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Lack of neurotensin type 1 receptor facilitates contextual fear memory depending on the memory strength

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ABSTRACT

Neurotensin is known to have antipsychotic-like behavioral and neurochemical effects, but its participation in fear memory has not been fully elucidated. Here, we report that a lack of type 1 neurotensin receptor (Ntsr1) increases the behavioral fear response elicited by weak fear memory. Adult Ntsr1-knockout (KO) mice and their wild-type (WT) littermates were compared in contextual fear conditioning. The mice were exposed twice for 3 min to the context 24 and 48 h after conditioning (first and second exposure, respectively), and freezing response of mice at the exposure was measured to evaluate fear memory. Ntsr1-KO mice showed a higher freezing rate than WT mice at both first and second exposures under the condition where a relatively weak unconditioned stimulus (footshock) was applied and thus elicited a relatively lower freezing rate. The difference in the first exposure between Ntsr1-KO and WT mice disappeared under the condition where a more intense unconditioned stimulus was used. The enhancement of freezing response in Ntsr1-KO mice at second exposure was abolished by propranolol, a β-adrenergic blocker that suppresses fear memory reconsolidation, and suppressed by MK-801, an NMDA receptor antagonist. These results suggest that Ntsr1 plays inhibitory roles in weak fear memory.

1. Introduction

Fear memory is critical for behavioral adaptation to the environment surrounding an individual. Identification of the neurotransmitter system that plays a modulatory role in fear memory formation, expression, and/or its changes by retrieval is fundamental to understanding the adjustment of these memory processes. Among the processes that occur following memory retrieval, reconsolidation and extinction are well known to modulate fear memory. That is, reconsolidation acts to stabilize (Sara, 2000; Nader et al., 2000), whereas extinction to reduce (Myers and Davis, 2002), the expression of the original fear memory depending on the duration of reexposure to a conditioned stimulus (CS) (Suzuki et al., 2004).

With respect to fear memory, various neurotransmitter systems are known to be involved in these processes (Davis, 2000, as review). Glutamatergic and catecholaminergic neurotransmissions are one of such systems. For instance, an antagonist for the NMDA receptor, 5-phosphonopentanoic acid (AP5), injected into the amygdala blocks acquisition or expression of fear memory (Fanselow and Kim, 1994; Lee and Kim, 1998; Maren et al., 1996). Intraperitoneal and intra-amygdala injections of the NMDA receptor co-agonist D-cycloserine facilitate not only reconsolidation (Lee et al., 2006) but also extinction of fear memory (Ledgerwood et al., 2003; Walker et al., 2002). Blockade of β-adrenergic receptor by propranolol (PROP) is known to inhibit fear memory consolidation and reconsolidation (Przybyslawski et al., 1999; Debiec and LeDoux, 2004), whereas the same blockade suppresses extinction of the fear memory (Ouyang and Thomas, 2005; Mueller et al., 2008). Local infusion of an antagonist of the dopamine D_1 receptor, SCH23390, into the amygdala blocks the acquisition and expression of the freezing response, whereas the same treatment by a D_1 receptor agonist, SKF82958, facilitates the acquisition and expression (Guarraci et al., 1999). Intra-amygdala infusion of an antagonist of dopamine D_2 receptor, eticlopride, attenuates the formation and/or consolidation of fear memory in tone-fear conditioning (Guarraci et al., 2000). The neurotransmissions that use lipids and neuropeptides as transmitters are also known to be involved in fear memory. For instance, the CB_1 cannabinoid receptor agonist anandamide, when administered by intra-hippocampal injection, blocks reconsolidation and facilitates extinction of contextual fear memory (De Oliveira Alvares et al., 2008). A lack of gastrin-releasing peptide receptor (GRPR) is known to result in more persistent fear memory in fear conditioning (Shumyatsky et al., 2002). Intra-hippocampal injection of GRPR antagonist, RC-3095, inhibits extinction and reconsolidation of memory in inhibitory avoidance task (Luft et al., 2006, 2008). Intra-amygdala injection of neuropeptide Y decreases the expression of fear memory in fear conditioning through a mechanism other than Y_1 receptor (Fendt et al., 2009).

