mice, predominantly depends on olfactory cues. This ability is needed for social familiarity and can be identified as a consistent decrease in olfactory investigation during repeated encounters with the same female in the social recognition test. In the social recognition test, *Erk2* CKO mice showed abnormal social memory (Fig. 7A; two-way RM ANOVA (trials 1–4), $F_{(1,22)} = 4.92, p < 0.05$ (genotype), $F_{(3,66)} = 14.38, p < 0.0001$ (trial), $F_{(3,66)} = 2.37, p > 0.05$ (interaction between genotype and trial)). Control mice showed a significant decline in the time spent investigating a female with subsequent presentation of the same female compared with *Erk2* CKO mice (Fig. 7A). This decrease was not due to a general decline in olfactory investigation because presentation of a novel female during trial five resulted in a similar amount of investigation as trial one with the original female. Therefore, it was concluded that *Erk2* CKO males, with persistent interest during repeated presentations, failed to develop social memory.

Mice are a social species and exhibit behavioral social interaction (Murcia et al., 2005; Kwon et al., 2006). Social interaction is thought to be a core paradigm to test autistic behavior in mice (Crawley, 2004; Moretti et al., 2005; Kwon et al., 2006). In the open field test for social versus inanimate preference (sociability), *Erk2* CKO mice exhibited abnormal social interaction (Fig. 7B; two-way ANOVA, $F_{(1,100)} = 6.13, p < 0.05$ (genotype), $F_{(1,100)} = 4.20, p < 0.05$ (interaction between genotype and preference). *Post hoc* comparison confirmed that control mice spent significantly more time interacting with the social target than with the inanimate target (social vs inanimate, $p < 0.0001$), indicating that control mice showed the normal preference for a social target over an inanimate target. In contrast to the control mice, *Erk2* CKO mice exhibited a significant decrease in interaction with the social target compared with controls (*post hoc* test, control vs *Erk2* CKO, $p < 0.01$). *Erk2* CKO mice showed no significant difference in the time they spent interacting with the social or inanimate targets (*post hoc* test, social vs inanimate, $p > 0.05$). The interaction with the inanimate target was similar in both *Erk2* CKO and control groups (*post hoc* test, control vs *Erk2* CKO, $p > 0.05$).

Additional social behavior tests gave similar results. In a sociability test for social versus empty preference using the three-roomed chamber, *Erk2* CKO mice exhibited abnormal social interaction (Fig. 7C; two-way ANOVA, $F_{(1,40)} = 5.04, p < 0.05$ (genotype), $F_{(1,40)} = 15.07, p < 0.001$ (interaction between genotype and preference)). The interaction with the empty cage was similar in both the *Erk2* CKO and control groups (*post hoc* test, control vs *Erk2* CKO, $p > 0.05$), but *Erk2* CKO mice exhibited significantly decreased interaction with the social target compared with controls (*post hoc* test, control vs *Erk2* CKO, $p < 0.0001$) and spent a similar amount of time interacting with both targets (*post hoc* test, social vs empty, $p > 0.05$). The control mice
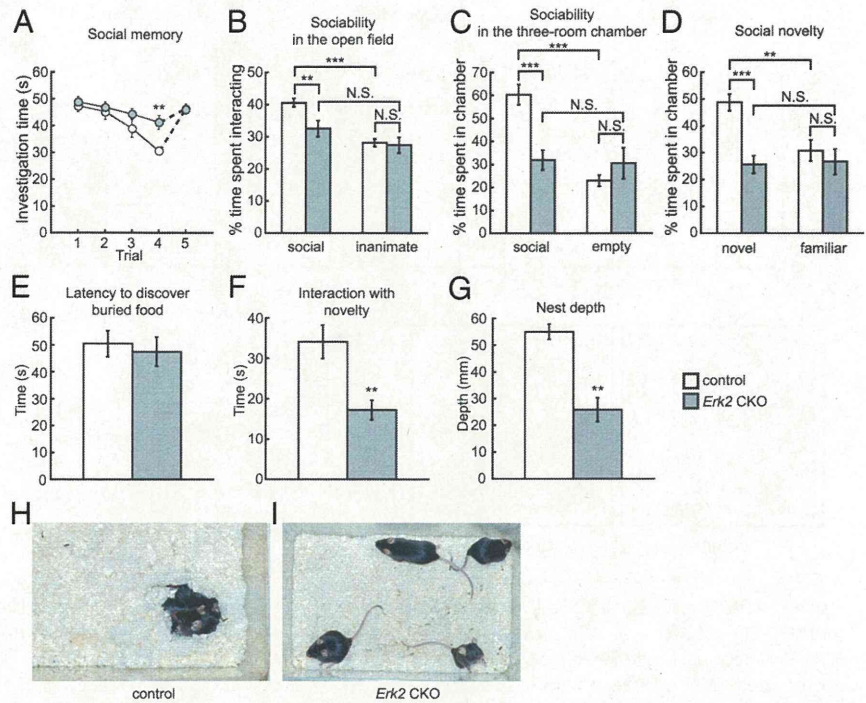

Figure 7. *Erk2* CKO mice are impaired in social behaviors. **A–D**, Abnormal behaviors in social interaction tests. **A**, Olfactory investigations in *Erk2* CKO mice are used for the social recognition test. Social memory by male mice is measured as the difference in investigation time (control, $n = 12$; *Erk2* CKO, $n = 12$). The data depict the amount of time spent investigating the same female during each of four successive 1 min trials. A fifth trial depicts the response to a new female. **B**, When exposed to caged social and inanimate targets in the open field, *Erk2* CKO mice show a decreased duration of interaction with the social target, and a similar duration of interaction with the inanimate object (control, $n = 29$; *Erk2* CKO, $n = 23$). Percentage time is depicted in **B–D**. **C**, In the sociability test in a three-room chamber, *Erk2* CKO mice spend less time than controls with the social target (control, $n = 11$; *Erk2* CKO, $n = 11$). **D**, In the social novelty test, controls show a preference for social novelty, while *Erk2* CKO mice show no preference between the novel and familiar targets. *Erk2* CKO mice spend significantly less time than controls interacting with the novel target. The same set of mice is used as in **C**. **E**, *Erk2* CKO mice are not significantly different from controls in the latency to find a buried treat following overnight food deprivation. The same set of mice is used as in **B**. **F**, *Erk2* CKO mice show a significant decrease in interaction with a novel object in their home cages. The same set of mice is used as in **B**. **G**, *Erk2* CKO mice show significant deficits in nest formation (control, 6 cages, $n = 4$ mice per cage; *Erk2* CKO, 6 cages, $n = 4$ mice per cage). **H, I**, Representative photographs of control (**H**) and *Erk2* CKO (**I**) cages, 45 min after the introduction of cotton nesting materials into each cage. Note the fluffy nest built in the control cage and the huddling of mice in this nest, in contrast to the poorly formed nests in the *Erk2* CKO cage. $**p < 0.01, ***p < 0.001$. N.S., Not significant.

spent significantly more time interacting with the social target than with the empty cage (*post hoc* test, social vs empty, $p < 0.0001$), indicating that control mice showed the normal preference for a social target over an empty cage. These results were consistent with those of the sociability test in the open field. Subsequently, we examined a preference for social novelty using the three-room chamber and found that *Erk2* CKO mice exhibited abnormal behaviors in this test (Fig. 7D; two-way ANOVA, $F_{(1,40)} = 12.61, p < 0.001$ (genotype), $F_{(1,40)} = 6.12, p < 0.05$ (interaction between genotype and preference). When control mice were exposed to a familiar mouse versus a novel mouse, control mice showed a clear preference for the novel mouse over the familiar mouse as expected (*post hoc* test, novel vs familiar, $p < 0.01$). On the other hand, the interaction with a familiar mouse was similar in both *Erk2* CKO mice and control groups (*post hoc* test, control vs *Erk2* CKO, $p > 0.05$), and *Erk2* CKO mice did not show a preference for social novelty (*post hoc* test, novel vs familiar, $p > 0.05$).

These results indicate that *Erk2* CKO mice have pervasive deficits in social interaction. We did not attribute the abnormalities in social memory and interaction to an overt loss of olfactory
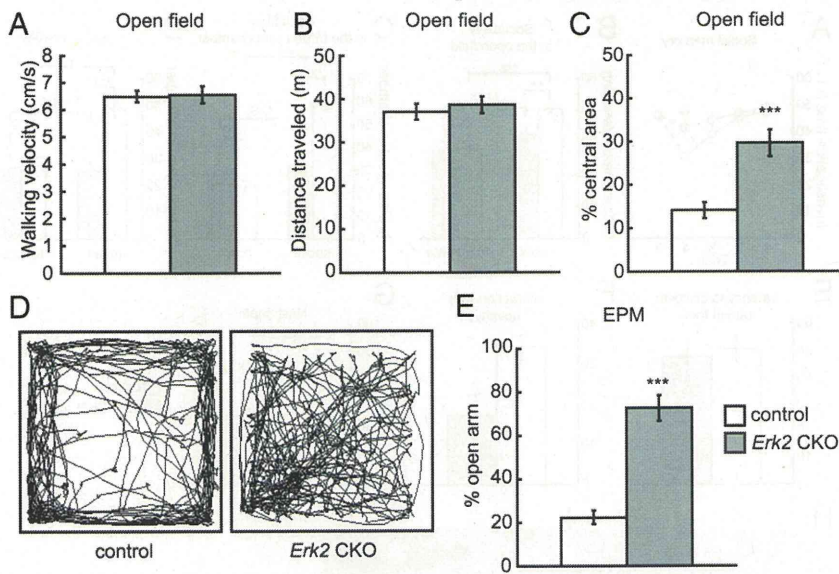
Figure 8. *Erk2* CKO mice exhibit normal locomotor activity but decreased anxiety-related behaviors. *A–D*, Open field test (control, $n = 19$; *Erk2* CKO, $n = 20$). *A*, The average speed without resting time is not different between control and *Erk2* CKO mice. *B*, The 10 min total path-length traveled is not significantly different between control and *Erk2* CKO mice. *C*, *Erk2* CKO mice exhibit reduced anxiety-like behavior, because they spend significantly more time in the central zone of the open field apparatus. *D*, Representative tracks of control and *Erk2* CKO mice in the open field chamber over 10 min. *E*, *Erk2* CKO mice exhibit reduced anxiety-like behavior in the elevated plus-maze test (the same set of mice as in *A*). ***$p < 0.001$.
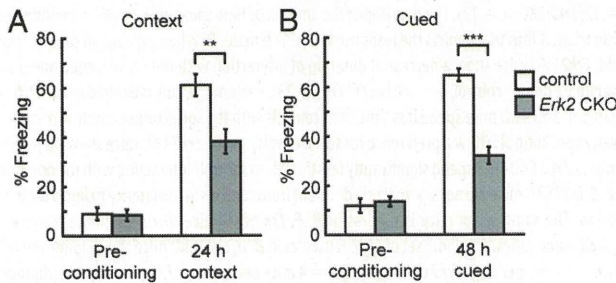
Figure 9. *Erk2* CKO mice show impaired memory performance in both contextual and cued tests. *A*, The freezing response is measured in the context before shock (basal freezing) and in the conditioning chamber (contextual fear response) 24 h after conditioning (control, $n = 12$; *Erk2* CKO, $n = 12$). *B*, The freezing response (the same set of mice as in *A*) is measured in an alternative context either without an auditory cue (basal freezing after conditioning) or with a cue 48 h after conditioning. **$p < 0.01$, ***$p < 0.001$.

function, since we did not detect any significant difference between groups in a test for olfaction (Fig. 7E; *t* test, $t = 0.40$, $p > 0.05$).

Erk2 CKO mice also exhibited a significant decrease compared with control mice in interaction with a novel object in a familiar cage compared with control mice (*t* test, $t = 3.37$, $p < 0.01$), suggesting a general disinterest in novelty (Fig. 7F). Thus the deficient social interactions might be at least partly attributable to a general lack of interest in novelty.

