SUPPLEMENTARY INFORMATION

Observational fear learning involves affective pain system and Ca\textsubscript{v}1.2 Ca\textsuperscript{2+} channels in ACC

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Supplementary Discussion

To optimize the conditioning we chose the 10-s interval for 2-s foot shock (1 mA) for 4-min training period from our pilot experiments with different protocols. When demonstrators received 0.7-mA foot shocks which are used in a typical fear conditioning, they showed heterogeneous behavioral responses: running, vocalization and/or jumping. However, foot-shock intensity with 1 mA always made them display a homogeneous response including all three behaviors, running, vocalization and jumping. Thus observers can always get similar inputs from demonstrators during the training. When the protocols with the 20-s interval for 2-s foot shock (1 mA) for 4-min training period (Supplementary Fig. 1a,b) and the 10-s interval for 2-s foot shock (1 mA) for 2-min training period (Supplementary Fig. 1c,d) were used, observers displayed low freezing levels during the training or in the 24-hr contextual memory test. However, high and stable freezing levels in the observers were induced by the protocol with the 10-s interval for 2-s foot shock (1 mA) for 4-min training period.

With this protocol we performed some control experiments and obtained the behavior profile of observer mice: 1) when there were no demonstrator mice, with or without foot shocks (Supplementary Fig. 1e), 2) simply in the presence of demonstrator mice for 9 min without foot shocks (Supplementary Fig. 1f,g), and 3) in a novel context (a different chamber from the one for training) 24 h after training (Supplementary Fig. 1h). The novel chamber was a black-colored plastic cylinder (diameter 20 cm and height 30 cm) and a small amount of mouse bedding was placed on the floor of the chamber. In experiments 1) and 2) the observers did not show freezing behavior, indicating that freezing response of the observers totally came from observation of freezing behaviors of the demonstrators. In experiment 3), the observers also did not show freezing behavior, indicating that the freezing response of the observers came from a specific contextual fear memory, rather than just an increase in generalized fear.

We also analyzed the freezing response of different demonstrators (siblings or couples vs. non-siblings or non-couples). There was no difference in the freezing levels among the demonstrators (Supplementary Fig. 9c,d). Therefore, we could not examine the possibility of a correlation between the intensity of the freezing response of observers and that of demonstrators, because the extensive training schedule might have induced maximum freezing response in the demonstrators.

To check a possible non-specific effect of cp-Cre on the animals, we microinjected cp-Cre into the ACC of wild-type mice (C57BL/6J), and then performed several behavioral experiments. The mice treated with cp-Cre showed similar levels of behavioral responses to those of non-treated mice in observational fear conditioning (Supplementary Fig. 11a,b), elevated plus-maze test (Supplementary Fig. 11c) and the light-dark transition task (Supplementary Fig. 11d), indicating a lack of non-specific effects of cp-Cre proteins on those behaviors.
Supplementary Figure 1. Pilot experiments with different protocols and control behavioral profiles in observational fear conditioning. (a,b) When the protocols with the 20-s interval for 2-s foot shock (1 mA) for 4-min training period were used, observers showed low freezing levels during the training and in the 24-hr contextual memory test. (c,d) When the protocols with the 10-s interval for 2-s foot shock (1 mA) for 2-min training period were used, observers displayed low freezing levels in the 24-h contextual memory test. (e) Freezing behavior of observer mice, when there are no demonstrator mice, with or without foot shocks. (f,g) Freezing behavior of observer mice, simply in the presence of demonstrator mice for 9 min without foot shocks. (h) Context specificity. Freezing behavior in a different chamber from the training chamber (a novel context, not the training context), 24 h after training.
Supplementary Figure 2. The number of fecal droppings (feces #) during observational fear learning. (a,b) When siblings or female mating partners were used as demonstrators, observers shed more fecal droppings during training than was the case when unrelated mice were demonstrators. (a) The number of droppings from subjects in siblings (n = 20) and non-siblings (n = 17) experiments ($F_{1,35} = 9.32, P = 0.0043$, one-way ANOVA). *$P < 0.01$. (b) The number of droppings from subjects from in couples (n = 15) and non-couples (n = 14) experiments ($F_{1,27} = 4.76, P = 0.038$, one-way ANOVA). *$P < 0.05$. Similar number of droppings was obtained in tests with mating partners co-housed for 10 to 15 weeks or 20 to 36 weeks, and therefore the results were pooled for analysis. (c) $\text{Ca}_v1.2^{\text{ACC/Cre}}$ mice (n = 22) shed fewer fecal droppings during training than the other groups ($\text{Ca}_v1.2^{\text{ACC/PBS}}$, n = 22; $\text{Ca}_v1.2^{\text{Floxed/Floxed}}$, n = 13) *$P < 0.01$, one-way ANOVA.
Supplementary Figure 3. Classical fear conditioning. (a) Administration of lidocaine into the ACC before training had no influence on the acquisition of fear (lidocaine, n = 10; saline, n = 8) ($F_{1,16} = 3.21$, $P = 0.092$ two-way repeated ANOVA). The horizontal line indicates the duration of a tone (28 s) and the vertical arrow indicates the time of foot shocks (2 s). (b) There was no difference between groups in 24-h contextual memory ($F_{1,16} = 0.16$, $P = 0.69$, two-way repeated ANOVA). (c) No difference was also found between groups in 24-h cued memory. The horizontal line indicates the duration of a tone (3 min).
a

