Orexin Receptor-1 in the Locus Coeruleus Plays an Important Role in Cue-Dependent Fear Memory Consolidation

【総説】

第15回　高安賞最優秀論文賞受賞

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「青班核のオレキシン1受容体は手がかり依存的な恐怖記憶の形成に重要な役割を果たす」

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Orexins[1] are implicated in regulation of sleep/wakefulness, energy homeostasis, and the reward system[2]. Orexin-immunoreactive fibers are observed in almost the entire brain regions, with especially dense projections in monoaminergic nuclei in the brainstem[3]. The distribution of the two orexin receptors is consistent with these projection sites. It has been thought that orexin-2 receptor (OX2R) plays a critical role in sleep–wake regulation. Metabolic and feeding regulation by orexins is also suggested to be regulated through an OX2R-mediated pathway. However, very limited information is available regarding the physiological role of the orexin-1 receptor (OX1R). OX1R is abundantly expressed in the locus ceruleus (LC), which plays important roles in many physiological functions via its widespread projections. The LC mainly contains tyrosine hydroxylase (TH)-positive noradrenergic (NA) neurons. Because orexin neurons send rich projections to the LC, and OX1R is expressed in noradrenergic neurons in the LC, the physiological function of OX1R might be closely related to the function of NA neurons in the LC (LC^{NA} neurons). Consistently, orexin potently excited LC^{NA} neurons. There is much evidence suggesting the importance of the NA system in emotional memory formation. Conditioned fear stress caused a robust increase in the firing rate of LC^{NA} neurons and induced Fos expression. LC^{NA} neurons project to the lateral amygdala (LA), an important structure for emotional memory. These observations suggest that NA input from the LC to the LA is one of the key factors in fear memory formation. Also, orexin neurons receive input from the limbic system including amygdala, raising the possibility that orexin neurons may be activated by emotional information transmitted from the amygdala, and in turn send excitatory output to the LC, and this connection plays an important role in modulating emotional memory.

Differential role of OX1R and OX2R in fear conditioning

To evaluate the possibility that OX1R-mediated pathways are involved in expression of fear-related behavior and/or fear memory formation/acquisition, we tested $OX1R^{-/-}$ mice and $OX2R^{-/-}$ mice in cued and contextual fear conditioning tests. $OX1R^{-/-}$ mice displayed a significant decrease in freezing behavior during both cued and contextual fear tests compared with wild-type littermates, while $OX2R^{-/-}$ mice showed a decreased freezing response only in the presence of contextual stimuli. In the cued fear conditioning, $OX1R^{-/-}$ mice already showed significant impairment of freezing behavior in the learning period, while $OX2R^{-/-}$ mice also showed a tendency for a shorter freezing time, although it did not reach significance. In the conditioning session, although all groups showed a gradual increase in freezing time in response to CS paired with US, $OX1R^{-/-}$ mice showed a significantly shorter freezing time in each epoch. In the cued test period, $OX1R^{-/-}$ mice also showed a significantly shorter freezing time in the periods of both presence and absence of CS compared with wild-type littermates. In the contextual fear conditioning test, both $OX1R^{-/-}$ and $OX2R^{-/-}$ mice showed significant impairment in freezing compared to wild type mice during conditioning period. Same tendency was observed during the test session. The degree of impairment in $OX1R^{-/-}$ mice compared with wild-type mice was larger in the test session than in the conditioning period. These results suggest that the decrease of freezing behavior in $OX1R^{-/-}$ mice is due to abnormalities both in the mechanisms that evoke

fear-related behavior itself and in the formation of fear memory. While $OX1R^{-/-}$ mice showed decreased freezing behavior in both cued and contextual fear conditioning tests, $OX2R^{-/-}$ mice showed impairment only in the contextual fear-conditioning test. This suggests that OX1R is involved in both cued and contextual fear memory, while OX2R plays a role only in contextual fear memory.

Impaired response of LC^{NA} neurons of $OX1R^{-/-}$ mice after exposure to fearful situations

Since OX1R is most abundantly expressed in the LC, we examined the activity of LC^{NA} neurons, in which the largest source of NA neurons in the brain is located. In this experiment, we examined Fos expression in LC^{NA} neurons after each session of cued and contextual fear paradigms. Mice were killed 90 min after the conditioning or test and their brains were subjected to analysis. Double-labeling immunofluorescence analysis with TH and Fos antibodies revealed that the number of Fos- positive LC^{NA} neurons was very low and comparable in both genotypes in the naive condition. After the cued or contextual conditioning, the number was increased in both genotypes. However, $OX1R^{-/-}$ mice showed a significantly lower number of double-labeled cells in the LC compared with wild-type after both cued and contextual conditioning. The number of double-positive cells was also increased after the test sessions compared with basal conditions, but $OX1R^{-/-}$ mice again showed a lower response compared with wild-type. These observations suggest that an OX1R-mediated pathway activates LC^{NA} neurons in emotionally relevant situations such as formation and retrieval of fear memory.

