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Observational fear learning involves affective pain system and $Ca_V1.2$ Ca^{2+} channels in ACC

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Fear can be acquired vicariously through social observation of others suffering from aversive stimuli. We found that mice (observers) developed freezing behavior by observing other mice (demonstrators) receive repetitive foot shocks. Observers had higher fear responses when demonstrators were socially related to themselves, such as siblings or mating partners. Inactivation of anterior cingulate cortex (ACC) and parafascicular or mediodorsal thalamic nuclei, which comprise the medial pain system representing pain affection, substantially impaired this observational fear learning, whereas inactivation of sensory thalamic nuclei had no effect. The ACC neuronal activities were increased and synchronized with those of the lateral amygdala at theta rhythm frequency during this learning. Furthermore, an ACC-limited deletion of $Ca_V1.2$ Ca^{2+} channels in mice impaired observational fear learning and reduced behavioral pain responses. These results demonstrate the functional involvement of the affective pain system and $Ca_V1.2$ channels of the ACC in observational social fear.

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Fear is a biological response to dangerous, threatening situations or stimuli. Fear can be acquired and expressed in a variety of ways[1]. First, fear can be learned from direct experience of an adverse situation (for example, an unconditioned stimulus in classical Pavlovian fear conditioning). In a classical conditioning experiment, pairing of a neutral, conditioned stimulus (for example, a tone) with an aversive, unconditioned stimulus (for example, a foot shock) causes an animal to express fear behaviors when the animal is later exposed to the conditioned stimulus in the absence of the unconditioned stimulus. The neural mechanism and circuitry of this fear has been well studied across species, including rodents[2,3]. Fear can be socially acquired from instruction by verbal information (instructed fear) or from a vicarious observation of a conspecific's distress (observational fear) in primates, including humans[1,4,5]. For example, a higher primate can recognize fear by observing a conspecific's distressed face or a conspecific suffering from an enemy attack[1,6–12]. Previous studies using a bar-pressing protocol found that rats seeing a distressed conspecific (by electric shocks) display fearful behavioral responses, such as crouching or motionlessness[13,14]. A recent study found that C57BL/6J mice that observed unfamiliar mice experiencing classical fear conditioning displayed freezing behaviors when they were later exposed to the conditioned stimulus alone[15]. These findings demonstrate social transfer of fear in rodents. Unlike classical fear conditioning, however, the neural substrate and mechanism underlying observational social fear has not been well defined.

ACC is known to receive sensory signals from the somatosensory cortices and other cortical areas, including the anterior insular cortex[16–20]. Brain-imaging studies in humans have shown that the neuronal activities of the ACC and the amygdala change during observation of others experiencing fear or others' fearful facial expressions[6,8,10,11]. In addition, animal studies have suggested that the ACC is involved in pain affection or emotion behavior, as well as pain sensation[21–23]. Thus, the ACC is considered to be an important brain region for the convergence of sensory and emotional information and may mediate affective or emotional responses to noxious stimuli. However, the functional involvement of the ACC in observational social fear learning in animals remains unknown.

Brain-imaging studies in humans have shown that some brain regions related to the affective or emotional dimension of pain are activated during observation of distress in individuals suffering from noxious stimuli[20,24–26]. There are at least two pathways of pain processing via the thalamus in the CNS[16,17,26,27]. One is the lateral pain system, which expresses the sensory or discriminative dimension of pain and involves the perception of intensity, location and quality of pain stimuli. This pathway projects from the spinal cord dorsal horn to the ventral posterolateral (VPL) and posteromedial (VPM) thalamic nuclei, which relay nociceptive information to the somatosensory cortices. The other is the medial pain system, which represents the affective or emotional dimension of pain and involves perception of the unpleasantness of pain. In this pathway, the spinal nociceptive inputs project to the midline and intralaminar thalamic nuclei (MITN), including parafascicular and mediodorsal thalamic nuclei, and then proceed to limbic cortical areas, including the ACC.

We hypothesized that the medial pain system, such as the ACC, representing affective or emotional components of pain might

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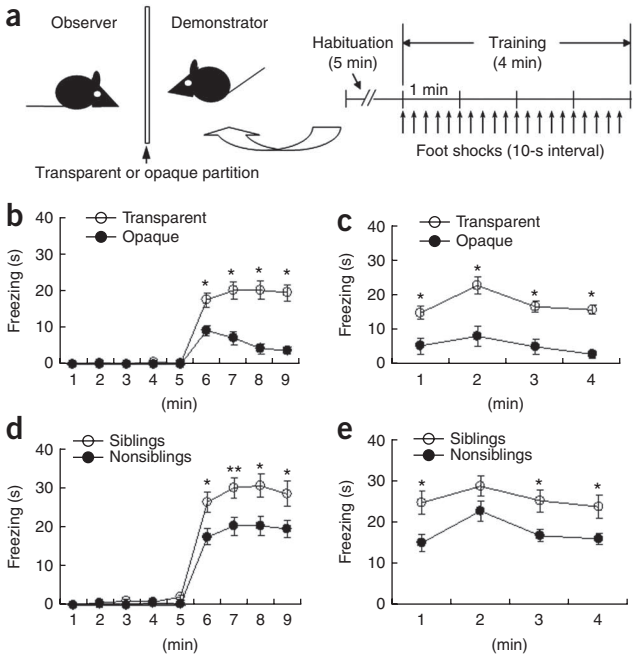

Figure 1 Observational fear learning in the mouse. (**a**) Diagram of the apparatus used for observational fear conditioning and the scheme of the behavioral assay. (**b**,**c**) Observational fear learning in the mouse (nonsiblings) using a transparent ($n = 21$) or opaque ($n = 8$) partition. A significant difference in the level of freezing behavior was apparent depending on whether a transparent or an opaque partition was used for the conditioning experiment on both the training day (**b**) and 24 h after training (**c**). $^*P < 0.01$, Scheffe's *post hoc* test. (**d**,**e**) Observational fear learning with siblings. We examined freezing behavior on the day of training ($F_{1, 45} = 9.41$, $P = 0.0036$, two-way repeated ANOVA, **d**) and 24 h after training ($F_{1, 45} = 11.48$, $P = 0.0015$, two-way repeated ANOVA, **e**) in siblings ($n = 26$) and nonsiblings ($n = 21$) using a transparent partition. $^*P < 0.05$, $^{**}P < 0.01$, Scheffe's *post hoc* test. Error bars represent s.e.m.

contribute to social learning of fear by observation. From previous studies[13,14], we developed an assay system to evaluate observational fear conditioning in the mouse and confirmed our hypothesis. In addition, we found that the $Ca_v1.2$ (α1C, *Cacna1c*) subunit of the L-type Ca^{2+} channel, which is known to contribute to synaptic transmission and neuronal excitability[28–31], in the ACC could be required in social fear learning.

