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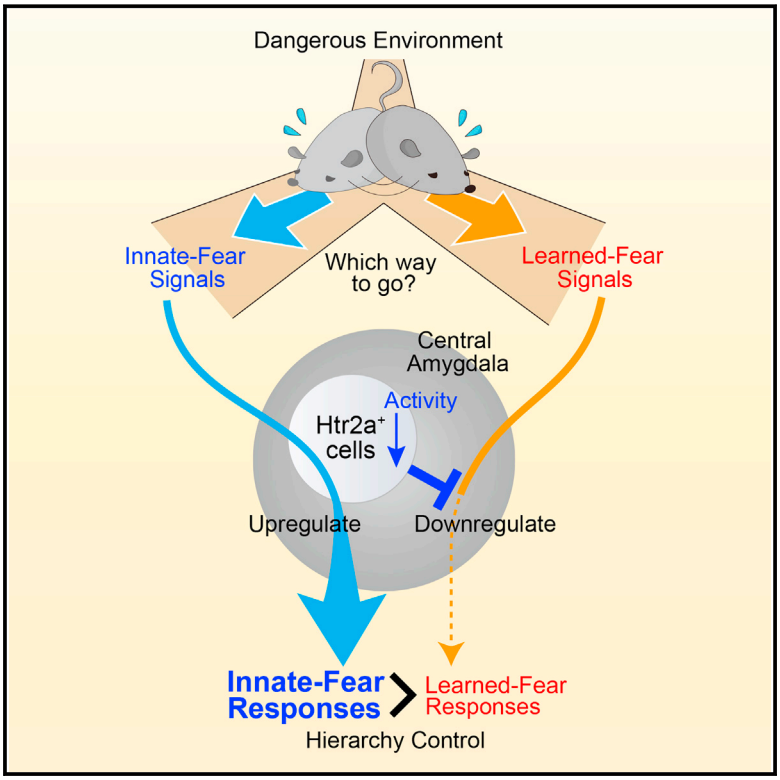
Htr2a-Expressing Cells in the Central Amygdala Control the Hierarchy between Innate and Learned Fear

Graphical Abstract



Highlights

- A hierarchical relationship exists between innate- and learned-fear responses
- Innate but not learned-fear stimuli suppress the activity of CeA Htr2a$^+$ cells
- CeA Htr2a$^+$ cell inhibition up/downregulates innate/learned freezing, respectively
- CeA Htr2a$^+$ cells act as a hierarchy generator prioritizing innate over learned fear

Authors

Tomoko Isosaka, Tomohiko Matsuo, Takashi Yamaguchi, Kazuo Funabiki, Shigetada Nakanishi, Reiko Kobayakawa, Ko Kobayakawa

Correspondence

kobayakr@hirakata.kmu.ac.jp (R.K.), kobayakk@hirakata.kmu.ac.jp (K.K.)

In Brief

The integration of innate and learned information processing is fundamental to controlling behavior. A population of serotonin 2A receptor-expressing cells in the central amygdala acts as a hierarchy generator by prioritizing innate over learned fear.

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Htr2a-Expressing Cells in the Central Amygdala Control the Hierarchy between Innate and Learned Fear

Tomoko Isosaka,[1,2] Tomohiko Matsuo,[1,2] Takashi Yamaguchi,[3] Kazuo Funabiki,[3] Shigetada Nakanishi,[3] Reiko Kobayakawa,[1,2,*] and Ko Kobayakawa[1,2,*]

[1]Institute of Biomedical Science, Kansai Medical University, Osaka 573-1010, Japan

[2]Department of Functional Neuroscience, Osaka Bioscience Institute, Osaka 565-0874, Japan

[3]Department of Systems Biology, Osaka Bioscience Institute, Osaka 565-0874, Japan

*Correspondence: kobayakr@hirakata.kmu.ac.jp (R.K.), kobayakk@hirakata.kmu.ac.jp (K.K.)

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SUMMARY

Fear is induced by innate and learned mechanisms involving separate pathways. Here, we used an olfactory-mediated innate-fear versus learned-fear paradigm to investigate how these pathways are integrated. Notably, prior presentation of innate-fear stimuli inhibited learned-freezing response, but not vice versa. Whole-brain mapping and pharmacological screening indicated that serotonin-2A receptor (Htr2a)-expressing cells in the central amygdala (CeA) control both innate and learned freezing, but in opposing directions. In vivo fiber photometry analyses in freely moving mice indicated that innate but not learned-fear stimuli suppressed the activity of Htr2a-expressing CeA cells. Artificial inactivation of these cells upregulated innate-freezing response and downregulated learned-freezing response. Thus, Htr2a-expressing CeA cells serve as a hierarchy generator, prioritizing innate fear over learned fear.

INTRODUCTION

Behaviors are controlled by innate and learned mechanisms. How the brain determines the appropriate behavior when both innate and learned sensory inputs are simultaneously presented is of great interest. Fear is a powerful emotion that greatly influences behaviors across species and can be induced by both innate and learned sensory inputs (Gross and Canteras, 2012; LeDoux, 2012). Both types of fear can be experienced simultaneously, in situations such as exposure to dangerous natural environments, raising the possibility that the integration of information processed by innate- and learned-fear pathways contributes to the selection of appropriate behaviors to promote organism survival. However, the cellular and molecular mechanisms underlying this process are not clear.

Although various therapeutic interventions have been tested, a considerable number of people experience fear- and anxiety-related disorders, such as phobia, posttraumatic stress disorder, and obsessive-compulsive disorder (Kessler et al., 2005; Dias et al., 2013). The pathogenesis and symptoms of these disorders are considered to be affected by innate and learned mechanisms (Rosen et al., 2008; Parsons and Ressler, 2013), but the precise contribution of each mechanism is still not understood. If innate and learned fears are controlled by synergistic mechanisms, administration of drugs that inhibit either fear mechanism is expected to alleviate advanced abnormal fear emotion. However, if both fears are controlled by antagonistic mechanisms, drugs that alleviate only one fear can aggravate the other fear, leading to paradoxical results. Thus, to formulate an effective drug discovery strategy, it is important to clarify the molecular targets that determine the relationship between innate and learned fears.

In mice, olfaction is the most important sensory system for detecting danger (Rottman and Snowdon, 1972; Apfelbach et al., 2005). Unlike other sensory systems, olfaction is unique in that different types of odorant molecules can induce either innate or learned-fear responses in mice. Thus, in this study, we focused on the olfactory system to clarify interaction mechanisms of innate- and learned-fear processing.

The amygdala is thought to be central to the mediation of both innate and learned fear. The amygdala consists of several subnuclei with distinct connections and functions. Like other sensory modalities, olfactory-mediated learned-fear information is conveyed to the lateral/basolateral amygdala (LA/BLA). Disruption of these nuclei affects learned-freezing behavior induced by olfaction (Cousens and Otto, 1998). Although it has not been reported for olfaction, auditory and visual conditioned information processed in the LA/BLA are then relayed to the CeA, which then regulates multiple fear responses (LeDoux, 2000; Davis, 2000; Maren and Quirk, 2004). 2,4,5-trimethyl-3-thiazoline (TMT) is a component of secretion products from the anogenital gland of foxes and induces innate-fear responses in mice through the main olfactory pathway (Vernet-Maury et al., 1984; Fendt et al., 2005; Kobayakawa et al., 2007). Presentation of TMT to mice upregulates the expression of immediate early genes (IEGs) in the medial amygdala (MeA) and the CeA (Day et al., 2004). Thus, among the subnuclei of the amygdala, the CeA is a candidate site involved in the interaction of olfactory-mediated innate- and learned-fear information. However, the cellular and molecular targets in the CeA underlying this interaction are not known.