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Neurotensin (NT) is a 13-amino-acid peptide that widely exists in many brain regions (Emson et al., 1982). Three subtypes of NT receptors (Ntsr1, Ntsr2, and Ntsr3) are known thus far. Both Ntsr1 and Ntsr2 are G-protein-coupled receptors (Chalon et al., 1996; Tanaka et al., 1990), whereas Ntsr3 is a single transmembrane domain protein that mainly localizes intracellularly (Mazella et al., 1998; Sarret et al., 2003). A population of neurons in the ventral tegmental area (VTA) contains NT, and a subpopulation of these neurons projects to the amygdala (Asan, 1998), a brain region that plays a central role in association learning in fear conditioning (Davis, 1997; Fendt and Fanselow, 1999; LeDoux, 2000; Maren, 1999). In the amygdala, the basolateral nucleus (BLA) and the central nucleus (CeA) have NT binding sites (Moyse et al., 1987) and Ntsr1-positive cells (Alexander and Leeman, 1998). In addition, Ntsr1 interacts with the dopamine system (Binder et al., 2001, as review), and the dopamine system is known to participate in fear memory as mentioned above. In this relation, we previously reported that the lack of Ntsr1 increases the activity of NMDA receptors in the amygdala by enhancing dopamine D_2 receptor activity (Amano et al., 2008). These data imply the possibility that Ntsr1 may participate in fear memory. However, there is only one recently published study reporting the participation of Ntsr1 in fear memory. Namely, the Ntsr1 agonist PD149163, systemically administered in rats, blocks fear-potentiated startle (Shilling and Feifel, 2008).

The purpose of the present study is to further elucidate the role of Ntsr1 in fear memory. For this purpose, we have compared performance of Ntsr1-knockout (KO) mice and their wild-type (WT) littermates in fear conditioning. As a result, we found that a lack of Ntsr1 enhanced expression of conditioned fear memory depending on the memory strength without any alterations in either locomotor activity or sensitivity to electrical footshock. This result from knockout mice, as well as a previous report using an Ntsr1 agonist (Shilling and Feifel, 2008), suggests that Ntsr1 inhibits fear memory.

2. Materials and methods

2.1. Animals

Ntsr1-KO mice were generated as previously described (Maeno et al., 2004) and backcrossed to C57BL/6 J mice 33–36 times. WT and Ntsr1-KO mice used in the present study were generated by mating heterozygous mice. The mice were housed four or five per cage under controlled conditions of temperature (25 ± 1 °C) and lighting (12-h light/dark cycle) and provided with food and water ad libitum. At the beginning of each experiment, mice were 15–19 weeks old. Animal procedures were in strict accordance with the guidelines of the National Institute of Neuroscience, National Center of Neurology and Psychiatry (Japan) and were approved by the Institutional Animal Investigation Committee (approved # 2007013).

2.2. Drugs

PROP [(±)-Propranolol hydrochloride (Sigma, St. Louis, MO, USA)] and MK-801 hydrogen maleate (Sigma) were both dissolved in saline (1 g/l and 10 mg/l, respectively). According to previous reports, these drug solutions were administered into mice at a dose of 10 mg/kg (PROP) and 0.1 mg/kg (MK-801) body weight (volume 10 ml/kg), respectively (Debiec and LeDoux, 2004; Lee et al., 2006; Wozniak et al., 1996).

2.3. Behavioral procedures

2.3.1. Contextual fear conditioning test

The contextual fear conditioning test was performed as previously described (Yamada et al., 2009). Briefly, mice were placed in the conditioning chamber (20×20-cm bottom, 35-cm high, illuminated at 200 lx; Muromachi Kikai, Tokyo, Japan) and permitted to explore the chamber. After 148 s, a single electrical footshock (0.8 mA, 2-s duration) was delivered. Under more intense conditioning condition, mice received a series of three or eight footshocks at 30-s intervals. After the end of conditioning, mice continued to be kept in the conditioning chamber for an additional 30 s and returned to their home cages. Twenty-four h later, mice were exposed to the chamber without footshock(s) to assess the contextual fear memory for 3 min (first exposure) as measured by freezing behaviors. On the next day, mice were exposed to the chamber for testing the memory again (second exposure). In the second and third experiments (shown in Figs. 2 and 3), the contextual fear conditioning (with a single footshock) was conducted as described above except for drug administrations. That is, WT and Ntsr1-KO mice were randomly divided into two groups, respectively [WT-sal, WT-drug, KO-sal, and KO-drug]. Immediately after the first exposure, the mice in the WT-sal and KO-sal groups were intraperitoneally administered saline, and the mice in the WT-drug and KO-drug groups were similarly administered PROP (10 mg/kg) or MK801 (0.1 mg/kg) in the respective experiment. For the extinction experiment that is shown in Fig. 4, mice were conditioned with a series of eight footshocks (0.8 mA, 1-s duration) at an average of 30-s intervals. The first footshock was delivered 59 s after placement in the chamber. After the end of the final footshock, mice continued to be kept in the conditioning chamber for an additional 30 s and returned to their home cages. On the next day, mice were exposed to the chamber for 6 min for extinction training (Ext training). Forty-eight h after the extinction training, mice were tested in the chamber for extinction for 6 min (Test).

