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Impaired Cognitive Function and Altered Hippocampal Synapse Morphology in Mice Lacking *Lrrtm1*, a Gene Associated with Schizophrenia

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Abstract

Recent genetic linkage analysis has shown that *LRRTM1* (*Leucine rich repeat transmembrane neuronal 1*) is associated with schizophrenia. Here, we characterized *Lrrtm1* knockout mice behaviorally and morphologically. Systematic behavioral analysis revealed reduced locomotor activity in the early dark phase, altered behavioral responses to novel environments (open-field box, light-dark box, elevated plus maze, and hole board), avoidance of approach to large inanimate objects, social discrimination deficit, and spatial memory deficit. Upon administration of the NMDA receptor antagonist MK-801, *Lrrtm1* knockout mice showed both locomotive activities in the open-field box and responses to the inanimate object that were distinct from those of wild-type mice, suggesting that altered glutamatergic transmission underlay the behavioral abnormalities. Furthermore, administration of a selective serotonin reuptake inhibitor (fluoxetine) rescued the abnormality in the elevated plus maze. Morphologically, the brains of *Lrrtm1* knockout mice showed reduction in total hippocampus size and reduced synaptic density. The hippocampal synapses were characterized by elongated spines and diffusely distributed synaptic vesicles, indicating the role of *Lrrtm1* in maintaining synaptic integrity. Although the pharmacobehavioral phenotype was not entirely characteristic of those of schizophrenia model animals, the impaired cognitive function may warrant the further study of *LRRTM1* in relevance to schizophrenia.

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Introduction

Elucidation of the genetic factors involved in schizophrenia is one of the major challenges in current neurobiology [1-6]. *LRRTM1* (*Leucine rich repeat transmembrane neuronal 1, OMIM 610867*) is an emerging candidate gene for schizophrenia. A three-marker haplotype upstream of *LRRTM1* on 2p12 is associated with schizophrenia/schizoaffective disorder when inherited paternally [7,8].

In biological terms, *LRRTM1* (humans) and *Lrrtm1* (mice) encode a single-membrane-spanning transmembrane protein with a leucine-rich repeat domain in its N-terminal side, and they are predominantly expressed in the nervous systems of humans and mice, respectively [7,9]. Tagged-rat Lrrtm1 protein is localized in the excitatory synapses of cultured hippocampal neurons and shows synaptogenic activity in neuron/fibroblast coculture assay [10]. Furthermore, the distribution of vesicular glutamate transporter (VGLUT1) is altered in $Lrrtm1^{-/-}$ mice [10]. These results raise the possibility that *Lrrtm1* is essential for higher brain function in mammals, but this possibility has not been addressed to date.

Schizophrenia is a relatively common mental disorder that affects 1% of the population worldwide. The disease is characterized by positive symptoms (delusions and hallucinations), negative symptoms (affective flattening and social withdrawal), and cognitive dysfunction (deficits in working memory, attention, processing speed, and executive function) [1,2]. Morphologically, there are abnormalities of the brain that are hallmarks of schizophrenia, such as enlarged ventricles, reduced hippocampal volume, dendritic changes in the pyramidal neurons, and alteration of specific subtypes of interneurons [11–14]. Several model mice that partially mimic these behavioral and morphological signs have been developed, contributing to our understanding of the pathophysiology of schizophrenia [3–6,15,16].

Here, we investigated the behavioral properties of *Lrrtm1* knockout (KO) mice. These mice showed deficits in behavioral responses to stressful situations and novel objects, together with spatial memory and social discrimination deficits. In addition, we clarified some of the morphological abnormalities of the mutant's hippocampus; these deficits may be related to the behavioral abnormalities found.

Results

Generation of *Lrrtm1*-null mutant mice

We generated an *Lrrtm1* null-type mutation ($Lrrtm1^{-}$) by homologous recombination in ES cells (Figure 1). Mating between heterozygotes ($Lrrtm1^{+/-}$) generated homozygotes ($Lrrtm1^{-/-}$,

Lrrtm1 KO) in an expected Mendelian ratio when examined at weaning (+/+, 23%, +/−, 50%; −/−, 27%; n = 205). The mice grew with normal body weight without any abnormalities in terms of external appearance (data not shown). They showed no obvious ataxic movements in observations during breeding and colony maintenance procedures.

Lrrtm1-deficient mice are impaired in adaptive behaviors to environmental changes

We first measured spontaneous activities in the home cages and in open-field (OF) boxes. Over 7 consecutive days of observation in a new cage, *Lrrtm1* KO mice showed 40% to 50% less activity than wild-type (WT, $Lrrtm1^{+/+}$) mice in the initial 2 h of the dark (night) phase (20:00 to 21:00, $P = 0.0085$; 21:00 to 22:00, $P = 0.022$) (Figure 2A), although mean activity did not differ significantly ($F(1,18) = 2.46$, $P = 0.13$). In the 15-min observation period in the OF box (Figure 2B), young adult KO mice (3 to 5 months old) showed significantly less locomotor activity than WT mice under bright illumination (250 lx) ($P = 0.046$) but not so under darker conditions ($P = 0.28$) (70 lx). Eight-month-old KO mice that had experienced several behavioral tests showed less locomotor activity ($P = 0.044$) than WT mice under 70 lx, as well as a significant preference to stay in the corners of the OF box ($P = 0.0053$) (Figure 2B). Thus, spontaneous activities differed between WT and KO mice in these two situations of environmental change.

In the light–dark box transition (LD) test (Figure 2C) mice were first placed in the light side of the box. WT mice moved to the dark box after a short while (mean 34 s), but the latency of the transition time in KO mice was much longer (mean 90 s,

