

# The 5-HT<sub>3A</sub> receptor is essential for fear extinction

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The 5-HT<sub>3</sub> receptor, the only ionotropic 5-HT receptor, is expressed in limbic regions, including the hippocampus, amygdala, and cortex. However, it is not known whether it has a role in fear memory processes. Analysis of 5-HT<sub>3A</sub> receptor knockout mice in fear conditioning paradigms revealed that the 5-HT<sub>3A</sub> receptor is not required for the acquisition or retention of fear memory but is essential for the extinction of contextual and tone-cued fear. Our data suggest that the 5-HT<sub>3A</sub> receptor could be a key molecule regulating fear memory processes and a potential therapeutic target for fear disorders.

[Supplemental material is available for this article.]

Fear is an emotion that is central to the organization of defensive behaviors in response to threat and, therefore, has an essential role in survival for animals. Unfortunately, in some cases, dysfunction in the fear system produces inappropriate and exaggerated fears that lead to psychiatric disorders, such as post-traumatic stress disorder (PTSD) (Johansen et al. 2011; Orsini and Maren 2012; Maren et al. 2013). These disorders severely affect the lives of patients and are an increasing burden on our societies. Treatment of such disorders generally involves the modulation of fear memory processes, such as promotion of fear extinction (Parsons and Ressler 2013). Therefore, understanding the molecular mechanisms underlying fear memory processes could help with the development of therapeutic strategies for fear disorders.

The 5-HT<sub>3</sub> receptor is the only ionotropic receptor in the family of 5-HT receptors (Derkach et al. 1989). The 5-HT<sub>3</sub> receptor comprises two subunits (5-HT<sub>3A</sub> and 5-HT<sub>3B</sub>), of which the 5-HT<sub>3A</sub> subunit is essential for formation of a functional receptor (Maricq et al. 1991; Davies et al. 1999). In the brain, the 5-HT<sub>3A</sub> receptor is mainly expressed on interneurons in limbic regions, such as hippocampus, amygdala, and cortex (Tecott et al. 1993; Morales et al. 1996b; Morales and Bloom 1997), suggesting its involvement in cognitive and emotional brain functions. Indeed, previous studies have indicated that the 5-HT<sub>3</sub> receptor plays roles in spatial learning and memory (Stäubli and Xu 1995; Naghdi and Harooni 2005), anxiety-like behavior (Kelly et al. 2003; Bhatnagar et al. 2004), and social behavior (Smit-Rigter et al. 2010). However, it is not known whether the 5-HT<sub>3</sub> receptor regulates fear memory processes. Therefore, to address this question, we used 5-HT<sub>3A</sub> receptor knockout (*5-ht3ar*<sup>-/-</sup>) mice and performed a contextual and tone-cued fear conditioning test. We also examined the fear extinction processes for contextual and tone-cued fear in *5-ht3ar*<sup>-/-</sup> mice.

The generation of *5-ht3ar*<sup>-/-</sup> mice was described previously (Zeitl et al. 2002). We confirmed no expression of 5-HT<sub>3A</sub> receptor in the brains of *5-ht3ar*<sup>-/-</sup> mice by RT-PCR analysis (Supplemental Fig. 1). The fear conditioning test was performed as described previously (Supplemental Methods; Kondo et al. 2012).

Because changes in pain sensitivity and abnormalities in motor activity affect the conditioning process and freezing assess-

ment, we examined whether the *5-ht3ar*<sup>-/-</sup> mutation altered nociceptive reactions to footshock or altered motor function (Kiyama et al. 1998). There were no significant differences in the current thresholds for common reactions (flinch, vocalization, and jump) to nociceptive shock (WT vs. KO [mA]: flinch, 0.1 vs. 0.1; vocal, 0.132 ± 0.012 vs. 0.138 ± 0.013, *P* = 0.7595; jump, 0.211 ± 0.014 vs. 0.207 ± 0.011, *P* = 0.8395). In addition, there were no significant differences in the observed values of spontaneous motor activity measured by means of a Supermex and a photocell beam system (Masuo et al. 1997) (WT vs. KO [counts/20 min], 5618 ± 61.86 vs. 5726 ± 84.04, *P* = 0.3134), or the latency to fall in the rotarod test (WT vs. KO [sec], 157.4 ± 17.3 vs. 165.0 ± 18.3, *P* = 0.7695) between wild-type and *5-ht3ar*<sup>-/-</sup> mice. These data indicate that *5-ht3ar*<sup>-/-</sup> mice show normal pain sensitivity, no abnormalities in spontaneous motor activity, and no motor dysfunction during rotarod performance.