RESULTS

Observational fear conditioning in the mouse

We developed an observational fear conditioning system to study social fear learning in the mouse. In this conditioning system, two male C57BL/6J mice were singly placed in each chamber of a double-chambered fear-conditioning apparatus separated by a transparent Plexiglas partition and one mouse (observer) was allowed to observe the other (demonstrator) as it was subjected to repetitive foot shocks in the opposite chamber (**Fig. 1a**; control experiments are described in **Supplementary Discussion** and **Supplementary Fig. 1**). The observer clearly showed freezing behavior as he saw the demonstrator suffering from foot shocks (**Fig. 1b**). When the observers alone were placed back into the same observing chamber 24 h after the training, they displayed freezing behaviors (**Fig. 1c**). However, when the observers were placed into a novel chamber 24 h after the training, they did not exhibit freezing behaviors (**Supplementary Fig. 1**), indicating that the conditioning was context specific[32]. These results indicate that observers learned, or were conditioned for, fear that was associated with the context. When the visual inputs were blocked by replacing the transparent partition with an opaque black panel, the fear response of the observers was significantly reduced ($F_{1,27} = 30.46$, $P < 0.0001$, two-way repeated ANOVA; **Fig. 1b**) under the otherwise similar situations and the 24-h contextual fear memory was also reduced ($F_{1,27} = 34.30$, $P < 0.0001$, two-way repeated ANOVA; **Fig. 1c**). Nevertheless, there was still residual freezing behavior when an opaque black partition prevented the observers from seeing the demonstrators (**Fig. 1b**,**c**). This indicates that other sensory modalities, such as olfactory and auditory cues, also contribute to the development of the conditioning. Taken together, these results indicate that mice can become conditioned to, or learn, fear by observing the behavior of a conspecific demonstrator.

To examine whether the relatedness of the demonstrator to the observer affects observational fear conditioning, we used siblings or female mating partners as demonstrators in the conditioning experiments. Notably, observers exhibited more freezing behavior during both conditioning (**Fig. 1d**) and the 24-h contextual memory test (**Fig. 1e**) when the demonstrators were siblings than they did when the demonstrators were unrelated. Next, we used female mating partners as demonstrators (couple and noncouple experiments) and tested whether the length of time that the couple was housed together affects the observational fear learning behavior of the observer male mice (**Fig. 2**). We did not observe any substantial differences in freezing in the observer mice during observational conditioning between couple and noncouple experiments after housing the mice together for 1 week (**Fig. 2a**,**b**) or 4–5 weeks (**Fig. 2c**,**d**). However, when female mating partners who lived together more than 10 weeks (10–15-week period and 20–36-week period; **Fig. 2e**–**h**) were used as demonstrators (couple experiments), male observers showed more freezing behaviors than was the case when unrelated female mice were demonstrators (noncouple experiments) during the training and the 24-h contextual memory test. Notably, the housing duration showed a graded effect on the development of observational fear (**Fig. 2i**). These results indicate that familiarity is a vital factor (fear was greater when the demonstrator was more familiar to the observer) in observational fear conditioning. The number of fecal droppings, an indirect measure of fear[33], also increased when the demonstrator was related to the observer (**Supplementary Fig. 2**). Taken together, these findings indicate that the magnitude of the fear response is dependent on the relatedness or familiarity of the demonstrator to the observer and suggest that social relationship is an important element in observational fear conditioning in the mouse.

Involvement of ACC and MITN in observational fear learning

Using this observational fear learning system, we investigated the neuronal substrate or mechanism underlying social fear learning. First, we examined the involvement of the ACC, which is thought to be a cortical substrate for the convergence of sensory and emotional information and may mediate affective or emotional responses to noxious stimuli. For local inactivation, we injected lidocaine (4%) through a cannula into the ACC of the observer 8 min before observational fear training. Lidocaine-injected observers exhibited impaired observational fear learning compared with control mice that were similarly treated with saline (**Fig. 3a**,**b**), indicating that the ACC is involved in observational fear learning. Administration of lidocaine to the ACC also impaired observational fear conditioning of mice observing siblings and female mating partners as demonstrators (**Fig. 3c**). In this experiment, similar freezing levels were obtained when siblings or mating partners were used as demonstrators and the results were thus pooled for analysis: siblings ($n = 12$) versus

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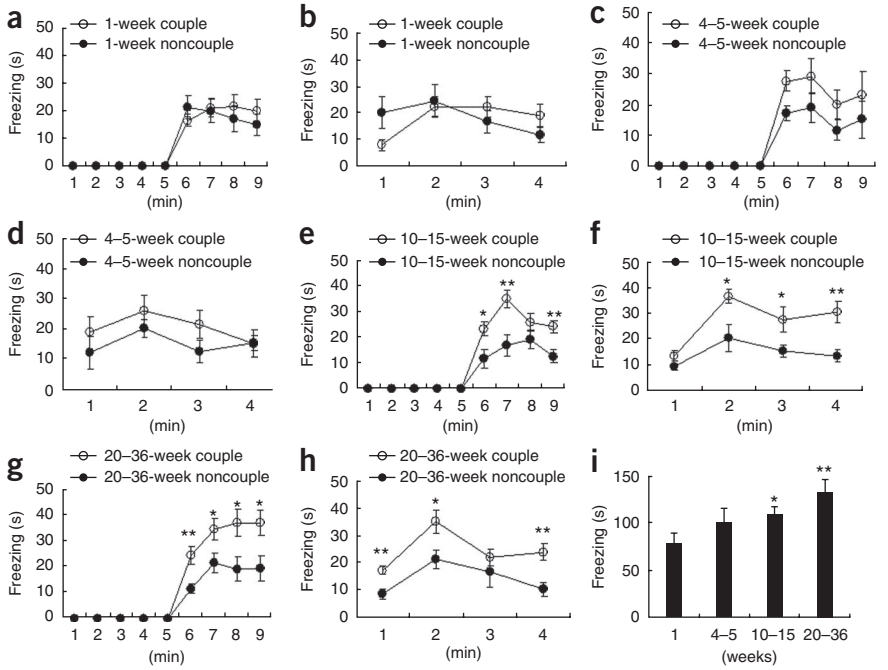

Figure 2 Observational fear learning with female mating partners as demonstrators: effect of the duration of co-housing period (familiarity). (**a**,**b**) Observational fear conditioning after 1 week of co-housing (couple, $n = 10$; noncouple, $n = 10$). There was no difference in the observational fear response (**a**) and the 24-h contextual memory (**b**) between couple and noncouple experiments. (**c**,**d**) Observational fear conditioning after a 4–5-week co-housing period (couple, $n = 6$; noncouple, $n = 9$). There was no difference in the observational training (**c**) and the 24-h contextual memory (**d**) between couple and noncouple experiments. (**e**,**f**) Observational fear conditioning after 10–15 weeks of co-housing (couple, $n = 12$; noncouple, $n = 7$). There were significant differences in the observational training ($F_{1,17} = 11.41$, $P = 0.0036$, two-way repeated ANOVA, **e**) and the 24-h contextual memory ($F_{1,17} = 11.77$, $P = 0.0032$, two-way repeated ANOVA, **f**) between couple and noncouple experiments. (**g**,**h**) Observational fear conditioning after 20–36 weeks of co-housing (couple, $n = 9$; noncouple, $n = 7$). There were significant differences in the observational training ($F_{1,14} = 8.62$, $P = 0.0109$, two-way repeated ANOVA, **g**) and the 24-h contextual memory ($F_{1,14} = 17.21$, $P = 0.001$, two-way repeated ANOVA, **h**) between couple and noncouple experiments. (**i**) The strength of the fear response was increased with as the duration of the co-housing periods increased. ANOVA ($F_{3,33} = 3.38$, $P = 0.029$) of the total freezing time revealed a graded effect of the duration of co-housing period on the development of observational fear and there was a significant difference in total freezing time between 1-week co-housing period group and 10–15-week or 20–36-week groups. $^*P < 0.05$, $^{**}P < 0.01$, Scheffe's *post hoc* test. Error bars represent s.e.m.