Neo

Frt site

LoxP site

b

Targeted Floxed Null

Floxed Wild type

400 bp 300 bp

c

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Wild type

D"c

e

Floxed

Wild type

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Supplementary Figure 4. Generation of conditional Ca_v1.2 mice and ACC-specific gene deletion of the Ca_v1.2 by cp-Cre. (a) Targeting strategy of Ca_v1.2 locus. Black box, the targeted exon (exon14 and 15); Neo, the NEO cassette; Frt, the Flipase Recognition Target; LoxP, the locus of X-over P1. (b) Southern blot (upper) and PCR analysis (lower) analysis for the targeting and genotyping. The 8.4 Kb segment corresponds to the wild-type allele; the 14.5 Kb, the targeted allele; the 12.6 Kb, the floxed allele (Neo-deleted); the 9.7 Kb, the null allele (Neo, Exon 14, 15 deleted). The floxed allele versus wild-type allele was detected by PCR with the following pair of primers (with annealing temperature at 60°C): The 5’ primer was 5’-CCT CCC TGT GAG CTG TTC-3’ and 3’ primer was 5’-CCT TTG ATG TGC CAG AGG-3’. The 300 bp band, the PCR product of the wild-type allele; the 350 bp band, the product of the floxed allele (Neo-deleted). (c) Gel stained with coomassie blue shows purified cp-Cre. The arrow indicates cp-Cre. (d) Immunohistological signals (green) for the GFP from the coronal brain section including ACC of ROSA-GFP reporter mouse after bilateral injection of the cp-Cre, which indicates that the cp-Cre is biologically active. (e) Immunohistological labeling for the Ca_v1.2 in the ACC of Ca_v1.2 Cre/LoxP conditional mouse. Animals were anesthetized and perfused through the heart with 50 ml of cold saline and 50 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were then removed and were post-fixed overnight. Coronal sections (30 μm) containing ACC were stained with anti-Ca_v1.2 (1:250, Alomone Labs, ACC-003) as a primary antibody. A biotinylated secondary antibody and the avidin/biotin system were used for antibody followed by a DAB reaction. Strong immunoreactivity for Ca_v1.2 was seen in the non-injected ACC area (middle, 200× or 400× magnified from left red rectangle in the upper), but the immunoreactivity for Ca_v1.2 was absent in the large part of the ACC that was injected with cp-Cre (down, 200× or 400× magnified from right blue rectangle in the upper). Yellow arrow indicates the ACC area injected with cp-Cre (upper, 40×).
Supplementary Figure 5. Observational fear learning (with an opaque partition) and pain behavioral responses in the Cav1.2ACC/Cre mice. (a,b) Observational fear learning of the Cav1.2ACC/Cre (n = 7), Cav1.2ACC/PBS (n = 6) and Cav1.2Floxed/Floxed (n = 7) mice with an opaque Plexiglas partition. (a) Freezing behavior on the day of training and (b) 24 h after training. (c,d) Normal responses in acute pain behaviors of the Cav1.2ACC/Cre mice. (c) Paw withdrawal responses to the mechanical stimuli with von Frey filaments in the Cav1.2ACC/Cre (n = 14), Cav1.2ACC/PBS (n = 13) and Cav1.2Floxed/Floxed (n = 9) mice. (d) Tail flick latency to radiant heat stimuli in the Cav1.2ACC/Cre (n = 10), Cav1.2ACC/PBS (n = 9) and Cav1.2Floxed/Floxed (n = 5) mice.