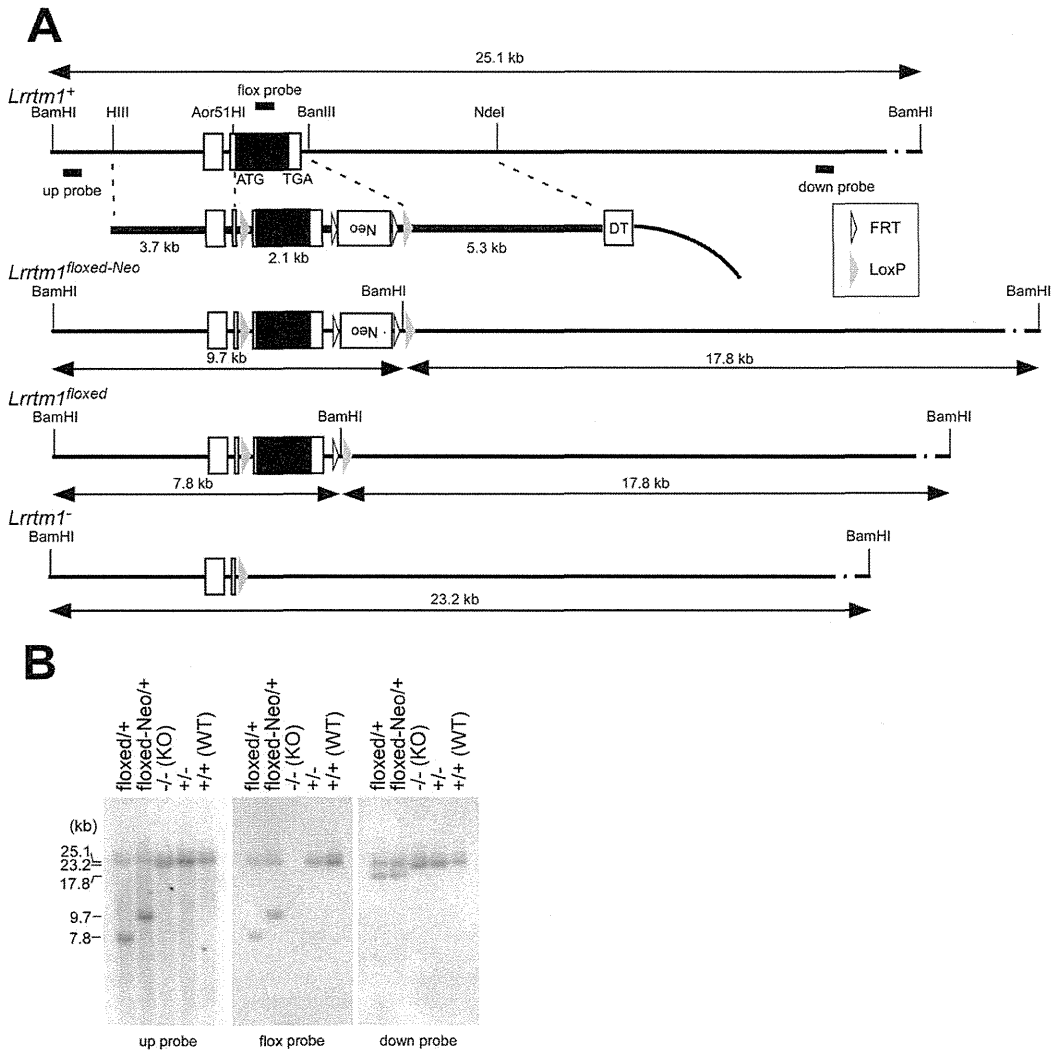

Figure 1. Targeted disruption of the *Lrrtm1* gene. (A) Structures of the *Lrrtm1* genomic locus, targeting vector, and mutated allele. Locations of the 5′ and 3′ probes for Southern blotting are shown. Solid box, protein coding region of the exons; open box, untranslated region of the exons; gray triangle, loxP site; open triangle, FRT site; DT, diphtheria toxin A; Neo, neomycin-resistance gene cassette; ATG, initiation codon; TGA, termination codon. Lines with double arrowheads indicate restriction fragment lengths. (B) Confirmation of homologous recombination of the mutant alleles by Southern blot. *Bam*HI-digested genomic DNA was hybridized with genomic fragments that corresponded to the genomic sequences of 5′ and 3′ outside the targeting vector (up probe and down probe, respectively) and an Lrrtm1 protein-coding region (flox probe).
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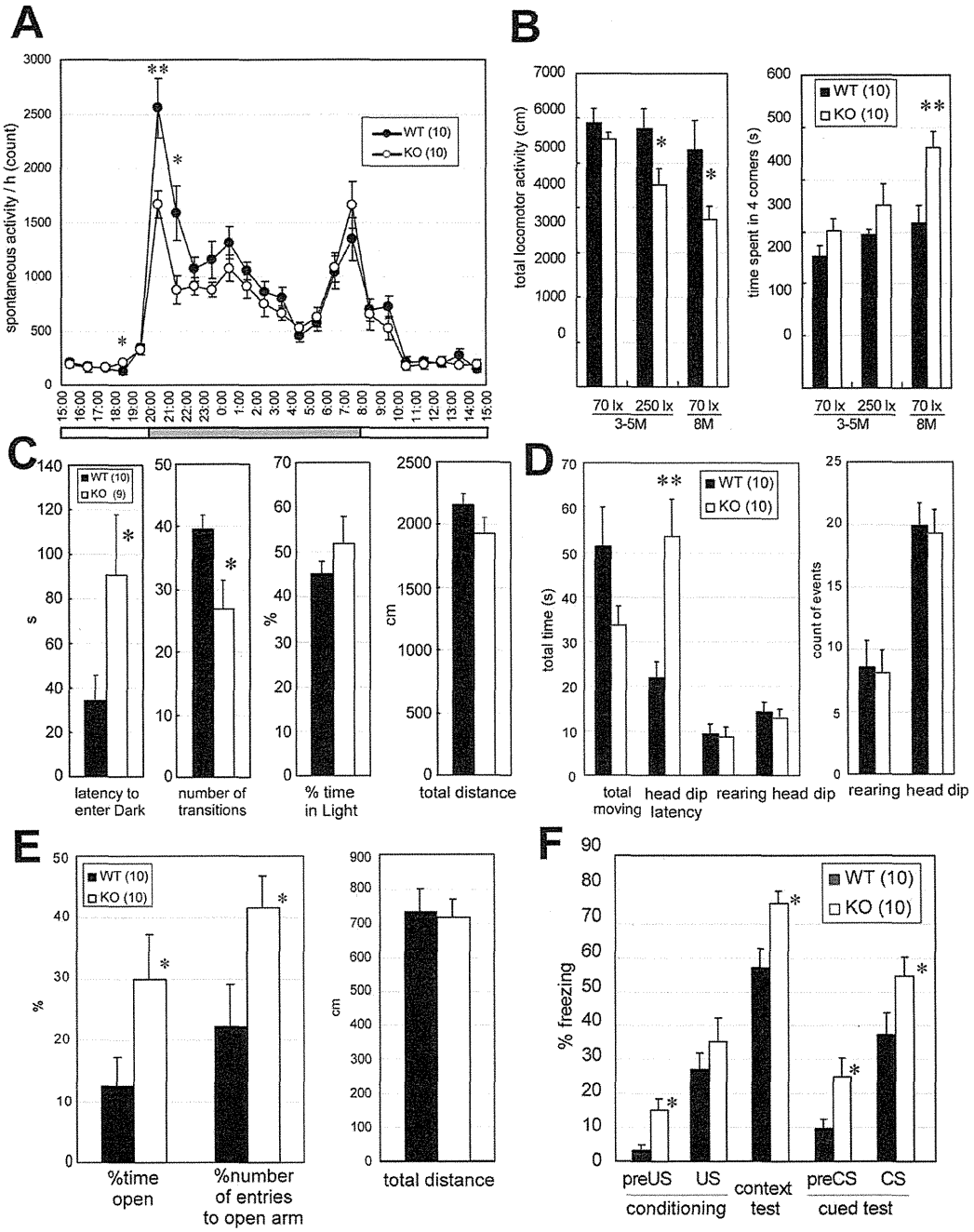

