Title: Processing of pain and itch information by modality-specific neurons within the anterior cingulate cortex in mice

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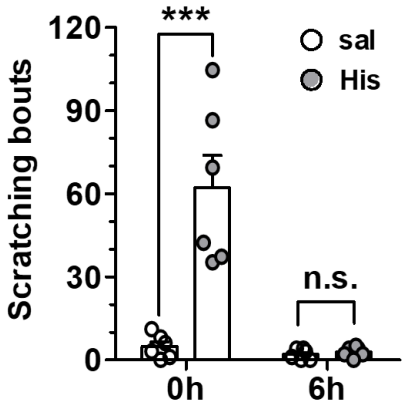
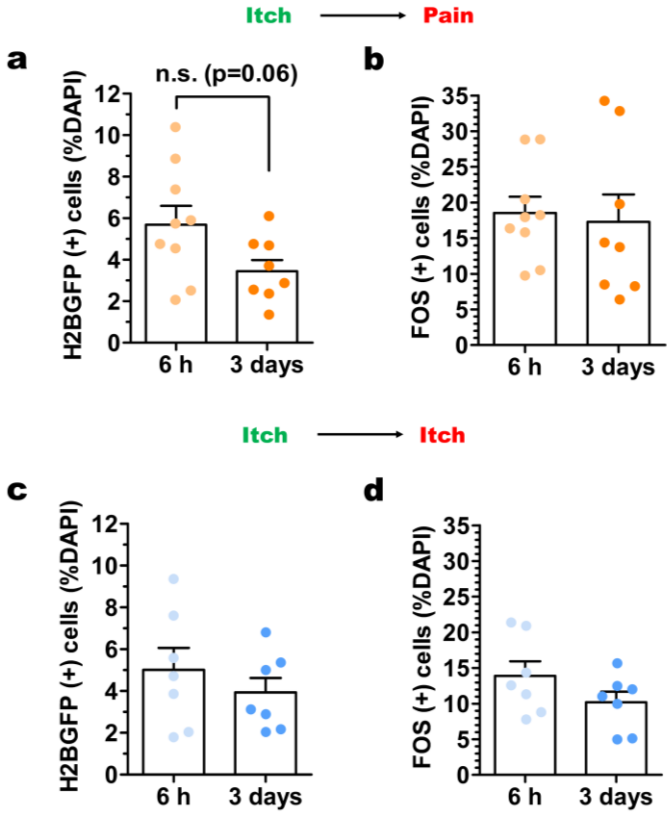
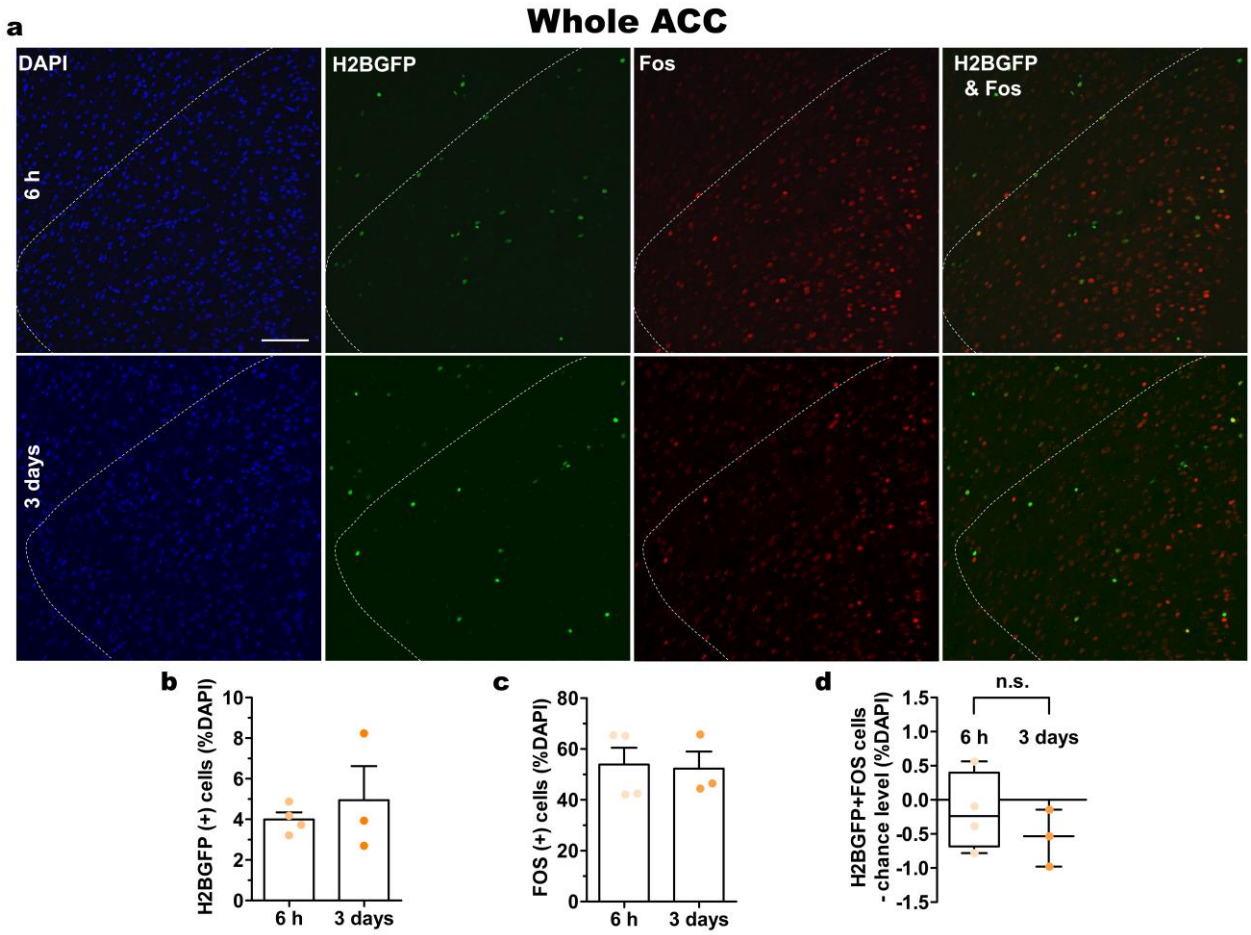


Supplementary Fig. 1. Itch sensation was not sustained at 6 h after histamine injection. Mice given intradermal histamine injections (40 mM, 20 μl) into their rostral backs exhibit robust scratching behavior for 30 min immediately after injection. This scratching behavior was no longer present 6 h after histamine injection (n = 6 mice/group; repeated-measures two-way ANOVA followed by Bonferroni post-test; effect of drug, $F_{(1,10)} = 25.28$, $p = 0.0005$; effect of time, $F_{(1,10)} = 25.64$, $p = 0.0005$; interaction effect, $F_{(1,10)} = 