Supplementary Figure 6. Ca,1.2^{ACC/Cre} mice showed a similar level of anxiety and locomotor activity with that of control mice. (a,b) Elevated plus maze test. (a) Total time spent in each arm and center was similar among the Ca,1.2^{ACC/Cre} (n = 15), Ca,1.2^{ACC/PBS} (n = 14) and Ca,1.2^{Floxed/Floxed} (n = 17) mice. (b) Total number of entries into each arm. (c) Light-dark transition test. There were no differences in the first time to entry into the illuminated, light compartment and the amount of time spent in each compartment among the Ca,1.2^{ACC/Cre} (n = 7), Ca,1.2^{ACC/PBS} (n = 8) and Ca,1.2^{Floxed/Floxed} (n = 12) mice. Inset, the total number of entry into the light compartment. (d,e) Open-field test. (d) The Ca,1.2^{ACC/Cre} (n = 10), Ca,1.2^{ACC/PBS} (n = 11) and Ca,1.2^{Floxed/Floxed} (n = 7) mice spent similar time in the center and (e) showed a similar locomotor activity in open-field box. However, all groups (Ca,1.2^{Floxed/Floxed}, Ca,1.2^{ACC/PBS}, and Ca,1.2^{ACC/Cre}) spent a little less time in the open arm in the elevated plus-maze test and in the center in the open-field box. This might be due to the mixed genetic background in conditional Ca,1.2 mice. Nevertheless, Ca,1.2^{ACC/Cre} have a similar level of anxiety responses as the control mice.
**Supplementary Figure 7.** Ca$_{v}$.1.2$^{ACC/Ca}$ mice showed normal behavioral responses in novel object recognition, predator exposure, or classical fear conditioning tasks. (a,b) Novel object recognition memory task. (a) Mean exploratory preference during training in the Ca$_{v}$.1.2$^{ACC/Ca}$ (n = 16) and Ca$_{v}$.1.2$^{ACC/PBS}$ (n = 14). Ca$_{v}$.1.2$^{ACC/Ca}$ mice and Ca$_{v}$.1.2$^{ACC/PBS}$ explored the two objects for equal time, which indicated no preference of the animals for either object. (b) Exploration to a novel object after each retention time. At 1 hr or 24 hr retention interval, when one of the familiar objects was replaced by a novel one, both the Ca$_{v}$.1.2$^{ACC/Ca}$ mice and the Ca$_{v}$.1.2$^{ACC/PBS}$ exhibited increased preference for the novel object to the familiar one, but no difference was found between the Ca$_{v}$.1.2$^{ACC/Ca}$ and the Ca$_{v}$.1.2$^{ACC/PBS}$ mice. (c) Predator exposure test for monitoring innate fear. The Ca$_{v}$.1.2$^{ACC/Ca}$ (n = 6), Ca$_{v}$.1.2$^{ACC/PBS}$ (n = 5) mice showed similar freezing behavior when a rat was located in opposite chamber as the control group. (d-f) Classical fear conditioning. (d) Freezing behavior on the day of training in the Ca$_{v}$.1.2$^{ACC/Ca}$ (n = 8) and Ca$_{v}$.1.2$^{ACC/PBS}$ (n = 9). Solid line indicates the duration of a tone (28 s) and the arrows indicate foot shocks (2 s). (e) Contextual fear conditioning 24 hr after training. (f) Cued fear conditioning 24 hr after training. A tone presentation is indicated by the horizontal line and foot shock, by vertical arrows.
Supplementary Figure 8 An inactivation of the PF thalamic nuclei did not affect classical fear conditioning (a–c), and an inactivation of the VPL/VPM thalamic nuclei reduced inflammatory pain responses to formalin (d,e). (a) Administration of lidocaine into the PF before training had no influence on the acquisition of fear (lidocaine, \( n = 8 \); saline, \( n = 6 \)). The horizontal line indicates the duration of a tone (28 s) and the arrow indicates the time of foot shocks (2 s). (b) There was no difference between groups in 24-hr contextual memory. (c) No difference was also found between groups in 24-hr cued memory. The horizontal line indicates the duration of a tone (3 min). (d) Behavioral responses to a formalin injection, plotted in 5 min intervals, in the mice injected with lidocaine in the VPL/VPM thalamic nuclei (\( n = 8 \)) compared with the saline group (\( n = 3 \)). The microinjection into was performed 3 times at 20-min interval. (e) Data from (d) grouped into five time intervals. (f) The averaged total freezing response among the observers with lidocaine injection into different brain areas (ACC, PF, MD, and LA) before the training.