Figure 2. *Lrrtm1* KO mice show adaptive behavior abnormalities. (A) Home-cage activities. The circadian profile of the locomotor activity (bin = 1 h) was first determined for each mouse. Then the mean and SEM of the locomotor activities per 1 h were calculated for each genotype. Statistical analysis was performed against the mean values for each mouse. The horizontal bar below the graph indicates the light–dark cycle (gray, dark phase; white, light phase). Values are presented as means ±SEM. * $P<0.05$; ** $P<0.01$. (B) OF test. (left) The locomotor activity indicates the total distance traveled (cm) in the test period. (right) Time spent in the four corner squares of a 5×5 subdivision of the field. Young adult mice (3 to 5 months (M)) that were new to the OF apparatus were subjected to the test at two different illuminances (70 lx or 250 lx, 3–5 M). Eight-month-old mice that had experienced several behavioral tests were also tested at 70 lx illuminance (70 lx, 8 M). Values are presented as means ±SEM. * $P<0.05$. (C) light–dark box transition test. Total distance traveled, % of time spent in the light box, number of transitions between the light and dark boxes, and the first latency period before entering the dark box are indicated as means ±SEM. * $P<0.05$. (D) Hole board test. Total moving time (s), latency until head-dipping (s), number of head-dips, duration of head-dips (s), duration of rearing (s), and number of rearings are indicated as means ±SEM. ** $P<0.01$. (E) Elevated plus maze test. Total distance traveled, % time spent in the open arms, and % of entries to the open arms were measured. Values are presented as means ±SEM. * $P<0.05$ in U-test. (F) Fear-conditioning test. In both contextual and cued (conditional) tests, *Lrrtm1* KO mice exhibited significantly greater freezing responses than WT mice. * $P<0.05$; U-test. US, unconditioned stimulus; CS, conditioned stimulus. The numbers in parentheses in the key boxes indicate those of WT and KO mice used in each experiment (common to all figures).
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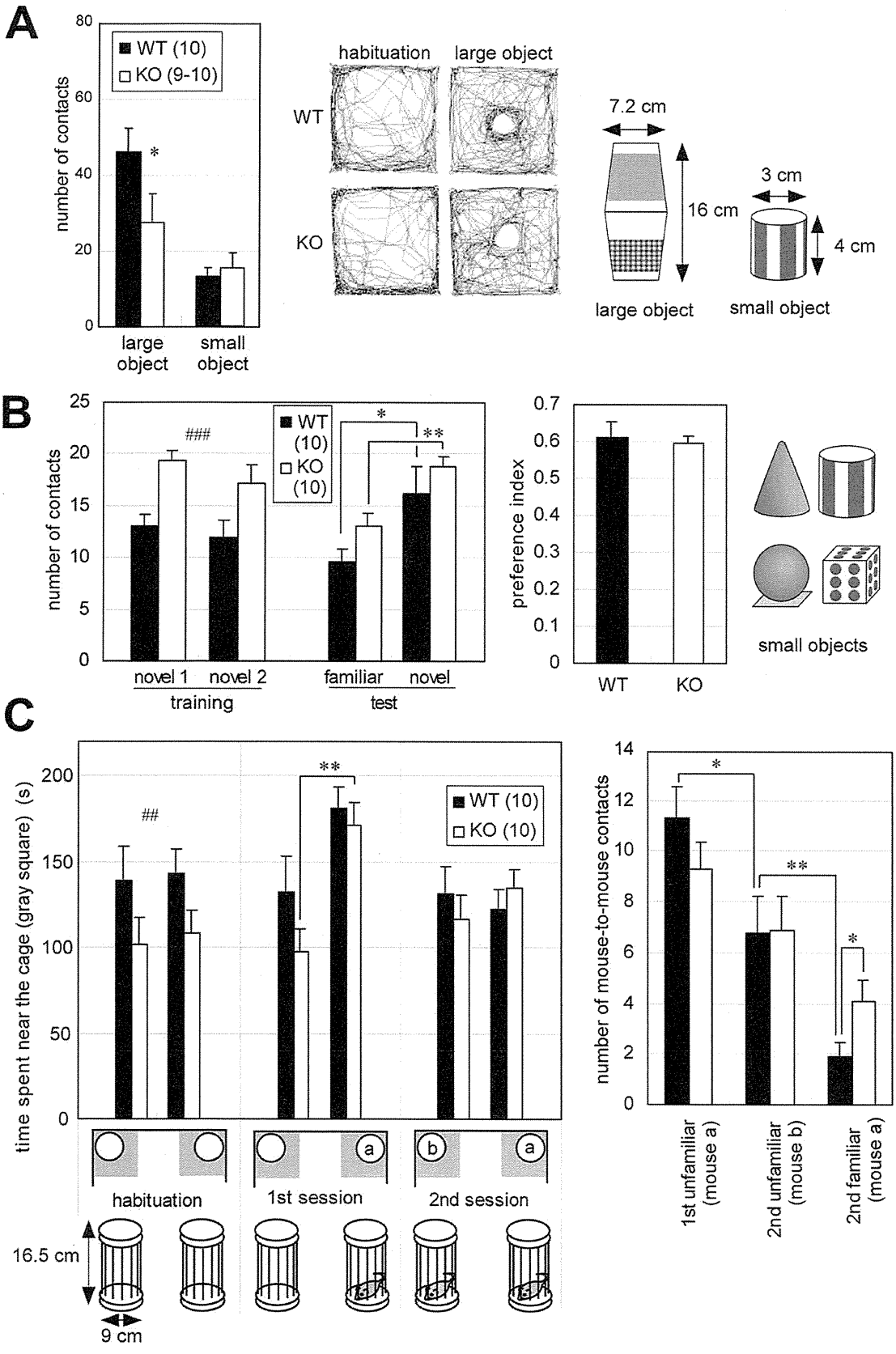
A
WT (10)
KO (9-10)
number of contacts
large object
small object
habituation
large object
WT
KO
7.2 cm
16 cm
3 cm
4 cm
large object
small object
B
WT (10)
KO (10)
number of contacts
novel 1
novel 2
familiar
novel
training
test
preference index
WT
KO
small objects
C
WT (10)
KO (10)
time spent near the cage (gray square) (s)
habituation
1st session
2nd session
16.5 cm
9 cm
number of mouse-to-mouse contacts
1st unfamiliar (mouse a)
2nd unfamiliar (mouse b)
2nd familiar (mouse a)