Behavior was video-recorded during each session and subsequently analyzed for freezing, defined as complete immobilization of the mouse except for respiration (Blanchard and Blanchard, 1972). The freezing response was scored as the time the mouse spent frozen during a total of 180-s or 360-s sessions or a 60-s bin, and the amount of freezing response was expressed as the percent freezing rate in the 180-s, 360-s, or 60-s time window.

2.3.2. Open-field test

The open-field test was performed using a computerized open-field apparatus (O'Hara & Co., Ltd., Tokyo, Japan). Naive mice were permitted to range freely for 5 min in a novel chamber (50×50-cm bottom, 40-cm high white polyvinyl chloride floor and walls, illuminated at 100 lx) that is placed inside a sound-attenuating box (Zushida et al., 2007). The total locomotion was video-recorded and automatically analyzed on a Macintosh computer using Image OF 2.15x (O'Hara & Co., Ltd.), a modified program based on the public domain software NIH Image program developed at the National Institutes of Health.

2.3.3. Shock sensitivity test

The shock sensitivity test was performed as previously described (Yamada et al., 2003). Briefly, six series of six footshocks (20, 40, 60, 80, 100 and 130 μA, 1-s duration) were delivered to mice in ascending and descending order of intensity via a floor grid. The inter footshock interval was 15 s. The shock threshold was defined as the least amount of electricity (μA) that causes an animal's hind paw left the floor. For each mouse, a mean shock threshold value was calculated as the average of the six thresholds recorded in the series.

2.4. Statistical analysis

All data are shown as means ± SEM. The two-tailed unpaired *t*-test was used for statistical comparisons between two groups. For more than three groups, the data were analyzed by two-way or three-way analysis of variance (ANOVA). In the three-way ANOVA, genotype (WT and Ntsr1-KO), session (first and second exposures), and number of footshock [one (1×), three (3×), and eight (8×)] were used as factors. The factors in the two-way ANOVA were genotype (WT and Ntsr1-KO) and drug [saline and drug (PROP or MK-801)] in the case of second and third experiments shown in Figs. 2 and 3, and genotype

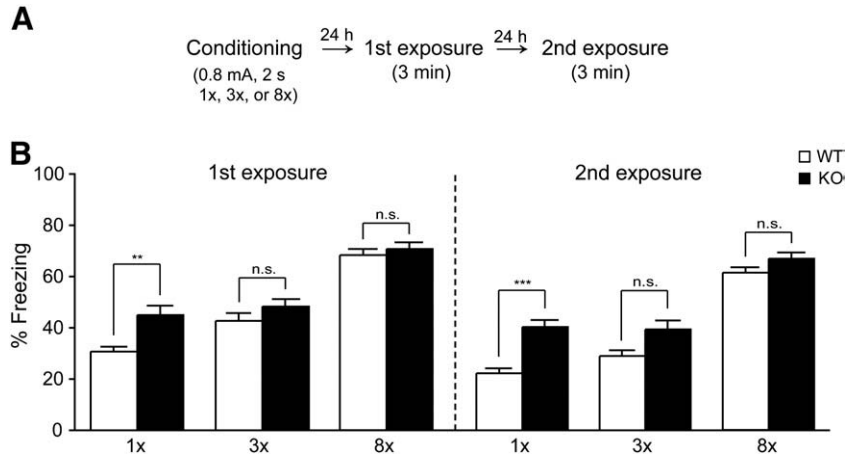

Fig. 1. Neurotensin type 1 receptor knockout (Ntsr1-KO) mice showed a facilitated freezing response, depending on the memory strength. (A) Experimental design. (B) Freezing rate of wild-type (WT, $n=10$ for single (1×), three (3×), and eight (8×) footshock condition, respectively) and Ntsr1-KO ($n=12$ for 1×; $n=10$ for 3×; and $n=11$ for 8×) mice during the first and second exposures. Data are expressed as mean ± SEM. Significant difference, $^{**}P<0.01$, $^{***}P<0.001$. n.s., not significant.

(WT and Ntsr1-KO) and session (extinction training and test) in the case of fourth experiment shown in Fig. 4. If the ANOVAs revealed a significant main effect or interaction among the factors, a post hoc Bonferroni's test was performed. $P<0.05$ was considered statistically significant.