OX1R in LC^{NA} neurons plays an important role in cued fear memory

We found that $OX1R^{-/-}$ mice showed lower activity of LC^{NA} neurons after cued or contextual fear-conditioning and tests. This suggests that OX1R-mediated activation of LC might play a role in establishing fear memory or fear expression. We next examined whether LC^{NA} neuron-specific restoration of OX1R expression in the LC could affect freezing behavior. To express OX1R specifically in LC^{NA} neurons, we used an AAV vector with the PRSx8 promoter, which directs expression of designated genes specifically in NA neurons (Fig. 1A). To examine the specific expression in LC^{NA} neurons, we confirmed the injection sites and expression of OX1R with double-labeling immunofluorescence analysis with anti-TH and anti-GFP antibodies. GFP was specifically observed in TH-positive cells (70.43 ± 15.62% of GFP-positive neurons were TH positive) in the LC (Fig. 1B). For the cued fear-conditioning test, we found that $OX1R^{-/-}$ mice with LC^{NA} neuron-specific expression of OX1R (KO-OX1R group) did not show any difference in freezing time in the conditioning session in the presence or absence of CS, compared with the control group ($OX1R^{-/-}$ mice with expression of ChR2 in LC^{NA} neurons, KO-ChR2 group)($F_{(1,13)} = 0.09$, $p = 0.7636$; Fig. 4C). However, the KO-OX1R group showed freezing behavior with a level comparable to that in wild-type controls in the test session ($F_{(1,13)} = 1.52$, $p = 0.2394$; Fig. 4D). Although both groups showed a significant increase of freezing times in response to CS presentation, the KO-OX1R group showed an increase of freezing time in the test period to a level comparable to that in wild-type mice, especially in the presence of CS, with a longer freezing time compared with that in the KO- ChR2 group ($t = 2.243$, $p = 0.0430$; Fig. 4E). These observations suggest that OX1R in LC^{NA} neurons does not play a major role in the emergence of fear-related behavior in unconditioned situations, but is likely to play an important role in consolidation, retrieval, and presentation of cue- dependent fear memory. AAV-mediated expression of OX1R in LC^{NA} neurons in wild-type mice (WT-OX1R) showed no difference in freezing response in both cued and contextual fear conditioning and testing compared with wild-type mice. Conversely, in the contextual fear conditioning test, we did not find any difference between the KO-OX1R and KO-ChR2 groups during both the conditioning and test periods ($F_{(1,12)} = 0.02$; $p = 0.8964$; $F_{(1,12)} = 0.03$; $p = 0.8711$, respectively; Fig. 4F- H). The KO-OX1R group showed significantly less freezing behavior during both the conditioning and test periods compared with wild-type ($F_{(1,22)} = 3.97$; $p = 0.0325$; $F_{(1,22)} = 4.73$; $p = 0.0407$, respectively). These results suggest that restoration of OX1R expression in the LC is not sufficient to rescue the formation of fear memory of contextual information. Restoration of OX1R expression in LC^{NA} neurons also did not affect freezing behavior during the contextual conditioning session, suggesting that the mechanisms in emergence of a behavioral response to unconditioned threats do not depend on OX1R in LC^{NA} neurons, but rather involve OX1R in other brain region (s).

OX1R in LC^{NA} neurons plays an important role in activation of the LA during fearful situations

To investigate whether OX1R in LC^{NA} neurons is involved in activation of the LA in fearful situations, we tracked the level of zif268 (Egr-1) protein expression in the LA. We analyzed zif268 expression in mice after cued or contextual fear tests. No difference was found in the level of zif268 in the LA

between genotypes in a naive condition. After cued or contextual tests, OX1R$^{-/-}$ mice showed fewer zif268-positive cells in the LA after cued test. We also observed a similar tendency in the contextual test, but the difference did not reach significance. Furthermore, rescue of OX1R in LC^{NA} neurons in OX1R$^{-/-}$ mice increased Fos expression in the LC and zif268 protein in the LA after cued testing, to levels comparable to those in wild-type mice. In contrast, ChR2 expression did not show any effect. OX1R rescue in LC^{NA} neurons did not normalize zif268 protein expression after the contextual test, although it increased the number of Fos-positive TH neurons in the LC. These observations suggest that OX1R in LC^{NA} neurons plays an important role in activation of the LA in response to an explicit cue, presumably through NA projections to the LA. However, this system is not sufficient to lead to LA activation in response to emotionally relevant contextual information.