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couples ($n = 7$) ($F_{1,17} = 0.38$, $P = 0.55$, two-way repeated ANOVA) with saline and siblings ($n = 7$) versus couples ($n = 6$) ($F_{1,11} = 0.20$, $P = 0.67$, two-way repeated ANOVA) with lidocaine (**Fig. 3c**). We then examined the involvement of parafascicular and mediodorsal thalamic nuclei, which are representative nuclei of MITN projecting to the ACC. When lidocaine was injected into the parafascicular (**Fig. 3d**,**e**) or mediodorsal thalamic nuclei (**Fig. 3f**,**g**), observers showed impaired observational fear learning compared with control mice that were similarly treated with saline. The impairment was also observed in the 24-h contextual memory test (parafascicular; **Fig. 3e**) or (mediodorsal; **Fig. 3g**). These results indicate that the parafascicular and mediodorsal thalamic nuclei are involved in observational fear learning.

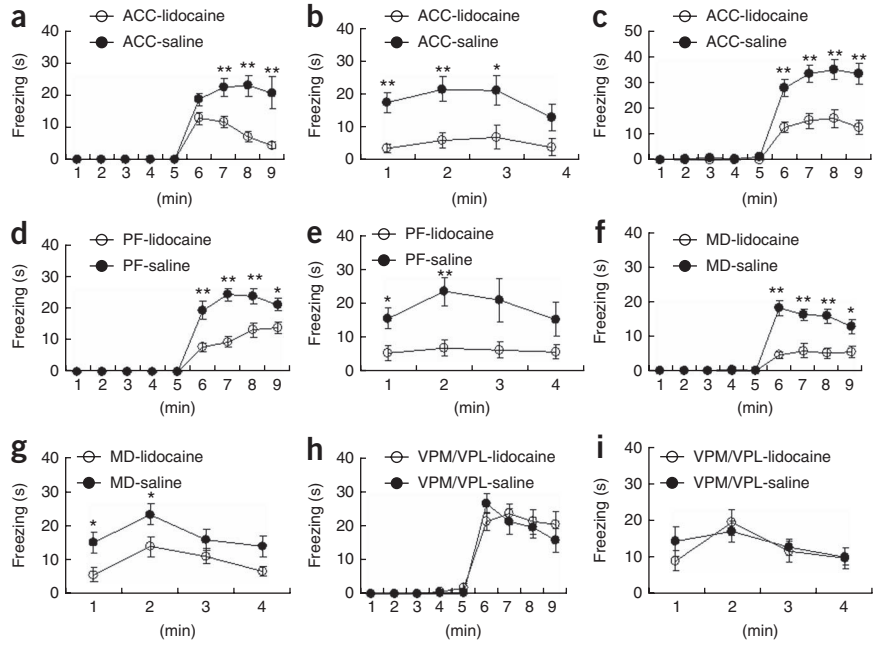

Figure 3 The ACC and MITN are involved in observational fear learning. (**a**) Mice with lidocaine injections into the ACC ($n = 12$) before training failed to acquire fear compared with those receiving saline injections ($n = 11$) ($F_{1,21} = 19.20$, $P = 0.0003$, two-way repeated ANOVA). (**b**) Contextual memory 24 h after the training in **a** ($F_{1,21} = 16.43$, $P = 0.0006$, two-way repeated ANOVA). (**c**) Mice with lidocaine injections into the ACC did not efficiently acquire fear by observation of siblings and mating partners (couples; $F_{1,30} = 18.25$, $P = 0.0002$, two-way repeated ANOVA). (**d**,**e**) Administration of lidocaine into the parafascicular (PF) thalamic nuclei ($n = 8$) before training led to impaired observational fear learning during training ($F_{1,15} = 43.84$, $P < 0.0001$, two-way repeated ANOVA, **d**) and 24 h after training ($F_{1,15} = 8.55$, $P = 0.0105$, two-way repeated ANOVA, **e**) as compared with those receiving saline injections ($n = 9$). (**f**,**g**) Administration of lidocaine into the mediodorsal (MD) thalamic nuclei ($n = 12$) before training caused impaired observational fear learning during training ($F_{1,28} = 24.11$, $P < 0.0001$, two-way repeated ANOVA, **f**) and 24 h after training ($F_{1,28} = 5.19$, $P = 0.0306$, two-way repeated ANOVA, **g**) as compared with those receiving saline injections ($n = 18$). (**h**,**i**) Administration of lidocaine into the VPL/VPM before training had no influence on the acquisition of observational fear (**h**) and 24-h contextual memory (**i**) (lidocaine, $n = 10$; saline, $n = 12$). $^*P < 0.05$, $^{**}P < 0.01$, Scheffe's *post hoc* test. Error bars represent s.e.m.