Additionally, we examined nest formation, a test for home cage behavior, of *Erk2* CKO mice. As well as social behavior, nesting has been proposed as a core paradigm to test autistic behavior in mice (Crawley, 2004), and has been used to measure autism-like behaviors in other mutant mouse models (Moy et al., 2004; Moretti et al., 2005; Kwon et al., 2006). In contrast to the immediate activity of nest building in control mice, *Erk2* CKO mice showed little nest-building activity (Fig. 7G–I). Control mice built nests from cotton nesting material that averaged >50

mm in depth, while *Erk2* CKO mice built significantly shallower nests, with depths that averaged <30 mm (Fig. 7G; *t* test, $t = 4.49$, $p < 0.01$). The poor nest building displayed by *Erk2* CKO mice also demonstrated an essential role for *Erk2* in social behavior in the home cage.

Erk2 CKO mice exhibit reduced anxiety

To examine responses to an unfamiliar environment, *Erk2* CKO mice were assayed in the open field test. Statistical analyses of walking velocity (Fig. 8A; *t* test, control vs *Erk2* CKO, $t = 0.13$, $t > 0.05$) and total path-length (distance traveled) (Fig. 8B; *t* test, control vs *Erk2* CKO, $t = 0.62$, $t > 0.05$) revealed that *Erk2* CKO mice did not differ from control animals in their exploratory behavior and locomotor activity. Next to study whether ERK2 abrogation affects anxiety-related behavior, *Erk2* CKO mice were subjected to the EPM test. *Erk2* CKO mice showed decreased anxiety-like behavior as they spent a significantly longer time in the open arms than did the controls (Fig. 8E; *t* test, $t = 6.96$, $p < 0.001$). Consistently, *Erk2* CKO mice showed decreased anxiety-like behavior in the open field test as indicated by prolonged localization in the central area (Fig. 8C,D). Normally, mice show a preference for the corners and peripheral areas over the central area of the open field, because they feel safer there (Fig. 8D, left). In contrast, *Erk2* CKO mice spent significantly more time in the central zone (Fig. 8D, right) compared with controls (Fig. 8C; *t* test, control vs *Erk2* CKO, $t = 4.24$, $t < 0.001$). Together with the finding that *Erk1* knock-out mice did not differ significantly from controls in the amount of time spent in the open arms in the EPM (Ailing et al., 2008), our results suggested that ERK2 might play an important role in anxiety-like behavior.

Erk2 CKO mice are impaired in long-term memory

To characterize the hippocampal-dependent and/or amygdala-dependent long-term memory in *Erk2* CKO mice, these mice were subjected to contextual/cued fear conditioning. We previously reported that *Erk2* knockdown mice exhibited impaired Pavlovian fear learning (Satoh et al., 2007). Other previous reports have revealed that preventing ERK activation with a MEK inhibitor impaired fear learning in mice (Atkins et al., 1998; Schafe et al., 1999). Fear memory was assessed based on the freezing behavior to the conditioned cue or the context to which the mice were previously exposed. The context freezing response of *Erk2* CKO mice was significantly reduced compared with that in controls after a 24 h retention delay (Figure 9A; *t* test, $t = 3.47$, $p < 0.005$). The response of *Erk2* CKO mice to the cued fear conditioning was also significantly reduced compared with control mice after a 48 h retention delay (Fig. 9B; *t* test, $t = 7.72$, $p < 0.0001$). These results suggest that ERK2 plays an important role in long-term fear memory.

Plasma Oxt levels in *Erk2* CKO mice are normal

Oxt belongs to the posterior pituitary hormone family and is essential for the induction of normal labor through uterine contraction and lactation in mammals (Hatton, 1990; Argiolas and
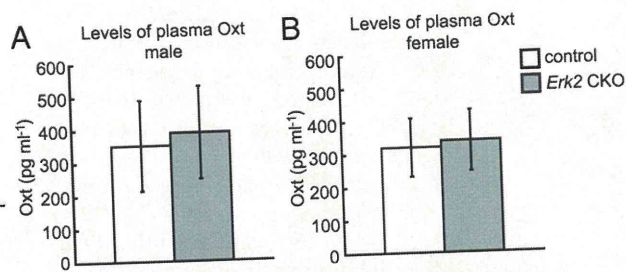
Figure 10. Plasma Oxt levels are not altered in *Erk2* CKO mice. *A, B,* Levels of plasma Oxt in male (control, $n = 10$; *Erk2* CKO, $n = 10$) (*A*), and female (control, $n = 8$; *Erk2* CKO, $n = 8$) mice (*B*). The levels in *Erk2* CKO mice are not different from those in controls.

Gessa, 1991; Russell et al., 2003). Several researchers have suggested that the Oxt signaling system also plays a crucial role in social interaction and it is implicated in the etiology of developmental psychiatric disorders characterized by deficits in social behavior (Argiolas and Gessa, 1991; Takayanagi et al., 2005; Jin et al., 2007). To investigate the possibility that abrogation of the ERK2 pathway affects the plasma concentration of Oxt, we examined the plasma concentration of Oxt in *Erk2* CKO mice. There was no significant difference in Oxt levels between control and *Erk2* CKO mice [Fig. 10 *A,B*; *t* test, $t = 0.61$, $p > 0.05$ (male); $t = 0.44$, $p > 0.05$ (female)], suggesting that the neuroendocrine system was not impaired in *Erk2* CKO mice and that ERK2 may not regulate Oxt secretion.

Oxt increases ERK phosphorylation in the hippocampus

Next, we examined whether there was a differences in the activation of ERK between control and *Erk2* CKO mice in response to Oxt treatment. Western blotting revealed that subcutaneous injection of Oxt (10 ng/kg body weight) significantly increased ERK1 phosphorylation levels in the hippocampus compared with in vehicle-injected mice 10 min after injection, regardless of genotype (Fig. 11 *A,B*). Two-way ANOVA indicated a main effect of Oxt treatment (vehicle vs Oxt: $F_{(1,12)} = 39.74$, $p < 0.001$) and of genotype (control vs *Erk2* CKO: $F_{(1,12)} = 61.06$, $p < 0.001$). Two-way ANOVA also revealed a significant interaction effect between Oxt treatment and genotype ($F_{(1,12)} = 9.27$, $p < 0.05$). There were no differences in total ERK1 expression levels between vehicle- and Oxt-treated mice both for controls and *Erk2* CKOs (Fig. 11 *A*).

Oxt also significantly increased ERK2 phosphorylation levels in control mice compared with vehicle-treated mice (Fig. 11 *A,C*). Two-way ANOVA indicated a main effect of Oxt treatment (vehicle vs Oxt: $F_{(1,12)} = 12.13$, $p < 0.01$), and of genotype (control vs *Erk2* CKO: $F_{(1,12)} = 218.00$, $p < 0.0001$), and an interaction between Oxt treatment and genotype ($F_{(1,12)} = 12.32$, $p < 0.01$). There were no significant differences in total ERK2 expression levels between vehicle- and Oxt-treated mice among controls (Fig. 11 *A*).

These results are consistent with the notion that Oxt signals through ERK activation. Furthermore, this result suggest that lack of ERK2 activation was compensated for by ERK1 activation in *Erk2* CKO mice. Although the amount of change in ERK1 phosphorylation was more robust in *Erk2* CKO mice than in controls (Fig. 11 *B*; *t* test, $t = 4.45$, $p < 0.01$), total phosphorylation level of ERK in response to Oxt was smaller in *Erk2* CKO mice than in controls (Fig. 11 *D*; two-way ANOVA, $F_{(1,12)} = 151.48$, $p < 0.001$ (genotype), $F_{(1,12)} = 30.91$, $p < 0.001$ (Oxt treatment), $F_{(1,12)} = 6.31$, $p < 0.05$ (interaction between Oxt treatment and genotype).
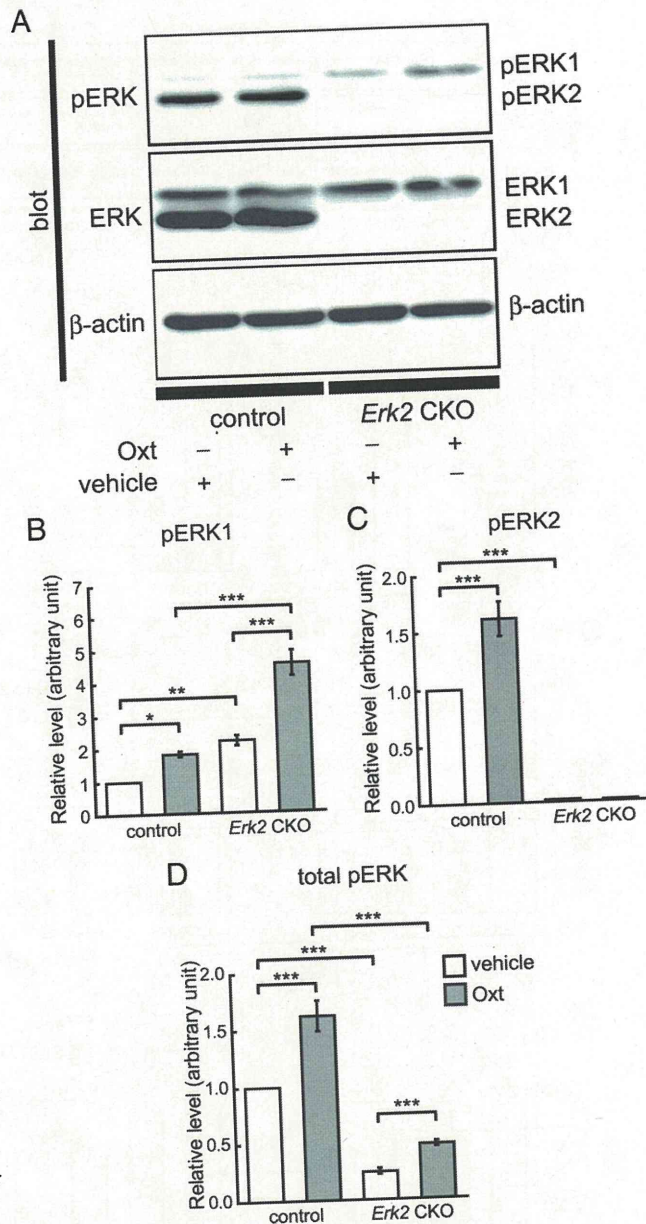
Figure 11. Subcutaneous injection of Oxt (10 ng/kg body weight) significantly increases phosphorylated ERK levels in the hippocampus. *A,* Hippocampus homogenates from vehicle-treated or Oxt-treated control ($n = 4$ for each) and *Erk2* CKO littermates ($n = 4$ for each) are analyzed simultaneously for phospho-ERK1/2 (pERK1/2) and ERK1/2 by quantitative Western blotting. In control mice, ERK1 phosphorylation level is significantly elevated 10 min after Oxt injection. Similarly, ERK1 phosphorylation is significantly elevated 10 min after Oxt injection in *Erk2* CKO mice. There is no difference in total ERK1 expression level among all groups. The ERK2 phosphorylation level is also elevated in control mice after Oxt injection without change of total ERK2 expression level. β-Actin serves as the control for protein loading. *B–D,* To evaluate phosphorylation, the intensities of the phospho-ERK1 and phospho-ERK2 bands are divided by their corresponding loading control (β-actin). Then, relative levels of ERK1 (*B*), ERK2 (*C*), and total ERK (*D*) phosphorylation are normalized to the mean control with vehicle injection. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Pharmacological blockade of ERK1 phosphorylation in *Erk2* CKO mice does not produce additional effect on impaired long-term memory