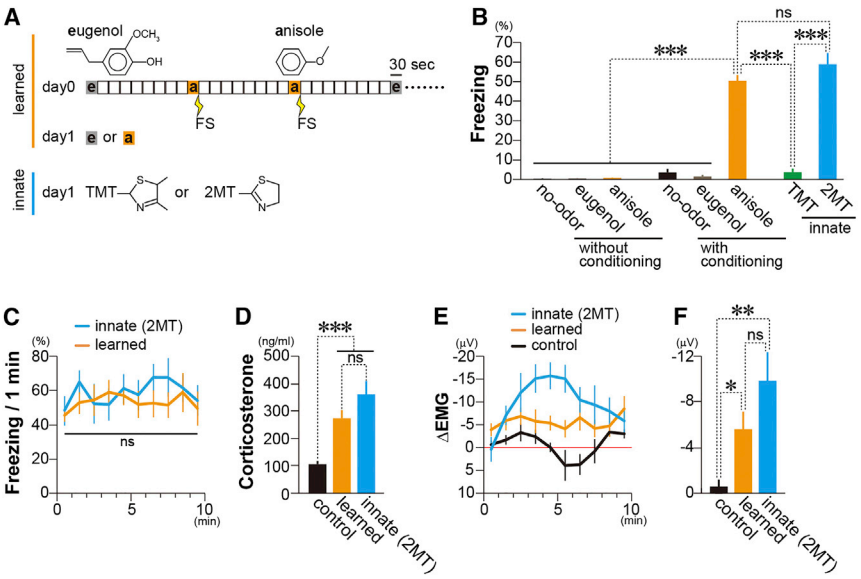

Figure 1. Odor-Induced Innate- and Learned-Freezing Behavior

(A) Induction methods of innate- and learned-freezing responses. Learned freezing was induced as follows: On day 0, eugenol (e) or anisole (a) was randomly presented for 30 s with a 4 min interval, and at the end of anisole presentation, electric foot shocks (FS) were delivered to mice. On day 1, eugenol or anisole was presented to the mice. For induction of innate freezing, TMT or 2MT was presented on day 1.

(B) The mean percentage of time spent freezing is shown for the no-odor control, eugenol, and anisole, with or without conditioning, and for innate-fear-inducing odorants (TMT and 2MT). Anisole previously paired with foot shocks and 2MT presentation induced robust freezing behavior.

(C) Temporal analyses of 2MT-induced innate- and learned-freezing responses indicate that the two freezing behaviors are indistinguishable.

(D) The mean levels of plasma corticosterone induced by no-odor (control), learned-freezing-inducing, and innate-freezing-inducing (2MT) odorants.

(E and F) The temporal changes (E) and the mean (F) of delta neck electromyography (ΔEMG) are shown for control odor (a spice odor, eugenol), learned-freezing-inducing, and innate-freezing-inducing (2MT) odorants. The mean EMG value prior to odor presentation was set to 0.

(B, D, and F) One-way ANOVA followed by Bonferroni correction. (C) Unpaired t test. Data are means ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, $p > 0.05$.

To clarify the relationship and potential integration mechanisms between innate- and learned-fear information processing, we developed an olfactory-mediated innate-fear versus learned-fear paradigm and established an anatomical, molecular, and cellular framework for the integration of innate- versus learned-fear information.

RESULTS

Induction of Innate- and Learned-Freezing Behavior by Olfaction

Freezing is a characteristic behavior that is closely linked to fear in mice and other species. It can be measured as the ratio of immobile time during a test period and is widely used as a quantitative marker for fear in various experimental paradigms (Blanchard and Blanchard, 1969; Bouton and Bolles, 1980; Bolles and Fanselow, 1980). A learned-freezing response is easily induced by pairing a neutral odorant with electric foot shocks (Figures 1A and 1B). Although the odorant-mediated induction of a potent innate-freezing response, comparable to that induced in learned freezing, was previously considered difficult, we recently developed potent innate-freezing inducers termed thiazoline-related fear odors (tFOs) (Kobayakawa and Kobayakawa, 2011) that enabled us to overcome this challenge. TMT is a widely used odorant molecule that induces innate-fear responses in rodents (Fendt et al., 2005; Takahashi et al., 2005). However, the level of innate-freezing response it induces is extremely weak compared to that induced in the learned-fear condition (Figures 1A and 1B) (Morrow et al., 2000; McGregor et al., 2002). tFOs were developed by optimization of the chemical structure of TMT. From our tFO catalog, we selected 2-methyl-2-thiazoline (2MT), which induces a level of freezing comparable to that induced in the learned condition (Figures 1A and 1B).

First, we used several fear indices to confirm whether 2MT actually induces fear responses comparable to those induced in the learned-fear condition. There were no significant differences in temporal patterns between the two freezing behaviors (Figure 1C). In addition to inducing freezing behavior, increases in plasma concentration of stress hormones and decreases in neck electromyography are used as fear indices (Steenland and Zhuo, 2009; Armario et al., 2012). Again, no significant differences were observed between 2MT-induced innate-fear and learned-fear conditions in terms of these fear indices (Figures 1D–1F). From these results, we concluded that 2MT induces innate-fear responses that are comparable to the learned-fear condition in terms of behavioral and physiological aspects. Thus, utilizing 2MT enabled us to fulfill the ideal experimental conditions for comparing the nature of innate and learned fear, in which both fear responses are induced by the same sensory modality and accompanied by the same behavior.

The Innate-Fear Response Is Prioritized over the Learned-Fear Response

Even under high-risk conditions, where innate- and learned-fear signals exist simultaneously, starving wild animals have to keep exploring to find food for survival. We reconstructed similar situations using two different behavior tests and explored the mutual effects of innate- and learned-fear inputs on behavioral outputs (Figure 2). Mice were classified into three groups. Food pellets were placed at both ends of two isles of a Y-maze, and either an innate-fear-inducing odorant or a learned-fear-inducing odorant, which had previously been linked to electric foot shocks, was presented in one aisle for the first and second groups, respectively. Then, food-deprived mice were placed at the maze entrance. Under these conditions, eating behavior

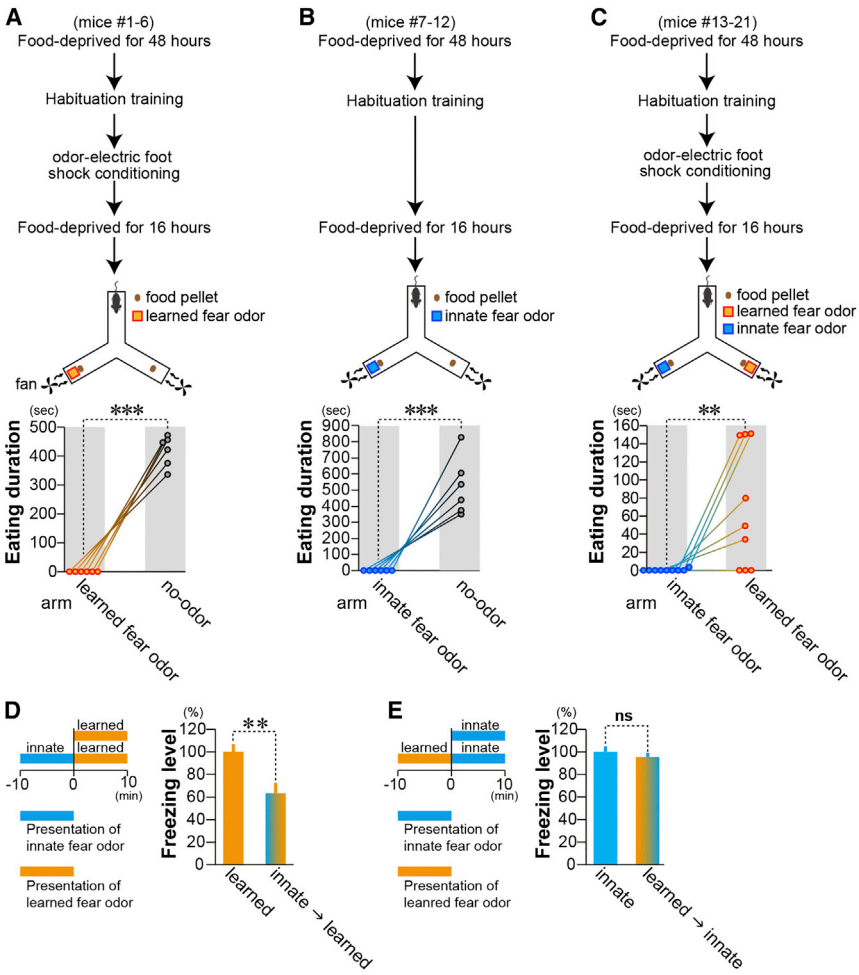

Figure 2. Hierarchical and Antagonistic Relationships between Innate- and Learned-Fear Responses

(A–C) Timelines of Y-maze experiments are shown in upper panels. Time spent eating by the individual mouse in each aisle is plotted (lower panels). Data points for the same individual are linked by a line.