Figure 3. Approach to inanimate and animate objects. (A) Behavioral tests of approach to inanimate objects in the OF. The mice were first placed in the OF box without the object (habituation), then placed again in the OF with the large or small object (*right*). Approach was measured by the numbers of direct contacts with the large or small object (*left*). The traces are representative ones of WT and KO mice during the habituation and the test session with the large object (*middle;* results are given for a pair that showed comparable trace patterns in habituation). Values are presented as means ±SEM. * $P<0.05$. (B) Novel object recognition test using four kinds of small object (*right*). *novel1* and *novel2* indicate that the same kinds of objects were placed in the left and right corners, respectively, of the cages in the training session. *familiar* and *novel* indicate respectively that the object was unchanged (*novel1*) and that a new, differently shaped object was added in place of *novel2* in the test session. A novel object *preference index* was calculated as follows: contacts with *novel* / total contacts with *novel* and *familiar*. Values are presented as means ±SEM. * $P<0.05$; ** $P<0.01$. ### $P<0.001$ (total contacts, comparison between WT and KO). (C) Social discrimination test. Approach to the cages was measured by the time spent in the rectangular region (indicated as gray squares below graph, 17.7 cm×17.7 cm) that included the cage (left). Mouse-to-mouse contacts (right). Values are presented as means ±SEM. * $P<0.05$; ** $P<0.01$. ## $P<0.01$ (total stayed time between WT and KO).
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$P=0.035$). In addition, the total number of transitions made by KO mice during the 10-min observation period was significantly lower than that by WT mice ($P=0.034$). The total time spent in the light side of the box and the total distance traveled did not differ significantly between the two genotypes. Similar abnormalities were found in the hole board (HB) test (Figure 2D) [17]. In this test, mice were placed in an OF-like apparatus with four holes (3 cm diameter) on the floor (50 cm×50 cm), and their behaviors were observed for 5 min. *Lrrtm1* KO mice showed a prolonged mean latency to the time of first head-dipping behavior ($P=0.0042$ by Welch's *t*-test), whereas the total duration and number of head-dipping behaviors were comparable with those in WT mice. There were no differences in terms of the duration and number of rearing behaviors. The LD and HB tests results suggested that the expected behavior responses in the novel environments were impaired in KO mice.

KO mice also showed behavioral abnormalities in stressful situations. In the elevated plus maze (EPM) test (Figure 2E), KO mice spent significantly more time on the open arms ($U=23$, $P=0.041$) and entered the open arms more frequently ($U=23$, $P=0.041$) than did WT mice. The total distance traveled by KO mice was comparable to that by WT mice. Although the increased time spent in the open arms and entering the open arms could be interpreted as indicating a decrease in anxiety-like tendencies, this seemed not to be the case. Because KO mice tended to freeze more frequently than WT at 1-m-high, 15-cm-diameter circle platform [freezing time (s, means ±SEM) in total 300 s observation: WT, 135±13.8 (n = 10); KO, 173±20.1 (n = 10); $U=34$, $P=0.082$], and we observed a significant increase in the number of feces in the EPM test [WT, 0.50±0.27 (n = 10); KO, 2.0±0.39 (n = 10); $U=18$, $P=0.0094$]. Accordingly, in the fear-conditioning (FC) test, KO mice showed greater freezing responses in conditioning (pre-US [unconditioned stimulus], $U=18$, $P=0.013$), a context test ($U=19.5$, $P=0.021$), and a cue test (pre-CS [conditioned stimulus], $U=23$, $P=0.041$; CS, $U=23.5$, $P=0.045$) (Figure 2F). Although our initial attempt was to assess fear memory by the FC test, this was hard to assess owing the consistently higher freezing responses.

In sum, the results of the LD, HB, EPM, and FC tests revealed behavioral deficits of *Lrrtm1* KO mice under stressful situations that urged the mice to execute adaptive responses.

Differential responses to both inanimate and animate objects are observed in *Lrrtm1* KO

To further clarify the adaptive behavior abnormalities, we investigated the mice's responses to inanimate and animate objects. We used two different-sized inanimate objects. The larger one was 16 cm high, with a cylindrical shape and the smaller one was 4 cm high, with a column shape (Figure 3A, far right panel). The objects was placed in the center of the OF test box (50 cm×50 cm). The number of contacts with the object were measured (Figure 3A). *Lrrtm1* KO mice contacted the large object significantly less frequently ($P=0.033$) than did WT mice. This result was also supported by trace pattern abnormality (Figure 3A, middle). In contrast, when small objects were placed in the OF box, KO and WT mice contacted the object equally (Figure 3A); this was significantly different from the case with the large object ($P=0.028$, $F(1,35)=5.4$, two-way ANOVA for genotype-object size interaction).

To test whether the perception of "novelty" was altered in *Lrrtm1* KO mice, we also used the small objects 3–4 cm high cone, sphere, and cube in addition to the column (Figure 3B, far right panel). The surfaces of these objects were differentially labeled with black or gray on a white background. In a home cage (17 cm×28 cm×12 cm [H]), contact with the small objects by KO mice was significantly more frequent than by WT mice (Figure 3B, *training*, $P=0.00024$), indicating that the approach to inanimate objects was context dependent. In the novel object recognition (NOR) test, two identical objects were first placed in the cage. After 15 min of exposure to the objects (Figure 3B, *training*), one object was replaced with a new one that differed in terms of shape and surface pattern. In the following 15 min, the mice were exposed to both the new, unfamiliar object and the familiar object (Figure 3B, *test*). The contacts with each object were counted in both sessions. In the NOR *test* session, both WT and *Lrrtm1* KO mice showed significantly more frequent contact with the novel object (WT, $P=0.033$; KO, $P=0.0022$) than with the familiar one, and the novel object preference indices of the WT and KO mice were almost the same (Figure 3B, *right*). The result suggested that an altered preference for "novelty" might not explain the above-described behavioral abnormalities.