3. Results

3.1. Ntsr1-KO mice showed a facilitated freezing response in fear conditioning, depending on the memory strength

Fig. 1 shows the freezing rates of mice during 3 min of exposure where they were conditioned with a single, three, or eight footshock(s). A three-way ANOVA (genotype × session × number of footshock) revealed significant main effects for genotype ($F(1, 127)=35.26$, $P<0.001$), session ($F(1, 127)=21.98$, $P<0.001$), and number of footshock ($F(2, 127)=160.49$, $P<0.001$), and a significant genotype × number of footshock interaction ($F(2, 127)=5.19$, $P<0.01$). Post hoc analysis revealed simple main effects for all factors, and the freezing rates of both WT and Ntsr1-KO mice increased as the number of footshocks was increased. Moreover, under the single footshock condition, the freezing rate of Ntsr1-KO mice ($n=10$) was significantly higher than that of WT mice ($n=12$) for both the first ($P<0.01$) and second ($P<0.001$) exposures (Fig. 1B). In contrast, under three and eight footshock conditions, post hoc test revealed no significant difference in freezing rates between WT ($n=10$ for each) and Ntsr1-KO ($n=10$ for 3×, $n=11$ for 8×) mice for both the first and second exposures ($Ps>0.05$; Fig. 1B).

In addition, the mean values of freezing rates in the group that received a single (30.67 ± 2.05%, WT and 44.91 ± 3.78%, KO) and three (42.67 ± 3.10%, WT and 48.28 ± 3.00%, KO) footshock(s) were lower than the freezing rate in the group that received eight footshocks (68.39 ± 2.51%, WT and 70.81 ± 2.61%, KO), respectively, suggesting that fear memory was enhanced as the number of footshocks was increased in our experimental conditions. Therefore, it is unlikely that the absence of augmentation of Ntsr1-KO mouse's freezing rate in the three footshock condition is due to saturation of freezing response in WT mice. These results suggested that the deficit of Ntsr1 in mice enhanced expression of fear memory only in the single footshock condition.

3.2. β-Adrenergic blocker PROP abolished the facilitated freezing response in Ntsr1-KO mice

As mentioned in the introduction, fear memory is stabilized by reconsolidation process after its retrieval. To test the possibility that facilitation of memory reconsolidation participates in facilitation of the freezing response of Ntsr1-KO mice at second exposure, we performed a single footshock experiment using PROP, a β-adrenergic blocker known to inhibit fear memory reconsolidation (Debiec and LeDoux, 2004).

Fig. 2 shows the freezing rates of mice in the first and second exposures. As we show in Fig. 1B (single footshock condition), the freezing rate of Ntsr1-KO mice ($n=21$) in the first exposure was significantly higher than that of WT mice ($n=17$) ($t(36)=6.64$, $P<0.001$). Just after the first exposure, mice were either administered PROP or saline control, and were tested in the second exposure after 24 h. A two-way ANOVA (genotype × drug) revealed significant main effects for genotype ($F(1, 34)=26.60$, $P<0.001$), drug ($F(1, 34)=26.00$, $P<0.001$), and a significant genotype × drug ($F(1, 34)=15.83$, $P<0.001$) interaction, suggesting the difference in effectiveness of PROP on the freezing rate between WT and Ntsr1-KO mice. Post hoc analysis showed a significant difference ($P<0.001$ for all) in the freezing rates in the saline-treated KO mouse group (KO-sal, $n=10$) versus the PROP-treated KO mouse group (KO-PROP, $n=11$), versus the saline-treated WT mouse groups (WT-sal, $n=8$), and versus the PROP-treated WT

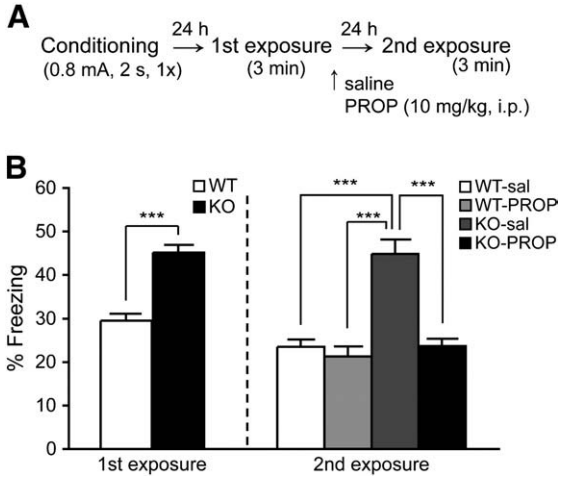

Fig. 2. The effect of intraperitoneal administration of β-adrenergic blocker propranolol (PROP) on freezing response in wild-type (WT) and neurotensin type 1 receptor knockout (Ntsr1-KO) mice. (A) Experimental design. (B) Freezing rates of WT [saline-treated (WT-sal), $n=8$ and PROP-treated (WT-PROP) $n=9$] and Ntsr1-KO [saline-treated (KO-sal), $n=10$ and PROP-treated (KO- PROP), $n=11$] mice during the first and second exposures. Note that the facilitated freezing response in Ntsr1-KO mice in the second exposure was abolished by intraperitoneal administration of PROP. Data are expressed as mean ± SEM. Significant difference, $^{***}P<0.001$.