(D and E) The mean percentages of time spent in learned freezing with and without prior induction of innate freezing (D) and spent in innate freezing with and without prior induction of learned freezing (E) are shown. The levels of freezing without prior induction of the other type of freezing were set at 100%. The experimental procedures are also shown in the left panels.

(A–C) Paired t test. (D and E) Unpaired t test. Data are means + SEM. $^{**}p < 0.01$; $^{***}p < 0.001$; ns, $p > 0.05$.

was completely suppressed in mice in both aisles in which a fear-inducing odorant was presented but not in the odor-free aisle (Figures 2A and 2B). For the third group, the learned-fear-inducing odorant that had previously been linked to electric shocks was presented in one aisle and the innate-fear-inducing odorant was presented in the other. Under this condition, we observed eating behavior in the aisle where the learned-fear-inducing odorant was presented, but this behavior was almost fully suppressed in the aisle where the innate-fear-inducing odorant was presented (Figure 2C). These results indicate that innate fear is prioritized over learned fear, at least under this condition.

Our findings also suggest that innate-fear-inducing odorants might suppress learned-fear behavior. To examine this possibility, we analyzed the effects of the sequential presentation of innate- and learned-fear-inducing odorants. Interestingly, prior presentation of an innate-fear-inducing odorant significantly decreased the learned-freezing response (Figure 2D). Conversely, prior induction of learned freezing did not affect the innate-freezing response (Figure 2E). These results suggest that the behavioral response to the presence of innate- and learned-fear stimuli is controlled through a hierarchical relationship in which innate fear predominates.

Serotonin 2A Receptors in the Central Amygdala Have Opposite Effects on Innate and Learned Freezing

We next aimed to clarify the cellular and molecular bases of this hierarchical control mechanism. To identify candidate molecules involved in the hierarchical control of innate- and learned-freezing responses, various antipsychotropic agents that affect different neurotransmitter systems were injected intraperitoneally (IP) in mice, and their effects on innate- and learned-freezing responses were analyzed (Figure 3A). Notably, injection of the atypical antipsychotic risperidone led to significantly downregulated learned freezing and significantly upregulated innate-freezing responses. The serotonin 2A receptor (Htr2a; Roth et al., 1998) is the major antagonistic target of risperidone, but the drug also affects the dopamine D2 receptor and other molecules, although with lower affinities (Binding DB: http://bindingdb.org/bind/index.jsp). Thus, we also analyzed the effects of IP injection of the Htr2a-selective antagonist glemanserin (Dudley et al., 1988) and observed the same results as those induced by risperidone (Figure 3B). This indicates that Htr2a controls both innate and learned freezing, but in opposing directions.

Next, a putative interaction site for innate- and learned-fear pathways was selected by whole-brain activity mapping of innate and learned freezing using mRNA expression levels of the immediate-early genes (IEGs) *arc* and *c-fos* as neuronal activity markers. We found that IEG expression levels were markedly upregulated in several brain regions, including the amygdala, extended amygdala, lateral septum, and hypothalamus during both innate and learned freezing.

In this study, we focused on the amygdala because it is the area most implicated in the processing of fear (LeDoux, 2000; Maren and Quirk, 2004). The amygdala is subdivided into anatomically

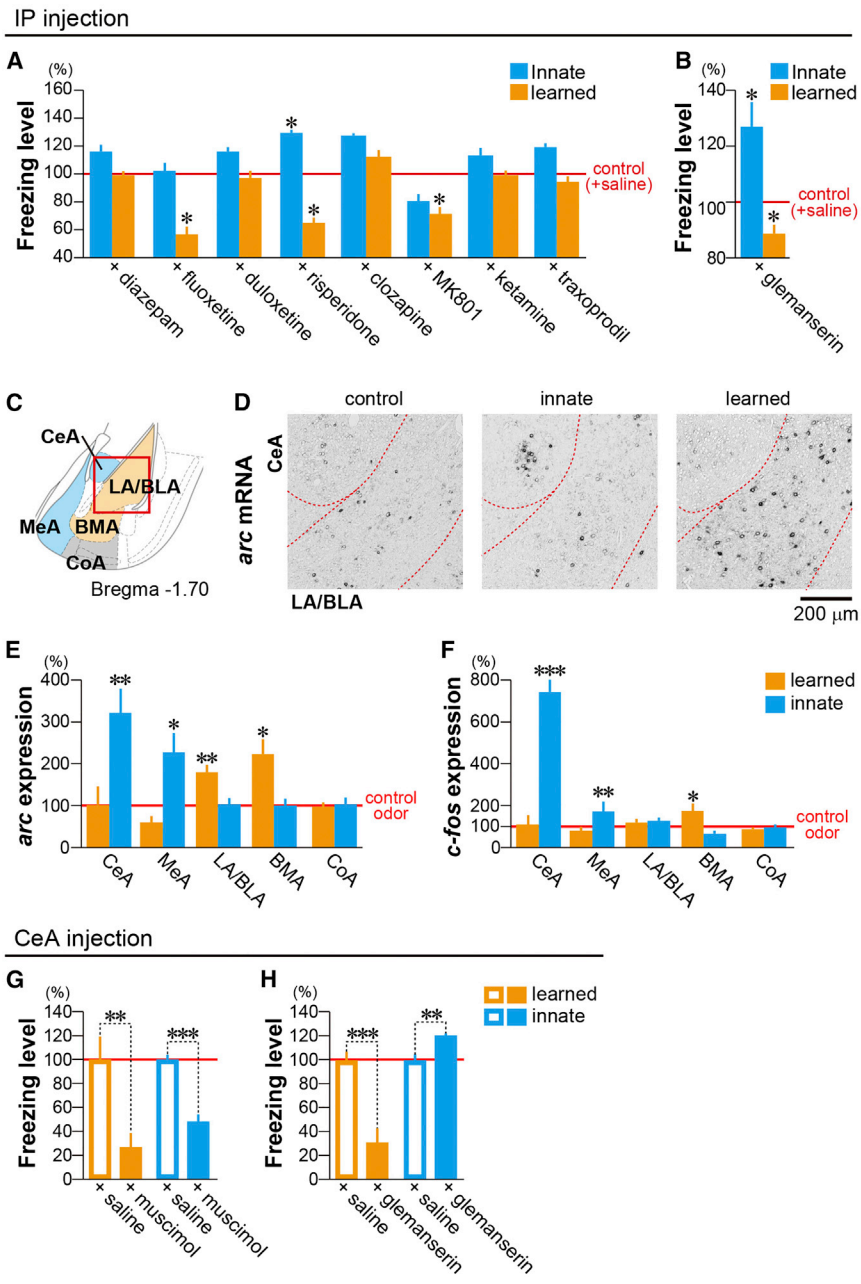

Figure 3. Screening for Molecules and Target Sites Involved in Differential Regulation of Innate and Learned Freezing

(A) The effect of intraperitoneal (IP) injection of psychotropics compared with that of saline (control value set at 100%) are shown for innate (blue) and learned (orange) freezing.

(B) The effect of IP injection of glemanserin compared with that of saline (control value set at 100%) is shown for innate (blue) and learned (orange) freezing.

(C) Schematic illustration of the structural organization of the amygdala. Blue and orange shaded areas represent areas in which *arc* mRNA was upregulated in the innate- and learned-freezing conditions, respectively.

(D) Representative images of in situ hybridization of *arc* mRNA following exposure to control, innate-, or learned-fear-inducing odorants. Scale bar, 200 μm.

(E and F) Levels of *arc* (E) and *c-fos* (F) mRNA following exposure to innate (blue) and learned (orange) fear-inducing odorants, as compared to control odorant (control values set at 100%).

(G and H) The CeA was infused with muscimol (G) or glemanserin (H), and the freezing levels were compared to the level seen in saline-infusion controls (control values set at 100%) for innate (blue) and learned (orange) freezing.