21.18$, $p = 0.0010$; saline vs. histamine at 0 h, *** $p < 0.001$). sal; Saline. His; Histamine. Data presented as mean ± SEM. Source data are provided as a Source Data file.



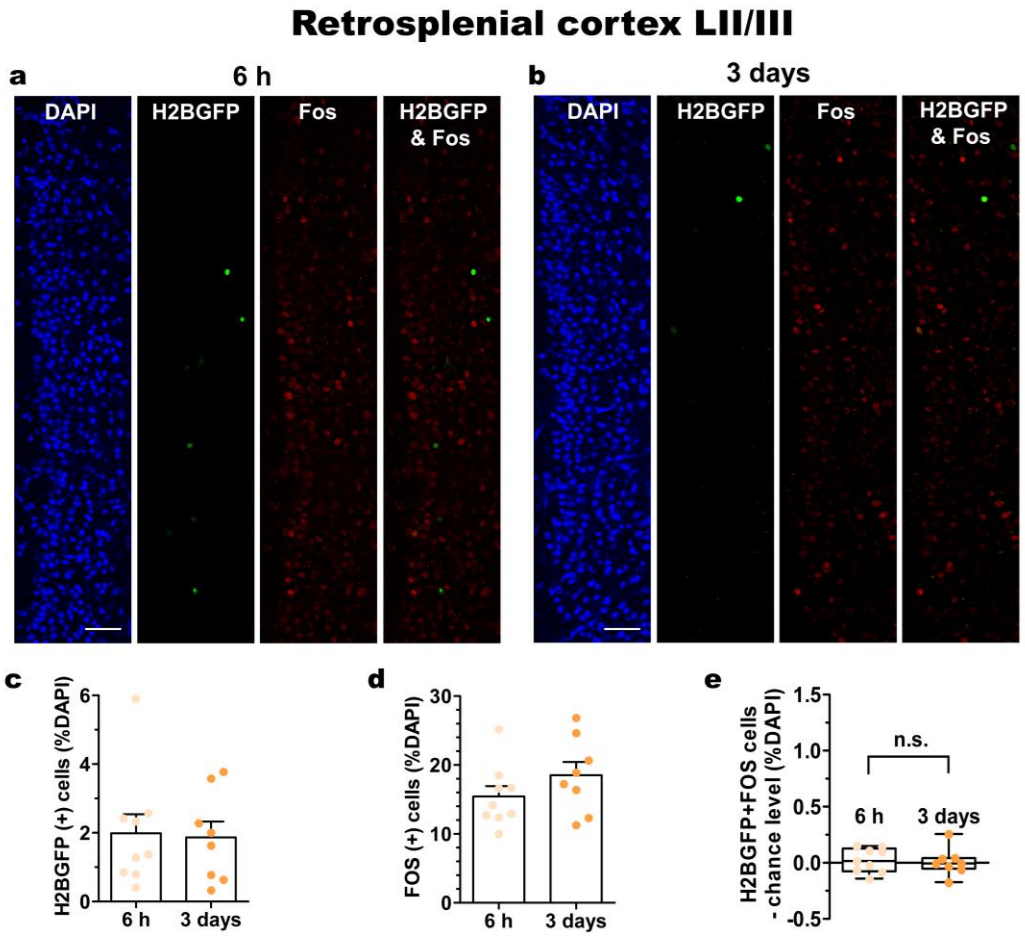
Supplementary Fig. 2. The proportion of ACC layer II/III neurons activated by a second stimulus did not depend on the time elapsed since the first stimulus. a. and **b.** are results from the Histamine → Formalin experiment. **c.** and **d.** are results from the Histamine → Histamine experiment. **a,** The proportion of H2BGFP-expressing neurons activated by the first (histamine) stimulus. There was no significant difference between the groups given a 6-h or 3-day interval between their first and second stimuli (n = 9 mice in 6-h interval, n = 8 mice in 3-day interval; two tailed unpaired t-test, $t_{15} = 2.033$, $p = 0.0601$). **b,** The proportion of Fos-expressing neurons activated by the second (formalin) stimulus. There was no significant difference between timing intervals (n = 9 mice in 6-h interval, n = 8 mice in 3-day interval; two tailed unpaired t-test, $t_{15} = 0.2891$, $p = 0.7765$). **c,** The proportion of H2BGFP-expressing neurons activated by the first (histamine) stimulus. There was no significant difference between timing intervals (n = 7 mice/group; two tailed unpaired t-test, $t_{12} = 0.8571\ 1$, $p = 0.4082$). **d,** The proportion of Fos-expressing neurons activated by the second (histamine) stimulus. There was no significant difference between timing intervals (n = 7 mice/group; two tailed unpaired

t-test, $t_{12} = 1.459$, $p = 0.1703$). Data presented as mean ± SEM. Source data are provided as a Source Data file.