To examine responses to animate objects, we performed a social discrimination (SD) test (Figure 3C). In this test, the mice were first habituated to empty cages (16.5 cm high, cylindrical) placed in two corners of the OF box. Before the first session, one empty cage was replaced with a cage containing a mouse. After the first session of 15 min, a new (unfamiliar) caged mouse and the familiar caged mouse were presented to the test mouse for 15 min as the second session. The results were quantified as the time spent near each cage and as the number of direct contacts through the wire slits. First, we noticed that *Lrrtm1* KO mice avoided approaching the empty cages in the habituation session ($P=0.0084$). This result seemed consistent with the avoidance of the large object (Figure 3A). However, the empty-cage-avoidance tendency disappeared in the second and third exposures to the empty cages in a control experiment (data not shown). KO mice showed a clear preference for the caged animals in the first session, in comparison with the empty cages ($P=0.0023$). In the second session, WT mice contacted the unfamiliar mice 3.6 times more frequently than the familiar mice. This preference was not as strong (1.7 times) in *Lrrtm1* KO mice; in fact, they contacted the familiar mice twice as frequently as did WT mice (Figure 3C) ($P=0.041$). The results suggested a deficit in social recognition performance in *Lrrtm1* KO mice.

Spatial memory deficits and other behavioral abnormalities in *Lrrtm1* KO mice

Having shown that adaptive behavior abnormalities were present in *Lrrtm1* KO mice, we then investigated other behavioral features. The Morris water maze (MWM) test is a useful common platform for assessing spatial memory. We performed 4 days of training sessions consisting of six trials per day. First, KO mice swam significantly farther than the WT mice on the first day of the 4 consecutive training days ($P = 0.0041$) (Figure 4A). In light of the above-mentioned results, we considered that this result reflected a delayed response to novel environments. In probe tests performed on the fifth day, the *Lrrtm1* KO mice showed significantly poorer performance, both in stay time in the target quadrant ($U = 109.5$, $P = 0.014$) and in crossing the position of the target platform ($U = 128.5$, $P = 0.048$) (Figure 4B and 4C). The results indicated that the KO mice had a spatial memory deficit. Notably, KO mice showed unusual behaviors during the MWM test, such as frequent dives to reach the platform (7 out of 10 KO mice but none of the WT mice showed diving behavior) and frequent rearing after reaching the platform (5 out of 10 KO mice but none of the WT mice showed rearing).

There were no significant differences between the two groups in the other behavioral tests (Table S1).

Morphological changes in the *Lrrtm1* KO hippocampus

Histological examination of *Lrrtm1* KO adult brain sections stained with cresyl violet did not reveal any strong qualitative architectural abnormalities (Figure 5A). However, when we performed MRI scanning to search for volume changes, the *Lrrtm1* KO brain showed significant reductions in hippocampus volume ($P = 0.029$) and in the volume of the hippocampus relative to the total brain volume ($P = 0.046$) (Figure 5B). Measurement of cortical thickness indicated that there was a slight (6.6%) but significant reduction ($P < 0.001$) in the thickness of the somatosensory cortex (Figure 5C).

The above findings led us to further morphologically analyze the *Lrrtm1* KO hippocampus by examining Golgi-stained and electron microscopic images. We found a 7.3% increment in spine length ($P = 0.0084$) (Figure 5D and 5E), a 16% decrement in synaptic density in the stratum radiatum ($P = 0.032$) (Figure 6A and 6B), and increments in the mean inter-vesicular distance in both the stratum radiatum (10%, $P < 0.001$) and the stratum oriens (7.4%, $P < 0.001$) (Figure 6A and 6B). There were no strong differences in the other structural parameters, including width and density of the dendritic spines (Figure 5D and 5E), postsynaptic density (PSD) length, PSD thickness, and synaptic cleft size (Figure 6A and 6B).

Difference in effects of MK-801 administration in *Lrrtm1* KO and WT mice

The above-mentioned morphological alteration in the hippocampal synapses, together with the spatial memory deficit, raises the possibility of altered hippocampal synaptic transmission. In light of the fact that there is also an altered distribution of VGLUT1-immunopositive signals in *Lrrtm1* KO mice [8], we hypothesized that an altered excitatory synaptic function could underlie some of the behavioral abnormalities in *Lrrtm1* KO mice. To test this hypothesis, we examined the effects of administration of an NMDA receptor blocker, MK-801, on the behavior of KO mice. Ten-month-old mice were injected intraperitoneally with 500 μg/kg of MK-801 or saline during an OF test. Analysis of locomotive behaviors before and after MK-801 administration revealed that, in KO mice, the duration of a single movement was significantly lower ($P = 0.034$) ($F(1,18) = 4.5$, $P = 0.049$, two-way ANOVA with repeated measures for genotype ×drug interaction), and the number of episodes of movement was significantly higher ($P = 0.0059$) ($F(1,18) = 4.3$, $P = 0.052$, two-way ANOVA with repeated measures for genotype × drug interaction) than in WT mice after MK-801 administration (Figure 7A). The total distance moved and the number of turns were non-significantly greater in WT mice than in KO mice after MK-801 administration, whereas the reverse was true for the number of rotations. These changes may reflect the enhanced locomotor activity and stereotypy found with the administration of a similar dose of MK-801 to C57BL/6 mice in previous studies [18,19]. After the OF test, we also tested the approach to the large object (Figure 7B) that was less frequently contacted by *Lrrtm1* KO mice in the above-described experiments (Figure 3A). After MK-801 administration, the time spent near the object became comparable to that spent by WT mice (Figure 7B, top left), and the number of contacts with the large object by KO mice tended to be even higher than in WT mice ($P = 0.15$) (Figure 7B, bottom left). Two-way ANOVA with repeated measures revealed that there was a significant genotype × MK-801 treatment interaction ($F(1,17) = 5.41$, $P = 0.033$). The traces of KO mice during the test were also similar to those of WT mice (Figure 7B, right), in contrast to those without MK-801 administration (Figure 3A). The total distance moved and the number of turns did not show genotype-specific effects of MK-801 (Figure 7B, bottom center and right), suggesting that the increment in approach behavior was not due to an alteration in general locomotor properties. Furthermore, this change was not caused by mere habituation to the object, because a follow-up test performed 2 weeks after MK-801 administration reproduced the changes seen soon after MK-801 treatment (*44 weeks*, Figure 7B). In sum, MK-801 administration induced differences in locomotor activity and attenuated the abnormality in large-object approaching behavior in a genotype-specific manner.