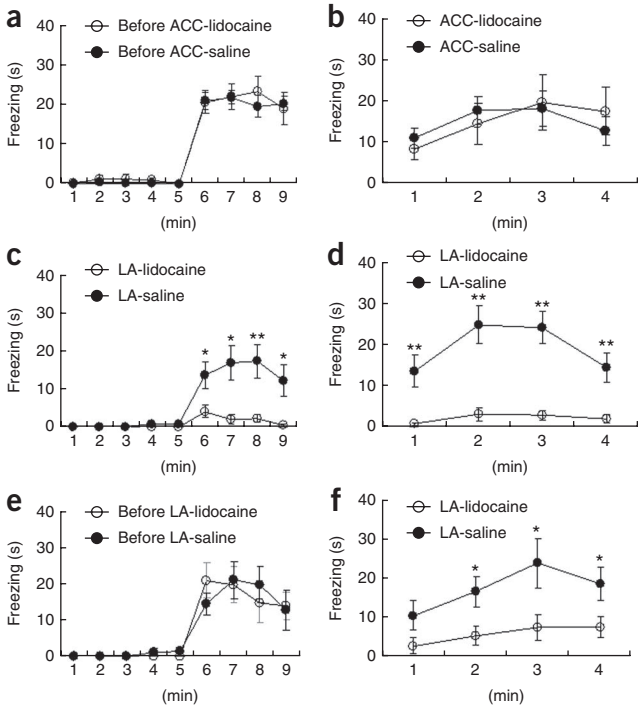

Figure 4 The ACC is involved in the acquisition of observational fear, but not in memory retrieval of observational fear and in classical fear conditioning. (**a**) Mice ($n = 9$) were trained with observational fear learning. (**b**) Local inactivation of the ACC 8 min before the 24-h contextual memory test did not affect the expression of fear in observational fear–conditioned mice as compared with fear expression by saline-injected mice ($n = 14$) ($F_{1, 21} = 0.001$, $P = 0.99$, two-way repeated ANOVA). (**c**,**d**) The contribution of the lateral amygdala (LA) to observational fear conditioning. Mice with lidocaine injections into the ACC ($n = 8$) before training failed to acquire fear compared with those receiving saline injections ($n = 10$) ($F_{1, 16} = 11.46$, $P = 0.004$, two-way repeated ANOVA, **c**); the same was true for contextual memory 24 h after training ($F_{1, 16} = 21.34$, $P = 0.0003$, two-way repeated ANOVA, **d**). (**e**,**f**) Local inactivation of the lateral amygdala ($n = 8$) before the 24-h contextual memory test (**f**) disrupted the expression of fear in observational fear-conditioned mice (**e**), as compared with fear shown by saline-injected mice ($n = 7$) ($F_{1, 13} = 7.66$, $P = 0.016$, two-way repeated ANOVA). $^*P < 0.05$, $^{**}P < 0.01$, Scheffe's *post hoc* test. Error bars represent s.e.m.

In contrast, a similar inactivation of the VPL/VPM thalamic nuclei, which belong to the lateral, sensory pain system, did not affect observational fear learning (**Fig. 3h**,**i**), indicating that these VPL/VPM thalamic nuclei may not be important for the observational fear learning. Taken together, these findings indicate that the ACC, parafascicular and mediodorsal nuclei, but not VPL/VPM nuclei, are involved in observational fear learning and that fear learning requires the medial pain system representing the affective or emotional dimension of pain, but not the lateral system for sensory dimension of pain.

Differential roles of ACC and amygdala in fear conditioning

To further study the role of the ACC in long-term memory of observational fear, we subjected mice to observational fear conditioning (**Fig. 4a**) and then microinjected lidocaine into the ACC 8 min before the 24-h contextual memory test (**Fig. 4b**). No difference in the freezing level was found between the lidocaine and the control groups (**Fig. 4b**). The amygdala is believed to be responsible for behavioral reactions to emotional changes, such as noxious stimuli or situations causing aversive or unpleasant consequences[2,3]. Many studies have found that the amygdala is involved in the recognition of fear or anxiety by observing faces bearing fearful expressions[6,8–11]. In addition, excitatory anatomical connections have been known to exist between the ACC and the amygdala, including lateral nucleus[34]. These raise the possibility that there is interaction between the lateral amygdala and the ACC during social fear learning. To test this possibility, we inactivated the lateral amygdala, a brain area that is important for fear learning and memory storage, before the training (**Fig. 4c**,**d**) or 24-h contextual memory test (**Fig. 4e**,**f**). Inactivation of the lateral amygdala led to disruption of both the acquisition of observational fear (**Fig. 4c**) and the expression of this fear memory 24 h later (**Fig. 4f**). These findings are different from the results of ACC inactivation, where the acquisition of observational fear, but not the 24-h memory retrieval or recall was impaired, and thus suggest that the long-term storage of this observational fear memory is not in the ACC, but is instead elsewhere, perhaps in the lateral amygdala.

We further investigated the role of the ACC in observational fear conditioning and classical fear conditioning. In contrast with its effect on observational fear conditioning, inactivation of the ACC before classical fear conditioning did not affect the mice's fear response during the training (**Supplementary Fig. 3**), in 24-h context-dependent memory (**Supplementary Fig. 3**) or in the 24-h cued memory (**Supplementary Fig. 3**). This result suggests that ACC is involved in vicarious fear learning by observation, but not in classical conditioning that relies on direct experience of the noxious stimuli of foot shocks, and also strengthens the idea that the ACC is generally associated with the emotional or affective aspects of noxious stimuli.

Synchronized theta activity in ACC and lateral amygdala

To confirm the functional connectivity of the ACC and the lateral amygdala, we simultaneously measured electrical activities of the ACC and the lateral amygdala by recording local field potentials in freely behaving observers (**Fig. 5**). We compared the local field potentials before (habituation; **Fig. 5a–c**) and during observational fear learning (conditioning; **Fig. 5d–f**). Colored power spectra analysis revealed that the moderate and dispersed neuronal activities in the ACC and lateral amygdala during habituation (**Fig. 5b**) became intensive and concentrated activities at theta frequency during observational fear learning (**Fig. 5e**). Theta rhythms became substantially stronger in both the ACC (**Fig. 5g**) and the lateral amygdala (**Fig. 5h**) during observational fear learning than they were before the learning. More importantly, nonsynchronized theta activities before training (**Fig. 5c**) became synchronized theta oscillations between the ACC and the lateral amygdala (**Fig. 5f**).

Thus, a cross-correlation analysis[35] revealed significant synchronization of theta rhythms (at 5.9 ± 0.19 Hz) between the ACC and the lateral amygdala during fear learning (the second peak value after the last foot shock, 0.217 ± 0.08; before the first foot shock, 0.01 ± 0.03; $P < 0.05$, Student's t test) (**Fig. 5i**). These results suggest that the ACC and the lateral amygdala may closely interact for dynamic information transfer during observational fear learning.

ACC–limited deletion of $Ca_v1.2$ on fear and pain behavior

To further examine the ACC in social fear learning, we generated *Cacna1c* conditional knockout mice using *cre-loxP* and *flp-Frt* (**Supplementary Fig. 4**), as the $Ca_v1.2$ Ca^{2+} channel is highly expressed and is known to contribute to synaptic transmission and neuronal excitability in the ACC[28,29]. To delete $Ca_v1.2$ locally, we injected purified cell-permeable Cre recombinase (cp-Cre)[36] into the ACC (**Supplementary Fig. 4**). The deletion of $Ca_v1.2$ in the ACC was confirmed by immunostaining (**Supplementary Fig. 4**).