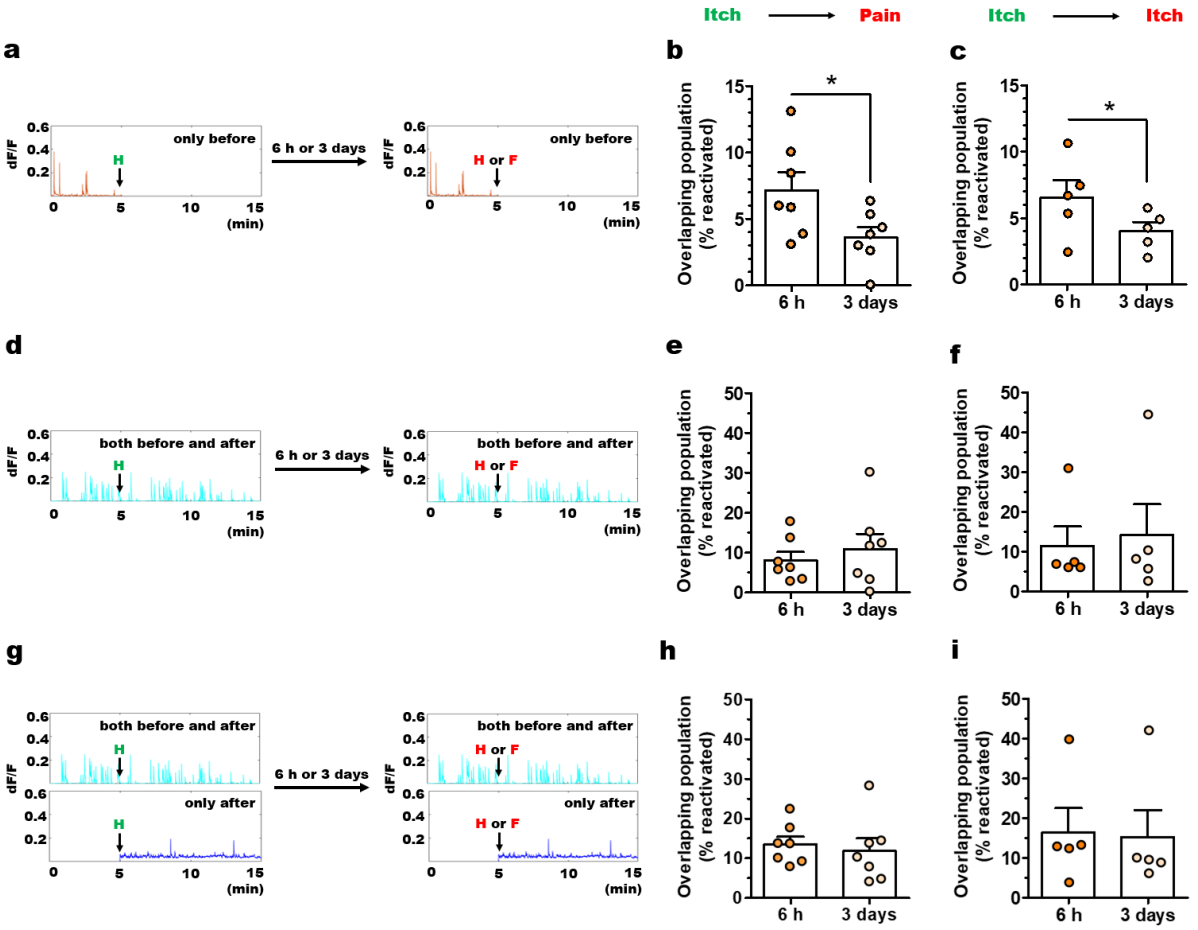
Supplementary Fig. 3. Excitability-based activation of neurons for pain and itch stimuli is specific to layer II/III of the ACC. a, Representative images of the entire ACC in mice with an interval of 6 h (top row) or 3 days (bottom row) between itch and pain stimuli. The white dashed line indicates the boundary of the ACC. Scale bar, 100 μm. **b,** The proportion of H2BGFP-expressing neurons activated by the first (histamine) stimulus. There was no significant difference between the groups given a 6-h or 3-day interval between the first and second stimuli (n = 4 mice in 6-h interval, n = 3 mice in 3-day interval; two tailed unpaired t-test, $t_5 = 0.6500$, $p = 0.544$). **c,** The proportion of Fos-expressing neurons activated by the second (formalin) stimulus. There was no significant difference between treatment intervals (n = 4 mice in 6-h interval, n = 3 mice in 3-day interval; two tailed unpaired t-test, $t_5 = 0.1640$, $p = 0.8762$). **d,** The proportion of neurons activated by both itch and pain stimuli was not different between the groups given a 6-h or 3-day interval between stimuli (n = 4 mice in 6-h interval, n

= 3 mice in 3-day interval; two tailed unpaired t-test, $t_5 = 0.9656$, $p = 0.3768$). Data presented as mean ± SEM (**b, c**) or Box and whiskers with 5-95 percentile (**d**). Source data are provided as a Source Data file.