As described above, phosphorylation level of ERK1 was increased in *Erk2* CKO mice although the total ERK1 expression levels were
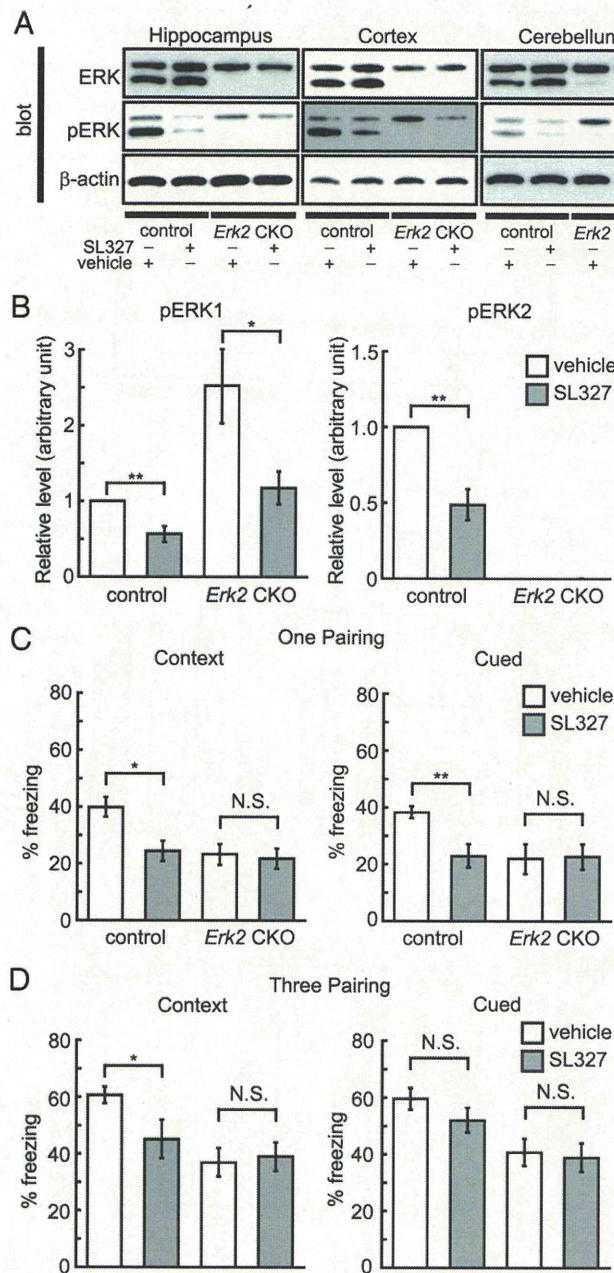
Figure 12. Pharmacological blockade of ERK1 phosphorylation in *Erk2* CKO mice does not additionally affect long-term memory. *A*, ERK1 and 2 phosphorylation levels in the hippocampus, cortex, and cerebellum are significantly inhibited 60 min after injection of 30 mg/kg MEK inhibitor SL327 in the hippocampus, cortex and cerebellum. There is no concurrent decrease in the total expression level of either isoform. *B*, The phosphorylation levels of ERK1 and 2 in the hippocampus as analyzed by band density (control, $n = 5$; *Erk2* CKO, $n = 5$). β-Actin serves as the control for protein loading. *C*, In control mice, SL327 attenuates freezing in the contextual and cued fear conditioning after receiving one pairing of a tone and shock, compared with vehicle (vehicle, $n = 10$; SL327, $n = 10$). However, no significant difference is observed between animals treated with SL327 or vehicle for *Erk2* CKO mice (vehicle, $n = 10$; SL327, $n = 10$). *D*, In control mice, SL327 attenuates freezing in contextual fear conditioning after receiving three pairings of a tone and shock compared with vehicle, but the additional pairings eliminated the effect of SL327 on freezing in response to the cue (vehicle, $n = 10$; SL327, $n = 10$). There is no significant difference in freezing in response to context and cue between *Erk2* CKO mice treated with SL327 or vehicle (vehicle, $n = 10$; SL327, $n = 10$). $*p < 0.05$, $**p < 0.01$. N.S., Not significant.

mg/kg) was administered intraperitoneally to subjects 1 h before sample preparation. Western blot analysis revealed that SL327 attenuated phosphorylated ERK levels (Fig. 12 *A,B*) as described previously (Selcher et al., 1999). SL327 administration affected ERK1 phosphorylation levels 1 h later in the hippocampus (Fig. 12 *A*). Two-way ANOVA confirmed this difference, indicating a main effect of SL327 treatment (Fig. 12 *B*; $F_{(1,16)} = 9.28$, $p < 0.05$). Administration of SL327 also significantly reduced ERK2 phosphorylation levels (Fig. 12 *A*). Two-way ANOVA confirmed this difference, indicating a main effect of SL327 treatment (Fig. 12 *B*; $F_{(1,16)} = 24.90$, $p < 0.01$), although ERK2 phosphorylation was undetectable in *Erk2* CKO mice (Fig. 12 *A,B*). There was no concurrent decrease in ERK1 and 2 expression levels (Fig. 12 *A*).

We investigated whether the administration of SL327 (30 mg/kg) to *Erk2* CKO mice could produce additional effects in the fear conditioning test. SL327 was administered intraperitoneally to subjects 1 h before cue and contextual fear conditioning. It was reported that 30 mg/kg SL327 did not impair basal activity in control mice (Selcher et al., 1999). For these experiments, foot shock was paired either once or three times with an auditory CS. With a one pairing protocol, SL327 administration affected the contextual conditioning response after a 24 h retention delay (Fig. 12 *C*). Two-way ANOVA indicated a main effect of SL327 administration ($F_{(1,36)} = 6.54$, $p < 0.05$) and that the genotype effect had a significant interaction with SL327 administration ($F_{(1,36)} = 4.48$, $p < 0.05$). In control mice, *post hoc* test revealed that injection of SL327 significantly reduced the contextual freezing response after a 24 h retention delay compared with vehicle (vehicle vs SL327, $p < 0.05$). However, in *Erk2* CKO mice, there was no significant difference in freezing between animals treated with vehicle and SL327 in contextual conditioning (*post hoc* test, vehicle vs SL327, $p > 0.05$).

SL327 administration also affected the cued freezing response after a 48 h retention delay (Fig. 12 *C*). Two-way ANOVA indicated a main effect of SL327 administration ($F_{(1,36)} = 4.44$, $p < 0.05$), and that a genotype effect showed a significant interaction with SL327 administration ($F_{(1,36)} = 7.37$, $p < 0.05$). In control mice, *post hoc* test revealed that the response of control mice to the cued fear conditioning was reduced significantly (vehicle vs SL327, $p < 0.01$). However, in *Erk2* CKO mice, there was no significant

unchanged. To identify putative ERK2 functions that could be compensated for, and to confirm that the abnormal behaviors in *Erk2* CKO mice were caused by the loss of ERK2 per se rather than a secondary upregulation of ERK1 activity, we examined the effect of the MEK inhibitor SL327 in *Erk2* CKO mice. SL327 (30

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difference in freezing between animals treated with vehicle and SL327 in the cued response (*post hoc* test, vehicle vs SL327, $p > 0.05$).

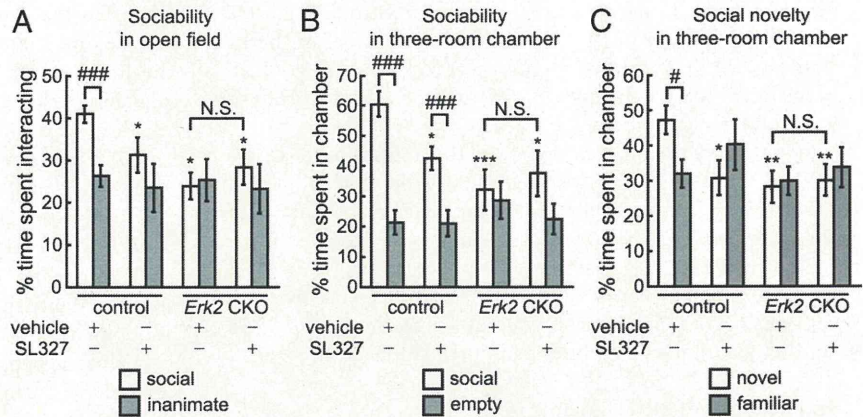When a three pairing protocol was used, SL327 administration affected the contextual conditioning response after a 24 h retention delay (Fig. 12 *D*). Two-way ANOVA indicated a main effect of SL327 administration ($F_{(1,36)} = 16.18$, $p < 0.001$), and that a genotype effect had a significant interaction with SL327 administration ($F_{(1,36)} = 11.42$, $p < 0.01$). In control mice, *post hoc* test revealed that SL327 reduced the contextual freezing response compared with vehicle (vehicle vs SL327, $p < 0.05$). In *Erk2* CKO mice, there was no significant difference in freezing between animals treated with vehicle and SL327 in the contextual response (*post hoc* test, vehicle vs SL327, $p > 0.05$). On the other hand, SL327 administration did not affect the cued conditioning response (two-way ANOVA, $F_{(1,36)} = 2.48$, $p > 0.05$ (SL327 treatment)) (Fig. 12 *D*).

These results suggest that the deficit in ERK2 function in long-term memory was not compensated for by upregulation of ERK1 activity as a result of ERK2 deletion. Moreover, these results suggest that the deficits are probably not the result of a secondary upregulation of ERK1 activity.

Figure 13. Pharmacological blockade of ERK1 phosphorylation in *Erk2* CKO mice does not additionally affect social behaviors. *A–C*, Treatment with the MEK inhibitor SL327 (30 mg/kg) reduces social behaviors in control mice, but does not additionally affect impaired social behaviors in *Erk2* CKO mice. *A*, When SL327 in control mice are exposed to caged social and inanimate targets in the open field, the duration of interaction with the social target is significantly decreased compared with vehicle ($n = 14$ mice for each). On the other hand, there is no significant difference in the spent with the social target between SL327- and vehicle-treated *Erk2* CKO mice ($n = 14$ mice for each). Percentage time is depicted in *A–C*. *B*, In the sociability test in the three-room chamber, SL327 reduces the time spent with the social target compared with vehicle in control mice. On the other hand, there is no significant difference in the time spent with the social target between SL327- and vehicle-treated *Erk2* CKO mice. The same set of mice is used as in *A*. *C*, In the social novelty test, vehicle-treated controls show preference for the novel target, while SL327-treated control mice does not. Furthermore, SL327 in control mice reduces the time spent with the social target compared with vehicle. On the other hand, there is no significant difference in the time spent with the novel target between SL327- and vehicle-treated *Erk2* CKO mice. The same set of mice is used as in *A* and *B*. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with vehicle-injected control mice; #$p < 0.05$; ###$p < 0.001$. N.S., Not significant.

Pharmacological blockade of ERK1 phosphorylation in *Erk2* CKO mice does not produce additional effect on abnormal social behaviors

Next, we examined the effect of a MEK inhibitor SL327 (30 mg/kg) on social behaviors. In the sociability test in the open field, two-way ANOVA indicated that there was a significant main effect of genotypes ($F_{(1,52)} = 8.29$, $p < 0.01$) and a significant interaction between genotype and SL327 treatment ($F_{(1,52)} = 4.23$, $p < 0.05$), although there was no significant main effect of SL327 treatment ($F_{(1,52)} = 0.65$, $p > 0.05$). In control mice, SL327 reduced the duration of interaction with the social target compared with vehicle (Fig. 13*A*). On the other hand, there was no significant difference in the duration of interaction with the social target between SL327- and vehicle-treated *Erk2* CKO mice, although both SL327- and vehicle-treated *Erk2* CKO mice showed reduced social interaction compared with vehicle-treated control mice (Fig. 13*A*). Vehicle-treated controls showed a preference for the social target, while SL327-treated control mice did not (Fig. 13*A*). Neither SL327- nor vehicle-treated *Erk2* CKO mice showed a significant preference for the social target (Fig. 13*A*).

In the sociability test in the three-room chamber (Fig. 13*B*), two-way ANOVA indicated that there was a significant main effect of genotype ($F_{(1,52)} = 8.24$, $p < 0.01$) and a significant interaction between genotype and SL327 treatment ($F_{(1,52)} = 4.05$, $p < 0.05$), although there was no significant main effect of SL327 treatment ($F_{(1,52)} = 1.21$, $p > 0.05$). While SL327 reduced the time spent with the social target in control mice, there was no significant difference between SL327- and vehicle-treated *Erk2* CKO mice (Fig. 13*B*). Both SL327- and vehicle-treated *Erk2* CKO mice showed reduced social inter-action compared with vehicle-treated control mice and did not show a significant preference for the social target (Fig. 13*B*).