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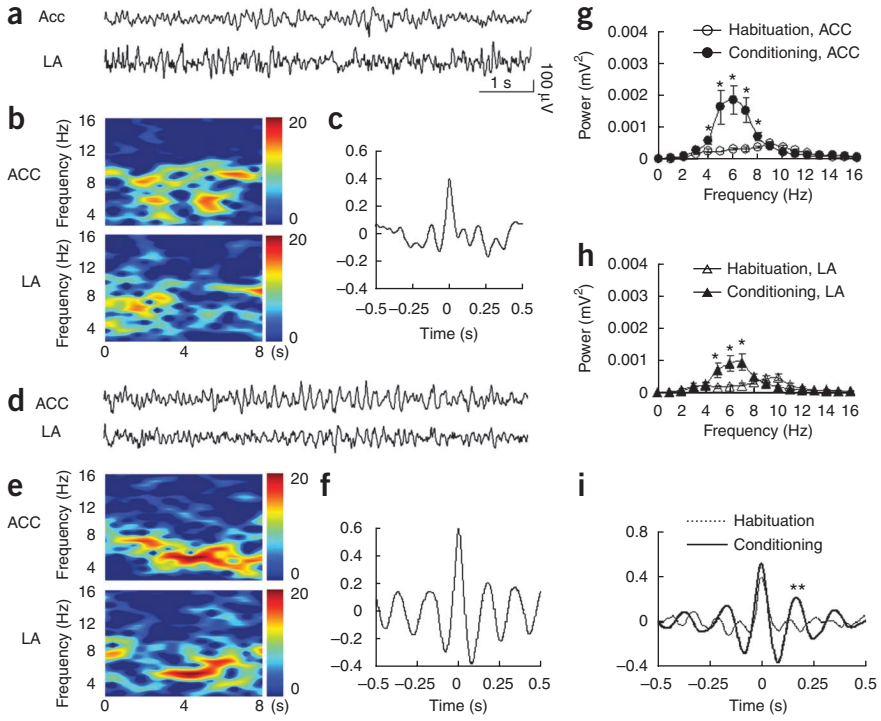

Figure 5 Synchronized theta activity between the ACC and lateral amygdala during learning of fear by observation. (**a**) Representative original traces of field potential recordings (8 s) in the ACC (upper) and lateral amygdala (bottom) during habituation. (**b**) Colored power spectra of the traces shown in **a**. (**c**) Cross-correlation analysis revealed no correlated neuronal activity in the two brain areas. (**d**) Representative original traces of field potential recordings in the ACC (upper) and lateral amygdala (bottom) during training. (**e**) Colored power spectra of the traces shown in **d**. Note the increased theta rhythms at 4–7 Hz. (**f**) Cross-correlation analysis revealed correlated neuronal activities in the two brain areas. (**g**,**h**) Averaged power spectra of neuronal activities ($n = 7$) in the ACC (**g**) and lateral amygdala (**h**) taken over an 8-s period just before delivery of the first foot shock (habituation) and after the last foot shock (conditioning). $^*P < 0.05$, one-way ANOVA. (**i**) Averaged cross-correlograms of neuronal activities in the ACC and lateral amygdala taken over an 8-s period just before delivery of the first foot shock (habituation) and after the last foot shock (conditioning) ($n = 7$). ** indicates a significant difference in the amplitude of the second peaks between the two ($P < 0.05$, Student's t test).

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We generated locally deleted *Cacna1c* ($Ca_v1.2^{ACC/Cre}$) mice and used them for various behavioral assays. The $Ca_v1.2^{ACC/Cre}$ observers exhibited impaired observational fear compared with control observers injected with phosphate-buffered saline (PBS, $Ca_v1.2^{ACC/PBS}$) or observers without any treatment (*Cacna1c*$^{loxP/loxP}$, referred to as $Ca_v1.2^{loxP/loxP}$ mice) (**Fig. 6a**,**b**). In addition, the $Ca_v1.2^{ACC/Cre}$ observers shed fewer fecal droppings during observational fear conditioning than the other groups (**Supplementary Fig. 2**). When an opaque partition was used during observational conditioning, all groups displayed similarly decreased levels of freezing behavior compared to those with a transparent partition (**Supplementary Fig. 5**). Notably, the $Ca_v1.2^{ACC/Cre}$ mice displayed reduced pain behavioral responses to formalin (**Fig. 6c**,**d**) and acetic acid (**Fig. 6e**,**f**), as compared with $Ca_v1.2^{ACC/PBS}$ mice. However, there was no difference between the two groups in acute pain behavior, either mechanical or thermal (**Supplementary Fig. 5**), which is known to be mediated by a spinal reflex[37]. These results suggest that functional $Ca_v1.2$ type 1 Ca^{2+} channels in the ACC are required for observational social fear learning and are critical for pain response modulation at the supraspinal levels, further supporting the involvement of the ACC in affective or emotional dimension of noxious or aversive stimuli[20,24–26]. In contrast, $Ca_v1.2^{ACC/Cre}$ mice showed normal behavioral responses in elevated plus maze (**Supplementary Fig. 6**), light/dark transition (**Supplementary Fig. 6**),