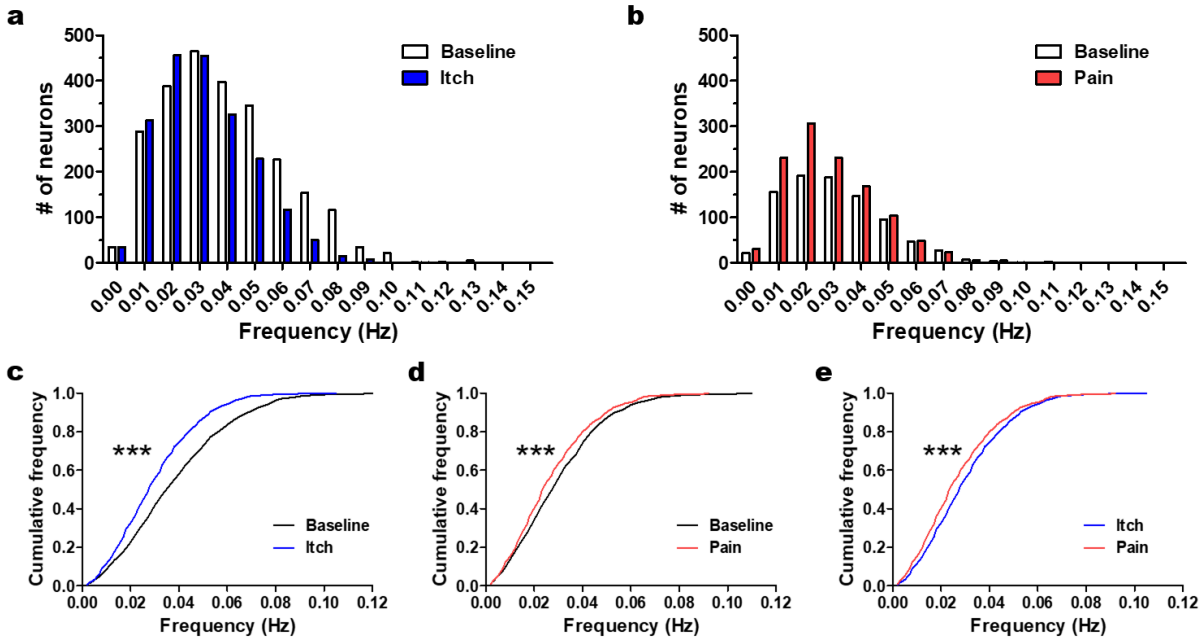
Supplementary Fig. 4. Layer II/III neurons in the retrosplenial cortex did not show excitability-based activation for pain and itch stimuli. a, Representative images of the retrosplenial cortex in mice with a 6-h interval between itch and pain stimuli. Scale bar, 50 μm. **b,** Representative images of the retrosplenial cortex in mice with a 3-day interval between itch and pain stimuli. Scale bar, 50 μm. **c,** The proportion of H2BGFP-expressing neurons activated by the first (histamine) stimulus. There was no significant difference between groups (n = 9 mice in 6-h interval, n = 8 mice in 3-day interval; two tailed unpaired t-test, $t_{15} = 0.1619$, p = 0.8736). **d,** The proportion of Fos-expressing neurons activated by the second (formalin) stimulus. There was no significant difference between groups (n = 9 mice in 6-h interval, n = 8 mice in 3-day interval; two tailed unpaired t-test, $t_{15} = 1.265$, p = 0.2253). **e,** The proportion of neurons activated by both itch and pain stimuli was not different in mice given a 6-h or 3-day interval between first and second stimuli (n = 9 mice in 6-h interval, n = 8 mice in 3-day

interval; unpaired t-test, $t_{15} = 0.2713$, $p = 0.7899$). Data presented as mean ± SEM (**c, d**) or Box and whiskers with 5-95 percentile (**e**). Source data are provided as a Source Data file.