(A) One-way ANOVA and unpaired t test. (B, G, and H) Unpaired t test. (E and F) One-way ANOVA followed by Bonferroni correction. Data are means + SEM. *p < 0.05; **p < 0.01; ***p < 0.001. See also Figure S1.

defined subnuclei (Figure 3C) (Pitkänen et al., 1997). The LA/BLA, basomedial amygdala (BMA), and CeA have been reported to be involved in the regulation of learned freezing (LeDoux, 2000; Maren and Quirk, 2004). The MeA and cortical amygdala (CoA) are considered to play crucial roles in regulating fear-related innate behaviors, such as risk-assessment and avoidance behaviors induced by predator odors (Li et al., 2004; Martinez et al., 2011; Root et al., 2014). Our analyses revealed that *arc* mRNA expression was significantly upregulated in both the MeA and CeA in innate-freezing mice, whereas it was significantly upregulated in the LA/BLA and BMA in learned-freezing mice (Figures 3D and 3E). Similar results were obtained for *c-fos* (Figure 3F).

Previous studies have also shown that IEGs are not upregulated in the CeA during the learned-freezing condition (Pezzone et al., 1992; Campeau et al., 1997). Nevertheless, it is widely accepted that the CeA regulates the expression of learned freezing (Medina et al., 2002). Electric ablation of the CeA decreases secretion of adrenocorticotropic hormone (ACTH) that is induced by forced immobilization stress (Beaulieu et al., 1986) and decreases tone-enhanced excitability of the nictitating membrane response (Weisz et al., 1992). These results indicate that the CeA also contributes to the regulation of innate responses induced by various fear-related stimuli. In this study, we first confirmed the possibility that the CeA regulates both innate- and learned-freezing behavior induced by olfaction. Stereotaxic injection of muscimol, a gamma-aminobutyric acid (GABA)-A receptor agonist, into the CeA significantly downregulated both innate and learned freezing, indicating that the CeA is involved in controlling both behaviors (Figures 3G and S1A). This result suggests that the CeA potentially works as an integrator of odor-induced innate- and learned-fear information.

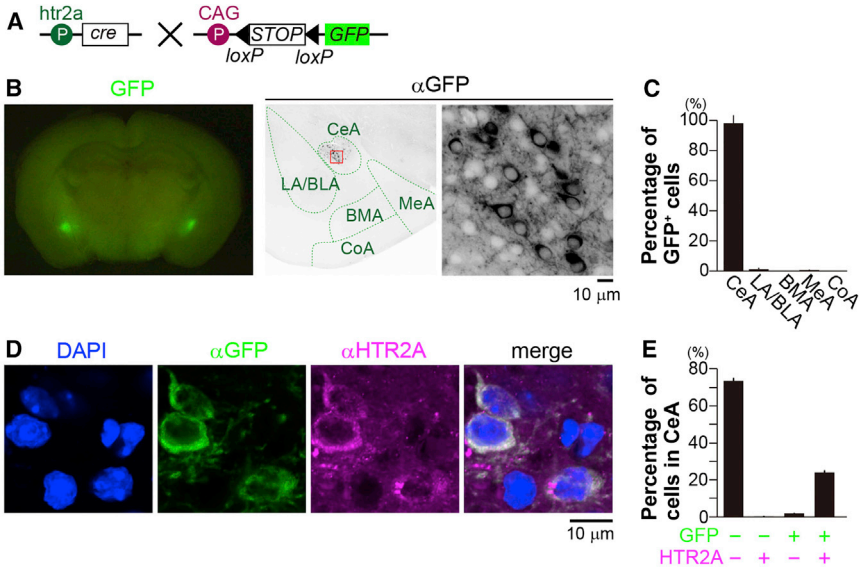

Figure 4. Expression Analysis of *Cre* in *Htr2a-Cre* BAC Transgenic Mice

(A) Strategy for selective labeling of CeA Htr2a$^+$ cells using *Htr2a-Cre* and *floxed-GFP* mice.

(B and C) Transgene expression, visualized by GFP immunofluorescent labeling, is shown (B). A low-magnification view is shown in the left panel. The area in the red box in the middle panel is enlarged in the right panel. The percentage of GFP$^+$ cells in amygdala subnuclei is shown (C).

(D and E) Transgene expression was compared with endogenous HTR2A expression detected by anti-HTR2A antibodies (D). Quantifications of the HTR2A ± and GFP ± cells are shown (E).

Data are means + SEM. Scale bars, 10 μm. See also Figure S2.

Next, we analyzed the function of the Htr2a receptor in the CeA for induction of innate- and learned-freezing behavior. Stereotaxic injection of glemanserin into the CeA significantly downregulated the learned-freezing and upregulated the innate-freezing response (Figures 3H and S1B). This suggests the possibility that the CeA is one of the major target sites for the differential control of innate and learned freezing mediated by Htr2a. However, it is difficult to confirm that neuronal inactivation is limited to the CeA using pharmacological methods. To overcome this challenge, we utilized Cre/loxP technology to manipulate Htr2a-expressing cells in the CeA (CeA Htr2a$^+$ cells).

Induction of *Cre* Gene Expression in CeA Htr2a$^+$ Cells

In many biological contexts, Htr2a increases neural activities by coupling with Gq (Roth et al., 1998). Therefore, injection of glemanserin is likely to decrease the activity of Htr2a-expressing neurons. Thus, it is possible that inactivation of CeA Htr2a$^+$ cells upregulates the innate-freezing response and downregulates the learned-freezing response. To test this hypothesis at the cellular level, we obtained *Htr2a-Cre* BAC transgenic mice (GENSAT, line KM208) in which Cre is selectively expressed in the CeA. We crossed *Htr2a-Cre* mice with floxed green fluorescent protein (GFP) mice to confirm Cre expression (Figure 4A). GFP signals were detected in the CeA, but not in the LA/BLA, BMA, MeA, or CoA (Figures 4B and 4C). GFP expression was compared with that of endogenous HTR2A using anti-HTR2A antibodies. Almost all GFP-positive cells were also HTR2A-positive, and almost all GFP-negative CeA cells were HTR2A-negative (Figures 4D and 4E). Therefore, Cre expression correctly recapitulated the pattern of endogenous Htr2a in the CeA. Detailed anatomical analyses indicated that CeA Htr2a$^+$ cells were mainly located in the CeL, and the majority of these cells co-expressed somatostatin (SOM) but not protein kinase C-δ (PKCδ) (Figure S2).

Innate- but Not Learned-Fear Stimuli Suppress the Activity of CeA Htr2a$^+$ Cells

To monitor the neuronal activity of CeA Htr2a$^+$ cells in parallel with the behavioral response in mice, in vivo photometry using a fiber-bundle probe was performed in freely moving mice (Goto et al., 2015). *Htr2a-Cre* mice were injected with a Cre-dependent adeno-associated virus (AAV) encoding GCaMP6. At 3 weeks after injection, a fiber-bundle probe was stereotaxically implanted above the CeA to monitor the GCaMP6 signal in the CeA (Figures 5A and 5B). GCaMP6 transients were occasionally observed without odor presentation, but these were absent in mice without AAV infection (Figure 5C). GCaMP6 transients were significantly reduced in the innate-freezing condition compared to the no-odor condition, but they were not significantly changed in the learned-freezing condition (Figures 5C, 5D, 5F, and 5G). Freezing levels were not significantly different between the innate- and learned-fear conditions in the photometry sessions analyzed (Figure 5E), suggesting that the observed difference in GCaMP6 transients was not due to motion artifacts. Glemanserin administration reduced GCaMP6 transients (Figures 5H and 5I), confirming that neuronal activity in CeA Htr2a$^+$ cells is controlled by Htr2a. These results, combined with our pharmacological analysis (Figure 3H), indicate that the activity of CeA Htr2a$^+$ neurons is downregulated by innate-fear-inducing odorants, which would result in upregulation of the innate-freezing response and downregulation of the learned-freezing response.