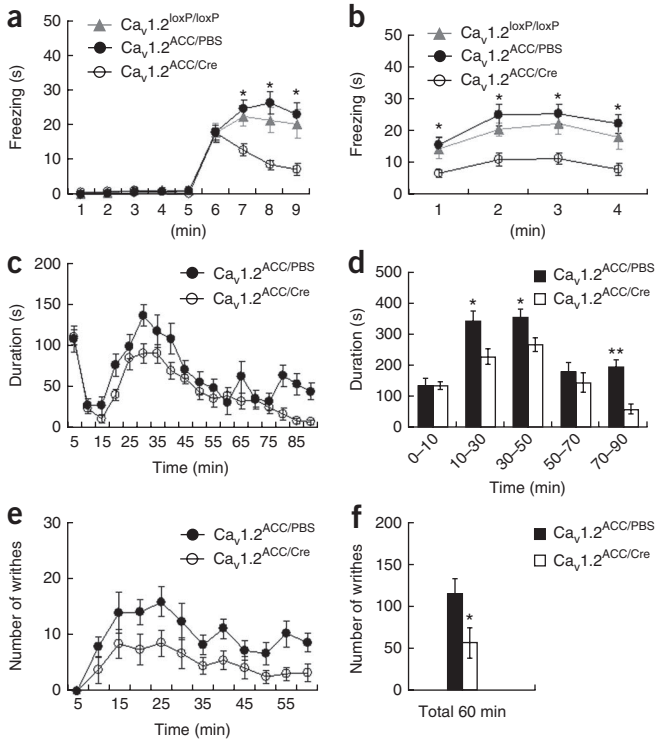

Figure 6 $Ca_v1.2^{ACC/Cre}$ mice showed impaired observational fear learning and reduced pain responses. (**a**,**b**) Observational fear conditioning of $Ca_v1.2^{ACC/Cre}$ ($n = 22$), $Ca_v1.2^{ACC/PBS}$ ($n = 22$) and $Ca_v1.2^{loxP/loxP}$ ($n = 13$) mice. Similar freezing levels were seen during training ($F_{1, 33} = 0.48$, $P = 0.49$, two-way repeated ANOVA) and in the 24-h contextual memory test ($F_{1,33} = 0.95$, $P = 0.34$, two-way repeated ANOVA) between $Ca_v1.2^{ACC/PBS}$ (PBS injected) and $Ca_v1.2^{loxP/loxP}$ (non-injected) observers, and the results were pooled for analysis. The $Ca_v1.2^{ACC/Cre}$ observers exhibited impaired observational fear learning during training ($F_{1, 55} = 17.47$, $P < 0.0001$, two-way repeated ANOVA, **a**) and 24-h contextual memory ($F_{1, 55} = 20.85$, $P < 0.0001$, **b**). $^*P < 0.01$, Scheffe's *post hoc* test. (**c**,**d**) Reduced inflammatory pain responses to formalin in $Ca_v1.2^{ACC/Cre}$ mice. Behavioral responses to a formalin injection, plotted in 5-min intervals, in $Ca_v1.2^{ACC/PBS}$ mice ($n = 9$) compared with $Ca_v1.2^{ACC/Cre}$ mice ($n = 15$) are shown in **c**. Data from **c** were grouped into five time intervals (**d**). $^*P < 0.05$, $^{**}P < 0.0001$, one-way ANOVA. (**e**,**f**) Reduced behavioral responses to acetic acid–induced visceral pain in $Ca_v1.2^{ACC/Cre}$ mice. Behavioral responses to acetic acid, plotted in 5-min intervals, in $Ca_v1.2^{ACC/PBS}$ mice ($n = 7$) and $Ca_v1.2^{ACC/Cre}$ mice ($n = 6$) are shown in **e**. The total numbers of writhing events over 60 min are shown in **f**. $^*P < 0.05$, one-way ANOVA. Error bars represent s.e.m.

open field (**Supplementary Fig. 6**), novel object recognition (**Supplementary Fig. 7**), predator exposure (**Supplementary Fig. 7**) and classical fear conditioning tasks (**Supplementary Fig. 7**).

DISCUSSION

We developed and characterized a behavioral assay system for observational fear conditioning in the mouse. Using this behavioral assay, we further investigated the neuronal substrate and mechanism underlying observational social fear learning. We found that the ACC and the MITN (parafascicular and mediodorsal thalamic nuclei), which consist of the medial pain system representing the affective or emotional dimension of pain, are involved in observational fear. In contrast, the VPL/VPM thalamic nuclei that belong to the lateral, sensory pain system may not be involved in the process. Notably, the lateral amygdala, but not the ACC, was involved in the expression of the observational fear memory. Augmented and synchronized theta activities between the ACC and the lateral amygdala were observed during this social fear behavior. In addition, the $Ca_v1.2$ type 1 Ca^{2+} channels in the ACC were required for the observational fear behavior. These data suggest a neural substrate and a cellular or molecular mechanism of social fear learning in the mouse.

Lesion and acute inactivation studies have shown that the ACC is involved in modulating the efficiency of trace auditory fear learning requiring high attention[34,38]. However, those lesions did not affect the expression of the memory[34]. In addition, other lesion studies on avoidance learning found that an ACC lesion after training did not impair the expression of conditioned place aversion, indicating that the ACC was involved in producing an aversive teaching signal, but not in memory retrieval for context[39,40]. Similarly, our results indicate that lateral amygdala inactivation disrupts both the acquisition and the retrieval of observational fear, whereas ACC inactivation only affects acquisition. These results suggest that the lateral amygdala is essential for the learning and memory storage of fear, whereas the ACC may have a modulatory role in the development of fear by integrating sensory and affective components of information.

Inactivation of the MITN decreased observational fear behavior to a similar extent as inactivation of the ACC (**Fig. 3**). However, inactivation of the parafascicular nuclei had no influence on classical fear conditioning (**Supplementary Fig. 8**), similar to that of the ACC (**Supplementary Fig. 3**). On the other hand, an inactivation of VPL/VPM thalamic nuclei, the sensory pain system, had no effect on social fear learning by observation, although the same inactivation decreased the pain response behavior of the mice (**Supplementary Fig. 8**). These results indicate that the medial pain system, but not the lateral sensory pain system, is involved in observational social fear learning. At the moment, however, we cannot rule out nonserial involvements of the ACC and the MITN. In fact, there was a difference in the freezing levels among the observers with lidocaine injection into different brain areas (ACC, parafascicular, mediodorsal and lateral amygdala) before training (**Supplementary Fig. 8**). The interpretation of these results, however, is limited by the lack of evidence for a complete inactivation of each nucleus. Further studies with crossed inactivation or disconnection of the ACC or lateral amygdala and the MITN will provide valuable information regarding whether these brain areas are involved in observational fear learning in serial processing.

It has been shown that synchronization of neuronal activities is a potential mechanism linking anatomically and functionally related regions of the brain[35,41,42]. The amygdala is widely believed to be responsible for behavioral reactions to emotional changes, such as noxious stimuli or situations causing aversive or unpleasant consequences, and previous work using images of facial expression has shown that activation of the amygdala, as well as the ACC, is necessary for fear recognition by observation of others experiencing fear or others' fearful facial expressions[6,8,10,11]. Thus, investigation of the neuronal activity between the ACC and the amygdala can provide important information on observational fear learning. The ACC is also known to be involved in executive processes, attentional processes and decision making, and a number of electroencephalography studies have shown that theta (4–7 Hz) oscillations around the ACC and prefrontal cortex occur in a variety of behavioral tasks, particularly in tasks involving working memory and mental efforts in humans[18,43–45]. Our results suggest that theta oscillations are also involved in observational fear recognition and that the synchronized activity in the ACC–lateral amygdala occurs during this behavior. Therefore, the increased coherent theta activities in the ACC–lateral amygdala may represent and promote neuronal communication required for recognition and expression of social fear.

We generated a mouse model for studying impaired social fear learning, a mouse with an ACC-limited deletion of $Ca_v1.2$ Ca^{2+} channels. The $Ca_v1.2^{ACC/Cre}$ mice showed not only disrupted social fear learning by observation, but also reduced pain responses. Notably, the $Ca_v1.2^{ACC/Cre}$ mice showed a level of anxiety (**Supplementary Fig. 6**) and innate fear (**Supplementary Fig. 7**) that was similar to that of control mice. Thus, these results suggest that the neural mechanisms underlying observational social fear behavior may be different from those for anxiety or innate fear. In addition, the $Ca_v1.2^{ACC/Cre}$ mice showed normal performance in the object recognition task with inanimate objects (**Supplementary Fig. 7**), suggesting that the processing of individual information obtained by observing different objects varies depending on the objects (a conspecific, a live predator and an inanimate object). The $Ca_v1.2^{ACC/Cre}$ mice had a normal fear response in classical fear conditioning (**Supplementary Fig. 7**), as did the ACC-inactivated mice (**Supplementary Fig. 3**). At this point, we do not know the role of $Ca_v1.2$ in observational fear. One possibility is that the effects of the $Ca_v1.2$ deletion might be the result of a general decrease of excitability in the ACC. Further electrophysiological or pharmacological experiments will reveal the role of $Ca_v1.2$ in this process. To the best of our knowledge, this is the first animal behavioral study to use the cp-Cre recombinase in the *cre-loxP* site-specific recombination system *in vivo*, suggesting that direct application of cp-Cre can be a useful tool for studying the time- and tissue-specific role of a gene of interest.