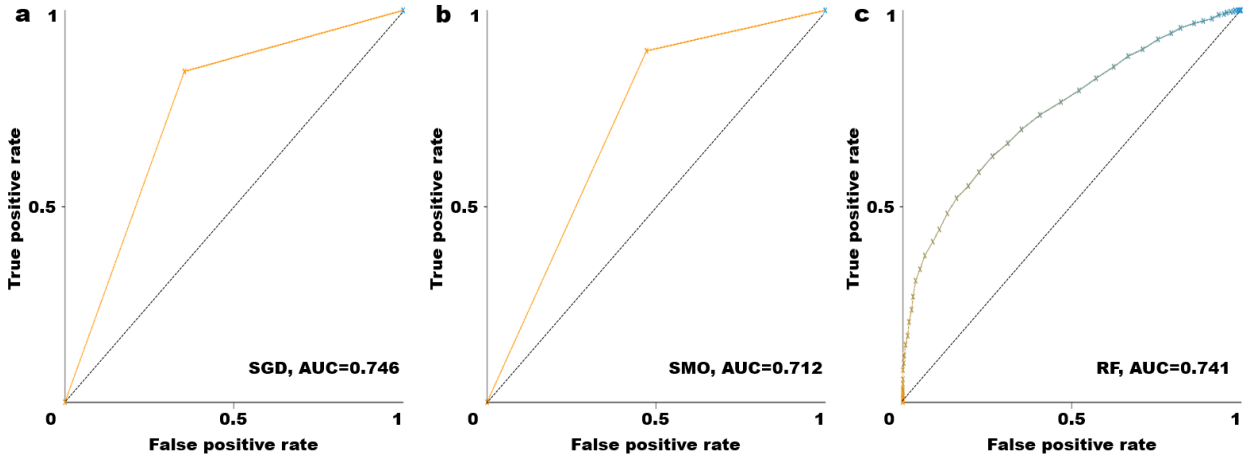


Supplementary Fig. 5. Overlap analysis of neurons classified as responding only before, only after, or both before and after histamine/formalin stimuli. a, Schematic view of the analysis for overlapping neurons activated "only before" histamine or formalin was given at 6-h or 3-day intervals. **b,** Percentage of overlapping neurons activated "only before" histamine and formalin, given at 6-h or 3-day intervals (n = 7 mice/group; two tailed unpaired t-test, t_{12} = 2.293, * p = 0.0407). **c,** Percentage of overlapping neurons activated "only before" two consecutive histamine injections, given at 6-h or 3-day intervals (n = 5 mice/group; two tailed paired t-test, t_4 = 2.816, * p = 0.0480). **d,** Schematic view of the analysis for overlapping neurons activated "both before and after" histamine or formalin given at 6-h or 3-day intervals. **e,** Percentage of overlapping neurons activated "both before and after" histamine or formalin, given at 6-h or 3-day intervals (n = 7 mice/group; two tailed unpaired t-test, t_{12} = 0.6697, p = 0.5157). **f,** Percentage of overlapping neurons activated "both before and after" two consecutive histamine injections, given at 6-h or 3-day intervals (n = 5 mice/group; two tailed paired t-test,

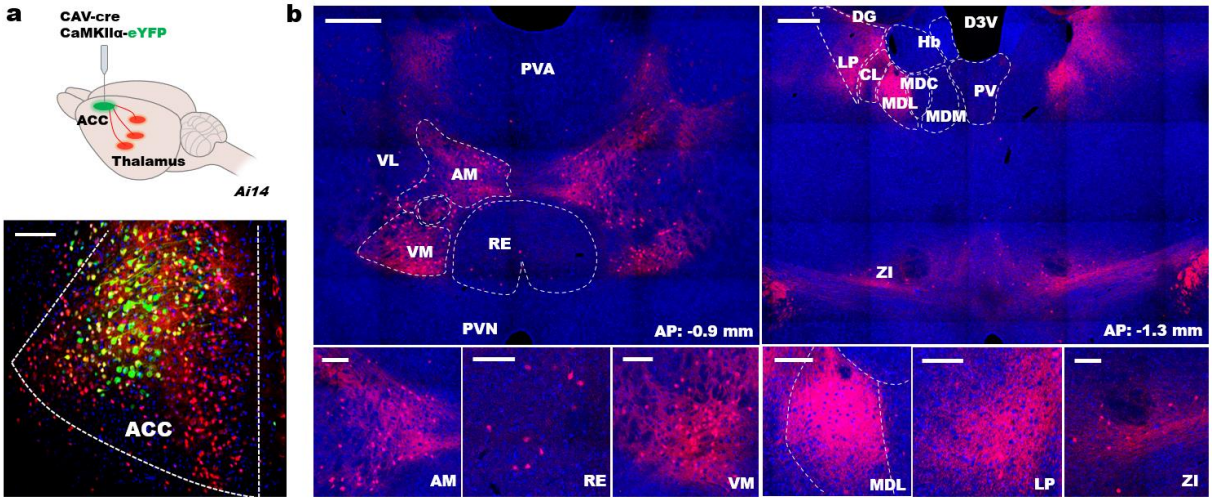
$t_4 = 0.9240$, $p = 0.4078$). **g,** Schematic view of the analysis for overlapping neurons activated "both before and after" and "only after" histamine or formalin given at 6-h or 3-day intervals. **h,** Percentage of overlapping neurons activated "both before and after" and "only after" histamine or formalin, given at 6-h or 3-day intervals (n = 7 mice/group; two tailed unpaired t-test, $t_{12} = 0.4349$, $p = 0.6714$). **i,** Percentage of overlapping neurons activated "both before and after" and "only after" two consecutive histamine injections, given at 6-h or 3-day intervals (n = 5 mice/group; two tailed paired t-test, $t_4 = 0.7834$, $p = 0.4772$). H; Histamine. F; Formalin. Data presented as mean ± SEM. Source data are provided as a Source Data file.



