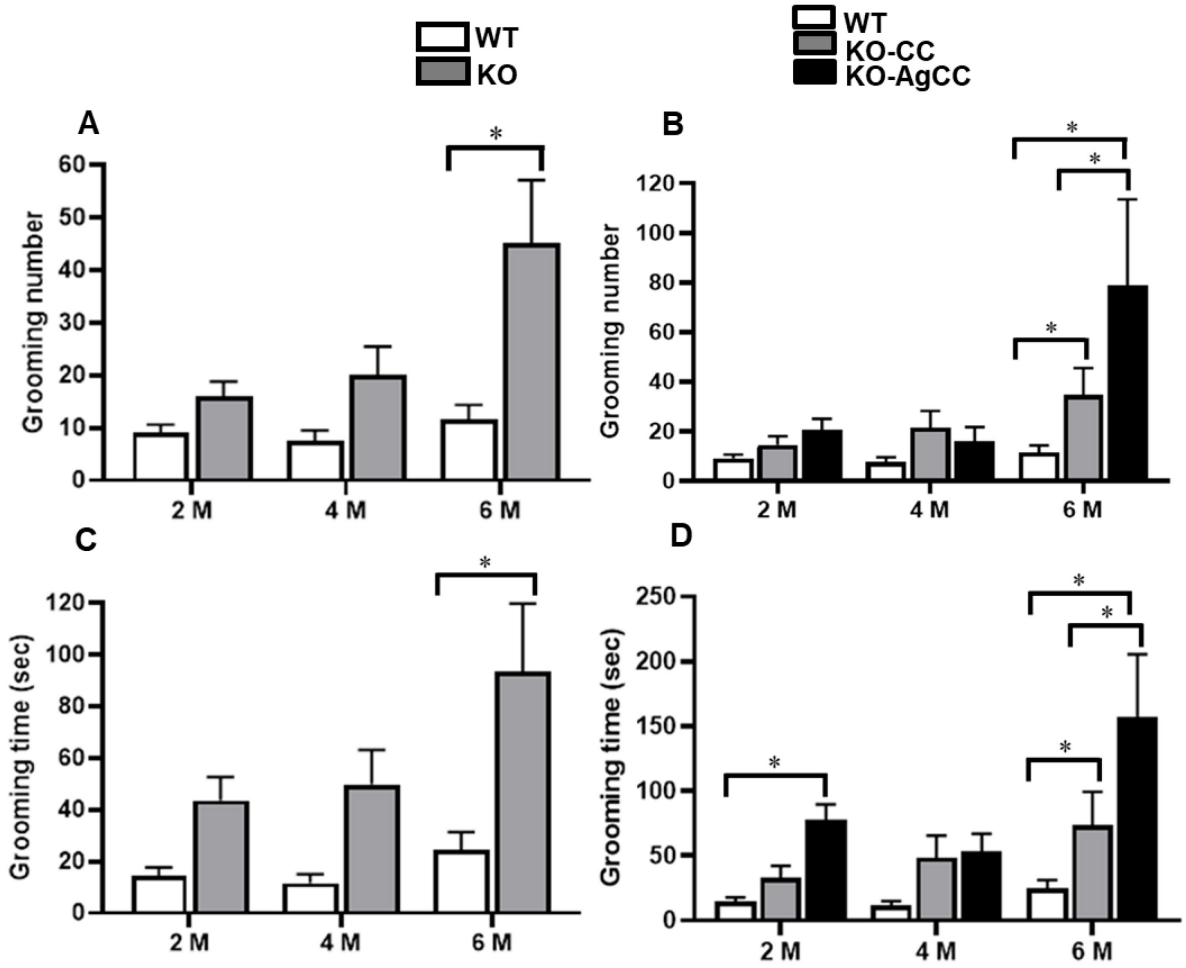


Fig. 6. Excessive grooming behavior by PlxnA1 KO mice in the open field.

(A) PlxnA1 KO mice at 6-month old exhibited a significantly increased number of grooming compared to WT mice. (B) PlxnA1 KO-CC at 6-month old exhibited significantly higher number of grooming than WT mice. PlxnA1 KO-AgCC at 6-month old showed significantly higher numbers of grooming compared to PlxnA1 KO-CC and WT mice. (C) PlxnA1 KO mice at 6-month old exhibited significantly longer grooming times than WT mice. (D) PlxnA1 KO-CC mice at 6 months exhibited significantly longer grooming times than WT mice. PlxnA1 KO-AgCC mice also exhibited significantly longer grooming times at 2 and 6 months old than age-matched WT mice. WT (n = 23); PlxnA1 KO (n = 21); PlxnA1 KO-CC (n = 16); PlxnA1 KO-AgCC (n = 5). (Data presented as mean ± SEM; *p < 0.05, **p < 0.01, ****p < 0.0001).

3.5. Grooming behavior in the home cage

Open field environment may induce stress and may alter a broad range of spontaneous behaviors, thus, grooming in the home cage was observed during the active (dark) phase from 20:00 to 8:00 using another cohort of WT and PlxnA1 KO mice. Consistent with previous findings in the open field, there was a significant main effect of genotype (Fig. 7A; $F(1, 43) = 10.87$, $p = 0.0020$) on grooming time as well as a main effect of time bin (hour) within the observation period (Fig. 7A; $F(5, 215) = 33.89$, $p < 0.0001$) and a significant genotype × hour interaction ($F(5, 215) = 4.598$, $p = 0.0005$). PlxnA1 KO mice demonstrated significant longer grooming times during the 20:00–22:00 period than WT mice (Fig. 7A; $p < 0.0001$ by Bonferroni test). In addition, there were also significant main effects of genotype (Fig. 7B; $F(1, 43) = 286.6$, $p < 0.0001$) and hour (Fig. 7B; $F(5, 215) = 13.10$, $p < 0.0001$) on scratching time and a significant genotype × hour interaction ($F(5, 215) = 7.426$, $p < 0.0001$). Bonferroni's multiple comparison tests confirmed a significant increase in scratching time by PlxnA1 KO mice during all time bins compared to WT mice (Fig. 7B; all $p < 0.0001$).