In the social novelty test, two-way ANOVA indicated that there was a significant main effect of genotype ($F_{(1,52)} = 4.78$, $p < 0.05$) and significant interaction between genotype and SL327 treatment ($F_{(1,52)} = 4.15$, $p < 0.05$), although there was no significant main effect of SL327 treatment ($F_{(1,52)} = 1.11$, $p > 0.05$). SL327 reduced the time spent with the social target compared with vehicle in control mice (Fig. 13*C*). On the other hand, there was no significant difference in time spent with a novel target between SL327- and vehicle-treated *Erk2* CKO mice, although the time interacting with novel target was significantly less in *Erk2* CKO mice than in vehicle-treated control mice (Fig. 13*C*). Vehicle-treated controls showed a preference for the novel target, while SL327-treated control mice did not (Fig. 13*C*). Neither SL327- nor vehicle-treated *Erk2* CKO mice showed a preference for the novel target. These results indicate that blockade of ERK1 phosphorylation causes no additional effects on the impaired social behaviors in *Erk2* CKO mice.

Discussion
The ERK signaling pathway and social behaviors
Using a conditional genetic approach, we have demonstrated important roles for the ERK2 signaling pathway *in vivo*: *Erk2* CKO mice exhibited pervasive deficits in social behaviors as well as anomalous affective behaviors and cognitive functions. This suggests that ERK2 plays complex and multiple roles in brain function, with important implications for human psychiatric disorders characterized by deficits in social behaviors. Our findings of pervasive deficits in social behaviors in *Erk2* CKO mice are consistent with those of some previous reports. For instance, it was reported that ERK was activated in the dorsal medial preoptic area neurons upon exposure to pups, leading to upregulation of

several genes critical for maternal behaviors (Kuroda et al., 2007). Genetic disruption of BDNF, a downstream target of the ERK pathway, caused increased aggression (Lyons et al., 1999). ERK is activated in social behavior circuits during resident-intruder aggression tests (Trainor et al., 2010).

On the other hand, it was reported that mice with the deletion of ERK2 in the CNS (Heffron et al., 2009) did not exhibit prominent anomalies in behavior tests, although they did not perform social behavior tests. They used breeding pairs consisted of male *nestin-cre+*; *Erk2*$^{flox/+}$ mated with female *nestin-cre−*; *Erk2*$^{flox/flox}$ mice: all mice in their study were borne and reared by control mothers. Thus, abnormal maternal behaviors of *Erk2* CKO mother would not be manifested in their studies.

Implications of ERK signaling for ASD

With the obvious interpretative limitation of translating mouse phenotypes to clinical symptoms in humans, it is still interesting to note that *Erk2* deficiency in the CNS caused behavioral phenotypes associated with ASD. ASD is a neuropsychiatric disorder characterized primarily by impairments in social, communicative, and behavioral functioning, although the molecular mechanisms of ASD remain largely unknown. While we found that *Erk2* CKO mice exhibited pervasive deficits in social behaviors that are core features of ASD, *Erk2* CKO mice also exhibited decreased general interest in novelty, deficits in nest-building and deficits in long-term memory, all of which also have great relevance to ASD. In humans, deletion of chromosome 16p11.2, on which *Erk1* is located is associated with autism (Kumar et al., 2008), although *Erk1* ablation in mice produced a behavioral excitement profile similar to bipolar disorder (Engel et al., 2009). *Erk2* gene is located on human chromosome 22q11.2, deletion of which has also been reported to be associated with autism (Vorstman et al., 2006; Mukaddes and Herguner, 2007). With relevance to human psychiatric disorders, it might be important to dissect the brain functions of ERK1 and ERK2 in animal models to understand the functional link between the ERK cascade and neuropsychiatric disorders.

The EPM and open field test revealed that *Erk2* CKO mice exhibited reduced anxiety. There is an interpretative limitation of this finding because the underlying mechanisms for anxiety in human and the anxiety-like behavior of rodents in the EPM would be different. Rodents' anxiety-like behavior in the EPM reflects the natural balance between the exploratory and escaping drives. Thus, entry into the open arm could also be conceptualized as risk-taking behavior. In our experiment, *Erk2* CKO mice might not recognize the risk rather than anxiety is reduced in them. In this context, it is interesting to note that ASD patient sometimes have deficits in recognizing the risk.

Oxt signaling and ERK

Our results revealed that *Erk2* CKO mice exhibited marked abnormalities in several social behaviors, including abnormal nurturing behavior and high levels of aggression, significantly similar to mice with disrupted Oxt signaling (Ferguson et al., 2000; Takayanagi et al., 2005). Mice deficient for the *Oxt* gene (Ferguson et al., 2000) or the *Oxt* receptor gene (Takayanagi et al., 2005) failed to develop social memory or maternal care, respectively. Oxt signaling system plays a crucial role in social interaction and it is implicated in the etiology of ASD (Jin et al., 2007). In humans, the Oxt signaling system regulates a wide range of social behaviors including nursing, social recognition, and pair binding in rodents and love, trust and fear in humans (Keverne and Curley, 2004; Kirsch et al., 2005; Kosfeld et al., 2005). It was also reported that individuals diagnosed with ASD showed significantly lower levels of plasma Oxt compared with age-matched healthy controls (Modahl et al., 1998). Furthermore, the Oxt signaling system is thought to have some relationship to human diseases associated with abnormal social behavior (Lim et al., 2005). Thus, one is prompted to speculate that the disruption of Oxt signal might be related to the abnormal social behaviors in *Erk2* CKO mice. However, the plasma Oxt level was not altered in *Erk2* CKO mice at 9–11 weeks of age. Thus, it is unlikely that ERK2 signaling regulates Oxt levels, although we cannot exclude the possibility that there are differences in Oxt levels during development or in the postpartum period that could account for some of the behavioral differences. Instead, ERK might mediate Oxt signal to induce enduring changes at the behavioral and physiological levels. Consistently, our data indicated that subcutaneous injection of Oxt increased the phosphorylation levels of ERK1 and ERK2 in control mice. Notably, in *Erk2* CKO mice, the increase in ERK1 phosphorylation was larger than that in control mice, suggesting that compensatory regulation of ERK signaling occurs upon Oxt stimulation. It may mean that, in the Oxt-activated state, the compensatory mechanism tries to maintain the total amount of ERK1 and ERK2 phosphorylation. Further analysis is required to understand the role of ERK in the regulation of Oxt signaling.

Central contribution of ERK2 to brain functions

Previous reports have suggested that ERK1 is not critical for associative learning (Selcher et al., 2001), but that ERK2 might be crucially involved in learning and memory (Satoh et al., 2007; Samuels et al., 2008). In this report, we demonstrated that long-term memory was impaired in *Erk2* CKO mice, consistent with these reports.

As described above, the phosphorylation level of ERK1 was greater in *Erk2* CKO mice than in controls. The administration of the ERK cascade inhibitor SL327 to *Erk2* CKO mice did not additionally affect long-term memory although the level of ERK1 phosphorylation was reduced. Thus, the deficiency in long-term memory in *Erk2* CKO mice was caused by the loss of ERK2 per se, rather than by a secondary upregulation of ERK1 activity. It was also reported that in mice lacking ERK1, ERK2 did not only compensate for the lack of ERK1, but also exhibited stronger biological activity in some regions (Mazzucchelli et al., 2002; Tronson et al., 2008). *Erk1* KO mice exhibited dramatic enhancement of striatum-dependent long-term memory likely due to enhanced activation of ERK2 (Mazzucchelli et al., 2002). These results indicated an unexpected complexity of ERK signaling. Together with these reports, our findings suggest that ERK2, but not ERK1, might provide a central and specific contribution to long-term memory. Furthermore, our study extended these findings by demonstrating pleiotropic and central involvement of ERK2 in many neuronal functions.

One possibility for the mechanism underlying the distinct roles of ERK2 and ERK1 would be resulted from the differences in the N terminus between ERK1 and ERK2, which affect the shuttling rates between the cytoplasm and the nucleus (Marchi et al., 2008). Another possibility is that ERK1 exclusively interacts with MP1, which forms a scaffold for MEK-ERK signaling proteins (Schaeffer et al., 1998). However, we could not exclude the possibility that the deficit was attributable to developmentally related structural changes, for instance in astroglial cells in the cortex.

Conclusions

Our findings demonstrated that ERK2 plays a critical role in regulating not only long-term memory but also social behaviors and anxiety and that ERK2 may be a factor in human neurodevelopmental or psychiatric disorders. Further investigation is required to dissect the roles of ERK1 and ERK2 and determine whether psychiatric disorders are linked to defects in the ERK signaling cascades.

References

Ailing F, Fan L, Li S, Manji S (2008) Role of extracellular signal-regulated kinase signal transduction pathway in anxiety. J Psychiatr Res 43:55–63.

Alonso M, Medina JH, Pozzo-Miller L (2004) ERK1/2 activation is necessary for BDNF to increase dendritic spine density in hippocampal CA1 pyramidal neurons. Learn Mem 11:172–178.

Argiolas A, Gessa GL (1991) Central functions of oxytocin. Neurosci Biobehav Rev 15:217–231.

Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM, Sweatt JD (1998) The MAPK cascade is required for mammalian associative learning. Nat Neurosci 1:602–609.

Calverley RK, Jones DG (1990) Contributions of dendritic spines and perforated synapses to synaptic plasticity. Brain Res Brain Res Rev 15: 215–249.

Crawley JN (2004) Designing mouse behavioral tasks relevant to autistic-like behaviors. Ment Retard Dev Disabil Res Rev 10:248–258.

Engel SR, Creson TK, Hao Y, Shen Y, Maeng S, Nekrasova T, Landreth GE, Manji HK, Chen G (2009) The extracellular signal-regulated kinase pathway contributes to the control of behavioral excitement. Mol Psychiatry 14:448–461.

Ferguson JN, Young LJ, Hearn EF, Matzuk MM, Insel TR, Winslow JT (2000) Social amnesia in mice lacking the oxytocin gene. Nat Genet 25:284–288.

Goldin M, Segal M (2003) Protein kinase C and ERK involvement in dendritic spine plasticity in cultured rodent hippocampal neurons. Eur J Neurosci 17:2529–2539.

Hatton GI (1990) Emerging concepts of structure-function dynamics in adult brain: the hypothalamo-neurohypophysial system. Prog Neurobiol 34:437–504.

Heffron DS, Landreth GE, Samuels IS, Mandell JW (2009) Brain-specific deletion of extracellular signal-regulated kinase 2 mitogen-activated protein kinase leads to aberrant cortical collagen deposition. Am J Pathol 175:2586–2599.

Jin D, Liu HX, Hirai H, Torashima T, Nagai T, Lopatina O, Shnayder NA, Yamada K, Noda M, Seike T, Fujita K, Takasawa S, Yokoyama S, Koizumi K, Shiraishi Y, Tanaka S, Hashii M, Yoshihara T, Higashida K, et al. (2007) CD38 is critical for social behaviour by regulating oxytocin secretion. Nature 446:41–45.

Kelleher RJ 3rd, Govindarajan A, Jung HY, Kang H, Tonegawa S (2004) Translational control by MAPK signaling in long-term synaptic plasticity and memory. Cell 116:467–479.

Keverne EB, Curley JP (2004) Vasopressin, oxytocin and social behaviour. Curr Opin Neurobiol 14:777–783.

Kirsch P, Esslinger C, Chen Q, Mier D, Lis S, Siddhanti S, Gruppe H, Mattay VS, Gallhofer B, Meyer-Lindenberg A (2005) Oxytocin modulates neural circuitry for social cognition and fear in humans. J Neurosci 25:11489–11493.