Pharmacogenetic and Optogenetic Regulation of CeA Htr2a$^+$ Cells

To confirm the possibility described above, we utilized designer receptors exclusively activated by a designer drug (DREADD) (Alexander et al., 2009) to artificially control the activity of CeA Htr2a$^+$ cells. A Cre-dependent AAV encoding hM3Dq (a chemogenetic activator) fused with mCherry (AAV-DIO-hM3Dq), or hM4Di (a chemogenetic silencer) fused with mCherry (AAV-DIO-hM4Di), was injected into the bilateral CeA of the *Htr2a-Cre* transgenic mice and control (*Htr2a-Cre*$^-$) mice (Figure 6A). At 3 weeks after injection, the respective hM4Di-mCherry and hM3Dq-mCherry expression was detected in the CeA of

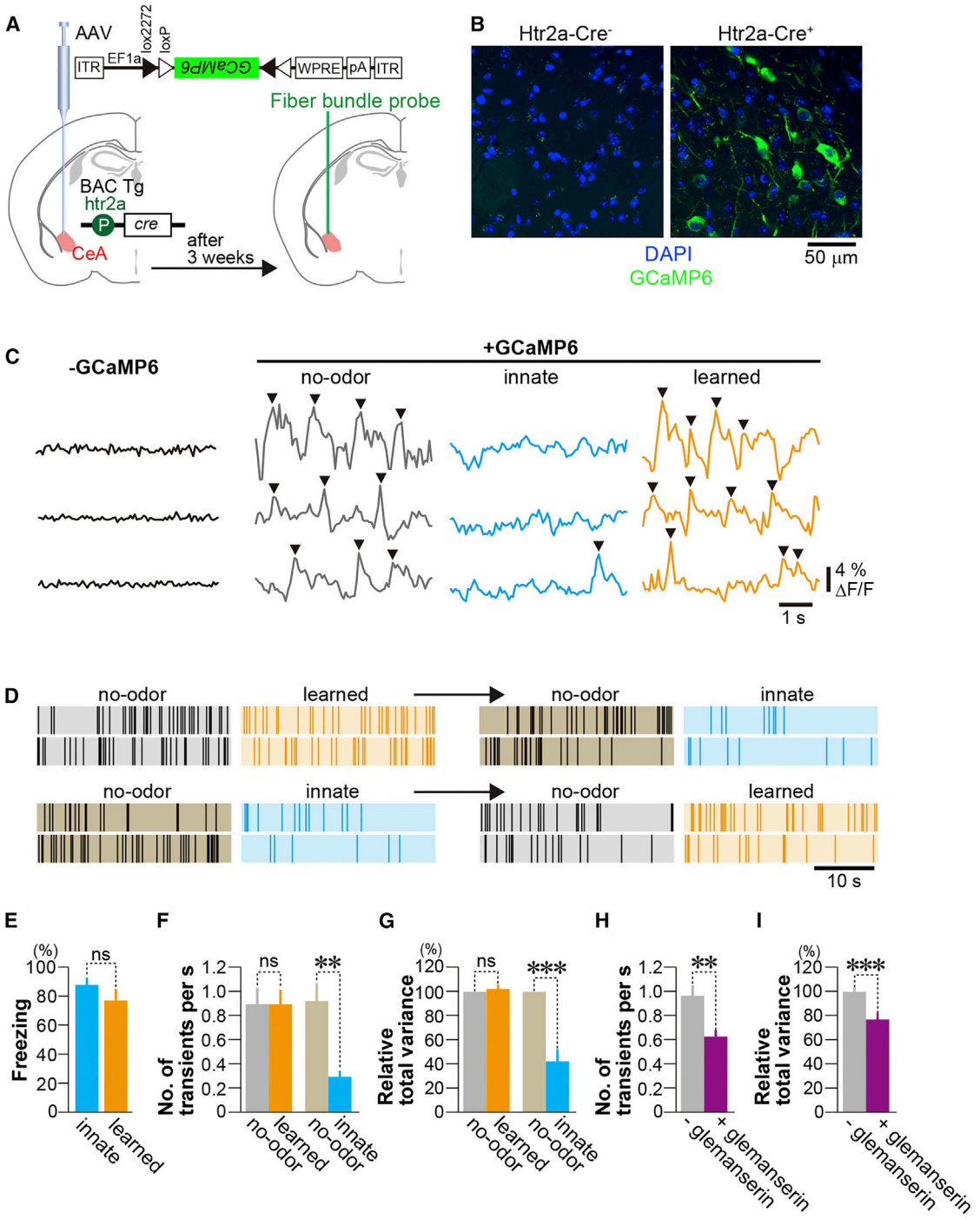

Figure 5. In Vivo Imaging of CeA Htr2a$^+$ Cells in Freely Moving Mice

(A) Experimental design of in vivo fiber photometry assays.

(B) Representative images of GCaMP6 expression in the CeA of control (*Htr2a-Cre*$^-$) and *Htr2a-Cre*$^+$ mice are shown. GCaMP6 signals were detected only in the *Htr2a-Cre*$^+$ mice. Scale bar, 50 μm.

(C) Examples of GCaMP6 fluorescence following exposure to innate- and learned-fear-inducing odorants. Mice without adeno-associated virus (AAV) infection (−GCaMP6) and no-odor control are also shown. Arrowheads indicate GCaMP transients that exceeded the arbitrary threshold (4% ΔF/F).

(D) Representative raster plots of GCaMP6 transients following exposure to innate- and learned-fear-inducing odorants. Two individuals first presented with the learned-fear-inducing odorant (upper panels) and two individuals first presented with the innate-fear-inducing odorant (lower panels) are shown.

(E) Levels of freezing during transient measurement induced by innate- and learned-fear-inducing odorants are shown.

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Htr2a-Cre$^+$ mice but not in that of *Htr2a-Cre*$^-$ mice (Figure 6B). In vivo photometry demonstrated that GCaMP6 transients in the CeA Htr2a$^+$ cells were significantly reduced by IP injection of clozapine-N-oxide (CNO) in AAV-DIO-hM4Di-treated mice (Figure 6C), indicating that this treatment artificially downregulates the neuronal activity of CeA Htr2a$^+$ cells as expected.

Following IP injection of CNO in AAV-DIO-hM4Di-treated mice, the innate-freezing response was significantly upregulated and the learned-freezing response was significantly downregulated in *Htr2a-Cre*$^+$ mice compared to *Htr2a-Cre*$^-$ mice (Figure 6D). These results are consistent with our pharmacological experiments (Figure 3H). On the other hand, IP injection of CNO into AAV-DIO-hM3Dq-treated mice significantly downregulated innate freezing but did not affect learned freezing (Figure 6E).

The opposing effects of CeA Htr2a$^+$ cells on innate and learned freezing were further confirmed using optogenetic methods (Boyden et al., 2005). A Cre-dependent AAV encoding archaerhodopsin (eArch3.0) fused with enhanced yellow fluorescent protein (EYFP), or channelrhodopsin (hChR2(H134R)) fused with EYFP, was injected into the bilateral CeA of the *Htr2a-Cre* transgenic mice and control (*Htr2a-Cre*$^-$) mice (Figure 6F). At 3 weeks after injection, eArch3.0-EYFP and hChR2-EYFP expressions were detected in the CeA of *Htr2a-Cre*$^+$ mice but not in that of *Htr2a-Cre*$^-$ mice (Figure 6G). Next, we investigated the effects on innate- and learned-freezing behaviors by optogenetic alteration of the activity of CeA Htr2a$^+$ cells. Levels of odor-induced innate- and learned-freezing behaviors before and during light stimulation were compared. Following artificial inactivation of CeA Htr2a$^+$ cells by light stimulation of eArch3.0, upregulation of the innate-freezing response and downregulation of the learned-freezing response in *Htr2a-Cre*$^+$ transgenic mice compared to *Htr2a-Cre*$^-$ mice were observed (Figure 6H). On the other hand, artificial activation of CeA Htr2a$^+$ cells by light stimulation of ChR2 significantly downregulated innate freezing but did not affect learned freezing (Figure 6I). Our pharmacological, pharmacogenetic, and optogenetic analyses confirmed that artificial inactivation of CeA Htr2a$^+$ cells upregulates innate freezing and, in parallel, downregulates learned freezing.

Unlike innate-fear-inducing odorants, learned-fear-inducing odorants did not influence CeA Htr2a$^+$ cell activity (Figures 5F and 5G). Moreover, pharmacogenetic activation of hM3Dq as well as optogenetic stimulation of ChR2 in CeA Htr2a$^+$ cells did not upregulate learned freezing; thus, hierarchical control between innate and learned freezing mediated by CeA Htr2a$^+$ cells is asymmetric. This can contribute to stabilizing the one-way hierarchical control of innate-fear over learned-fear responses, which may determine the behavior of mice in dangerous situations.