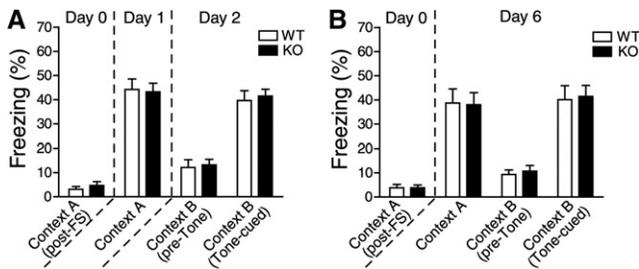
We then examined whether the 5-HT<sub>3A</sub> receptor plays a possible role in the acquisition and retention of fear memory using the fear conditioning test. On the conditioning day (Day 0), the mice showed few freezing responses, with no differences between wild-type and *5-ht3ar*<sup>-/-</sup> mice (WT vs. KO, post-first footshock, 3.15% ± 1.16% vs. 4.71% ± 1.54%, *P* = 0.4214) (Fig. 1A). After the conditioning day, we performed the contextual fear test on Day 1 and the tone-cued fear test on Day 2. There were no significant differences in contextual freezing responses under context A (Day 1) (WT vs. KO, 44.26% ± 4.30% vs. 43.24% ± 3.58%, *P* = 0.8566), or in tone-cued freezing responses under context B (Day 2) (WT vs. KO, 39.66% ± 4.07% vs. 41.50% ± 2.81%, *P* = 0.7151) between wild-type and *5-ht3ar*<sup>-/-</sup> mice (Fig. 1A). To examine the long-term retention of fear memory, we performed the contextual fear test and the tone-cued fear test after an interval of 6 d. To rule out the possibility that the previous fear tests could affect the behavior of mice in the subsequent tests on Day 6, we used another cohort of mice. There were also no significant differences in contextual freezing responses under context A (Day 6) (WT vs. KO, 38.69% ± 5.84% vs. 37.98% ± 5.07%, *P* = 0.9271), or in tone-cued freezing responses under context B (Day 6) (WT vs.

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**Figure 1.** The 5-HT<sub>3A</sub> receptor is not required for the acquisition or retention of fear memory. (A,B) Contextual and tone-cued freezing responses after conditioning (WT,  $n = 18$ ; KO,  $n = 17$  mice) (A), and both groups ( $n = 14$  mice) (B). (FS) Footshock. Means  $\pm$  SEM are shown in all histograms.

KO,  $40.08\% \pm 5.80\%$  vs.  $41.47\% \pm 4.53\%$ ,  $P = 0.8517$ ) between wild-type and *5-ht3ar*<sup>-/-</sup> mice (Fig. 1B). These data suggest that the *5-ht3ar*<sup>-/-</sup> mutation does not affect the acquisition or retention of fear memory, irrespective of the fear conditioning paradigm.