Effects of antipsychotics and selective serotonin reuptake inhibitor (SSRI)

We next evaluated the effect of the antipsychotic clozapine [1], which has been widely used in both clinical and preclinical studies of schizophrenia, on the behavioral abnormalities in *Lrrtm1* KO mice. For the evaluation, we performed EPM tests in which KO mice showed strong reproducible abnormalities in repeated pilot experiments (data not shown). A low dose (0.4 mg/kg) was chosen, because administration of higher doses inhibits all active behavior in mice in the EPM [20]. The time spent in the open arm was not influenced by a single dose of clozapine at 0.4 mg/kg (Figure 8A). Because the impaired behavioral response in a stressful situation looked like a panic-type reaction, we also tested fluoxetine, an SSRI and a first-line drug in panic disorder patients [21]. KO mice given a single dose of 10 mg/kg fluoxetine spent significantly less time in the open arm than did saline-injected KO mice ($U = 19$, $P = 0.011$), but there was no effect on total distance traveled (Figure 8B). Consistent with the results of a previous study in C57BL/6 mice [22], the time spent in the open arm by WT mice was not significantly affected by 10 mg/kg fluoxetine. Collectively, these experiments revealed that the SSRI effectively rescued the behavioral abnormalities in the EPM test. To determine the effectiveness of antipsychotics on the KO behavioral abnormalities, more systematic analyses with multiple drugs and multiple doses are needed before a conclusion can be drawn.

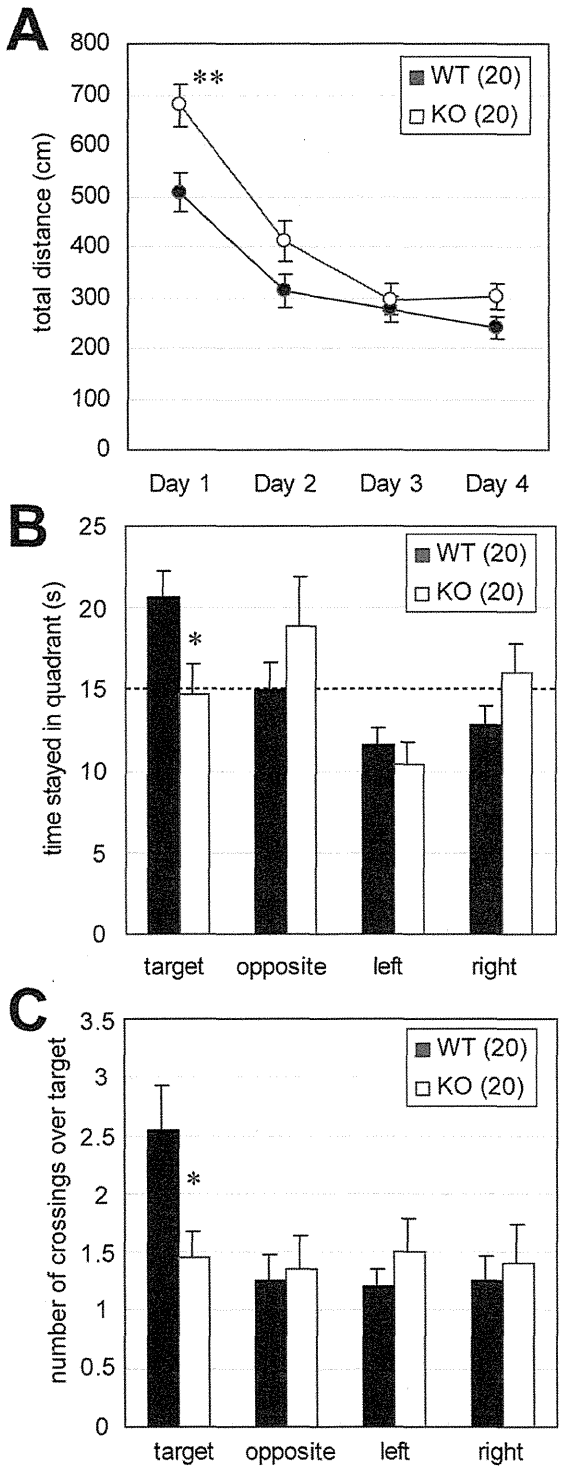

Figure 4. Spatial memory deficits in *Lrrtm1* KO mice. (A) Morris water maze training session. The total distance swum before reaching the target was significantly greater in KO mice than in WT mice on the first day, whereas it was comparable to that in WT mice on the second to fourth days. Values are presented as means ±SEM. * $P<0.05$; ** $P<0.01$. (B, C) Morris water maze probe test. Both the time spent in the target quadrant (B) and the number of crossings over the targets (C) were lower in *Lrrtm1* KO mice than in WT mice. Dotted line indicates the chance level. Values are presented as means ±SEM. * $P<0.05$ in U-test.
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Discussion

Lrrtm1 KO behavioral abnormalities

Lrrtm1 KO mice exhibited abnormalities in several behavioral tests. As a frequently observed behavioral abnormality in this study, we emphasize altered behavioral responses to environmental change. The results of the OF, LD, EPM, HB, FC, and MWM tests may be considered in relation to this key concept, as described above. The results of the inanimate object approach experiments may also be considered in this context from a broader perspective, because contact with objects can be regarded as a behavioral response to environmental change. The environmental changes in these tests may have exposed the mice to stressful situations in which they had to evoke behavioral responses. We speculate that Lrrtm1 is necessary for some versatile perception or executive functions required for the appropriate behavioral responses.

We also identified other behavioral abnormalities through our behavioral analysis. One was a social discrimination performance defect in the SD test. Because the test was conducted soon after the training session, the increased response to the familiar mice may indicate impairment of social perception, disturbance of short-term memory formation, or altered emotional status. However, the possibility of the latter two abnormalities may be low, considering that the other behavioral tests did not show abnormalities closely related to these two. The other suggestive abnormality is the spatial memory deficit shown in the MWM test. Although we cannot exclude the influence of altered adaptive response in the training process, the longer distance swum by KO mice was limited to the first day (Figure 4A), and the other parameters—latency in approach to the goal, and no movement time—were not significantly altered in the MWM test (data not shown). We therefore considered that a spatial memory deficit did exist in the *Lrrtm1* KO analysis. On the whole, the behavioral abnormalities in *Lrrtm1* KO mice could be summarized as indicating impaired cognitive function.

Morphological alteration of hippocampal synapses

The morphological analysis revealed altered synaptic density and morphology in the *Lrrtm1* KO hippocampus. The decrement in synapse density may represent the absence of *Lrrtm1* synaptogenic activity [10]. The longer spines are considered to indicate an abnormality related to postsynaptic differentiation. YFP-tagged Lrrtm1 is known to localize to excitatory synapses in cultured hippocampal neurons and can induce postsynaptic differentiation upon being subjected to an artificial clustering stimulus [10]. On the other hand, the increased inter-synaptic vesicle distances seemed to be consistent with the increment in the size of VGLUT1-immunopositive puncta in the hippocampus of another *Lrrtm1* KO strain [10]; punctum size may be influenced by the distributional area of the synaptic vesicles. Taken together, both the in vivo and the in vitro results indicate that Lrrtm1 exerts important roles in establishing or maintaining synaptic integrity of the hippocampus.