Kosfeld M, Heinrichs M, Zak PJ, Fischbacher U, Fehr E (2005) Oxytocin increases trust in humans. Nature 435:673–676.

Kumar RA, KaraMohamed S, Sudi J, Conrad DF, Brune C, Badner JA, Gilliam TC, Nowak NJ, Cook EH Jr, Dobyns WB, Christian SL (2008) Recurrent 16p11.2 microdeletions in autism. Hum Mol Genet 17:628–638.

Kuroda KO, Meaney MJ, Uetani N, Fortin Y, Ponton A, Kato T (2007) ERK-FosB signaling in dorsal MPOA neurons plays a major role in the initiation of parental behavior in mice. Mol Cell Neurosci 36:121–131.

Kwon CH, Luikart BW, Powell CM, Zhou J, Matheny SA, Zhang W, Li Y, Baker SJ, Parada LF (2006) Pten regulates neuronal arborization and social interaction in mice. Neuron 50:377–388.

Lefloch R, Pouysségur J, Lenormand P (2008) Single and combined silencing of ERK1 and ERK2 reveals their positive contribution to growth signaling depending on their expression levels. Mol Cell Biol 28:511–527.

Lijam N, Paylor R, McDonald MP, Crawley JN, Deng CX, Herrup K, Stevens KE, Maccaferri G, McBain CJ, Sussman DJ, Wynshaw-Boris A (1997) Social interaction and sensorimotor gating abnormalities in mice lacking Dvl1. Cell 90:895–905.

Lim MM, Bielsky IF, Young LJ (2005) Neuropeptides and the social brain: potential rodent models of autism. Int J Dev Neurosci 23:235–243.

Lyons WE, Mamounas LA, Ricaurte GA, Coppola V, Reid SW, Bora SH, Wihler C, Koliatsos VE, Tessarollo L (1999) Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. Proc Natl Acad Sci U S A 96:15239–15244.

Marchi M, D'Antoni A, Formentini I, Parra R, Brambilla R, Ratto GM, Costa M (2008) The N-terminal domain of ERK1 accounts for the functional differences with ERK2. PLoS One 3:e3873.

Mazzucchelli C, Vantaggiato C, Ciamei A, Fasano S, Pakhotin P, Krezel W, Welzl H, Wolfer DP, Pagès G, Valverde O, Marowsky A, Porrazzo A, Orban PC, Maldonado R, Ehrengruber MU, Cestari V, Lipp HP, Chapman PF, Pouysségur J, Brambilla R (2002) Knockout of ERK1 MAP kinase enhances synaptic plasticity in the striatum and facilitates striatal-mediated learning and memory. Neuron 34:807–820.

Meng Y, Zhang Y, Tregoubov V, Janus C, Cruz L, Jackson M, Lu WY, MacDonald JF, Wang JY, Falls DL, Jia Z (2002) Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. Neuron 35:121–133.

Modahl C, Green L, Fein D, Morris M, Waterhouse L, Feinstein C, Levin H (1998) Plasma oxytocin levels in autistic children. Biol Psychiatry 43:270–277.

Moretti P, Bouwknecht JA, Teague R, Paylor R, Zoghbi HY (2005) Abnormalities of social interactions and home-cage behavior in a mouse model of Rett syndrome. Hum Mol Genet 14:205–220.

Moy SS, Nadler JJ, Perez A, Barbaro RP, Johns JM, Magnuson TR, Piven J, Crawley JN (2004) Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. Genes Brain Behav 3:287–302.

Mukaddes NM, Herguner S (2007) Autistic disorder and 22q11.2 duplication. World J Biol Psychiatry 8:127–130.

Murcia CL, Gulden F, Herrup K (2005) A question of balance: a proposal for new mouse models of autism. Int J Dev Neurosci 23:265–275.

Nakata T, Yorifuji H (1999) Morphological evidence of the inhibitory effect of taxol on the fast axonal transport. Neurosci Res 35:113–122.

Paquin A, Barnabé-Heider F, Kageyama R, Miller FD (2005) CCAAT/enhancer-binding protein phosphorylation biases cortical precursors to generate neurons rather than astrocytes in vivo. J Neurosci 25:10747–10758.

Russell JA, Leng G, Douglas AJ (2003) The magnocellular oxytocin system, the fount of maternity: adaptations in pregnancy. Front Neuroendocrinol 24:27–61.

Samuels IS, Karlo JC, Faruzzi AN, Pickering K, Herrup K, Sweatt JD, Saitta SC, Landreth GE (2008) Deletion of ERK2 mitogen-activated protein kinase identifies its key roles in cortical neurogenesis and cognitive function. J Neurosci 28:6983–6995.

Satoh Y, Endo S, Ikeda T, Yamada K, Ito M, Kuroki M, Hiramoto T, Imamura O, Kobayashi Y, Watanabe Y, Itohara S, Takishima K (2007) Extracellular signal-regulated kinase 2 (ERK2) knockdown mice show deficits in long-term memory; ERK2 has a specific function in learning and memory. J Neurosci 27:10765–10776.

Satoh Y, Kobayashi Y, Takeuchi A, Pagès G, Pouysségur J, Kazama T (2011) Deletion of ERK1 and ERK2 in the CNS causes cortical abnormalities and neonatal lethality: Erk1 deficiency enhances the impairment of neurogenesis in Erk2-deficient mice. J Neurosci 31:1149–1155.

Satomoto M, Satoh Y, Terui K, Miyao H, Takishima K, Ito M, Imaki J (2009) Neonatal exposure to sevoflurane induces abnormal social behaviors and deficits in fear conditioning in mice. Anesthesiology 110:628–637.

Schaeffer HJ, Catling AD, Eblen ST, Collier LS, Krauss A, Weber MJ (1998) MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. Science 281:1668–1671.

Schafe GE, Nadel NV, Sullivan GM, Harris A, LeDoux JE (1999) Memory consolidation for contextual and auditory fear conditioning is dependent on protein synthesis, PKA, and MAP kinase. Learn Mem 6:97–110.

Selcher JC, Atkins CM, Trzaskos JM, Paylor R, Sweatt JD (1999) A necessity for MAP kinase activation in mammalian spatial learning. Learn Mem 6:478–490.

Selcher JC, Nekrasova T, Paylor R, Landreth GE, Sweatt JD (2001) Mice

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lacking the ERK1 isoform of MAP kinase are unimpaired in emotional learning. Learn Mem 8:11–19.

Sweatt JD (2004) Mitogen-activated protein kinases in synaptic plasticity and memory. Curr Opin Neurobiol 14:311–317.

Takayanagi Y, Yoshida M, Bielsky IF, Ross HE, Kawamata M, Onaka T, Yanagisawa T, Kimura T, Matzuk MM, Young LJ, Nishimori K (2005) Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice. Proc Natl Acad Sci U S A 102:16096–16101.

Thomas GM, Huganir RL (2004) MAPK cascade signalling and synaptic plasticity. Nat Rev Neurosci 5:173–183.

Trainor BC, Crean KK, Fry WH, Sweeney C (2010) Activation of extracellular signal-regulated kinases in social behavior circuits during resident-intruder aggression tests. Neuroscience 165:325–336.

Tronson NC, Schrick C, Fischer A, Sananbenesi F, Pagès G, Pouysségur J, Radulovic J (2008) Regulatory mechanisms of fear extinction and depression-like behavior. Neuropsychopharmacology 33:1570–1583.

Vernay B, Koch M, Vaccarino F, Briscoe J, Simeone A, Kageyama R, Ang SL (2005) Otx2 regulates subtype specification and neurogenesis in the midbrain. J Neurosci 25:4856–4867.

Vorstman JA, Morcus ME, Duijff SN, Klaassen PW, Heineman-de Boer JA, Beemer FA, Swaab H, Kahn RS, van Engeland H (2006) The 22q11.2 deletion in children: high rate of autistic disorders and early onset of psychotic symptoms. J Am Acad Child Adolesc Psychiatry 45:1104–1113.

Wu GY, Deisseroth K, Tsien RW (2001) Spaced stimuli stabilize MAPK pathway activation and its effects on dendritic morphology. Nat Neurosci 4:151–158.

PLoS one

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.

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 ions KO mice was much longer (mean 90 s,
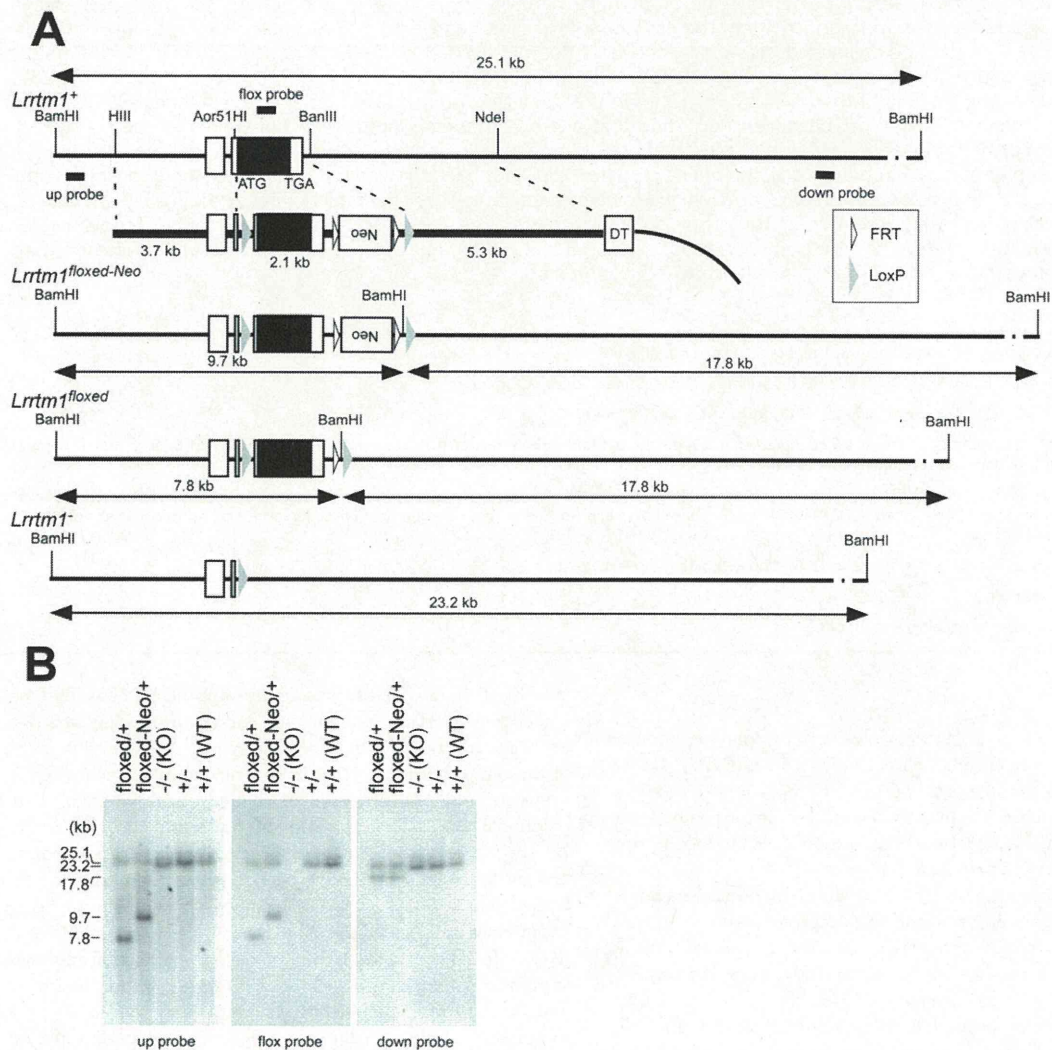
Figure 1. Targeted disruption of the *Lrrtm1* gene. (A) Structures of the *Lrrtm1* genomic locus, targeting vector, and mutated allele. Locations of the 5′ and 3′ probes for Southern blotting are shown. Solid box, protein coding region of the exons; open box, untranslated region of the exons; gray triangle, 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).
doi:10.1371/journal.pone.0022716.g001