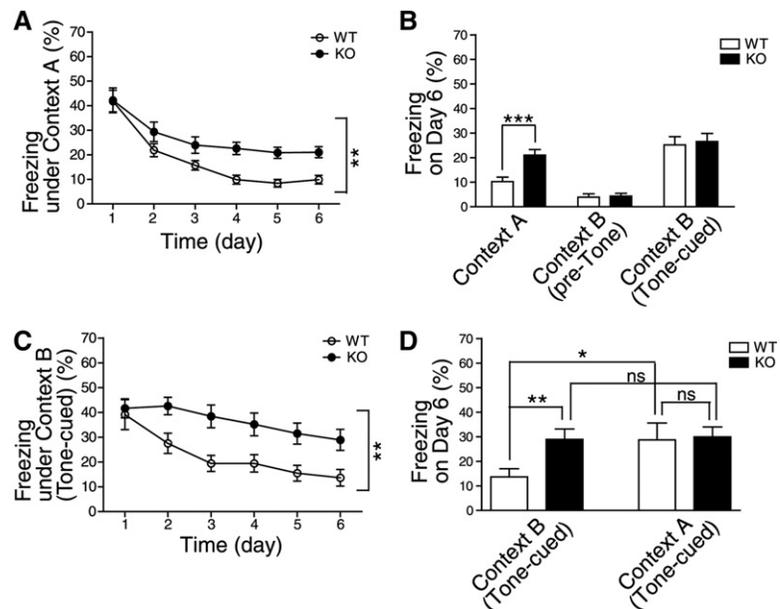
We next investigated the effects of the *5-ht3ar*<sup>-/-</sup> mutation on the processes underlying extinction of learned fear. The fear extinction protocol was performed as described in Supplemental Methods. In the first extinction paradigm, *5-ht3ar*<sup>-/-</sup> mice showed a significantly impaired reduction in freezing behavior, compared with wild-type mice (genotype,  $F_{(1,30)} = 8.016$ ,  $P = 0.0082$ ; time,  $F_{(5,150)} = 43.30$ ,  $P < 0.0001$ ; genotype  $\times$  time interaction,  $F_{(5,150)} = 2.126$ ,  $P = 0.0653$ ) (Fig. 2A), indicating that the extinction of contextual fear was impaired in *5-ht3ar*<sup>-/-</sup> mice. Meanwhile, in the post-extinction tests on Day 6, both wild-type and *5-ht3ar*<sup>-/-</sup> mice displayed similar freezing responses in a novel chamber (context B), which was different from the conditioned context (context A), following either exposure to the tone presentation or no exposure (WT vs. KO, pre-tone,  $3.89\% \pm 1.40\%$  vs.  $4.34\% \pm 1.10\%$ ,  $P = 0.8009$ ; tone-cued,  $25.2\% \pm 3.38\%$  vs.  $26.56\% \pm 3.27\%$ ,  $P = 0.7716$ ) (Fig. 2B). This suggested that the differential extinction responses between wild-type and *5-ht3ar*<sup>-/-</sup> mice were specific to the context of extinction trials. These data suggest that the 5-HT<sub>3A</sub> receptor is required for the extinction process for contextual fear.

Then, in the second extinction paradigm, *5-ht3ar*<sup>-/-</sup> mice showed a significantly impaired reduction in tone-cued freezing behavior, compared with wild-type mice (genotype,  $F_{(1,22)} = 10.78$ ,  $P = 0.0034$ ; time,  $F_{(5,110)} = 10.91$ ,  $P < 0.0001$ ; genotype  $\times$  time interaction,  $F_{(5,110)} = 1.750$ ,  $P = 0.1293$ ) (Fig. 2C), indicating that the extinction of tone-cued fear was impaired in *5-ht3ar*<sup>-/-</sup> mice. In the post-extinction tests on Day 6, both wild-type and *5-ht3ar*<sup>-/-</sup> mice displayed similar tone-cued freezing responses in context A, which was different from the context of extinction trials (context B) (WT vs. KO,  $28.70\% \pm 6.91\%$  vs.  $29.94\% \pm 4.08\%$ ,  $P = 0.8797$ ) (Fig. 2D), suggesting that the differential ex-

tingtion responses between wild-type and *5-ht3ar*<sup>-/-</sup> mice were specific to the context of extinction trials. These data suggest that the 5-HT<sub>3A</sub> receptor is required for the extinction process for tone-cued fear.

It is known that an extinguished conditional fear response reappears when the conditioned stimulus (CS) is presented in a context different from the one in which the extinction trials took place. This phenomenon is known as the renewal effect, one of the properties of context-specific extinction (Ji and Maren 2007; Myers and Davis 2007; Maren 2011; Maren et al. 2013). Wild-type mice showed significantly higher freezing responses in context A (i.e., a context different from the extinction context) than in context B (i.e., the extinction context) (context B vs. context A,  $13.66\% \pm 3.32\%$  vs.  $28.70\% \pm 6.91\%$ ,  $P = 0.0469$ ) (Fig. 2D), indicating the existence of a renewal effect. Interestingly, there was no significant difference in freezing responses between the contexts in *5-ht3ar*<sup>-/-</sup> mice (context B vs. context A,  $28.94\% \pm 4.22\%$  vs.  $29.94\% \pm 4.08\%$ ,  $P = 0.8696$ ) (Fig. 2D). These data support the idea that the 5-HT<sub>3A</sub> receptor contributes to the context-specificity of extinction processes.