It is interesting that another Lrrtm family, Lrrtm2 [9], can bind neurexin proteins, which are presynaptic transmembrane proteins involved in presynapse differentiation [23]. Considering the fact that the neurexin binding code is conserved in Lrrtm1 [23], Lrrtm1 may be involved in presynapse instruction through an interaction with neurexin-like proteins.

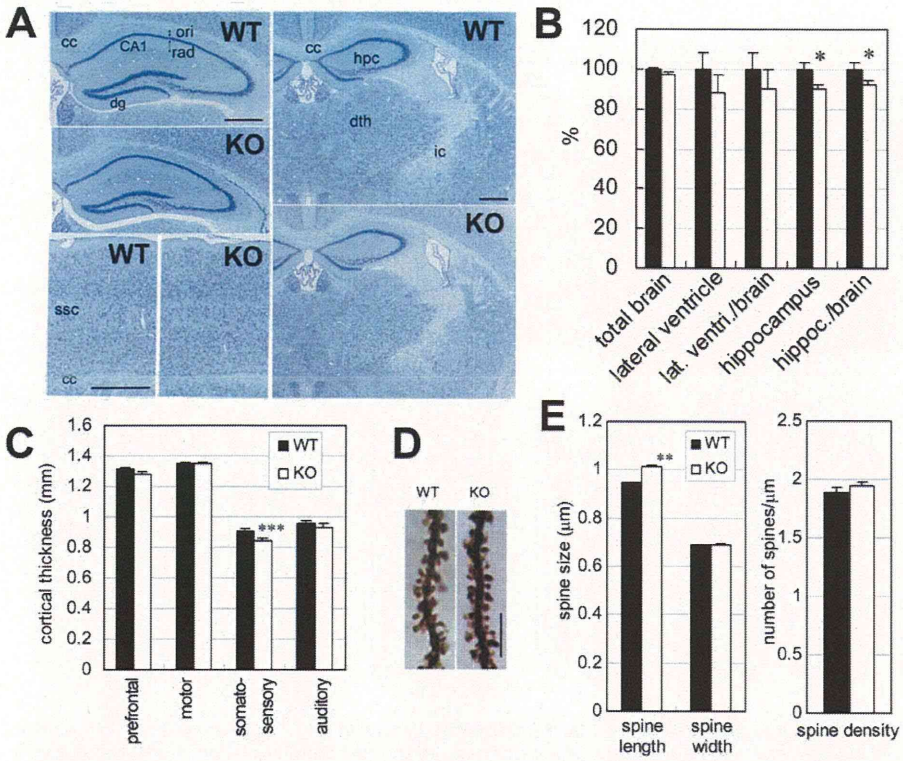

Figure 5. Morphological abnormalities in the *Lrrtm1* KO brain. (A) Histological examination of the hippocampus, thalamus, and cerebral cortex from WT and *Lrrtm1* KO mice. Scale bar, 0.5 mm. CA1, hippocampal CA1 area; cc, corpus callosum; dg, dentate gyrus; dth, dorsal thalamic nuclei; hpc, hippocampus; ic, internal capsule; ori, stratum oriens; rad, stratum radiatum; ssc, somatosensory cortex. (B) Volumetric analysis using MRI. Ten pairs of 36-week-old WT and *Lrrtm1* KO mice were subjected to in vivo analysis. (C) Thickness of cerebral cortices. Histological sections through prefrontal cortex, motor cortex, somatosensory cortex, and auditory cortex were subjected to morphometric analysis. (D) Spine morphology. Golgi-impregnation staining of hippocampal CA1 pyramidal neuron dendrites. Scale bar, 5 μm. (E) Length and width of spines (left) and number of spines (right) are quantified from secondary or tertiary dendrite segments (more than 20 μm; WT, 58 from 5 mice; KO, 53 from 4 mice). Mean values for each segment were analyzed. Black bars, WT; open bars, KO. Values are presented as means ±SEM. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.
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Lrrtm1 KO phenotypes and psychiatric disorders

Schizophrenia is characterized by positive symptoms, negative symptoms, and cognitive dysfunction [1,2]. The impaired cognitive function of *Lrrtm1* KO mice seems to be related to the cognitive dysfunction seen in schizophrenia patients. Furthermore, the increased time spent in the corners of the OF box and the reduction in home-cage activity could be regarded as negative-symptom-related behavioral abnormalities. However, it should also be noted that we did not find any signs suggesting positive-symptom-like abnormalities or sensorimotor gating deficits, which are often reported in mouse models of schizophrenia [24]. The behavioral phenotypes in *Lrrtm1* KO mice thus partly resemble the signs of schizophrenia. Morphologically, the reduction of hippocampal volume is analogous to that seen in first-episode schizophrenia patients [12].

In terms of the pathophysiological basis of the behavioral anomalies seen in the KO mice, alteration in NMDA transmission is suggested by the results of the MK-801 treatment experiment. Because specific malfunction of the glutamate receptor is proposed to be a potential pathogenic mechanism in schizophrenia [25,26], our results suggest that the involvement of *LRRTM1* dysfunction in schizophrenia needs to be considered. On the other hand, the effectiveness of fluoxetine in the recovery from behavioral response deficit in a stressful situation raises the possibility that a panic-like pathological status exists in *Lrrtm1* KO mice. Although panic disorder is generally considered to fall in the category of anxiety [27], the anxiety-like behaviors in *Lrrtm1* KO mice were not clear. The preference of *Lrrtm1* KO mice to stay in the corners of the OF box suggested enhanced anxiety; however, the LD and EPM tests did not reveal typical traits of enhanced anxiety. In this regard, hasty assumptions should be avoided in correlating the phenotype with the symptoms. It is essential to further clarify the biological role of *Lrrtm1* on the basis of a pharmacobehavioral analysis, longitudinal analysis, and conditional gene targeting. In light of the fact that *LRRTM1* is associated with schizophrenia [7,8], we suggest that the *Lrrtm1* KO mouse would be useful for further clarifying the involvement of *LRRTM1* in schizophrenia.