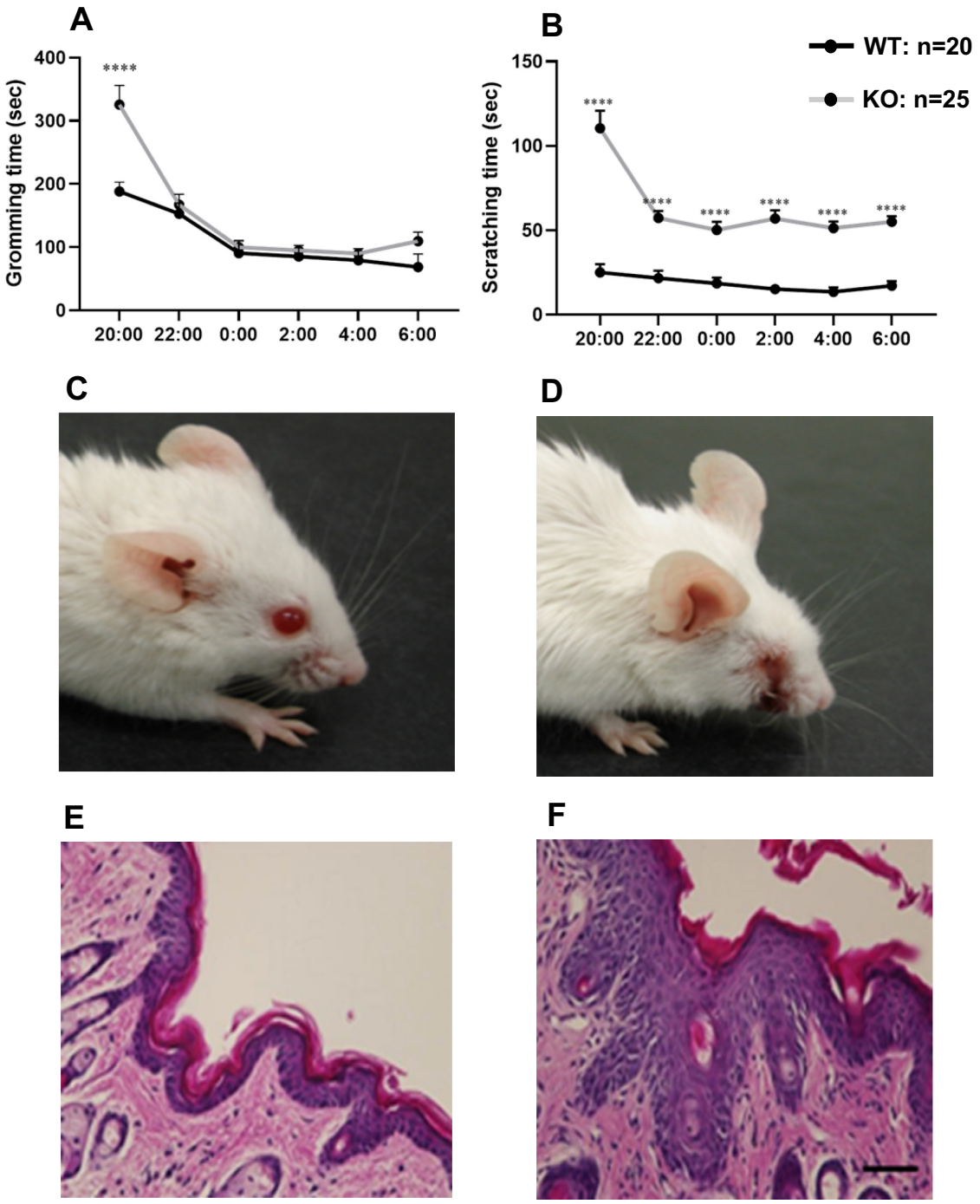


Fig. 7. Excessive grooming behavior of PlxnA1 KO mice in the home cage.

(A, B) PlxnA1 KO mice demonstrated significantly greater grooming behavior during the period from 20:00 to 22:00 and greater scratching behavior (B) during all time bins compared to WT mice. WT (n = 20); PlxnA1 KO (n = 25). (C, D) Severe skin lesions around the eyes, presumably caused by excessive grooming,

observed in several PlxnA1 KO mice (D) but absent in WT mice (C). (E, F) Hematoxylin-eosin staining confirmed increased thickness of the epidermis under the eyes of PlxnA1 KO mice (F) but not WT mice (E). (Data presented as mean ± SEM; ****p < 0.0001).

Table 1

Behavioral phenotypes of PlxnA1 KO mice in the open field test.

Behavioral phenotype	2 months of age			4 months of age			6 months of age		
	KO	KO-CC	KO-AgCC	KO	KO-CC	KO-AgCC	KO	KO-CC	KO-AgCC
Total distance	NS	NS	NS	NS	NS	NS	NS	NS	NS
Central zone (number)	NS	↑*	NS	↑*	↑*	NS	NS	NS	NS
Central zone (time)	NS	NS	NS	NS	NS	NS	NS	NS	NS
Grooming (number)	NS	NS	NS	NS	NS	NS	↑****	↑*	↑**¶
Grooming (time)	NS	NS	↑*	NS	NS	NS	↑****	↑*	↑****¶

NS, not significant; ↑: increased, * p < 0.05, ** p < 0.01, **** p < 0.0001: significantly increased compared with WT, ¶: significantly increased also in comparison with KO-CC in addition to the comparison with WT. KO, PlxnA1 KO mice; KO-CC, PlxnA1 KO mice with an intact corpus callosum (CC); KO-AgCC, PlxnA1 KO mice with substantial agenesis of corpus callosum (AgCC).