mouse group (WT-PROP, $n=9$). These results suggest that the augmented fear response in Ntsr1-KO mice at the second exposure could be attributable to facilitation of fear memory reconsolidation.

3.3. NMDA receptor-mediated signaling is involved in the facilitated freezing response in Ntsr1-KO mice

As mentioned in the introduction, neurotransmission mediated by NMDA receptors modulates the reconsolidation of fear memory (Lee et al., 2006), and NMDA receptor activity in the amygdala is facilitated in Ntsr1-KO mice (Amano et al., 2008). Taking these reports into consideration, we performed a single footshock experiment using the NMDA receptor antagonist MK-801 to explore the involvement of NMDA receptors in facilitated reconsolidation in Ntsr1-KO mice.

Fig. 3 shows the freezing rates of mice in the first and second exposures. As we show in Figs. 1B and 2B, the freezing rate of Ntsr1-KO mice ($n=38$) in the first exposure was significantly higher than that of WT mice ($n=33$) ($t(64)=4.20$, $P<0.001$). Just after first exposure, mice were either administered MK-801 or saline control, and were tested in the second exposure after 24 h. A two-way ANOVA (genotype × drug) revealed significant main effects for genotype ($F(1, 70)=21.89$, $P<0.001$) and drug ($F(1, 70)=8.77$, $P<0.01$), but no significant genotype × drug ($F(1, 70)=2.13$, $P>0.05$) interaction, suggesting the higher freezing rate in Ntsr1-KO mice than that in WT-mice, and the higher freezing rate in saline-treated mice than that in MK-801-treated mice. Post hoc analysis showed a significant difference in the freezing rates in the saline-treated KO mouse group (KO-sal, $n=20$) versus the MK-801-treated KO mouse group (KO-MK, $n=21$) ($P<0.01$), versus the saline-treated WT mouse groups (WT-sal, $n=15$) ($P<0.001$), and versus the MK-801-treated WT mouse group (WT-MK, $n=18$) ($P<0.001$). These results suggest that NMDA receptor activity participates in facilitation of fear memory reconsolidation in Ntsr1-KO mice.

3.4. Ntsr1-KO mice showed a normal extinction of conditioned fear

Given the participation of NMDA receptor activity in facilitation of reconsolidation in Ntsr1-KO mice, and because extinction is known to be modulated by pharmacological manipulation at NMDA receptor, we tested whether fear extinction is altered in Ntsr1-KO mice. For this purpose, we conducted a fear extinction experiment using a longer exposure protocol and more intense footshock condition (Yamada et al., 2009).

Fig. 4 shows the freezing rates of mice during the extinction training and test. A two-way ANOVA (genotype × session) revealed a significant main effect of session ($F(1, 26)=112.9$, $P<0.001$), but no significant main effect of genotype and genotype × session interaction ($Fs<1$), suggesting that WT and Ntsr1-KO mice showed similar reduction in the freezing rates (i.e. extinction) and there is no difference in freezing rate between WT and Ntsr1-KO mice (Fig. 4B). Within-session analysis of freezing response (bin = 60 s) showed a substantially indistinguishable time course in Ntsr1-KO and WT mice (Fig. 4C).

3.5. Locomotor activity is identical in WT and Ntsr1-KO mice

It is possible that a deficit of Ntsr1 reduces locomotion in mice, which contributes to a higher freezing score during the tests conducted in the first to third experiment (shown in Figs. 1–3) because of activity-state-dependent effects. However, WT and Ntsr1-KO mice ($n=21$ for each) showed similar locomotor activity in the open-field test, at least for the 5 min after introducing the mouse to the apparatus ($t(40)=0.78$, $P>0.05$; Fig. 5A). Within-session analysis (bin = 60 s) also showed a substantially indistinguishable time course in the locomotor activity of the WT and Ntsr1-KO mice (Fig. 5B).