The observers showed higher levels of fear responses when siblings or female mating partners were used as the demonstrators than when the demonstrators were not siblings or related female mice. These results suggest that something more than just emotional contagion could be involved in observational social fear learning. Particularly, the strength of the fear response was increased with increasing familiarity of the observer to the demonstrator (couples experiments). Familiarity is considered to be a factor in making the observer more empathetic for the situation or state of the demonstrator in primates[46]. Therefore, if empathy is broadly used as a term representing affective behaviors focused on the response of the observer[47], empathy might be engaged in observational social fear learning, as in the case of the social modulation of pain[48]. However, although there were apparently no aggressive behaviors such as dashing toward the other mouse during the training, the responses of the demonstrators to repetitive shocks might be experienced as a dangerous (threatening) or stressful action by the observers, rather than the observers having an empathic fear response for the demonstrators. Future studies are necessary to clarify this point.

In our observational fear assay, it appears that the sensory modalities (olfactory, auditory and visual cues) could have a synergistic effect on the

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observers. This is supported by the results of the experiments in which an opaque partition selectively removed the visual input. Even under this condition, siblings or couples induced higher freezing levels in observers than nonsiblings or noncouples, respectively (**Supplementary Fig. 9**).

Here we developed a behavioral assay system to measure social fear conditioning by observation in the mouse and found that mice can learn fear without first-hand experience of noxious stimuli (for example, foot shocks). We found that the affective pain system is involved in social fear learning by observation and identified the $Ca_v1.2$ Ca^{2+} channel in the ACC as an essential element in the process. Many aberrant social behaviors associated with psychiatric conditions, including various psychopathic or mental disorders (for example, post-traumatic stress disorders, schizophrenia, autism and dementia), feature impairment of recognition of the emotions and feelings of others and dysfunctions in the ACC have been associated with these psychiatric conditions[19,49]. We suggest that this behavioral model for social fear can be a useful tool for developing measures to control such disorders.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

D. Jeon and H.-S.S. designed the experiments. D. Jeon purified Cre protein. D. Jo and H.E.R. made and provided the vector containing His_6-NLS-Cre-MTS. D. Jeon, S.K. and M.C. performed surgeries, microinjections and immunostainings and analyzed the data. D. Jeon and S.K. performed *in vivo* electrophysiology. S.-Y.L., D.R. and J.-P. K. generated the $Ca_v1.2$ conditional mice. D. Jeon and H.-S.S. wrote the manuscript. All of the authors commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Observational fear conditioning. The apparatus for observational fear conditioning consisted of two identical chambers (each, 18 × 17.5 × 38 cm) containing a transparent Plexiglas (or opaque, when required) partition in the middle and a stainless-steel rod floor (5-mm diameter rods, spaced 1 cm apart) (which are modified passive avoidance cages, Coulbourn Instruments). Sounds and smells could be transmitted between the chambers under the rod floor. For observational fear conditioning, mice (observer and demonstrator) were individually placed in apparatus chambers for 5 min and then a 2-s foot shock (1 mA) was delivered every 10 s for 4 min to one of the mice (demonstrator) via a computer-controlled animal shocker (WinLinc, Coulbourn Instruments). As the optimal conditioning protocol, we chose 10-s intervals for foot shocks and a 4-min training period based on our pilot experiments with different protocols, and performed some control experiments (**Supplementary Discussion** and **Supplementary Fig. 1**). To assess contextual memory, we placed the observers back into the training context 24 h after training and observed freezing behavior for 4 min. In all experiments, we used male C57BL/6J mice, except in the couple versus noncouple experiments (a male mouse observed a female mate). Siblings were maintained in groups of two or three mice, separate from their mother, after weaning. For the couple and noncouple experiments, mating cages were set up 7–8 weeks after birth, consisting of one male and two female mice. After housing periods of different length (1 week, 4–5 weeks, 10–15 weeks and 20–36 weeks), some male mice were randomly chosen and used as couple observers (co-housed female mice were used as demonstrators) and others used as noncouple observers (female mice co-housed with different male mice were used as demonstrators). Male and female mice were separated just before training and they were not housed together after the training. Fear response was video-recorded and quantified by an experimenter blind as to the condition and genotype by measuring the length of the time during which a mouse showed freezing behavior by hand, defined as lack of movement (except for respiratory movements) for longer than 2 s. All experiments were performed on 12–15-week-old mice, except those of the couple versus noncouple experiments. Animals were housed with a 12-h light/dark cycle and *ad libitum* access to food and water, and animal care and handling were carried out according to the guidelines from the Animal Care and Use Committee of the Korea Institute of Science and Technology.

Classical fear conditioning. Mice were placed in the fear-conditioning apparatus chamber for 5 min and a 28-s acoustic conditioned stimulus was delivered. Following the conditioned stimulus, a 0.7-mA shock, the unconditioned stimulus, was immediately applied to the floor grid for 2 s (WinLinc, Coulbourn Instruments). The conditioned stimulus–unconditioned stimulus coupling was performed three times at 60-s intervals. To assess contextual learning, we placed the mice back into the training context 24 h after training. To assess cued learning, we placed the mice in a different context (a novel chamber) 24 h after training and monitored their behaviors for 5 min. During the last 3 min of this test, the mice were exposed to the tone. Fear response was quantified as described above.

Cannula implantation and microinjection. For microinjection, a cannula (Plastic Products) was unilaterally implanted in the right hemisphere at anteroposterior (AP) +1.0 mm, lateral (L) 0.2 mm and dorsoventral (DV) 1.2 mm for the ACC, AP −2.3 mm, L 0.7 mm and DV 3.3 mm for the parafascicular nuclei, AP −1.4 mm, L 0.2 mm and DV 3.5 mm for the mediodorsal nuclei, AP −1.7 mm, L 1.8 mm and DV 3.5 mm for the VPL/VPM, and AP −1.8 mm, L 3.5 mm and DV 4.2 mm for the lateral amygdala from bregma using a stereotaxic apparatus (Kopf Instruments). Experiments began 14 d after surgery. Lidocaine (4%, vol/vol, 0.7 μl) or saline (0.9% NaCl, vol/vol, 0.7 μl) was infused into each brain area via an inner cannula (33 gauge) connected to a 25-μl Hamilton syringe; the flow rate (0.1 μl min^{-1}) was regulated by a syringe pump (SP100i, WPI). Experiments began 8 min after a single microinjection. The position of the cannula was verified histologically after experiments (**Supplementary Fig. 10**).