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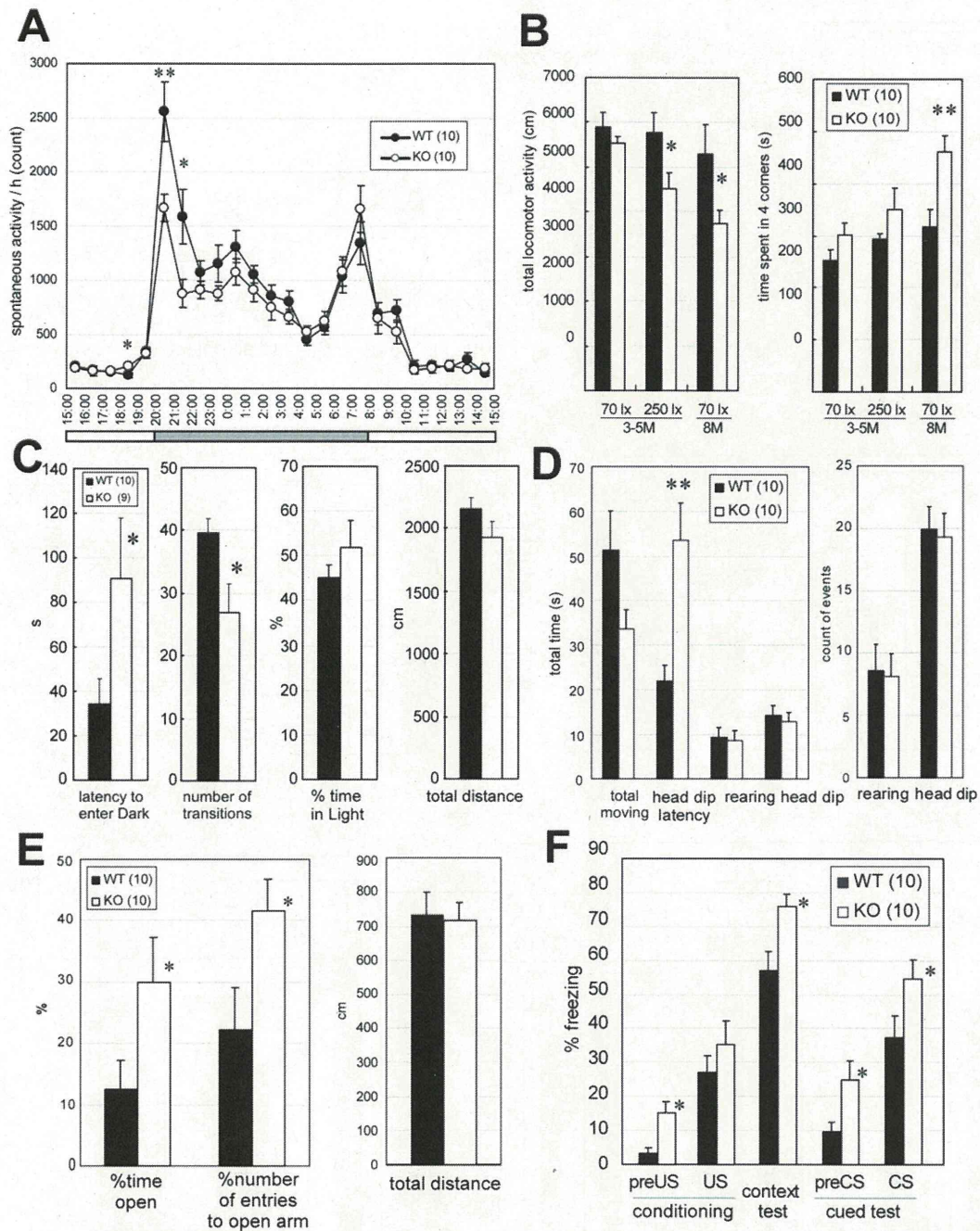
Figure 2. *Lrrtm1* KO mice show adaptive behavior abnormalities. (A) Home-cage activities. The circadian profile of the locomotor activity (bin = 1 h) was first determined for each mouse. Then the mean and SEM of the locomotor activities per 1 h were calculated for each genotype. Statistical analysis 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).
doi:10.1371/journal.pone.0022716.g002

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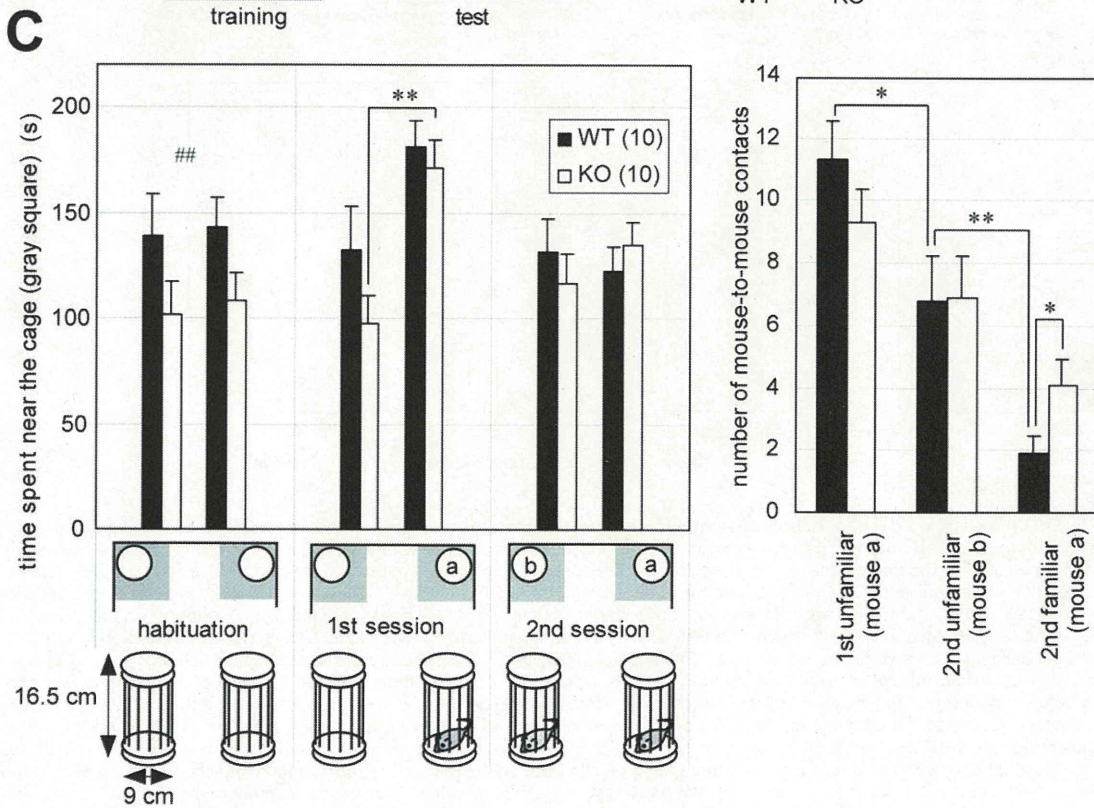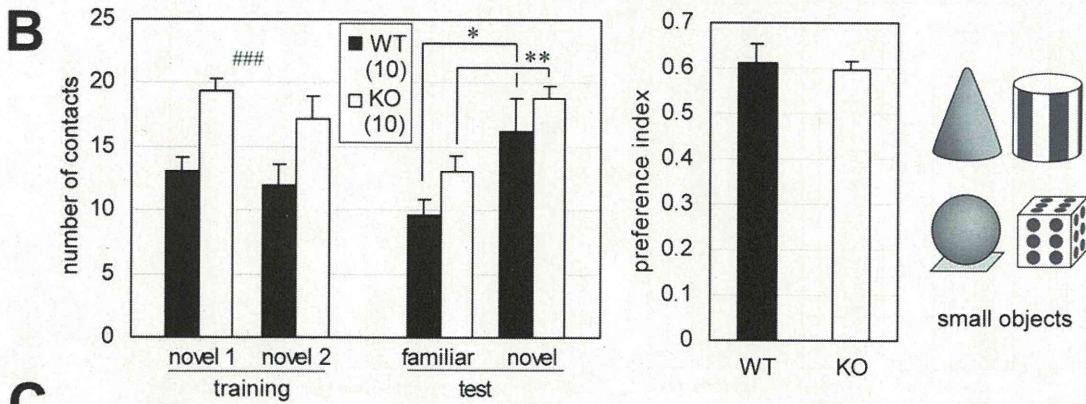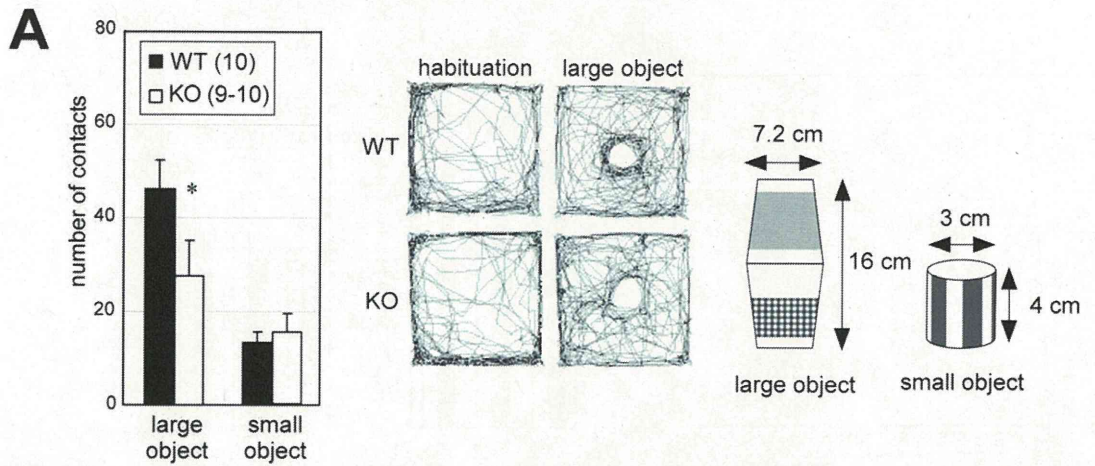
Figure 3. Approach to inanimate and animate objects. (A) Behavioral tests of approach to inanimate objects in the OF. The mice were first placed in the OF box without the object (habituation), then placed again in the OF with the large or small object (*right*). Approach was measured by the numbers of 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).
doi:10.1371/journal.pone.0022716.g003

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 to 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.

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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.