3.6. Sensitivity to footshock is not altered in Ntsr1-KO mice

Because augmentation of fear memory expression was not observed in the three- and eight-footshock condition, there is a possibility that pain threshold is reduced in Ntsr1-KO mice, which results in augmentation of the expression of fear memory in the single footshock condition. To confirm this possibility, we performed the

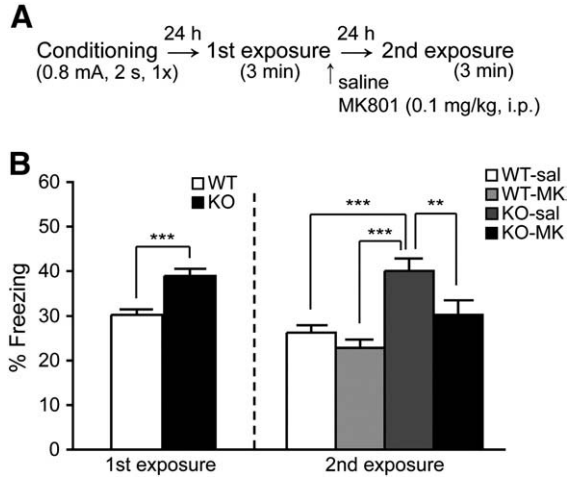

Fig. 3. The effect of intraperitoneal administration of NMDA receptor antagonist MK-801 on freezing response in wild-type (WT) and neurotensin type 1 receptor knockout (Ntsr1-KO) mice. (A) Experimental design. (B) Freezing rates of WT [saline-treated (WT-sal), $n=15$ and MK-801-treated (WT-MK) $n=18$] and Ntsr1-KO [saline-treated (KO-sal), $n=20$ and MK-801-treated (KO-MK), $n=21$] mice during the first and second exposures. Note that the facilitated freezing response in Ntsr1-KO mice in the second exposure was suppressed by intraperitoneal administration of MK-801. Data are expressed as mean ± SEM. Significant difference, $^{**}P<0.01$ and $^{***}P<0.001$.

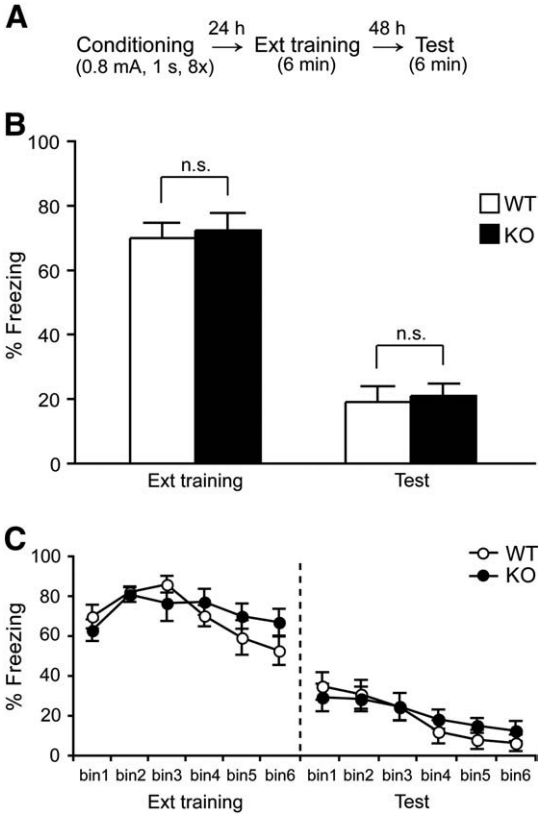

Fig. 4. Neurotensin type 1 receptor knockout (Ntsr1-KO) mice showed a normal extinction of contextually conditioned fear. (A) Experimental design. (B) Freezing rates of wild-type (WT, $n=7$) and Ntsr1-KO ($n=8$) mice during the Ext training and Test. (C) Time course of the freezing rates (bin = 60 s) of mice in each 60-s bin during the Ext training and Test. Data are expressed as mean ± SEM. n.s., not significant.