***In vivo* electrophysiology.** Field potential recording *in vivo* was performed as described previously[35]. Recordings were obtained using tungsten electrodes (0.005 inch, 2 MΩ) positioned unilaterally into the right hemisphere at AP +1.0 mm, L 0.2 mm and DV 1.2 mm (ACC), and AP −1.8 mm, L 3.5 mm and DV 4.2 mm (lateral amygdala) from bregma with grounding over the cerebellum. Electrode position was verified histologically after experiments (**Supplementary Fig. 10**). Electrical activities were recorded after being amplified (×5,000), bandpass-filtered from 0.1 to 100 Hz (QP511 QUAD AC), digitized with a 1-kHz sampling rate (DIGIDATA 1320A, Axon Instruments) and stored on a computer. Field potential waveforms were analyzed offline using Matlab and pClampfit 9. To obtain colored power spectra and for cross-correlation analyses, we filtered field potential waveforms from 2–40 Hz. Colored power spectra were calculated and drawn with Fourier transformation of 2-s window sizes. Cross-correlograms were averaged between mice after alignment to the maximal positive peak.

Generation of *cre-loxP* $Ca_v1.2$ conditional knockout mice. Murine *Cacna1c* gene sequences were isolated from a mouse strain 129/Sv genomic phage library. The construct targeted the same area (exons 14 and 15) as was done previously[50] with two differences: a 2-loxP-2-Frt vector was used instead of a 3-loxP vector and a *Bam*HI site was manipulated (removed in the 5′ isogenic arm and re-introduced into the 3′ isogenic arm) to allow us to differentiate between the four configurations, wild type (8.4 kb), *Frt* and *loxP* flanked (targeted, 14.5 kb), *loxP* flanked (12.6 kb) and null (9.7 kb) (**Supplementary Fig. 4**). To generate chimeric mice, we injected the targeted embryonic stem cell clones (14.5 kb) into blastocytes from BALB/c mice. The chimeric mice generated were then crossed with C57BL/6J mice to generate the heterozygous $Ca_v1.2^{Frt-loxP/+}$ mice (in B6/129 mixed background). FLPe mice (in B6 background) were crossed to the $Ca_v1.2^{Frt-loxP/+}$ mice to generate the $Ca_v1.2^{loxP/+}$ mice (the *Neo* gene was removed by *flp-Frt* recombination). After backcrossing with an inbred C57BL/6J over three generations, heterozygous $Ca_v1.2^{loxP/+}$ mice were intercrossed to derive homozygous $Ca_v1.2^{loxP/loxP}$ mice. $Ca_v1.2^{loxP/loxP}$ mice aged 8–10 weeks were injected with cp-Cre or PBS and the mice were used in experiments 5 weeks after the injection.

Microinjection and verification of cp-Cre recombinase. The construction of the cp-Cre (His_6-NLS-Cre-MTS) expression plasmid and the purification methods of cp-Cre were described previously[36]. Purified cp-Cre (1 μl, 1 mg ml^{-1}) was bilaterally infused, under a stereotaxic apparatus, into the ACC of the ROSA-GFP reporter mice and the $Ca_v1.2^{loxP/loxP}$ mice through a 30-gauge needle connected to a 25-μl Hamilton syringe. The ROSA-GFP reporter mice were used to confirm the biologically active transduction of cp-Cre (**Supplementary Fig. 4**). The nonspecific effect of cp-Cre on the mice was also investigated in behavioral tasks (**Supplementary Discussion** and **Supplementary Fig. 11**). The $Ca_v1.2$-deleted proportion of the ACC was examined by immunostaining with antibody to $Ca_v1.2$ (1:250, Alomone Labs, ACC-003) (**Supplementary Figs. 4** and **11**).

Nociception behaviors. The formalin test was carried out in a Plexiglas cylinder (diameter, 20 cm; height, 30 cm) positioned over a mirror angled at 45°. Video was recorded through the mirror from the bottom, which allows a clear observation of the movements of the paws. After a 1-h habituation, 10 μl of formalin (5%, vol/vol) was subcutaneously injected into the dorsal surface of a hind paw. The mouse's behavior (licking, biting and shaking the injected hind paw) was recorded for the next 90 min and the total time of licking and biting was monitored at 5-min intervals.

For visceral pain, acetic acid (0.6%, vol/vol, 10 ml kg^{-1}) was injected into the peritoneum. Writhing (abdominal stretching and constriction) was measured over 1 h. The number of writhing motions was counted at 5-min intervals. Visceral pain test was performed with the same apparatus that was used in the formalin test.

The mechanical pain was performed with von Frey filaments (Stoelting). The filament was applied from underneath the floor, through the mesh, to the plantar surface of the paw for each limb. The mechanical threshold was defined as the bending force of the filaments, in grams, at which the mouse withdrew its paw.

To inflict thermal pain, we used a constant-intensity radiant heat source (7371 Plantar Test, UGO BASILE S.R.L.). Mice were placed and habituated in an open-top, glass-bottom, transparent plastic square chamber (14 × 22 × 17 cm) for 1 h. A mobile radiant heat across the glass floor was delivered to the underside of the last 2 cm of the mice's tails. Two different infrared intensities were applied, 20 and 50, and a 15-s cut-off time was used to prevent tissue damage.

Anxiety and locomotor activity tasks. The elevated plus maze was made of plastic and consisted of two white open arms (25 × 8 cm), two black enclosed arms (25 × 8 × 20 cm) and a central platform (8 × 8 × 8 cm) in the form of a cross. The maze was placed 50 cm above the floor. Mice were individually placed

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in the center with their heads directed toward one of the closed arms. The total time spent in each arm or center, and total number of entries into each arm was analyzed by video monitoring for 5 min.

The light/dark box (30 × 45 × 27 cm) was made of plastic and had a dark compartment (one-third of the total area) and a light compartment with a hole in the middle. The light compartment was illuminated at 600 lx. Both the elapsed time to entry into the light compartment and the amount of time spent in each compartment were measured over a 5-min period by video monitoring.

The open-field box was made of white plastic (40 × 40 × 40 cm) and the open field was divided into a central field (center, 20 × 20 cm) and an outer field (periphery). Individual mice were placed in the periphery of the field and the paths of the animals were recorded by a video camera. The total distance traveled was analyzed by using EthoVision XT (Noldus).

Predator exposure task. The same apparatus used in observational fear conditioning was used for the predator exposure task. A live Sprague-Dawley rat (280–330 g) was used as a predator giving threat. To assess innate fear, we placed the predator in one of chambers and then introduced the mice into the remaining chamber. Fear response was video-recorded for 4 min and quantified as described for observational fear conditioning.

Novel object recognition memory task. During the training trial, two objects were placed in an open-field box (40 × 40 × 40 cm) and mice were allowed to explore them for 5 min. A mouse was considered to be exploring the object when its head was facing the object to within 1 inch. After retention intervals (1 or 24 h), mice were placed back in the box with two objects in the same locations, but one of the familiar objects was replaced by a novel object, and the mice were then allowed to explore the two objects for 5 min. The preference percentage, ratios of the amount of time spent exploring any one of two objects or the novel one over the total time spent exploring both objects, was used to assess the recognition memory.

Statistics. Statistical analyses were conducted using SAS. Two-way repeated ANOVA, one-way ANOVA and Student's *t* test were used for behavioral analyses. $P < 0.05$ was considered to be statistically significant. All data are shown as means ± s.e.m.

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