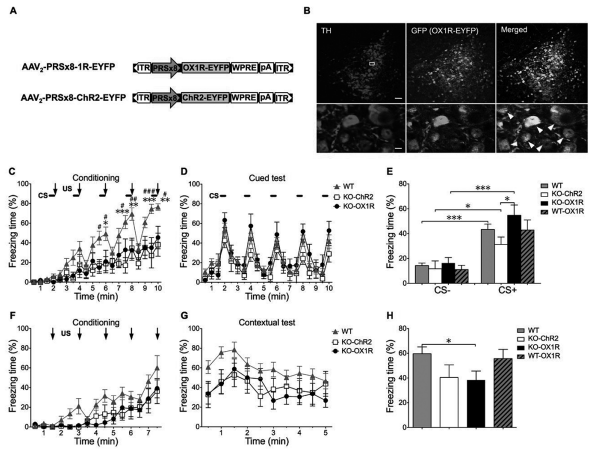

Figure 1. OX1R in the LC is involved in consolidation of cued fear memory. A, Constructs of recombinant AAV vectors carrying OX1R for rescued group or ChR2 for control group. B, A representative image of brain sections of AAV-OX1R-EYFP injected group labeled with TH and GFP. Immunoreactivity for TH is shown in red, while that of GFP (OX1R-EYFP) is shown in green. Arrows show examples of co-localization (yellow in merged images). Scale bars: 50 μm (top), 10 m (bottom). C, Freezing time of OX1R$^{-/-}$ mice with ChR2 expression in LC^{NA} neurons (KO-ChR2, n = 7) and with OX1R expression in LC-NA neurons (KO-OX1R, n = 8) during the cued fear-conditioning period. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, wild-type (WT) vs KO-OX1R, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, and WT vs KO-ChR2. D, Effects of OX1R injection on freezing time during test period of cued fear conditioning. E, Graphic representation of results in D. We also expressed OX1R in LC^{NA} neurons in WT mice (WT-OX1R, n = 7). F, There was no significant difference between KO-ChR2 (n = 7) and KO-OX1R (n = 7) groups in freezing time during the conditioning period. G, Effect of restored expression of OX1R in LC on freezing behavior during the test period of contextual fear conditioning compared with the control group. H, Graphic representation of the results in G. We also expressed OX1R in LC^{NA} neurons in WT mice (WT-OX1R, n = 7). Data are shown as mean ± SEM * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

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LH^{orexin}-LC^{NA}-LA circuit mediates behavioral fear expression

We reported that LC^{NA} neurons, which abundantly express orexin receptor-1 (OX1R), are involved in the expression and/or consolidation of cued fear memory via activation of the downstream LA neurons. These results suggest the potential role of LH^{orexin} neurons in the recruitment of LA-projecting LC^{NA} (LC^{NA} → LA) neurons to control expression of fear-related behavior. However, the role of this pathway on the behavioral fear expression itself, rather than consolidation of fear memory, has not been previously addressed. LC^{NA} neurons have divergent input/output architecture and are involved in various physiological functions such as stress response, attention, arousal, cognitive function, and reward-seeking behavior. In addition, NA transmission is involved in anxiety and phobia. In the rodent LA, noradrenaline is known to be a critical modulator of fear memory formation via-adrenergic receptor activation (βARs)[4]. While it had been suggested that the LH is not involved in Pavlovian fear learning, recent data demonstrate that LH^{orexin} neurons, which send dense projections to the LC, play an important role in fear memory learning and consolidation[5], as well as fear memory extinction[6]. From these findings, we hypothesized that LH^{orexin} neurons, which respond to emotional stimuli, can also modulate the expression of fear via noradrenergic signaling governed by LC^{NA} neurons projecting to the LA.

Role of OX1R in LC^{NA} neurons in fear expression

Mice with a global deletion of the OX1R gene demonstrated decreased freezing behavior in both cued and contextual fear tests, and focal expression of OX1R only in LC^{NA} neurons completely rescued the abnormalities in cued fear memory consolidation. Here, using mice lacking the OX1R receptor specifically in LC^{NA} neurons (*OX1R$^{f/f}$; NAT-Cre* mice), we first confirmed the role of OX1R in LC^{NA} neurons in cued fear-conditioning test with extended cue presentation (150 s), because we found this condition robustly activates LC^{NA} neurons. While both genotypes showed similar freezing levels during training (*OX1R$^{F/F}$*, n = 8; *OX1R$^{F/F}$; NAT-Cre*, n = 12: Two-Way RM ANOVA with Sidak's post-hoc test, $F_{(1, 18)} = 2.673$, $p = 0.1194$, Fig. 1c), when tested the next day *OX1R$^{f/f}$; NAT-Cre* mice showed a significant deficit in freezing (Two-Way RM ANOVA, Sidak's post-hoc test, $F_{(1, 18)} = 7.177$, $p = 0.0153$, Fig. 1d-left) during CS presentation. This phenotype of *OX1R$^{f/f}$; NAT-Cre* was highly similar to the findings in global *OX1R$^{-/-}$* mice in our previous work and suggests that OX1R in LC^{NA} neurons plays a major role in fear