3.6. Decreased prepulse inhibition in PlxnA1 KO mice

Defects of sensorimotor gating constitute one of the most robust behavioral phenotypes observed in schizophrenia and the rodent models (40, 41). Prepulse inhibition (PPI) test is used for the evaluation of sensorimotor gating, in which a startle reflex is normally attenuated in both humans and rodents when a startling stimulus is preceded by a weaker pre-stimulus or prepulse (42). PPI is decreased in both schizophrenic patients and rodent models of schizophrenia (41). To assess whether deficiency of PlxnA1 affects sensorimotor gating, PlxnA1 KO mice were subjected to PPI test of the acoustic startle response. The baseline acoustic startle response did not differ between both genotypes (Fig. 8A; $p > 0.05$ by Welch's *t*-test). However, there was a significant main effect of genotype (Fig. 8B; $F (1, 62) = 5.688$, $p = 0.0202$) and prepulse condition (Fig. 8B; $F (2, 124) = 56.47$, $p < 0.0001$) on %PPI, as well as a significant genotype × prepulse interaction ($F (2, 124) = 3.900$, $p = 0.0228$). Further, %PPI in the mutant mice was significantly lower than WT mice at a prepulse intensity of 86 dB (Fig. 8B; $p = 0.0068$ by Bonferroni test). There was also a significant main effect of AgCC group (Fig. 8C; $F (2, 61) = 3.451$, $p = 0.0380$) and prepulse intensity (Fig. 8C; $F (2,122) = 20.40$, $p < 0.0001$) but no significant group × prepulse intensity interaction ($F (4, 122) = 1.964$, $p = 0.1042$). PlxnA1 KO-CC mice demonstrated significantly reduced %PPI at both 78 dB and 86 dB prepulse intensities compared to WT mice (Fig. 8C; 78 dB: $p = 0.0310$ by Bonferroni test, 86 dB: $p = 0.0050$ by Bonferroni test), while there were no significant differences between WT and PlxnA1 KO-AgCC mice or between PlxnA1 KO-CC and PlxnA1 KO-AgCC mice for any test condition (Fig.7C).

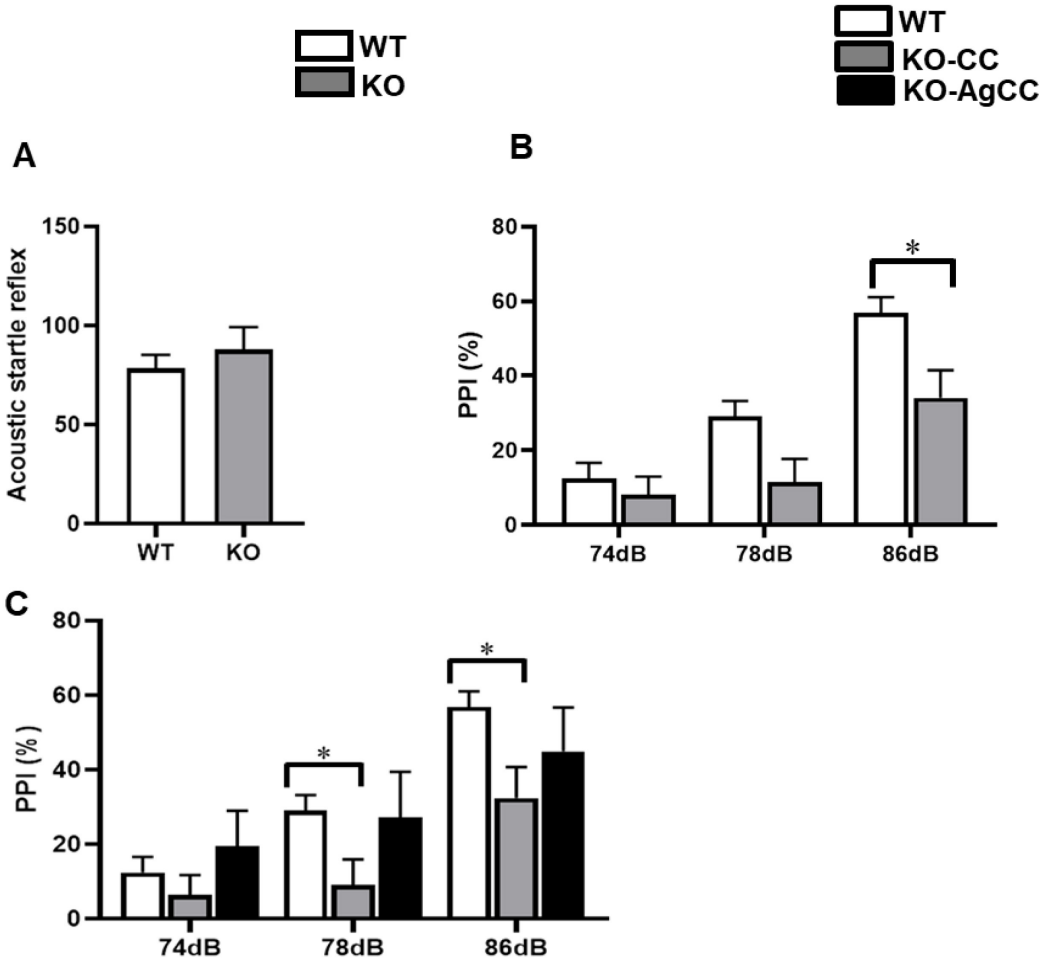


Fig. 8. Impaired prepulse inhibition (PPI) in PlxnA1 KO mice.