\* \( P < 0.05 \), \** \( P < 0.01 \), Student’s \( t \)-test.
Supplementary Figure 9. The fear responses of the siblings/couples observers in the presence of an opaque partition (a,b), and the freezing response of each demonstrator in a transparent partition during the training (siblings or couples vs. non-siblings or non-couples) (c,d). Freezing behavior (a) in siblings (n = 15) vs. non-siblings (n = 20) experiments ($F_{1,33} = 17.44$, $P = 0.0002$, two-way repeated ANOVA) and (b) in couples (n = 11) vs. non-couples (n = 7) experiments ($F_{1,16} = 10.93$, $P = 0.0045$, two-way repeated ANOVA) using an opaque partition. Note that observers exhibited higher freezing levels with siblings or couples as demonstrators than with non-siblings or non-couples, respectively. *$P < 0.05$, Scheffe’s post-hoc test. (c) There was no difference in the freezing levels of the demonstrators between siblings and non-siblings. (d) There was also no difference in the freezing levels of the demonstrators between couples and non-couples.
**Supplementary Figure 10.** Postmortem histology of the recording regions, cannula positions, and verification of local delivery by dye into the target brain area. (a) Cannula position. Coronal brain slices show the location implanted with a cannula. (b) Fluorescent dye (Vybrant® DiI cell-labeling solution) was used to monitor that our injection was appropriately done into the target anatomical structures. After the injection of DiI into the target regions with the same condition (0.7 μl, 0.1 μl min⁻¹), brain was removed and post-fixed with 4% paraformaldehyde, and then coronally sectioned (100 μm) by a vibratome (VIBRATOME®). The red fluorescent signals by injection of DiI dye solution indicate that drug was properly delivered into the each restricted brain area. (c) Recording electrode position. Coronal brain slices show the positions of electrodes in the ACC (left) and the LA (right). Arrows indicate where the electrodes and cannula tip were located.
**Supplementary Figure 11.** Normal behavioral responses in wild-type (C57BL/6J) mice injected with cp-Cre (a–d), and Ca_{1.2}-deleted proportion of the ACC (e,f). (a,b) Normal observational fear conditioning in wild-type (C57BL/6J) mice injected with cp-Cre. No obvious difference between wild-type (C57BL/6J) mice treated with cp-Cre and non-treated mice in (a) the training and (b) the 24-hr contextual memory. (b,c) Normal anxiety in wild-type mice injected with cp-Cre. The mice having the ACC microinjected with cp-Cre had a similar level of behavioral responses in (e) elevated plus maze and (d) light-dark transition tasks as the control mice, which indicates that cp-Cre injections did not cause a non-specific abnormal activity. (e) Schematic representative drawings of mouse brain coronal sections showing the boundary areas of Ca_{1.2}-negative nuclei (grey regions). (f) Ca_{1.2}-positive cells in the ACC. For the counting of Ca_{1.2}-positive cells, after staining with anti-Ca_{1.2} as a primary antibody, five coronal sections around the injection site were selected and analyzed. Square sampling regions (rectangular, 300 × 300 μm) in (e) were used for Ca_{1.2}-positive cell counting. Total counts in these sampling regions were converted into cell densities for quantification. *P < 0.001, Student’s *t*-test.