Materials and Methods

Animals

Mice were maintained by the Laboratory Animal Facility, RIKEN Brain Science Institute. All animal experiments were performed in accordance with the guidelines for animal experimentation at RIKEN. The mice were housed on a 12 h light–dark cycle, with the dark cycle occurring from 8:00 P.M. to 8:00 A.M. The behavior experiments were conducted in a light phase (10:00 AM to 7:00 P.M.). The mice were housed in groups until 1 week before the start of the behavioral experiments, and they were housed singly during the behavioral experiments. In total, 51 pairs

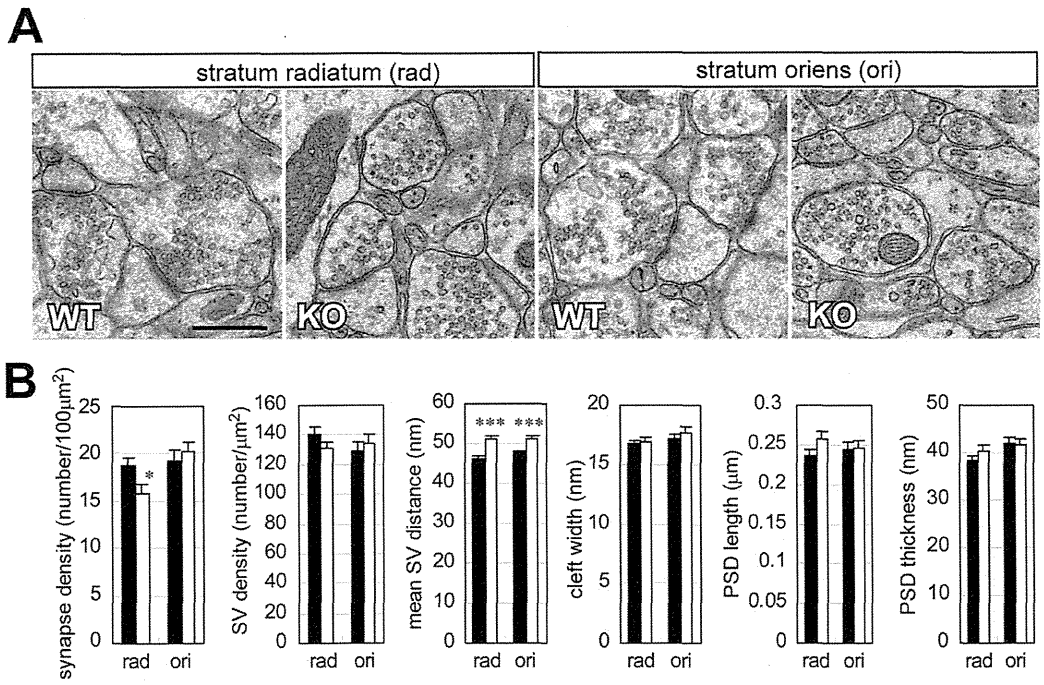

Figure 6. Electron microscopic analysis of hippocampal synapses. (A) Representative images of stratum radiatum and stratum oriens synapses. Scale bar, 500 nm. (B) Quantification of synapse number in 100 μm^2 of the entire images (synapse density), number of synaptic vesicles in 1 μm^2 of presynaptic bouton region (SV density), distance between synaptic vesicles (mean SV distance), cleft width, and postsynaptic density (PSD) width (length) and thickness. One hundred and thirty-three synapses from 3 KO mice and 126 synapses from 3 WT mice were analyzed. Black bars, WT; open bars, KO. Values are presented as means ±SEM. * $P<0.05$; *** $P<0.001$.
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of male *Lrrtm1* KO and WT control mice were subjected to the behavioral analysis. The experimental group, the number of KO and WT mice pairs in each group, and the type of behavioral experiment (listed in the order in which the experiments were performed), along with (age [weeks-old] at which the behavioral testing was performed), were as follows: Group 1, 10 pairs, home cage activity (10), OF test (12), LD test (12), EPM test (13), auditory startle response and prepulse inhibition (13), rotarod test (15), MWM test (16), FC test (17); Group 2, 10 pairs, OF test (21), social interaction in the OF (22), marble-burying test (29), OF test (32), resident–intruder test (35), social discrimination test (36), NOR test (37), OF test with MK801 (42), OF test (44); Group 3, 10 pairs, HB test (24), hotplate test (26), tail-flick test (27), MWM test (28), tail suspension test (30), forced swimming test (31); and Group 4, 21 pairs, OF test (14), EPM test (34), EPM test with clozapine (14–34), EPM test with fluoxetine (14–34). To minimize undesirable interexperimental influences, the intervals between the experiments were at least 3 days.

Generation of *Lrrtm1* KO mice

We generated a conditional knockout of *Lrrtm1*, and the null mutant. To construct the *Lrrtm1* targeting vector, overlapping *Lrrtm1* genomic clones were purchased from BACPAC Resources (Children's Hospital Oakland Research Institute, Oakland, CA, USA). The targeting construct contained the 3.7-kb 5′ and 5.3-kb 3′ homology regions, and the 2.1-kb fragment containing the open reading frame (ORF) of *Lrrtm1* was replaced by an area bounded by two LoxP sequences, together with a phosphoglycerol kinase (PGK) – neomycin-resistance-gene expression cassette flanked by an *FRT* sequence (Figure 1). Embryonic stem cells (EmbryoMax Embryonic Stem Cell Line – Strain C57BL/6, Millipore, Billerica, MA) were electroporated with the targeting construct and selected with G418. Drug-resistant clones were analyzed by Southern blotting. Chimeric mice were generated by injection of the targeted embryonic stem cells into BALB/c blastocysts. To excise the Lrrtm1 protein coding sequence and neo cassette, germline-transmitted mice were first mated with mice transgenic for Cre recombinase under the control of the cytomegalovirus immediate early enhancer – chicken β-actin hybrid (CAG) promoter [28]. Correct excision was confirmed by Southern blot. The resultant allele, which contained a LoxP sequence instead of the 2.1-kb Lrrtm1 ORF-containing region, is called the *Lrrtm1*$^-$ allele in this study. (*Lrrtm1*+/–, Cre-transgene) mice were backcrossed once to C57BL/6J mice to remove the Cre-transgenes. Lrrtm1 +/– heterozygotes were used to generate *Lrrtm1*–/– mice, which are called *Lrrtm1* KO mice in this study. In all experiments, we used age-matched male *Lrrtm1* KO and WT mice for the analyses. Genotyping was performed by Southern blot or PCR analysis of DNA isolated from tail samples; the PCR primers used were Lr1_5′loxP_F (5′ ATTACCCCGGCTTTGATCTT 3′) and Lr1_3′loxP_R (5′ AGGGAATGATAAAGGGCAGAGA 3′).

Home-cage activity

Spontaneous activity of mice in their home cages was measured by using a 24-channel Activity Monitoring System (O'Hara, Tokyo, Japan). Cages were individually set into compartments made of stainless-steel in a negative breeding rack (JCL, Tokyo, Japan). A piezoelectric sensor was added to the ceiling of each compartment; it scanned the movements of the mice (approximately 5 times/s). Home-cage activity was measured for 1 week