(A) The amplitude of the auditory startle reflex did not differ between PlxnA1 KO and WT mice. (B) %PPI was significantly reduced in PlxnA1 KO mice at a prepulse intensity of 86 dB compared to WT mice. (C) PlxnA1 KO-CC mice displayed significantly reduced %PPI at both 78 dB and 86 dB prepulse intensities compared to WT mice. WT (n = 34); PlxnA1 KO (n = 30); PlxnA1 KO-CC (n = 26); PlxnA1 KO-AgCC (n = 4). Data are presented as mean ± SEM. WT: wild type, KO: PlxnA1 knockout mice, PlxnA1 KO-CC: PlxnA1 KO mice with corpus callosum, PlxnA1 KO-AgCC: PlxnA1 KO mice with agenesis of corpus callosum. (Data presented as mean ± SEM; *$p < 0.05$).

3.7. Reduced anxiety-like behavior of PlxnA1 KO mice in the elevated plus maze test

Anxiety-like behaviors of PlxnA1 KO mice were evaluated in the elevated plus-maze tests. In the tests, PlxnA1 KO mice spent significantly more time on the open arms than WT mice (Fig. 9B; 197.3 ± 32.27 vs. 85.30 ± 21.20 s, p = 0.0096 by Student's *t*-test) and significantly less time on the closed arms (Fig. 9E; 99.95 ± 33.61 vs. 206.8 ± 22.71 s, p = 0.0140 by Student's *t*-test), suggesting reduced anxiety-like behavior (consistent with more numerous central zone entries in the open field) than WT control.

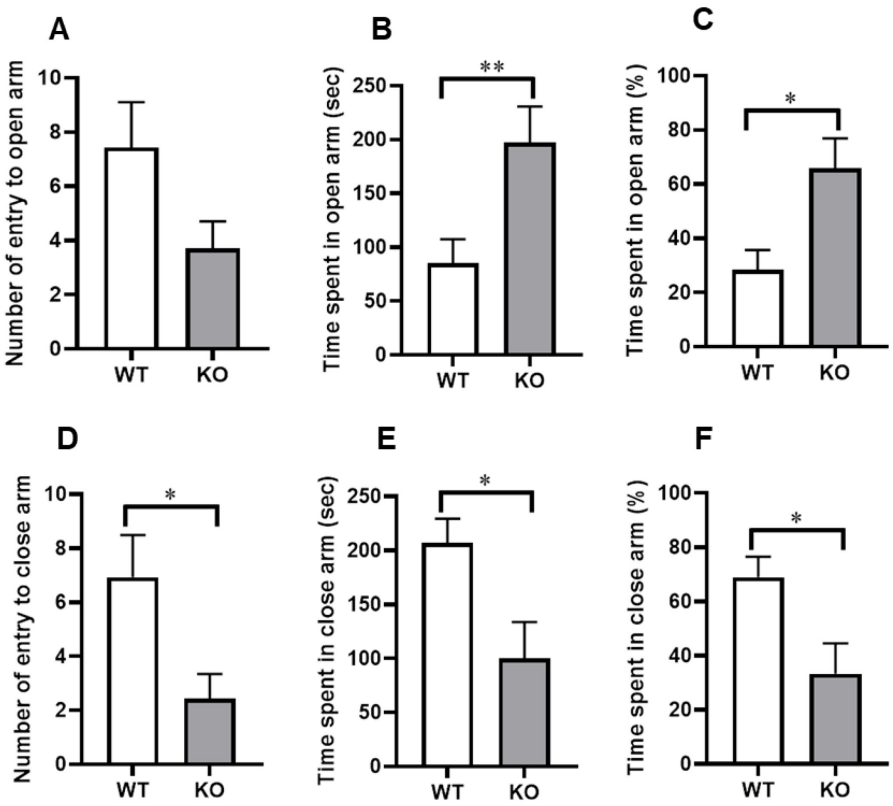


Fig. 9. Reduced anxiety-like behavior in the elevated plus maze.

PlxnA1 KO mice spent significantly more time on the open arms (B), and significantly less time on closed arms (E). A significantly higher percentage (%) of time on open arms (C) by PlxnA1 KO mice was observed and significantly lower percentage (%) of time on closed arms than WT (F) along with significantly fewer closed arm entries compared to WT (D). (Data presented as mean ± SEM; *p < 0.05, **p < 0.01).

3.8. Social interaction of PlxnA1 KO mice is not different from the WT mice

In social interaction test for sociability, both WT and PlxnA1 KO mice showed a significant preference for exploring the cage housing stranger mouse 1 compared to the empty cage (Fig. 10A; $F(3, 52) = 5.345$, $p = 0.0028$; WT: empty vs. stranger 1, $p = 0.0473$; KO: empty versus stranger 1, $p = 0.0335$) in one-way ANOVA comparison, however, post hoc analysis does not show any significant difference ($p > 0.05$) between WT and PlxnA1 KO mice in terms of sociability. In the following trail, with the original stranger 1 presented with a novel mouse (stranger 2), the number of interactions and time of close contact visitations were measured. For social novelty, both WT and PlxnA1 KO mice (Fig. 10B; $F(3, 52) = 2.825$, $p = 0.0476$; WT: stranger 1 vs. stranger 2, $p = 0.1880$; KO: stranger 1 vs. stranger 2, $p > 0.9999$) did not show any difference in number and time of visitations to each one. Thus, PlxnA1 KO mice have normal social interaction like WT control.