In this study, we found that the 5-HT<sub>3A</sub> receptor is not required for the acquisition or retention of fear memory, but is essential for the extinction of contextual and tone-cued fear. In contrast to our findings, Park and Williams (2012) reported that systemic injection of a 5-HT<sub>3</sub> receptor antagonist (granisetron) facilitated the memory of cued and contextual fear extinction in rats. However, there are several points of difference between our experiments and theirs, which could account for the differences between the results obtained. First, the species studied differed. Park and Williams (2012) used rats in their experiments, whereas we used mice. It is known that differences in the species, strains, gender, and ages of animals used in behavioral tests can influence



**Figure 2.** The 5-HT<sub>3A</sub> receptor is required for the extinction of contextual and tone-cued fear. The extinctions of contextual (A,B) and tone-cued (C,D) fear were impaired in *5-ht3ar*<sup>-/-</sup> mice. (A,C) Mean percentage freezing time averaged every day during the fear extinction trials (Days 1–6). (\*\*)  $P < 0.01$  (genotype effect in two-way repeated-measures ANOVA). (B,D) Contextual and tone-cued freezing responses in the fear extinction and post-extinction trials on Day 6. (\*)  $P < 0.05$ , (\*\*)  $P < 0.01$ , (\*\*\*)  $P < 0.001$ , (ns) not significant (two-tailed *t*-test) (A,B, for extinction trials, both groups,  $n = 16$  mice; for post-extinction test, WT,  $n = 15$ ; KO,  $n = 16$  mice; C,D, for extinction trials, both groups,  $n = 12$  mice; for post-extinction test, both groups,  $n = 9$  mice). Mean  $\pm$  SEM shown in all histograms.

the results (Whishaw 1995; Frick et al. 2000; Ammassari-Teule and Castellano 2004; Stranahan 2011). Second, the experimental paradigms differed. Park and Williams (2012) used passive avoidance paradigms to measure conditioned contextual fear, whereas we used the fear conditioning paradigms described above. These two behavioral tasks are known to be fear learning tests; however, the requirements vary across learning tasks. Therefore, it is possible that fear learning processes (including extinction processes of learned fear) are different between the two tasks. Furthermore, as an index of conditioned fear, Park and Williams (2012) used the latency to enter the dark compartment in the passive avoidance task, whereas we have used freezing behavior in the fear conditioning test. It has been reported that different types of conditioned fear behavior (i.e., avoidance behavior of the aversive stimulus vs. freezing responses) are mediated by distinct neural systems (Killcross et al. 1997). In addition, the conditions of extinction trials are different. Park and Williams (2012) performed tone-cued extinction trials, which gave 10 presentations of the CS tone with 60-sec intertrial intervals (ITIs) within 1 d. In contrast, we performed the extinction trials over six consecutive days with 24-h ITIs. It has been reported that ITIs in extinction trials can affect the processes of fear extinction (Bouton et al. 2006). Taken together, these differences in experimental paradigms (i.e., behavioral tasks, indices of conditioned fear, and extinction trials) could account for the differences between our results and theirs. Third, to block 5-HT<sub>3</sub> receptor function, Park and Williams (2012) administered a 5-HT<sub>3</sub> receptor antagonist before the extinction trials. In contrast, we used 5-HT<sub>3A</sub> receptor knockout mice, which are devoid of 5-HT<sub>3A</sub> receptors. The methodological differences between pharmacological blockage and gene knockout of 5-HT<sub>3</sub> receptor could also have contributed to the differences between our results and theirs.

Previous reports have indicated that the 5-HT<sub>3</sub> receptor is involved in anxiety-like behavior (Kelly et al. 2003; Bhatnagar et al. 2004). To examine anxiety in *5-ht3ar*<sup>-/-</sup> mice, we performed the elevated plus maze test as described previously (Wolf and Frye 2007). The results of the elevated plus maze test (Supplemental Table 1) suggest that *5-ht3ar*<sup>-/-</sup> mice exhibit indices of decreased anxiety compared with wild-type mice, consistent with previous reports (Kelly et al. 2003; Bhatnagar et al. 2004). This difference in anxiety could have affected the performance of mice in the fear conditioning test. Our results showed that the freezing behavior either before or after the footshock on the day of conditioning of *5-ht3ar*<sup>-/-</sup> mice was comparable to that observed in wild-type mice (Fig. 1A,B). In addition, there were no significant differences in freezing behavior under no exposure to the tone presentation in a novel chamber (context B), which was different from the conditioned context, between wild-type and *5-ht3ar*<sup>-/-</sup> mice (Fig. 1A,B). These data indicate that baseline freezing responses in the fear conditioning test did not differ between wild-type and *5-ht3ar*<sup>-/-</sup> mice. However, the difference in anxiety might potentially influence the acquisition of contextual and cued fear memory and the subsequent extinction of those memories in the fear conditioning paradigms.