We next confirmed whether CeA Htr2a$^+$ cells actually contribute to regulation of the hierarchical relationship between the innate- and learned-fear responses, rather than just regulating innate- and learned-freezing behavior. As we have shown in Figure 2D, prior presentation of innate-fear stimuli suppressed the learned-fear response. Presentation of innate-fear stimuli downregulated the activity of CeA Htr2a$^+$ cells (Figures 5C, 5D, 5F, and 5G). If this inactivation contributes to determining the hierarchical relationship between innate and learned fear, artificial activation of CeA Htr2a$^+$ cells during the presentation of innate-fear stimuli may affect the suppressing effect on learned-freezing behavior by prior presentation of innate-fear stimuli. To test this concept, CeA Htr2a$^+$ cells were artificially activated by pharmacogenetic and optogenetic methods. Interestingly and importantly, artificial activation of CeA Htr2a$^+$ cells by hM3Dq, as well as ChR2, clearly reversed the suppressing effect (Figures 6J and 6K). These results indicate that the effect of prior presentation of innate-fear stimuli on learned-fear responses can be bidirectionally controlled by CeA Htr2a$^+$ cells. Thus, CeA Htr2a$^+$ cells do not merely influence innate- and learned-fear responses in opposite directions but control the hierarchy and relationship between innate- and learned-fear responses.

Odor-Induced Innate- and Learned-Freezing Behaviors Are Regulated in Distinct Subnuclei in the Periaqueductal Gray

Opposite directional control of innate and learned freezing (upregulation of innate freezing in parallel with downregulation of learned freezing) may imply that both freezing behaviors are regulated by separate neuronal mechanisms. Learned-freezing behaviors are controlled by the ventral periaqueductal gray (vPAG) (LeDoux et al., 1988; De Oca et al., 1998; Vianna et al., 2001; Gross and Canteras, 2012; LeDoux, 2012). On the contrary, the dorsal periaqueductal gray (dPAG) regulates innate-avoidance and risk-assessment behaviors induced by presentation of predator animals (Aguiar and Guimarães, 2009; Sukikara et al., 2010; Silva et al., 2013). Furthermore, electrical stimulation of the dPAG induces freezing behavior (Vianna et al., 2001). Thus, it is possible that innate- and learned-freezing behaviors are separately controlled by the dPAG and vPAG, respectively. To confirm this possibility, induction of IEG expression in the PAG was analyzed in mice after induction of either innate- or learned-freezing behavior. In the dPAG, *arc* mRNA expression was significantly upregulated in the innate-freezing condition but not in the learned-freezing condition. In contrast, in the vPAG, *c-fos* mRNA expression was significantly upregulated in the learned-freezing condition but not in the innate-freezing condition (Figure S3). Stereotaxic injection of muscimol into the dPAG significantly downregulated innate-freezing behavior but not learned-freezing behavior (Figures S4A and S4B), whereas such an injection into the vPAG significantly downregulated learned-freezing behavior but not innate-freezing behavior (Figures S4C and S4D). These results indicate that odor-induced innate- and learned-freezing behaviors are separately controlled by the dPAG and vPAG, respectively.

We examined whether artificial inactivation of CeA Htr2a$^+$ cells affects IEG expression in the dPAG and vPAG in parallel with

(F–I) Changes in GCaMP6 transients (F and H) and in the mean total variance in the trace of GCaMP transients (G and I) induced by innate- and learned-fear-inducing odorants (F and G) and by IP injection of glemanserin (H and I) are shown. The mean total variances in control sessions (no-odor) prior to odor presentation were set at 100%.

(E–I) Unpaired t test. Data are means + SEM. $^{**}p < 0.01$; $^{***}p < 0.001$; ns, $p > 0.05$.

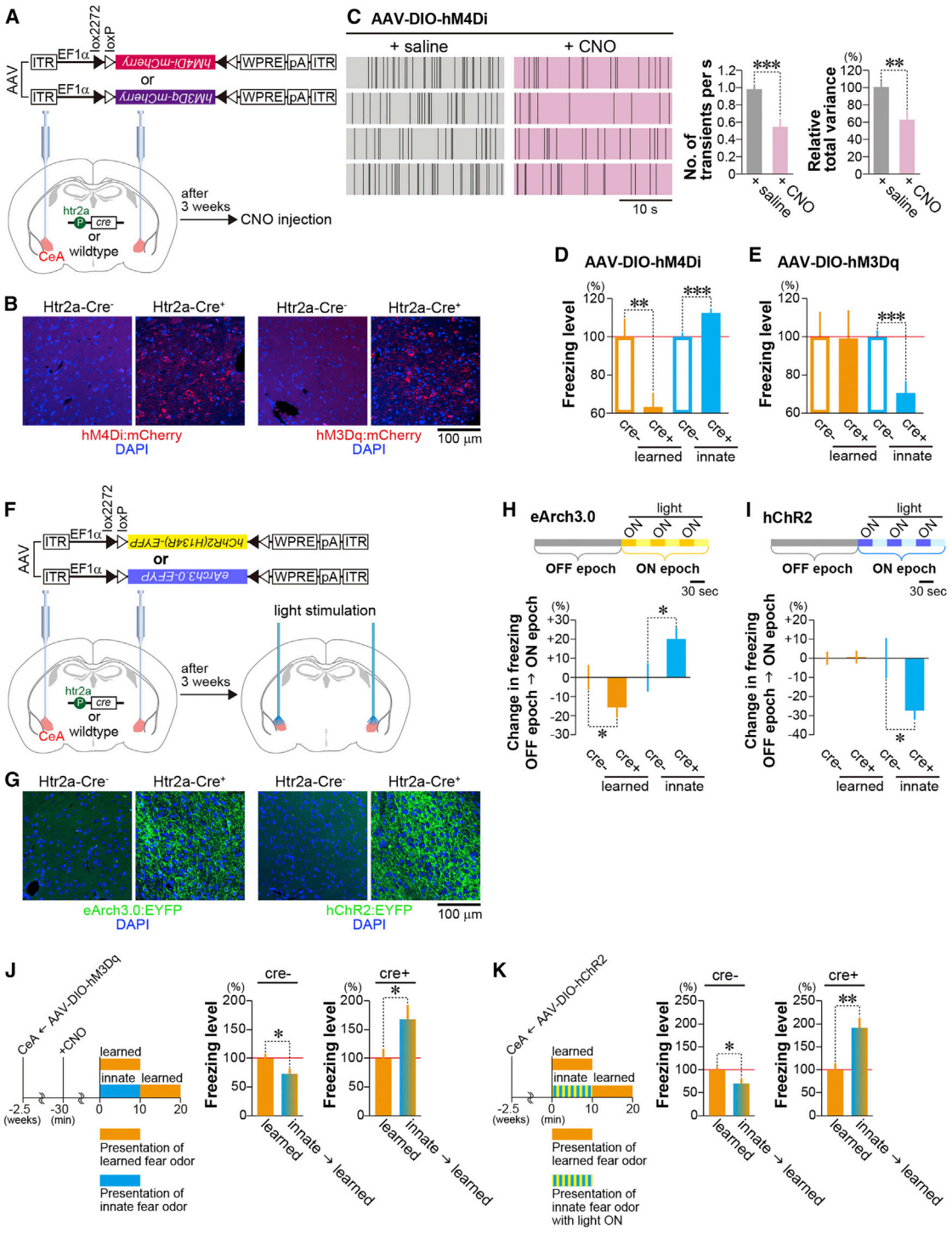

Figure 6. Chemogenetic and Optogenetic Manipulation of CeA Htr2a$^+$ Cells

(A) Experimental design of the chemogenetic activation and silencing of CeA Htr2a$^+$ cells.

(B) Representative images of hM4Di-mCherry and hM3Dq-mCherry expression in the CeA of control (*Htr2a-Cre*$^-$) and *Htr2a-Cre*$^+$ mice are shown.

(C) Representative raster plots (left), number of GCaMP6 transients (middle), and the mean total variance in the trace of GCaMP transients (right) induced by IP injection of saline and CNO in hM4Di-infected mice.

(D and E) Levels of freezing following exposure to innate- and learned-fear-inducing odorants are shown for hM4Di inhibition (D) and hM3Dq activation (E). The mean percentage of freezing in control mice (cre$^-$) following exposure to either the innate- or learned-fear-inducing odorant was set at 100%.

(F) Experimental design of the optogenetic activation and silencing of CeA Htr2a$^+$ cells.