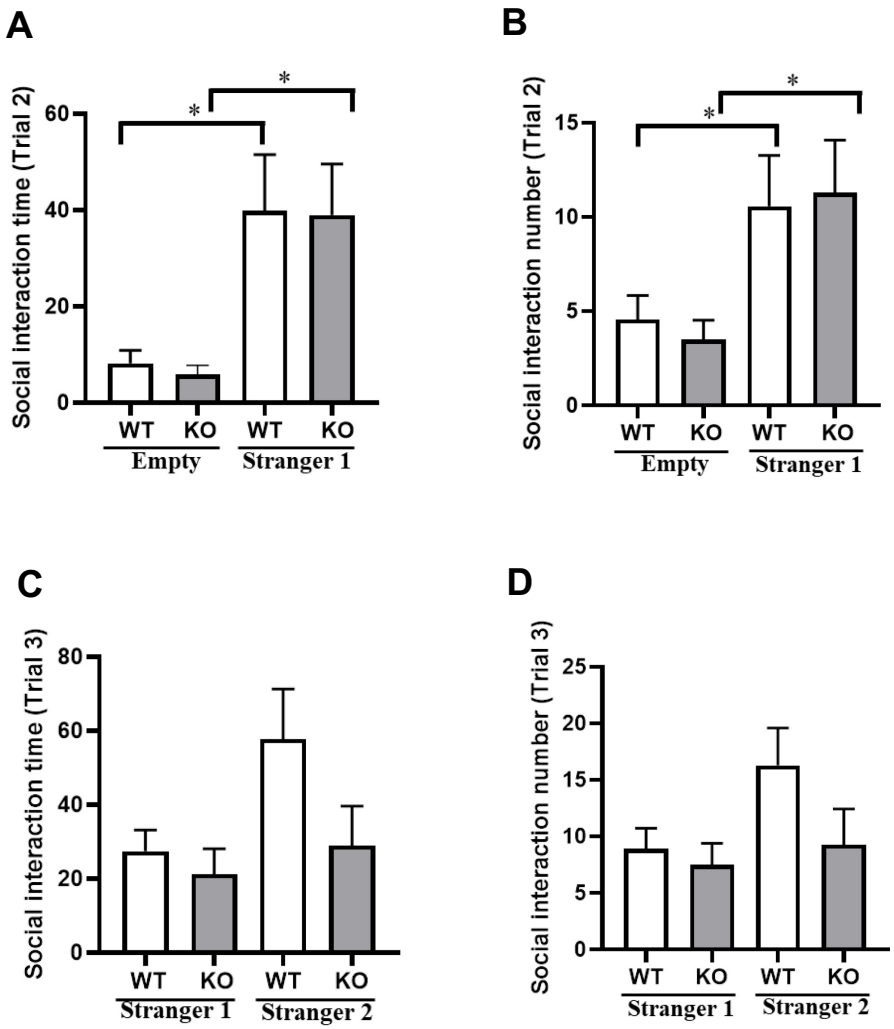


Fig. 10. Normal social behavior of PlxnA1 KO mice.

(A, B) Both WT and PlxnA1 KO mice demonstrated a significant preference for the cage containing a stranger mouse (1) compared to an empty cage. (C, D) Neither genotype demonstrated a significant preference for the cage containing a novel mouse (stranger 2) compared to stranger 1. WT (n = 14); PlxnA1 KO (n = 14). WT: wild type, KO: PlxnA1 knockout mice. (Data are presented as mean ± SEM; *p < 0.05).

3.9. Normal working and fear memory in PlxnA1 KO mice

In the Y maze spontaneous alternation test, PlxnA1 KO mice did not show significant differences in spatial working memory than WT controls (Fig. 11A; 56.77% ± 3.91% vs. 64.87% ± 2.85%, $p > 0.05$ by Student's *t*-test). However, PlxnA1 KO mice made significantly fewer total arm entries compared to WT mice (Fig. 11B; 20.07 ± 1.96 vs. 26.28 ± 1.49, $p < 0.05$ by Student's *t*-test), indicating lower exploratory activity.

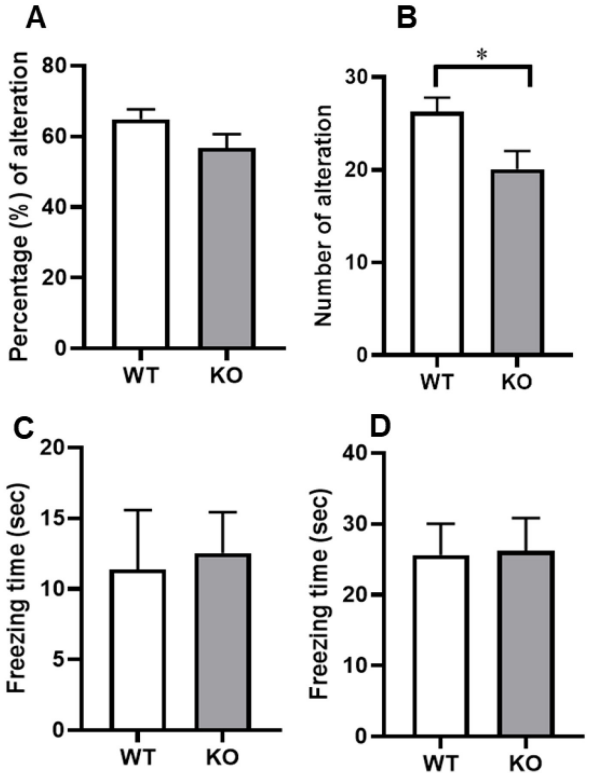


Fig. 11. Normal working and fear memory in PlxnA1 K O mice.