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B

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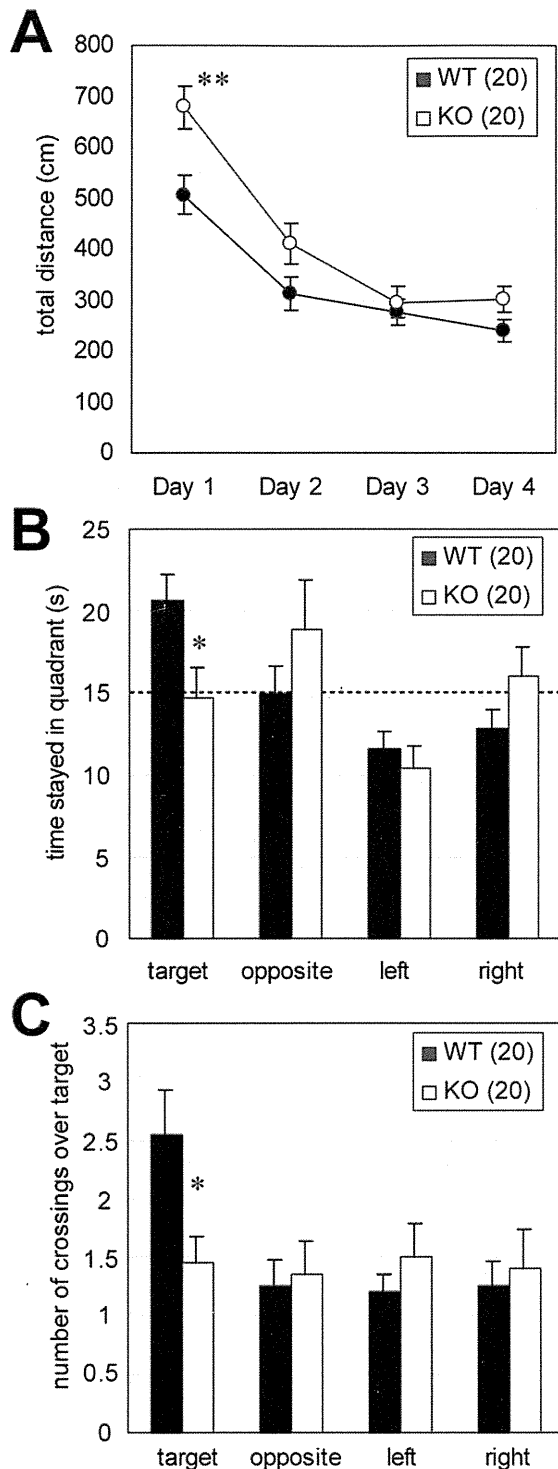
Figure 4. Spatial memory deficits in *Lrrtm1* KO mice. (A) Morris water maze training session. The total distance swum before reaching the target was significantly greater in KO mice than in WT mice on the first day, whereas it was comparable to that in WT mice on the second to fourth days. Values are 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.
doi:10.1371/journal.pone.0022716.g004

Discussion

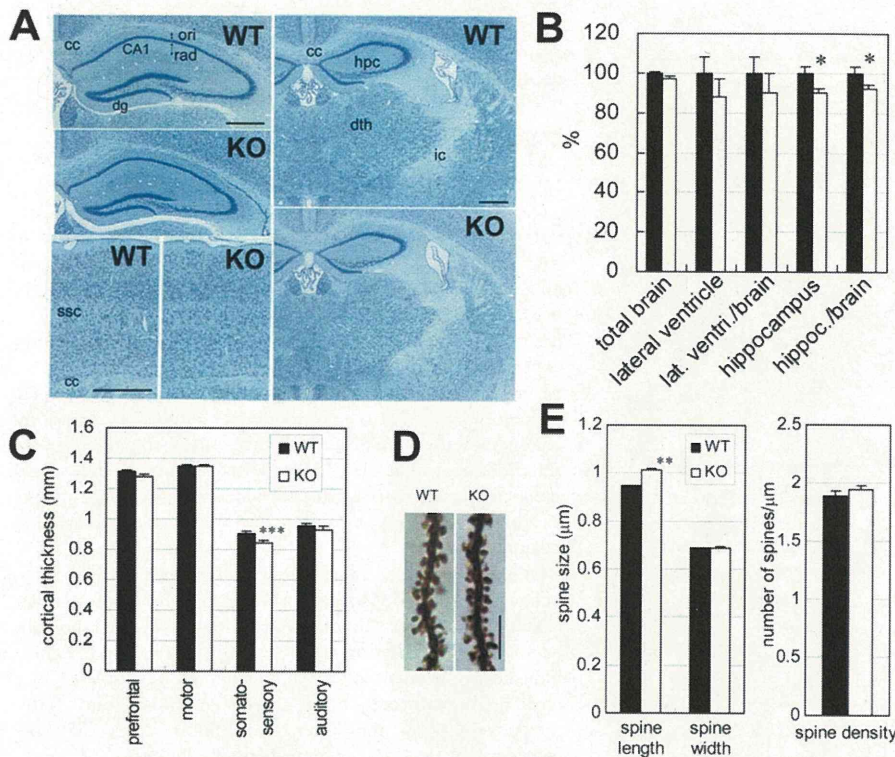### *Lrrtm1* KO behavioral abnormalities

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.

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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. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
doi:10.1371/journal.pone.0022716.g005

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
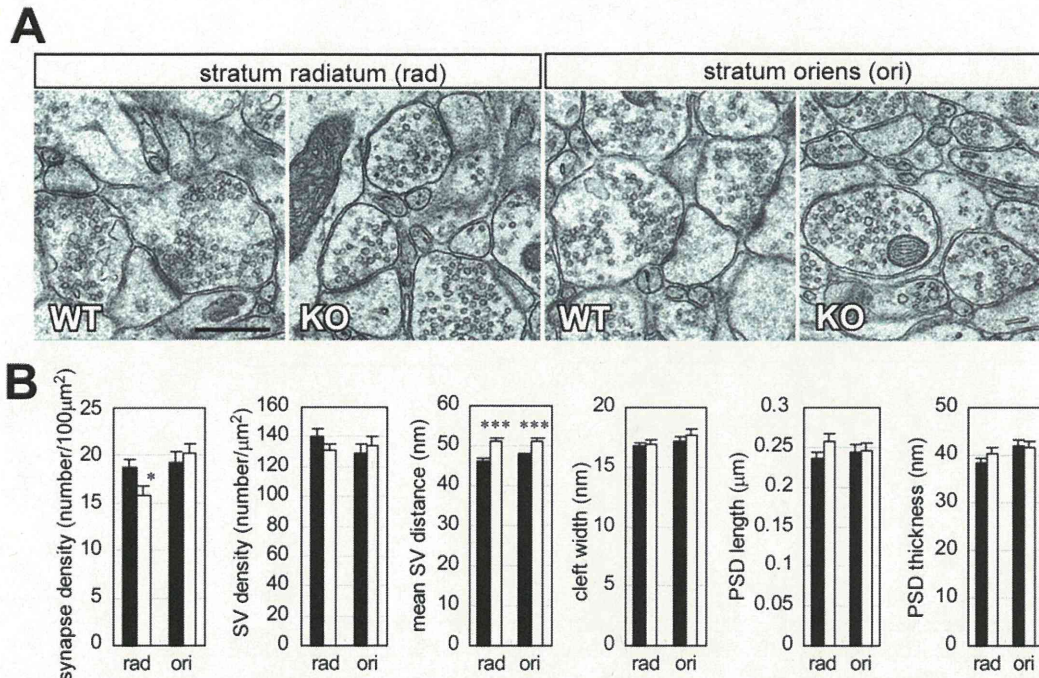
Figure 6. Electron microscopic analysis of hippocampal synapses. (A) Representative images of stratum radiatum and stratum oriens synapses. Scale bar, 500 nm. (B) Quantification of synapse number in 100 μm^2 of the entire images (synapse density), number of synaptic vesicles in 1 μm^2 of presynaptic 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. * $P<0.05$; *** $P<0.001$.
doi:10.1371/journal.pone.0022716.g006

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

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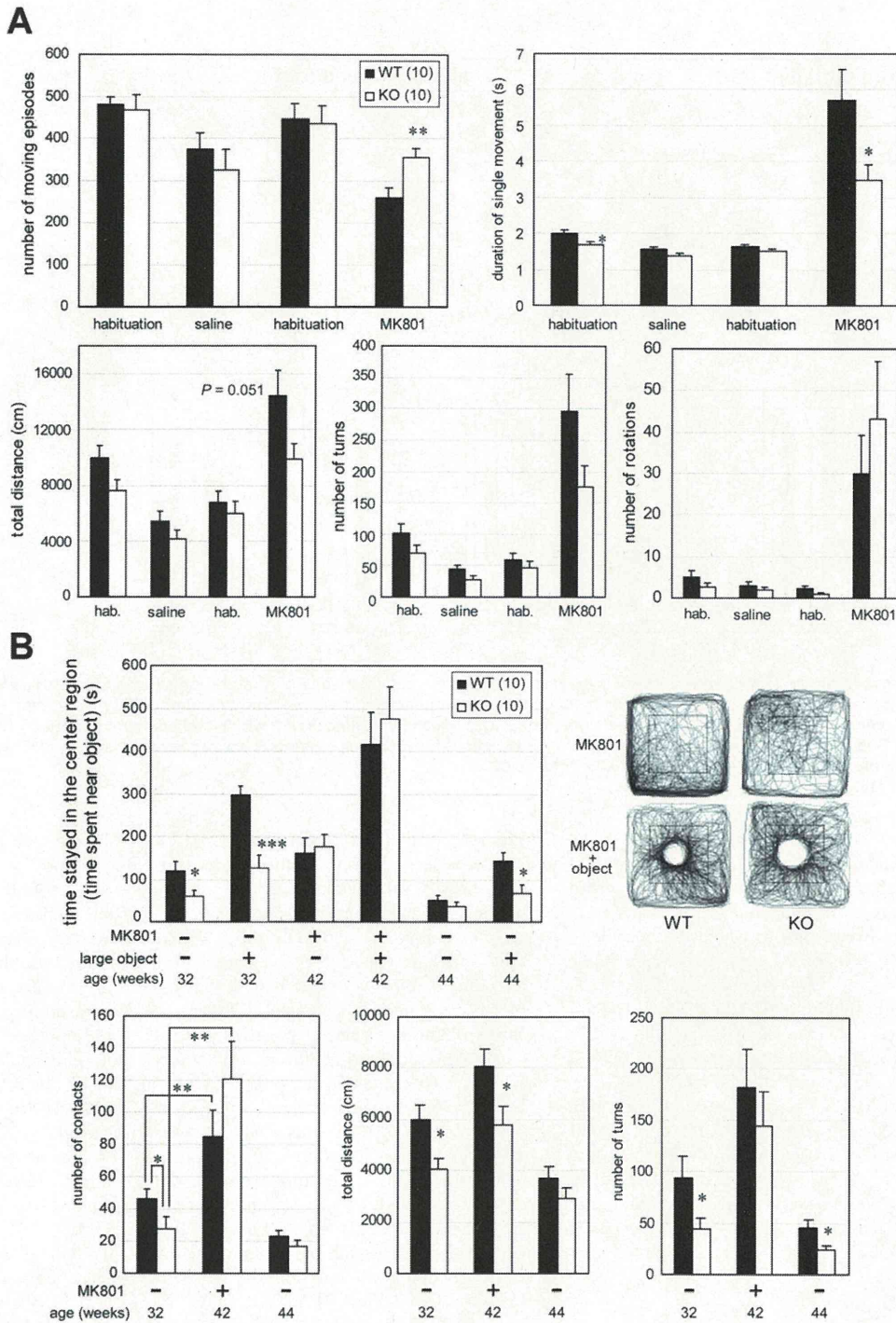
Figure 7. Effects of MK-801 administration on *Lrrtm1* KO behavior in the OF box. (A) Locomotor activities before (habituation) and 10 min after MK-801 treatment or saline treatment were examined in the OF apparatus. Saline injection was done once, followed by MK-801 injection the next day, using the same animals. Number of moving episodes, duration of a single movement, total distance, number of turns, and number of rotations were measured in each 30-min session. (B) Approach to the large object. (*top left*) Time spent in the central area (30% of the total area, indicated as squares in the representative traces at *top right*), which included the large, inanimate object, in the 15-min test period. Compare the traces with those in Figure 3A. As a control, we used the value of the latter half (15 min) of the preceding OF session (large object [–]). (*bottom*) Number of contacts with the large object before, soon after, and 2 weeks after MK-801 treatment. As controls, corresponding values in the large inanimate object approach test (Figure 3A) are indicated (*MK801-, 32 weeks*). The experiments were done in the same animals at the ages indicated. Values are presented as means ±SEM. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.
doi:10.1371/journal.pone.0022716.g007

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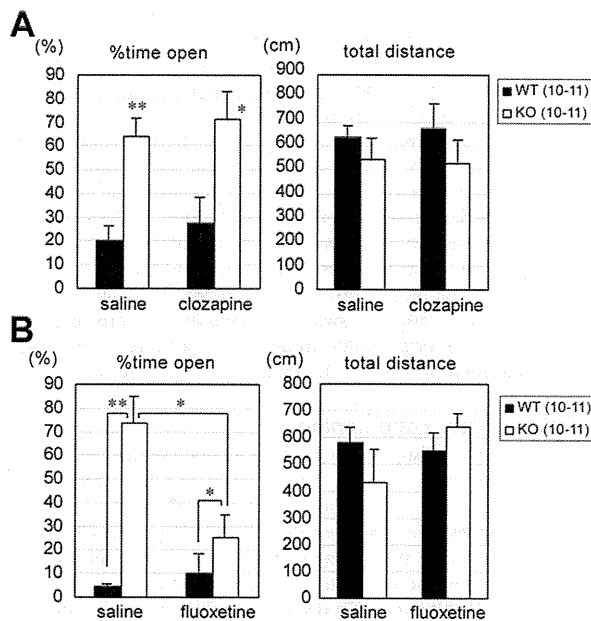
Figure 8. Effects of clozapine or fluoxetine administration on *Lrrtm1* KO behavior in the elevated plus maze test. (A, B) Percentage of time spent in the open arms and total distance traveled in the elevated plus maze test. WT and KO mice were subjected to the test 30 min after intraperitoneal injection of saline, 0.4 mg/kg clozapine, or 10 mg/kg fluoxetine. (A) clozapine treatment. (B) fluoxetine treatment. Values are presented as means ±SEM. * $P<0.05$; ** $P<0.01$ in U-test.
doi:10.1371/journal.pone.0022716.g008

from the afternoon of the day of transfer to the behavioral laboratory (Day 1) until the first day of the next week (Day 8). After the termination of home-cage activity measurement, cages and bedding materials were changed to fresh ones and the mice were maintained in the same type of micro-isolation rack (Allentown Inc., Allentown, NJ, USA) as used in the breeding rooms throughout the behavioral screening.