It is known that fear extinction is mediated by a distributed network, including the hippocampus, amygdala, and prefrontal cortex (Myers and Davis 2007; Maren 2011; Orsini and Maren 2012; Maren et al. 2013). Previous studies have shown that the 5-HT<sub>3A</sub> receptor is selectively expressed in GABA neurons in these brain areas (Morales et al. 1996a; Morales and Bloom 1997; Lee et al. 2010). Further, it has been demonstrated that activation of 5-HT<sub>3A</sub> receptors directly excites GABAergic interneurons (Kawa 1994; McMahan and Kauer 1997; Puig et al. 2004) and regulates GABA neurotransmission (Ropert and Guy 1991; Koyama et al. 2000; Katsurabayashi et al. 2003; Turner et al. 2004). Interestingly, the inhibitory neurotransmitter GABA system has

been implicated in fear extinction via modulation of neural circuits in limbic brain regions (Harris and Westbrook 1998; Makkar et al. 2010; Orsini and Maren 2012). Taken together, our results and those findings raise the possibility that the 5-HT<sub>3A</sub> receptor is involved in the processes of fear extinction through GABA neurotransmission. In addition, it was reported that the 5-HT<sub>3</sub> receptor is colocalized with cholecystokinin (CCK) immunoreactivity, and that CCK neurotransmission is highly regulated by the 5-HT<sub>3</sub> receptor (Paudice and Raiteri 1991; Férézou et al. 2002). Moreover, it was recently shown that CCK is involved in fear extinction (Joseph et al. 2013). Therefore, CCK transmission may also contribute to the 5-HT<sub>3A</sub> receptor-mediated extinction of fear memory. It has been suggested that 5-HT<sub>3</sub> receptor-containing interneurons are composed of a biochemically heterogeneous subpopulation of neurons that may be involved in different inhibitory circuits (Morales and Bloom 1997; Lee et al. 2010; von Engelhardt et al. 2011); however, the specific function of each subgroup in fear memory processes remains to be established. Therefore, detailed analysis based on the subgroups of 5-HT<sub>3</sub> receptor-expressing interneurons will provide further insight into their specific roles in the regulation of fear memory.

The process of extinction involves a complex neuronal circuitry in numerous brain regions (Myers and Davis 2007; Maren 2011; Orsini and Maren 2012; Maren et al. 2013); however, it remains to be determined what roles the 5-HT<sub>3A</sub> receptor plays in those brain areas during fear extinction, and which regions of 5-HT<sub>3A</sub> receptor expression are centrally involved in the 5-HT<sub>3A</sub> receptor-mediated extinction of fear memory. Further studies using conditional 5-HT<sub>3A</sub> receptor knockout mice should provide answers to these questions.

The processes of fear extinction share several attributes with other steps of fear memory formation (Makkar et al. 2010; Johansen et al. 2011; Maren 2011); however, there is some evidence that several cellular pathways are involved specifically in extinction, but not in the acquisition or consolidation of fear memory (Marsicano et al. 2002; Deng et al. 2009). It has been reported that PTSD is associated with impairment of fear extinction (Guthrie and Bryant 2006; Blechert et al. 2007; Parsons and Ressler 2013). Selective serotonin reuptake inhibitors (SSRIs) are considered to be first-line drug treatments for PTSD; however, it is not fully understood how serotonin neurotransmission is involved in the pathophysiology of PTSD (Sadock and Sadock 2007). This is the first study to demonstrate directly that the 5-HT<sub>3A</sub> receptor is involved specifically in the processes of fear extinction by behavioral analyses using *5-ht3ar*<sup>-/-</sup> mice. Moreover, recently, Harmer et al. (2006) reported a role for 5-HT<sub>3</sub> receptors in some elements of fear processing in humans. The 5-HT<sub>3A</sub> receptor could be a key molecule for regulation of fear extinction, and a potentially important therapeutic target for disorders of regulation in fear systems, such as PTSD.

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