(A, B) Percentage alternations in the Y maze test (A) and number of arm entries (B) by WT and PlxnA1 KO mice. (C, D) No significant difference in freezing time by PlxnA1 KO mice compared to WT mice in both the contextual fear conditioning test (C) and cued fear conditioning test (D). WT (n = 14); PlxnA1 KO (n = 14). WT: wild type, KO: PlxnA1 knockout mice. (Data presented as mean ± SEM; *$p < 0.05$).

Similarly, there were no differences in freezing time (fear memory) between PlxnA1 KO and WT mice under either the contextual condition (Fig. 11C; 12.54 ± 2.896 s vs. 11.40 ± 4.200 s, $p > 0.05$ by Student's *t*-test) or the cued condition (Fig. 11D; 26.18 ± 4.69 s vs. 25.63 ± 4.410 s, $p > 0.05$ by Student's *t*-test). In summary, these findings suggest that PlxnA1 deficiency had no effects on spatial working memory, long-term contextual fear memory, or long-term cued fear memory.

Taken together, measures of motor performance in wire hang test and rotarod test, working memory in Y maze test, fear memory, sociability and social novelty did not differ significantly between genotypes., while PlxnA1 KO mice displayed excessive self-grooming, impaired PPI, and lower anxiety in the open field and the elevated plus maze test (Table 1, Table 2).

Table 2

Behavioral phenotypes of PlxnA1 KO mice in other tests

Measurement	6 months of age
Wire hang test	NS
Rotarod test	↑ * trial 1, trial 2 and trial 3
Home cage grooming	↑ **** grooming time (20.00-22.00) ↑ **** scratching time (all time bin, 20.00-6.00)
Y maze test	NS (spontaneous alteration)
Elevated plus maze test	↑ ** more time in the open arms ↓ * less time in the closed arms
Social interaction test	NS (both sociability and social novelty)
PPI test	↓ * %PPI at 86dB, KO < WT ↓ * %PPI at 78dB and 86dB, KO-CC < WT
Fear conditioning test	NS (freezing time on both contextual and cued conditioning)

NS, not significant; ↑: increased, ↓: decreased, *p < 0.05, **p < 0.01, ****p < 0.0001: significantly increased compared with WT.
KO, PlxnA1 KO mice; KO-CC, PlxnA1 KO mice with an intact corpus callosum (CC).

4. Discussion

The present study of PlxnA1 KO mice under BALB/cAJ background confirmed the following findings: 1) *PlxnA1* mRNA is expressed in GABAergic interneurons in both prefrontal cortex and hippocampus of adult mice; 2) PlxnA1 KO mice demonstrated poor postnatal survival until weaning and impaired body and brain growth thereafter; 3) behavioral tests of PlxnA1 KO mice revealed multiple abnormalities in adulthood, including excessive stereotypic repetitive behaviors of self-grooming in both the open field and home cage as well as significantly decreased PPI representing a sensorimotor deficit.

4.1 Expression of PlxnA1 in adult mouse brain

Plxns are a family of molecules which are distributed in various animal species including vertebrates and invertebrates (43). In mouse and human, four members of the PlxnA subfamily (PlxnA1, A2, A3 and A4) have been isolated (10). In the present study, PlxnA1 protein was widely expressed in WT mouse brain, especially in the prefrontal cortex, hippocampus, and striatum, but was undetectable in all regions of PlxnA1 KO mice (Fig. 2A). In adult BALB/cAJ mice, expression of *PlxnA1* mRNA was enriched in GABAergic neurons of both prefrontal cortex and hippocampus (Fig. 2B, C). This finding of PlxnA1 expression is in consistent with previous immunohistochemical detection of PlxnA1 in developing GABAergic neurons of embryonic C57BL/6J mouse brain (18). In addition, expression of PlxnA1 is also observed in the globular structures on the plasma membranes of parvalbumin-positive GABAergic interneurons of adult mouse brain (44).

4.2. Decreased postnatal survival, body weight and brain weight

PlxnA1 KO mice were observed at the expected Mendelian ratio of ~25% at P0.5, and neither body nor head weight differed significantly from WT littermates. Thus, PlxnA1 deficiency did not have a lethal effect during BALB/cAJ mouse embryogenesis, but more than half died from P0.5 to weaning by an unknown cause. AgCC that was observed in PlxnA1 KO mice under BALB/cAJ strain at P0.5 may underlie one possible causes of the postnatal death in high frequency (20). Another possible explanation is seizure activity, as *PlxnA1* allelic variants are associated with intractable epilepsy in humans (30, 31, 32, 33). However, lethal complications like epilepsy have not been observed in adult PlxnA1 KO mice, even those with AgCC. Alternatively, AgCC or interneuronal abnormalities may affect maternal bonding or suckling. Further studies exploring the nutritional status of these mice is necessary to disclose the cause of early postnatal death.

4.3. Unimpaired motor function and hyper-grooming of PlxnA1 KO mice

Despite impaired postnatal growth, PlxnA1 KO mice showed normal limb muscle strength in the wire hang test (Fig. 4A) along with superior motor coordination in the rotarod test (Fig. 4B). Though lower body weight of PlxnA1 KO mice may facilitate rotarod performance, it is also possible that they improve limb coordination. Cortico–motoneuronal (CM) connections are critical for fine manual dexterity in primates (45, 46). Rodents showed a limited degree of manual dexterity without CM connections by using coordinated paw movements to manipulate food pieces (47). However, conditional PlxnA1 KO specific to *Emx1*-expressing excitatory neurons resulted in superior manual dexterity compared to WT mice by

maintaining stronger CM connections into adulthood (48). I speculate that constitutive PlxnA1 KO mice may switch hands more skillfully during rod rotation due to superior manual dexterity, although this remains to be examined.