(legend continued on next page)

controlling innate- and learned-freezing behaviors in opposite directions (Figure 7A). Upregulation of *arc* expression in the dPAG induced by an innate-fear-inducing odorant was further increased by selective inhibition of CeA Htr2a$^+$ cells using hM4Di. In contrast, upregulation of *c-fos* expression in the vPAG by a learned-fear-inducing odorant was inhibited by selective inhibition of CeA Htr2a$^+$ cells using hM4Di (Figures 7B–7D).

Collectively, our results indicate that innate-fear-inducing odorants inactivate CeA Htr2a$^+$ cells. Inactivation of these cells led to an increase of the innate-freezing response and IEG expression in the dPAG and a decrease of the learned-freezing response and IEG expression in the vPAG. Thus, CeA Htr2a$^+$ cells regulate the antagonistic and hierarchical relationship between innate- and learned-freezing responses, in which the innate-freezing response predominates (Figure 7E).

Discussion

It is widely accepted that innate and learned fears are regulated by distinct neural pathways (Gross and Canteras, 2012; LeDoux, 2012), but potential interactions between these pathways are still unclear. In this study, we illustrated that innate-fear-inducing odorants suppress learned-fear response via the activity of CeA Htr2a$^+$ cells. Although our finding of a hierarchical relationship between innate- and learned-fear responses was unexpected, this mechanism does seem advantageous for organism survival. It is conceivable that animals experience the two types of fear simultaneously in the natural environment and are forced to prioritize their response to one type of fear over the other (for example, when they have to explore dangerous environments for food). We modeled this scenario experimentally in this study as described in Figure 2C. Innate fear is induced by conserved dangers among species and is acquired over the course of evolution. In contrast, learned fear is acquired in response to an individual's fearful experiences and is therefore mutable. Thus, it is reasonable that innate fear has priority over learned fear if the risk levels of both fears are comparable, and our data support this idea.

The amygdala is proposed to work as a switchboard for separating innate- and learned-fear information into adjacent subnuclei connecting to different downstream pathways that induce distinct behavioral and physiological responses (Gross and Canteras, 2012; LeDoux, 2012). Contrary to this idea, we propose that the CeA works as an integrator for innate- and learned-fear information. It is widely accepted that the CeA contributes to the regulation of learned-freezing responses (LeDoux, 2000; Davis, 2000; Maren and Quirk, 2004); however, the function of the CeA in regulating innate-freezing responses has not been directly clarified. Our pharmacological, pharmacogenetic, and optogenetic experiments clearly show that CeA Htr2a$^+$ cells regulate the innate-freezing response. Moreover, we showed that CeA Htr2a$^+$ cells regulate both innate- and learned-freezing responses, in opposite directions, which contributes to establishing the hierarchical relationship in which the innate-fear response predominates over the learned-fear response.

It has been reported that olfactory-mediated innate-fear information is conveyed to the MeA and CoA to regulate fear responses (Li et al., 2004; Martinez et al., 2011; Root et al., 2014). However, the pathway conveying olfactory information to the CeA is not known. In this study, we demonstrated that inactivation of CeA Htr2a$^+$ cells by innate-fear information is a key process for determining the hierarchical relationship between innate- and learned-fear responses. To further confirm this idea, it is important to clarify the afferent pathway that conveys olfactory fear information to CeA Htr2a$^+$ cells. We also demonstrated that innate- and learned-freezing responses are separately processed in the dPAG and vPAG, respectively. Artificial inactivation of CeA Htr2a$^+$ cells inhibits upregulation of IEG expression in the vPAG induced by learned-fear odors, indicating that the vPAG is a downstream target of CeA Htr2a$^+$ cells. However, in this study we did not clarify anatomical connections of the CeA Htr2a$^+$ cells.

The majority of CeA Htr2a$^+$ cells are located in the lateral subnucleus of the CeA (CeL) (Figure S2A). In the CeL, two distinct cell populations (SOM$^+$ and PKCδ^+) have been reported to regulate learned-freezing behavior in opposing directions. Histological analyses indicated that the majority of CeA Htr2a$^+$ cells were also SOM$^+$ (Figure S2B). Inactivation of CeL SOM$^+$ cells has been reported to downregulate learned-freezing behavior (Li et al., 2013). Thus, it is suggested that suppression of learned-freezing behavior by the CeL Htr2a$^+$ cells is at least partly mediated by CeL SOM$^+$ cells. We also clarified that a considerable number of c-*fos*$^+$ cells induced by innate-fear input in the CeL were PKCδ^+ (Figure S2D). Inactivation of CeL PKCδ^+ cells has been reported to upregulate learned-freezing behavior, and, inversely, activation of these cells inhibits PAG-projecting CeM cells, which may then downregulate learned-freezing behavior (Haubensak et al., 2010). If this is the case, it is likely that innate-fear input suppresses learned-freezing behavior via activation of c-*fos*/PKCδ double-positive cells. In summary, suppression of learned-freezing behavior by innate-fear input may be mediated by two distinct cell populations (SOM$^+$ and PKCδ^+) in the CeL, which are reported to regulate learned-freezing behavior (Figure S5).

The CeL PKCδ^+ cells connect with CeM PAG-projecting output neurons, whereas CeL SOM$^+$ cells directly connect with the PAG (Haubensak et al., 2010; Penzo et al., 2014). These indirect and direct connections to the PAG may contribute to

(G) Representative images of eArch3.0-EYFP and hChR2-EYFP expression in the CeA of control (*Htr2a-Cre*$^-$) and *Htr2a-Cre*$^+$ mice are shown.

(H and I) Timelines of the experiments are shown in the upper panel. After odor presentation, freezing behavior in the 3 min light-OFF epoch and the subsequent light-ON epoch (3 times repeat of 30 s light ON, with 30 s interval) was measured. Delta freezing was calculated by subtracting freezing rate during light-OFF epochs from those during light-ON epoch. The mean delta freezing are indicated in bar graphs for eArch3.0 stimulation (H) and ChR2 stimulation (I). The mean delta freezing in control mice (cre$^-$) was set at 0%.

(J and K) Timelines of the experiments are shown in the left panels. The mean percentages of time spent in learned freezing with and without prior induction of innate freezing were analyzed for hM3Dq (J) and ChR2 (K) activation of CeA Htr2a$^+$ cells (*Htr2a-Cre*$^+$) and control (*Htr2a-Cre*$^-$). The levels of freezing without prior induction of innate freezing were set at 100%.

(C–E, and H–K) Unpaired t test. Data are presented as means + SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Scale bars, 100 μm.