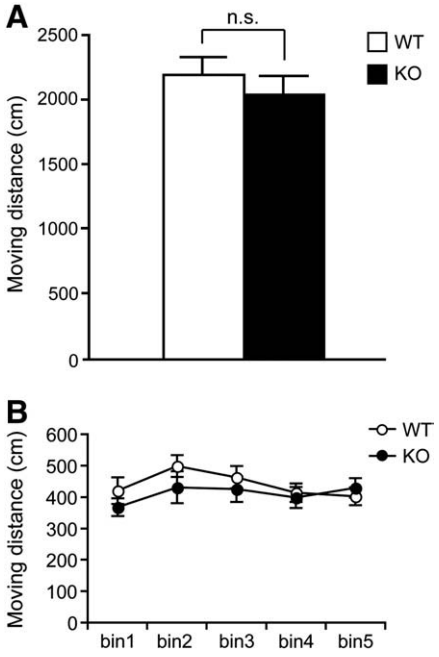

Fig. 5. Analysis of locomotor activity of wild-type (WT) and neurotensin type 1 receptor knockout (Ntsr1-KO) mice in the open-field test. (A) Total locomotor activity of WT and Ntsr1-KO mice ($n=21$ for each) during a 5-min session. (B) Time course of locomotor activity of mice in each 60-s bin during the total 5-min session. Data are expressed as mean ± SEM. n.s., not significant.

shock sensitivity test. Fig. 6 shows the shock threshold of the mice (43.50 ± 0.98 μA for WT and 44.55 ± 1.39 μA for Ntsr1-KO; Fig. 6). There is no significant difference between WT and Ntsr1-KO mice ($t(19)=0.61$, $P>0.05$).

4. Discussion

A main finding in the present study is that a deficit of Ntsr1 in mice facilitated fear memory in contextual fear conditioning dependently on memory strength. Namely, for weaker fear memory formed by a single footshock condition, both fear responses in the first and second exposures to the context were augmented in Ntsr1-KO mice, but in more intensive memory formed by three and eight footshock conditions, there was no significant difference in fear responses both in the first and second exposures. Moreover, we confirmed that facilitation of freezing rate in Ntsr1-KO mice was not due to decreased locomotor activity and hypersensitivity to the footshock.

This finding and a previous finding that the Ntsr1 agonist PD149163 blocks fear-potentiated startle in rats (Shilling and Feifel, 2008) consistently suggest a putative inhibitory role of Ntsr1 in fear memory. In contrast to these results, however, there are several reports that indicate the memory-enhancing effect of Ntsr1 in recognition memory tasks, such as social discrimination and object recognition (Feifel et al., 2009; Azmi et al., 2006). With respect to fear memory, inputs from the cortical area, including the visual and auditory cortices, thalamus, and hippocampus to the basolateral nucleus of the amygdala are necessary to form the memory (Davis, 2000). In contrast, inputs from the olfactory system, such as the main and accessory olfactory bulb to the medial and cortical nuclei of the amygdala are suggested in processing of olfactory information that is required for social discrimination in rodents (Cooke et al., 1998; Richter et al., 2005). The hippocampus, perirhinal and entorhinal cortices participate in object recognition (for review, see Winters et al., 2008). In these brain regions, expression of Ntsr1 mRNA is observed (Alexander and Leeman, 1998). Therefore, the discrepancy in the role of Ntsr1 between fear memory and social discrimination and object recognition memory may be due to the difference in neural circuits that participate in respective memory.

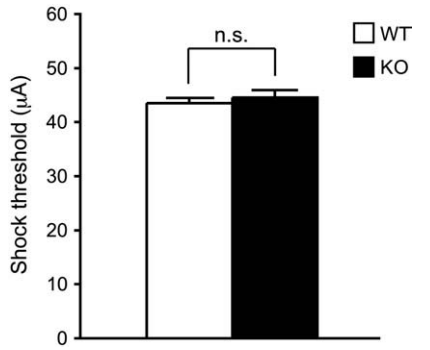

Fig. 6. Analysis of shock sensitivity of wild-type (WT, $n=11$) and neurotensin type 1 receptor knockout (Ntsr1-KO, $n=10$) mice in the shock sensitivity test. Shock threshold (μA) of mice was presented as means ± SEM. n.s., not significant.

Because the freezing rate of Ntsr1-KO mice was higher than that of WT mice even in the first exposure, there is a possibility that the augmented fear response in Ntsr1-KO mice in the second exposure was due to facilitation of acquisition and/or consolidation processes. Although we did not directly examine this possibility in the present study, our pharmacological analysis using PROP suggested that augmentation of fear response at least in the second exposure to the context, in the case of weak memory, was attributed to facilitation of fear memory reconsolidation because PROP inhibits fear memory reconsolidation (Debiec and LeDoux, 2004). Previous studies have suggested that activation of β-adrenergic and NMDA receptors plays crucial roles in fear memory reconsolidation and that intracellular signaling mechanisms activated by these receptors crosstalk with each other (for review, Tronson and Taylor, 2007; Sara, 2009). In the present study, antagonists of these receptors, PROP and MK-801, suppressed augmentation of freezing response at second exposure in the single-shock protocol, when administered in Ntsr1-KO mice just after first exposure. Therefore, there is a possibility that the activity of β-adrenergic and/or NMDA receptors is elevated in Ntsr1-KO mice. In this relation, we have previously reported that NMDA receptor activity in BLA pyramidal neurons is elevated in Ntsr1-KO mice, compared with WT mice, via disinhibition of the dopamine D_2 receptor by a deficit of Ntsr1 (Amano et al., 2008). This elevation of NMDA receptor activity causes enhanced long-term potentiation of synaptic response in these neurons (Amano et al., 2008). Because the BLA is known to play a central role in fear memory reconsolidation (Duvarci and Nader, 2004; Nader et al., 2000), electrophysiological abnormalities in the BLA of Ntsr1-KO mice may participate in the enhanced fear memory reconsolidation in Ntsr1-KO mice.