While most behavioral metrics did not differ from WT littermates, significant hyper-grooming behavior in both the open field and the home cage was observed in PlxnA1 KO mice (Fig. 6, 7). Grooming is an important part of rodent behavioral repertoire, which involves a series of individual movements that form functional sequences, including stereotyped patterns (49). However, hyper-grooming is often regarded as a sign of anxiety and stress in mice (50). PlxnA1 KO mice made more frequent entries into the central zone of the open field than WTs (Table 1, Fig. 4C, 4D), behaviors associated with reduced anxiety in mice (51). Furthermore, PlxnA1 KO mice spent significantly more time on the open arms and significantly less time on the closed arms of an elevated plus maze (Fig. 9), again indicative of reduced anxiety (52). Thus, hyper-grooming behavior observed in the present study is unlikely attributable to greater trait anxiety in this mice model.

The significantly enhanced self-grooming in the home cage was associated with epidermal lesions around the eyes (Fig. 7), indicating its severity. A significant increase of epidermal thickness was observed (WT: 12.01 ± 1.59 μm vs. KO: 25.79 ± 3.23 μm; $p < 0.01$ by Student's *t*-test) in PlxnA1 KO mice than WTs, which was likely associated with excessive grooming behavior. Similar excessive self-grooming in the home cage was observed in mice with a deficiency in neuropilin 2, a coreceptor of PlxnA family (53, 54, 55). Excessive self-grooming in rodents is regarded as a valuable endophenotype for pathological statuses

characterized by repetitive behaviors (49, 52). However, these PlxnA1 KO mice showed a behavioral profile different from other such models. For instance, both Sema5A KO mice (56) and interneuron-specific Sema3F KO mice (57) displayed social deficits but no hyper-grooming, while PlxnA1 KO mice did not exhibit social deficits (Fig. 10). Repetitive behaviors including excessive self-grooming have been reported in several autism spectrum disorder (ASD) models (58, 59). Thus, the mechanisms underlying hyper-grooming activity in PlxnA1 KO mice may still be related to neurodevelopmental disorders characterized by stereotypic behavior.

The open-field test disclosed the significant increase of self-grooming in PlxnA1 KO mice at 6 months of age, and thus the hyper-grooming phenotype in PlxnA1 KO mice developed along with age (Fig. 6). Similar findings with the age-dependent increase in the duration of self-grooming was observed in mice deficient of the SAP90/PSD-95 associated protein (Sapap3; DLGAP3; GKAP3) (60). The age-dependent increase in the duration of self-grooming in Sapap3 KO mice is suggested to associate with an age-dependent decrease in mGluR5 availability in the cortex, striatum, hippocampus, and amygdala which are related to obsessive compulsive disorder (60). Thus, the effect of ligands for PlxnA1 in PlxnA1 KO mice at young age may be maintained in brain areas related to the control of grooming behavior by the compensatory function of other PlxnA family members (PlxnA2, A3, A4). However, the effect of ligands for PlxnA1 may be attenuated in PlxnA1 KO mice with increasing age because of the decompensation of PlxnA1 function, and significant augmentation of self-grooming may be evident in PlxnA1 KO mice at 6 months of age.

AgCC significantly augmented the level of hyper-grooming behavior in PlxnA1 KO mice at 6 months of age (Fig. 7B, 7D). Similar significant increase of self-grooming behaviors is also observed in BTBR T+tf/J (BTBR) strain of mice in conjunction with a complete congenital AgCC (61), which is consistent with other reports (62, 63, 64). However, subtle disruptions of neurodevelopment other than AgCC are indicated to underlie excessive self-grooming and lower sociability in BTBR strain of mice (65). Since PlxnA1 KO mice with an intact CC (PlxnA1 KO-CC) displayed a significant increase of grooming as compared with WT at 6 months (Fig. 6B and 6D), abnormalities other than CC may underlie the increase of grooming in PlxnA1 KO mice. Therefore, in PlxnA1 KO mice, AgCC may intensify the stereotypic hyper-grooming behavior due to disordered function of neural circuitry or subtle abnormalities in neurodevelopment other than AgCC.

4.4. PPI deficits in PlxnA1 KO mice

PlxnA1 KO mice under BALB/cAJ background exhibited significant PPI impairment. PPI impairment is regarded as an endophenotype of the sensorimotor gating dysfunction observed in schizophrenia patients (66, 67, 68, 69, 70). Among mice harboring mutations of *PlxnA* family members, both constitutive *Plxna2*$^{-/-}$ mice and PlxnA2-GAP-deficient mice exhibit PPI deficits (71). Behavioral studies with *Plxna2*$^{-/-}$ mice revealed deficits in sensorimotor gating traits, associative learning and sociability commonly observed in neuropsychiatric disorder (71). Further, allelic variants of human *PlxnA2* are associated with schizophrenia (24). Thus, PlxnA1-Sema signaling may be critical for the development of neural circuitry necessary for PPI.

As to the relationship between PPI and AgCC, BTBR mice with AgCC shows normal PPI (72). Thus, AgCC itself may not be responsible for the impairment of PPI. Since PlxnA1 KO-CC (PlxnA1 KO mice

with an intact CC) displayed the significant decrease of PPI and PlxnA1 KO-AgCC (PlxnA1 KO mice with substantial AgCC) did not exhibit the significant differences in PPI as compared with either WT or KO-CC (Fig. 8C), contribution of AgCC to the PPI deficit may be low in PlxnA1 KO mice.