memory consolidation and/or expression. To examine whether the function of OX1R is necessary particularly during the fear memory retrieval period, we next used acute pharmacological blockade by a selective OX1R antagonist (SB334867)(Fig. 1e). One day after fear conditioning, in which freezing was comparable between genotypes (Vehicle, n = 13; SB334867, n = 13, Two-Way RM with Sidak's post-hoc test, $F_{(1, 24)}$ = 1.086, p = 0.3077, Fig. 1f), mice receiving an intraperitoneal (i.p.) injection of SB334867 (5 mg/kg) 1 hour before the test session. The effect of the antagonist was not evident for first 30 s of CS presentation, but we observed dramatic reduction in freezing behavior for the remainder of the tone (Two-Way RM ANOVA with Sidak's post-hoc test, $F_{(1, 24)}$ = 11.47, p = 0.0024, 3.5-5 min in Fig. 1g-left). Average freezing during CS period also showed a significant reduction as compared with mice receiving vehicle (unpaired two-tailed Student's t-test, t = 4.411, p = 0.0002, Fig.1g-right). These results demonstrate that OX1R activation modulates the LC to sustain behavioral fear expression. In other words, animals cannot maintain the appropriate behavioral expression of fear without the function of OX1R.

Concluding remarks

We found a prominent role of OX1R in cued fear conditioning and retrieval. Our results suggest that OX1R expressed in several regions of the brain plays different roles in fear-related behavior and memory through independent mechanisms. Especially, the orexin system is involved in the consolidation of cue-dependent fear memory via OX1R expressed in LC^{NA} neurons. OX2R may also be involved in establishing contextual fear memory, suggesting a further complex mechanism involving the hippocampus or other OX2R expressing regions. In addition, we found that OX1R expressed in LC^{NA} neurons plays an important role in sustaining cue-induced behavioral fear expression, suggesting the mechanism of establishing and retrieving fear memory critically involves the orexin system. These findings might contribute to further understanding of the neural mechanism of fear memory, and suggest the inhibition of OX1R is a promising avenue for treating psychiatric conditions with exaggerated and/or inappropriate fear-related responses triggered by external cues, such as panic disorder and PTSD.

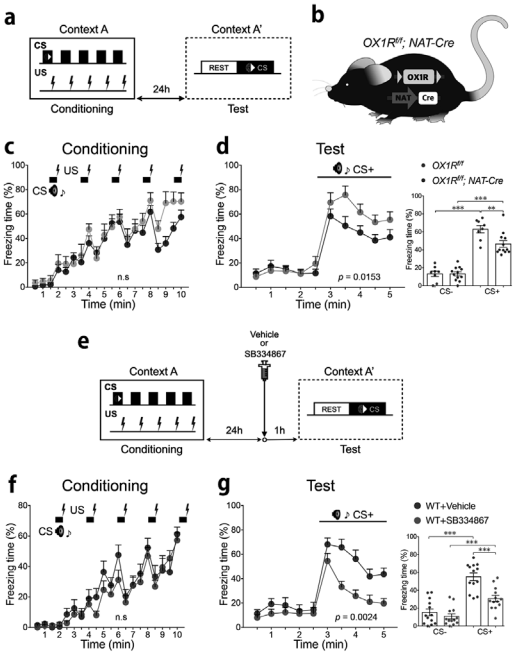

Figure 2. Role of OX1R in LC^{NA} neurons in cued fear memory retrieval. (a) Schematic drawing of experimental protocol. (b) *LoxP* sites are introduced in the *OX1R* allele in *OX1R*$^{f/f}$ mice to delete exons 5 and 6 by Cre-mediated recombination. (c, d) After cued fear conditioning, cell type-selective deletion of OX1R in LC^{NA} neurons reduced freezing responses in test session. (e) Experimental protocol for pharmacological blockade of OX1R by OX1R antagonist (SB334867) in cued fear conditioning paradigm. (f, g) After cued fear conditioning, i.p. administration of SB334867 1 hour before the test session significantly attenuated cued fear memory retrieval. ** p < 0.01, *** p < 0.001. Values are mean ± SEM.

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Profile

2016年6月　金沢大学大学院医薬保健学総合研究科博士課程修了
2016年7月　日本学術振興会特別研究員(PD)
2017年4月～　筑波大学 国際統合睡眠医科学研究機構

現在、恐怖記憶の形成および想起におけるオレキシンの役割を薬理遺伝学や光遺伝学を用いて解析している。