OF test

The OF test was performed as previously described [29]. Each mouse was placed in the center of an OF apparatus [50×50×40 (H) cm] illuminated by light-emitting diodes (LEDs; 70 lx at the center of the field) and then allowed to move freely for 15 min. Distance traveled (cm) and time spent (%) in the central area of the field (30% of the field) or in the four corner squares of the 5×5 subdivisions were adopted as indices, and the relevant data were collected every 1 min. Data were collected and analyzed by using Image J OF4 (O'Hara).

Hole-board test

An OF system made of gray plastic (50×50×40 (H) cm) with four equally separated holes (3 cm diameter with infra-red sensor) on the floor was used (Model ST-1/WII, Muromachi-kikai, Tokyo, Japan). The field was illuminated by fluorescent light (180 lx, at the center of the field), and the level of background noise was approximately 50 dB. The behavior of each mouse was monitored by a CCD camera located about 1.5 m above the field. In the HB test, mice were individually introduced into the center of the field and were then allowed to explore freely for 5 min. Total moving time (s), distance traveled (cm), latency of head-dipping (s), number

of head-dips, duration of head-dipping (s), duration of rearing (s), and number of rearings were measured as indices. Data were collected and analyzed by using a CompACT VAS system (Muromachi-Kikai, Tokyo, Japan).

Light–dark box test

A four-channel LD-box system was added to the same soundproof room as the OF. Each light box was made of white plastic [20×20×20 (H) cm] and illuminated by LEDs (250 lx at the center of the box); a CCD camera was attached to the ceiling. Each dark box was made of black plastic [20×20×20 (H) cm]; an infra-red camera was attached to the ceiling. There was a tunnel for transition on the center panel between the light box and dark box (3×5 cm) via a sliding door. In the LD test, mice were individually introduced into the light box, and the door of the tunnel automatically opened immediately after the software detected the mouse. The mice were then allowed to move freely in the LD box for 10 min. Total distance traveled, percentage of time spent in the light box, number of transitions between the light and dark boxes, and the duration of the first latency period before entry to the dark box were measured as indices. Data were collected and analyzed by using Image J LD4 (O'Hara).

Elevated plus maze test

A single channel of EPM [closed arms: 25×5×15 cm (H); open arms 25×5×0.3 cm (H)) was placed in the same soundproof room that was used for the OF and LD tests. The floor of each arm was made of white plastic, and the wall of the closed arms and the ridge of the open arms were made of clear plastic. The closed arms and open arms were arranged orthogonally 60 cm above the floor. The illuminance at the central platform of the maze (5×5 cm) was 70 lx. In the EPM test, mice were individually placed on the central platform facing an open arm and were then allowed to move freely in the maze for 5 min. Total distance traveled, % of time spent in the open arms, and number of open arm entries as a percentage of the total number of entries were measured as indices. Data were collected and analyzed by using Image J EPM (O'Hara).

Inanimate object approach tests

This test was performed in the OF apparatus. A mouse was first placed in the OF with 70 lx illumination for 15 min (habituation session). After the habituation session, the mouse was returned to its home cage and an inanimate object was placed in the center of the field. In the next test session, the mouse was placed again in the OF with the novel object. The large object was prepared by joining two paper cups by their openings (see Figure 3A). Inside the bottom of one cup, a metal block was placed to give stability, and gray monotone and check-patterned printed papers were wrapped around the external surfaces of the cups. Each large object was discarded after use and a new object that had had no contact with the experimental animals was used. The mean time interval between two sessions was 4 min. The total distance traveled and % of time spent in the central area (30% of the field), which included the object and the area around it, were analyzed by using Image J OF4 (O'Hara). Contacts with the novel object were counted on the video records by an observer who was blind to the genotypes. Contact was defined as a forward movement toward the object and subsequent direct contact using the head.

Novel object recognition test

The experiments were done in accordance with the method of Yoshiike et al. [30]. The test is based on the innate tendency of

rodents to differentially explore novel objects over familiar ones. Briefly, the mice were habituated for 15 min to a cage (17 cm×28 cm×12 cm [H]) without bedding materials. After the habituation session, the mice were exposed to two identical small objects for 15 min (training session). Soon after the training session, the mice were presented again with two objects, one used in the training session and a novel object (test session). The used small objects were spherical, conical, cube-shaped, or columnar, made of metal painted black or white in patterns, and generally consistent in their heights and volumes (Figure 3B). The behavior of the mice was video-recorded and the contact with each object was assessed with the naked eye, as in the inanimate object approach test.

Social discrimination test

This test was performed in the OF test apparatus with 70 lx luminance. The test consisted of a habituation session, first test session, and second test session. Each session continued for 15 min and took place in the following order. In the habituation session, two empty cylindrical wire cages (inner size, 7 cm×15 cm [H]; outer size, 9 cm×16.5 cm [H], with twenty-one 3-mmvertical stainless wires longitudinally and gray polyvinyl discs on the top and the bottom, manufactured by the RIKEN Rapid Engineering Team) were placed in two adjacent corners. In the first test session, a mouse (7-week-old male DBA2, purchased from Nihon SLC, Shizuoka, Japan) that was new to the test mouse was put in one of the two cylindrical cages. In the second test session, another mouse that was also new to the test mouse was put in the remaining cylindrical cage. Between the three sessions there were 4-min intervals, during which the test mouse was returned to its home cage. The three sessions were video-recorded from above, and the times spent in the two corner squares containing the cylinders within the 3- ×3-square subdivision (17.7×17.7 cm square) were measured with Image J OF4 (O'Hara). For the two test sessions, video recording was also done from an obliquely upward position to observe contact between the test mouse and the in-cage mouse. Contact with the in-cage mouse was defined as a forward movement toward the mouse in the cage and subsequent direct contact using the head. The position and posture of the in-cage mouse were observable through the slits of the wires. The contacts were counted on the video records by an observer who was blind to the genotypes. Each in-cage mouse was used once a day; when the habituation session began, the mouse was simultaneously placed in its cylindrical cage on the corners of an OF box that was not being used for the tests. These rules were thought to minimize the difference between the two in-cage mice in the second test sessions in regard to their acclimation to the cylindrical cage and the OF-box environment. After each use, the cylindrical cage was extensively washed with water and rinsed with 90% ethanol, which was then evaporated off, to minimize the effects of remnant materials.

Morris water maze test

A circular maze made of white plastic (1 m diameter, 30 cm depth) was filled with water to a depth of about 20 cm (22 to 23°C). The water was colored by the addition of white paint to prevent the mice from seeing the platform (20 cm high, 10 cm diameter; 1 cm below the surface of water) or other cues under the water. Some extra-maze landmark cues (i.e. a calendar, a figure, and a plastic box) were visible to the mice in the maze. The movements of the mice in the maze were recorded and analyzed with Image J WM (O'Hara). Mice received six trials (= 1 session) per day for 4 consecutive days. Each acquisition trial was initiated by placing an individual mouse into the water facing the outer

edge of the maze at one of four designated starting points quasi-randomly; the position of the submerged platform remained constant for each mouse throughout the testing. A trial was terminated when the mouse reached the platform, and the latency and distance swum were measured. The cut-off time of the trial was 60 s; mice that did not reach the platform within 60 s were removed from the water and placed on the platform for 30 s before being toweled off and placed back into their home cages. The inter-trial interval was about 6 min. After 4 days of training, a probe test was conducted on day 5. In the probe test, the platform was taken away; each mouse was placed into the water at a point opposite to the target platform and allowed to swim in the maze for 60 s. The distance swum, the number of crossings of the position of the target platform and the other three platforms, and the time spent in each of the four quadrants were measured.

Classical fear conditioning

This test consisted of three parts: a conditioning trial (Day 1), a context test trial (Day 2), and a cued test trial (Day 3). Fear conditioning was performed in a clear plastic chamber equipped with a stainless-steel grid floor [34×26×30 (H) cm]. A CCD camera was mounted on the ceiling of the chamber and connected to a video monitor and computer. The grid floor was wired to a shock generator. White noise (65 dB) was supplied from a loudspeaker as an auditory cue [i.e. the conditioned stimulus (CS)]. The conditioning trial consisted of a 2-min exploration period followed by two CS–US pairings separated by 1 min. A US (foot-shock: 0.5 mA, 2 s) was administered at the end of the 30-s CS period. Twenty-four hours after the conditioning trial, a context test was performed in the same conditioning chamber for 3 min in the absence of the white noise. A cued test was also performed in an alternative context with distinct cues; the test chamber was different from the conditioning chamber in terms of luminance (about 0 to 1 lx), color (white), floor structure [no grid but with thin bedding material (Alpha-Dri: Shepherd, TN, USA)], and shape (triangular). The cued test was conducted 24 h after the contextual test was finished; it consisted of a 2-min exploration period (no CS) to evaluate nonspecific contextual fear, followed by a 2-min CS period (no foot shock) to evaluate the acquired cued fear. The rate of freezing response (immobility, except for respiration and heartbeat) of mice was measured as an index of fear memory. Data were collected and analyzed with Image J FZ2 (O'Hara).

Acoustic startle response and prepulse inhibition

For startle response testing, each mouse was put into a small cage (30 or 35 mm diameter, 12 cm long) and the cage was placed on a sensor block in a soundproof chamber [60×50×67 cm (H)]. A dim light was mounted on the ceiling of the soundproof chamber (10 lx at the center of the sensor block), and 65-dB white noise was presented as background noise. In the auditory startle response test, mice were acclimatized to the experimental conditions for 5 min, and then the experimental session began. In the first session, 120-dB startle stimuli (40 ms) were presented to the mice 10 times, with random inter-trial intervals (10 to 20 s). In the second session, startle responses to stimuli at various intensities were assessed. Five white noise stimuli (each 40 ms) at 70 to 120 dB (70, 75, 80, 85, 90, 95, 100, 110, or 120 dB) were presented in quasi-random order and with random inter-trial intervals (10 to 20 s). In the prepulse inhibition session, mice experienced five types of trial: no stimulus; startle stimulus (120 dB, 40 ms) only; prepulse 70 dB (20 ms, lead time 100 ms) and pulse 120 dB; prepulse 75 dB (20 ms, lead time 100 ms) and pulse 120 dB; and prepulse 80 dB (20 ms, lead time 100 ms) and pulse 120 dB. Each trial was performed 10 times in quasi-random order and with random inter-trial intervals (10 to 20 s). In the final session, a 120-dB

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