4.5. PlxnA1 KO mice recapitulate only limited features of developmental encephalopathy and schizophrenia

Three humans with distinct missense mutations of *PlxnA1* exhibited developmental encephalopathy or schizophrenic symptoms (30, 31, 32, 33). Thus, it is of interest to assess the degree to which the neurobehavioral phenotypes of PlxnA1 KO mice may overlap with the symptoms of these diseases. Developmental encephalopathies involve an extensive and genetically heterogeneous range of diseases associated with global developmental delay, intellectual disability, frequent epilepsy, and other neurofunctional impairments (33, 73, 74). Heterozygous missense mutation of *PlxnA1* in a human showed developmental encephalopathy characterized by intractable infantile-onset epilepsy and intellectual disability with ASD. The patient also displayed features suggestive of Dubowitz syndrome characterized by growth failure, cutaneous symptoms, and dysmorphic facial features (33). While PlxnA1 KO mice exhibited body and brain growth impairments, the present study did not detect seizure activity, abnormal sociability, impaired working memory, or deficient long-term memory in PlxnA1 KO mice.

Nonetheless, the substantial behavioral and information processing deficits displayed by Plxn1A1 KO mice may provide a valuable model for mechanistic investigations. Prepulse inhibition is considered as a useful,

reliable, robust, and quantitative phenotype for exploring the neurobiology and genetics of gating disorder in schizophrenia (75). A PPI deficit observed in a genetic mouse model could indicate that the gene may play a crucial role in the neural circuitry known to regulate PPI (41). Recent methylation and gene ontology enrichment analyses have recognized several biological processes with potential relevance to schizophrenia pathophysiology (76). Hyperlocomotor activity in mice is also recognized as an endophenotype of mania and positive symptoms of schizophrenia (77), but this was not observed in PlxnA1 KO mice (Table 1, Fig. 5A and 5B).

PlxnA1 KO mice exhibited reduced anxiety in the open field and elevated plus maze test, which suggests that hyper-grooming observed in the KO mice was unrelated to anxiety. Reduced anxiety and normal sociability of PlxnA1 KO mice also suggest that hyper-grooming is not associated with pathomechanisms relevant to ASD or obsessive-compulsive disorder. Alternatively, stereotypical behavior like hyper-grooming has been considered as a positive symptom in rodent models of schizophrenia (78, 79). Thus, increased self-grooming and excessive scratching behavior in PlxnA1 KO mice may be related to the stereotypical behaviors characteristic of schizophrenia, although PlxnA1 KO mice did not display other schizotypic phenotypes such as impaired sociability (Fig. 10) and memory (Fig. 11). Taken together, PlxnA1 KO mice seem to display the two specific features relevant to the pathophysiology of schizophrenia. PlxnA1 KO mice may thus be a unique mouse model to investigate the mechanisms of stereotypical behaviors and impaired PPI fundamental to neurodevelopmental or psychiatric disorders.

5. Summary

In the present study, western blot analyses confirmed the expression of PlxnA1 protein widely in WT mouse brain, especially in the prefrontal cortex, hippocampus, and striatum, but was undetectable in all regions of PlxnA1 KO mouse brain. Further, RNAscope *in situ* hybridization confirmed the expression of *PlxnA1* mRNA in most GFP-positive GABAergic (GAD67-GFP KI mice) neurons in the medial prefrontal cortex and hippocampus of the adult brain. Even though PlxnA1 KO mice born from heterozygous parents were obtained at P0.5 following the Mendelian rule，but more than half of PlxnA1 KO mice died during the early postnatal period before weaning by an unknown cause.

At 6 months of age, PlxnA1 KO mice showed a significantly lower body weight than that of WT littermates ($p < 0.0001$ by Student's *t*-test). Similarly, brain weight at 6 months was significantly lower compared to WT controls ($p < 0.05$). Neither body nor brain weight were differed at P0.5 ($p > 0.05$) between WT and PlxnA1 KO mice. Thus, under the genetic background of BALB/cAJ mice, PlxnA1 KO mice demonstrated poor survival rate during the early postnatal period as well as impaired body and brain growth into adulthood. On the other hand, AgCC that was frequently observed in PlxnA1 KO mice at both embryonic day 17.5 (E17.5) and P0.5, but only 13 out of 80 adult (6-month-old) PlxnA1 KO mice exhibited AgCC as evidenced by immunohistochemical staining with myelin basic protein. Thus, PlxnA1 KO mice showed poor postnatal survival rate before weaning and impaired body and brain growth with morphological deficits of CC. Comprehensive behavioral test battery in my study was open field assessment of spontaneous locomotion, central zone entry and time, grooming numbers and duration, home cage grooming, the wire hang test of

muscle strength, motor coordination on the rotarod task, elevated plus maze, sociability and social novelty test, Y maze alternation task, cued and contextual fear conditioning, and PPI test. PlxnA1 KO mice displayed excessive self-grooming behavior, impaired PPI, and slightly lower anxiety than WT control, while other behavioral phenotypes like motor performance, working memory, fear memory, and sociability did not differ between genotypes. These outcomes indicate a critical role for PlxnA1 in the development and function of brain regions controlling self-grooming and PPI. Thus, PlxnA1 KO mice may be a significant model to research neural mechanisms underlying neurodevelopmental and neuropsychiatric disorders.

6. Conclusion

PlxnA1 KO mice demonstrated poor survival during the early postnatal period as well as impaired body and brain growth into adulthood. Behavioral testing revealed excessive self-grooming in both the open field and home cage as well as impaired PPI. Thus, PlxnA1 KO mice may be a suitable model to explore the mechanisms of stereotypic behaviors and PPI deficits fundamental to several neurodevelopmental and psychiatric disorders.

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