Participation of the circuit consisting of the amygdala, hippocampus, and medial prefrontal cortex (mPFC) is established in the extinction learning of fear memory (Myers and Davis, 2007; Quirk and Mueller, 2008). Because Ntsr1 is expressed in all of these brain regions (Alexander and Leeman, 1998), the lack of changes in extinction in Ntsr1-KO mice in the present study was somewhat unexpected. In particular, exogenously applied NT stimulates tetrodotoxin-sensitive release of GABA in the mPFC via Ntsr1 (Petrie et al., 2005). In this study, the release of GABA is monitored by in vivo microdialysis, and the exchanging area by dialysis is prelimbic and infralimbic regions of the mPFC. In the mPFC, the infralimbic region is important for the consolidation and retrieval of extinction, and, in contrast, the prelimbic region rather plays a role in fear memory expression in rats (Corcoran and Quirk, 2007; Milad and Quirk, 2002; Quirk et al., 2000; Vidal-Gonzalez et al., 2006). Therefore, for the interpretation of the absence of changes in extinction in Ntsr1-KO mice, whether endogenous NT is released and stimulates GABAergic interneurons in the infralimbic region of mPFC during extinction learning would be informative. Yet, the existence of a mechanism where Ntsr1 activates GABAergic

interneurons is informative for the interpretation of augmentation of fear expression in Ntsr1-KO mice because activation of $GABA_A$ receptor in the BLA decreases expression of contextual fear memory (Helmstetter and Bellgowan, 1994). A similar mechanism via Ntsr1 is also known in the globus pallidus (Ferraro et al., 1997) and is possible also in the BLA because NT/DA afferent fibers project to BLA non-pyramidal neurons (Asan, 1998). However, further investigations are needed to identify the brain region that participates in augmentation of fear expression in Ntsr1-KO mice.

We previously reported that Ntsr2-KO mice showed reduced freezing response in a test conducted 24 h after fear conditioning (Yamauchi et al., 2007). Therefore, there is a possibility that Ntsr1 and Ntsr2 functionally diverge with respect to participation in fear memory. These two receptors are both diffusely expressed in the brain, including the cerebral cortex, hippocampus, amygdala, hypothalamus, thalamus, substantia nigra, and septal region (Alexander and Leeman, 1998; Mazella et al., 1996; Moyse et al., 1987; Sarret et al., 1998), but a main receptor subtype that plays a critical role in NT-dopamine interaction is thought to be Ntsr1 (Binder et al., 2001, as review). The interaction with the dopamine system may be a main factor underlying functional divergence of Ntsr1 and Ntsr2 in fear memory. As mentioned above, pharmacological inhibition of dopamine D_2 receptors in the amygdala reduces the formation and/or consolidation of freezing responses in fear conditioning (Guarraci et al., 2000). Therefore, facilitation of freezing response in fear conditioning in Ntsr1-KO mice could be explained by the impairment of Ntsr1-induced inhibition of dopamine D_2 receptors. As for the mechanism underlying Ntsr1 inhibits D_2 receptors, it is known that allosteric Ntsr1-D_2-like receptor interaction decreases the agonist-binding affinity of D_2-like receptors (Diaz-Cabiale et al., 2002; von Euler et al., 1989).

It is noteworthy that patients with post-traumatic stress disorder (PTSD) showed higher serum prolyl endopeptidase, which is one of the NT-degradation enzymes (Maes et al., 1999). Although it has not been demonstrated, higher activity of the degradation enzyme for NT would reduce NT content in the brain, which in turn would downregulate NT-signaling via Ntsr1. Because uncontrollable augmentation of fear memory is hypothesized to be involved in anxiety-related disorders including PTSD (Amstadter et al., 2009, as review), reduction of endogenous Ntsr1-mediated signaling may participate in the pathophysiology of PTSD via augmentation of fear memory.

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