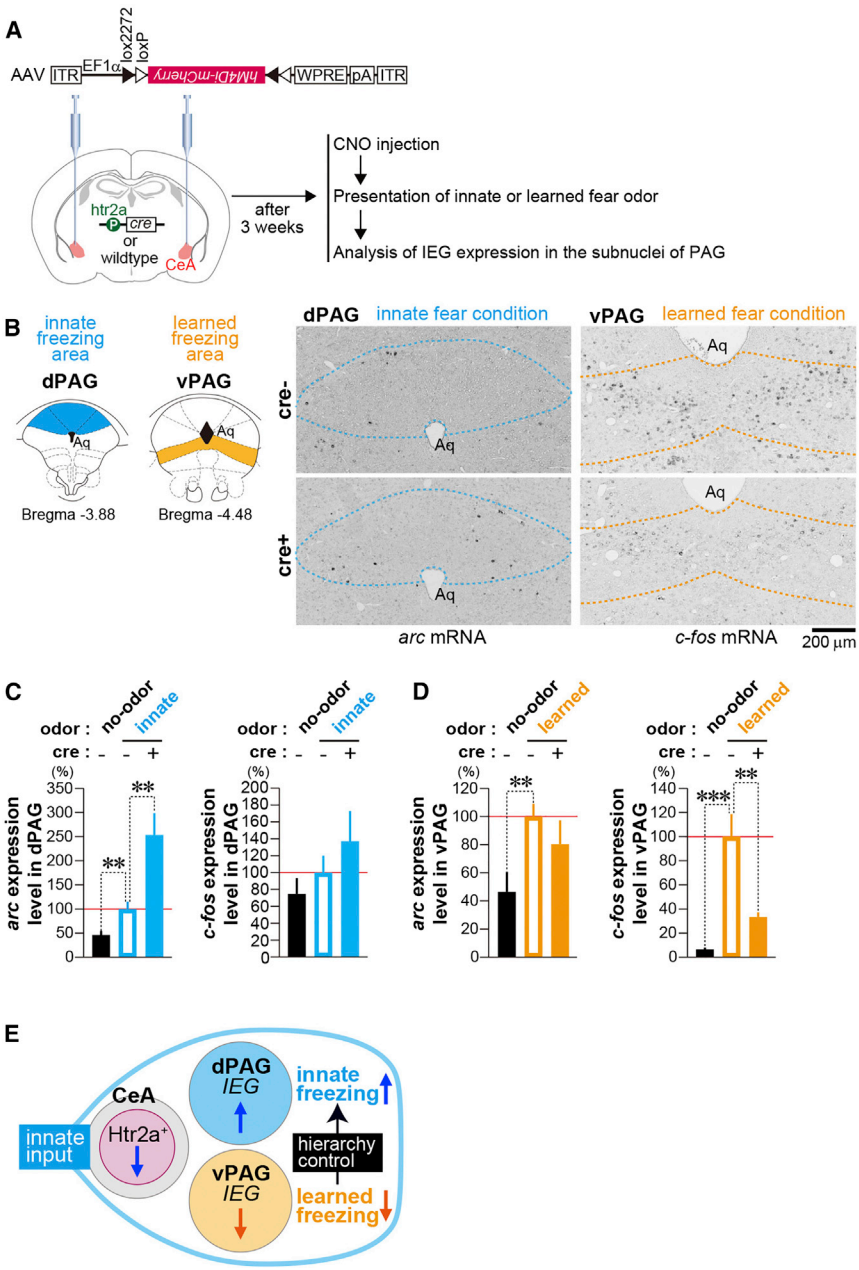

Figure 7. Effect of Artificial Inactivation of CeA Htr2a$^+$ Cells on IEG Expression in the Subnuclei of the PAG

(A) Design of the experiment. At 3 weeks after injection of a cre-dependent AAV-encoding hM4Di-mCherry to the CeA, CNO was intraperitoneally injected, and IEG expression in the subnuclei of the PAG induced by innate- or learned-fear odors was analyzed.

(B) IEG expression in the dorsal PAG (dPAG, blue area) and ventral PAG (vPAG, orange area) was analyzed with and without artificial inactivation of CeA Htr2a$^+$ cells following exposure to innate-fear-inducing and learned-fear-inducing odorants, respectively. Representative images of *arc* mRNA in the dPAG (left) and *c-fos* mRNA in the vPAG (right) following exposure to innate- and learned-fear-inducing odorants with (cre$^+$) and without (cre$^-$) hM4Di silencing of CeA Htr2a$^+$ cells are shown. Scale bar, 200 μm.

(C and D) Levels of *arc* and *c-fos* mRNA in the dPAG (C) and in the vPAG (D) compared to the no-odor control following exposure to innate-fear-inducing odorants (C) and learned-fear-inducing odorants (D) with (cre$^+$) and without (cre$^-$) hM4Di silencing of CeA Htr2a$^+$ cells are shown. The levels of mRNA following exposure to innate-fear-inducing odorant (C) and learned-fear-inducing odorant (D) without hM4Di silencing were set at 100%.

(E) Model of hierarchical control of innate- and learned-freezing responses by CeA Htr2a$^+$ cells.

(C and D) One-way ANOVA followed by Bonferroni correction. Data are presented as means + SEM. **p < 0.01; ***p < 0.001. See also Figures S3 and S4.

suppress learned-freezing behavior by CeA Htr2a$^+$ cells. We showed that the dPAG contributes to regulating odor-induced innate- but not learned-freezing behavior (Figures S3 and S4). Moreover, artificial inactivation of CeA Htr2a$^+$ cells upregulated odor-induced innate-freezing behavior (Figures 6D and 6H) and *arc* mRNA expression in the dPAG (Figure 7C). These data suggest that CeA Htr2a$^+$ cells control innate-freezing behavior through the dPAG. However, the anatomical connection between CeA Htr2a$^+$ cells and dPAG is still unclear. Innate-fear responses are controlled by the medial amygdala–hypothalamus–dPAG pathway (Gross and Canteras, 2012). It may be possible that CeA Htr2a$^+$ cells control innate-freezing behavior via this pathway.

The BLA is proposed to connect the processing of unconditioned stimuli (US) and conditioned stimuli (CS) (LeDoux, 2000; Davis, 2000; Maren and Quirk, 2004). US induce c-*fos* expression in the BLA, and these c-*fos*$^+$ cells contribute to regulation of learned-freezing behavior (Gore et al., 2015). Thus, in the BLA, innate- and learned-fear circuits are synergistically integrated to form conditioned fear memory. In contrast, in the CeA, innate sensory inputs have an antagonistic effect on learned-fear responses to determine the behavioral hierarchy. Thus, there are two distinct modes for integration between innate- and learned-fear information processing: synergistic and antagonistic. These modes are separately regulated in the different subnuclei in the amygdala.

Our finding that innate fear affects learned fear antagonistically, but not synergistically, provides new insight not only for understanding the emotion of fear but also for the development of psychotropic medications. For instance, our data indicate that Htr2a antagonists, such as risperidone, which can alleviate learned fear, may in turn aggravate innate fear. Accordingly, our results suggest that it is important to dissect and analyze

the contribution of innate and learned systems in mental disorders and identify appropriate molecules for their treatment.

EXPERIMENTAL PROCEDURES

Mice

Male C57BL/6NCr mice were purchased from Japan SLC, Inc. The *Htr2a-Cre* BAC transgenic line (STOCK Tg[Htr2a-cre] KM208Gsat/Mmucd) was imported from the Mutant Mouse Regional Resource Center. *Rosa-CAG-LSL-GCaMP3-WPRE* mice (stock number 14538) were purchased from The Jackson Laboratory to monitor Cre recombination; these are referred to as *floxed-GFP* mice in this study. All animals were maintained on a 12 hr light−dark schedule (lights on at 7:00 a.m.) with food and water available ad libitum at the Osaka Bioscience Institute and Kansai Medical University animal house. Mice were 9–13 weeks old at the start of testing. All tests were performed between 9:00 a.m. and 7:00 p.m. The protocols used for all animal experiments in this study were approved by the Animal Research Committee of the Osaka Bioscience Institute and Kansai Medical University.

Viral Production

AAV expression vectors were created by subcloning GCaMP6 (Ohkura et al., 2012) into the AscI-NheI site of pAAV-Ef1a-DIO-hChR2(C128S/D156A)-EYFP vector (Addgene #35503). The resulting AAV-EF1a-DIO-GCaMP6 construct was packaged and serotyped as described previously (Hikida et al., 2010). AAVs expressing hM4Di (AAV-EF1α-DIO-hM4Di-mCherry), hM3Dq (AAV-EF1α-DIO-hM3Dq-mCherry), ChR2 (AAV-EF1α-DIO-hChR2 (H134R)-eYFP), or Arch (AAV-EF1α-DIO-eArch3.0 -eYFP) were obtained from the UNC Vector Core Facilities (Chapel Hill, NC, USA).

Freezing-Behavior Analysis

For analyses of freezing behavior induced by innate (2MT or TMT) or learned (anisole, previously paired with electric foot shocks) fear-inducing odorants, mice were individually placed in a test cage (28 × 18 × 13.5 cm) and habituated for 10 min. Each subject received test odor presentations for 10 min. Each odorant (271 μmol) was pipetted onto a filter paper (2 × 2 cm). For the no-odor control condition, a plain filter paper was presented.

Odor presentation was performed in the chemical fume hood. Mouse behavior was recorded and quantified using a video-based measurement system (Freeze Frame2, Actimetrics). The mice were considered to freeze if movement was not detected for 2 s.

Further methods, including behavioral assays, in vivo fiber photometry, and histological procedures, can be found in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.10.047.

AUTHOR CONTRIBUTIONS

K.K. designed the experiments. T.I., R.K., and K.K. performed most of the experiments. T.M. performed most of the histological analyses. T.I., T.Y., K.F., and S.N. performed in vivo photometry. K.K., R.K., and T.I. analyzed the data. The manuscript was written by K.K., R.K., and T.I. All authors discussed the results and commented on the manuscript.

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