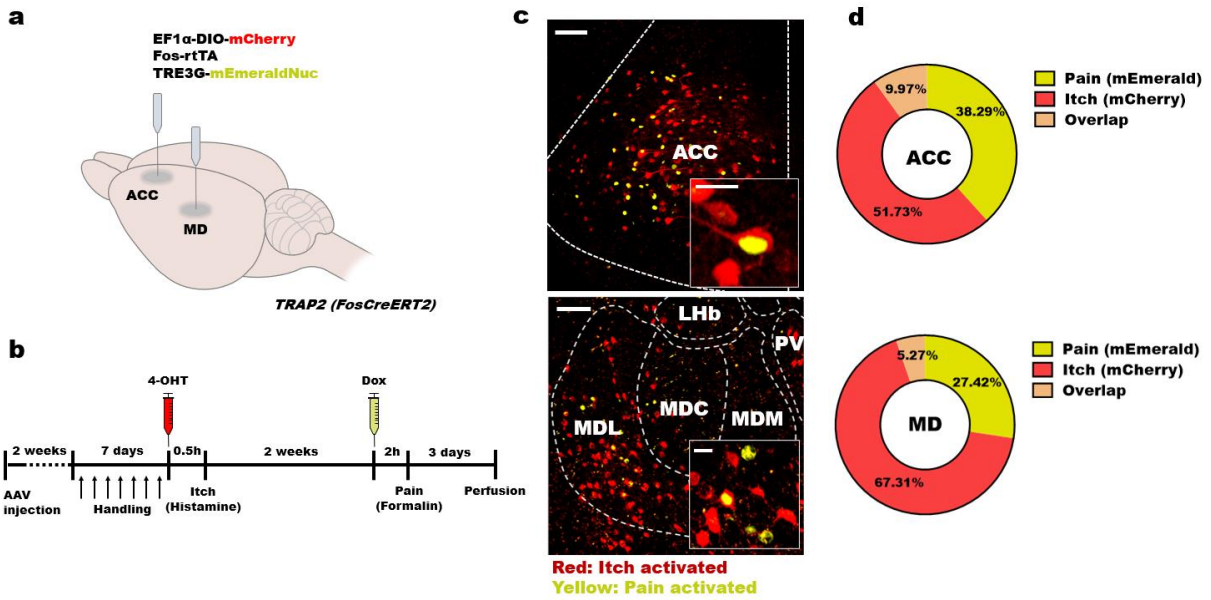
Supplementary Fig. 6. Mean firing frequency of ACC neurons before and after giving itch or pain stimuli. a-b, Histograms showing the mean firing frequency of ACC neurons during the 5 min before and 10 min after histamine (**a**) or formalin (**b**) injection. For the baseline group, neurons that fired only during the 5 min before histamine or formalin injection were included. For the itch or pain group, neurons that fired only during the 10 min after histamine or formalin injection were included. **c,** A cumulative distribution showing a significant increase in mean firing frequency for neurons that responded to histamine injection (n = 2484 neurons in the baseline group, n = 2005 neurons in the itch group; two-tailed Mann-Whitney test, *** $p < 0.0001$). **d,** A cumulative distribution showing a significant increase in mean firing frequency for neurons that responded to formalin injection (n = 890 neurons in the baseline group, n = 1155 neurons in the pain group; two-tailed Mann-Whitney test, *** $p < 0.0001$). **e,** A cumulative distribution showing significant differences in the mean firing frequency of neurons responding to formalin injection compared to neurons responding to histamine injection (two-tailed Mann-Whitney test, *** $p < 0.0001$). Source data are provided as a Source Data file.



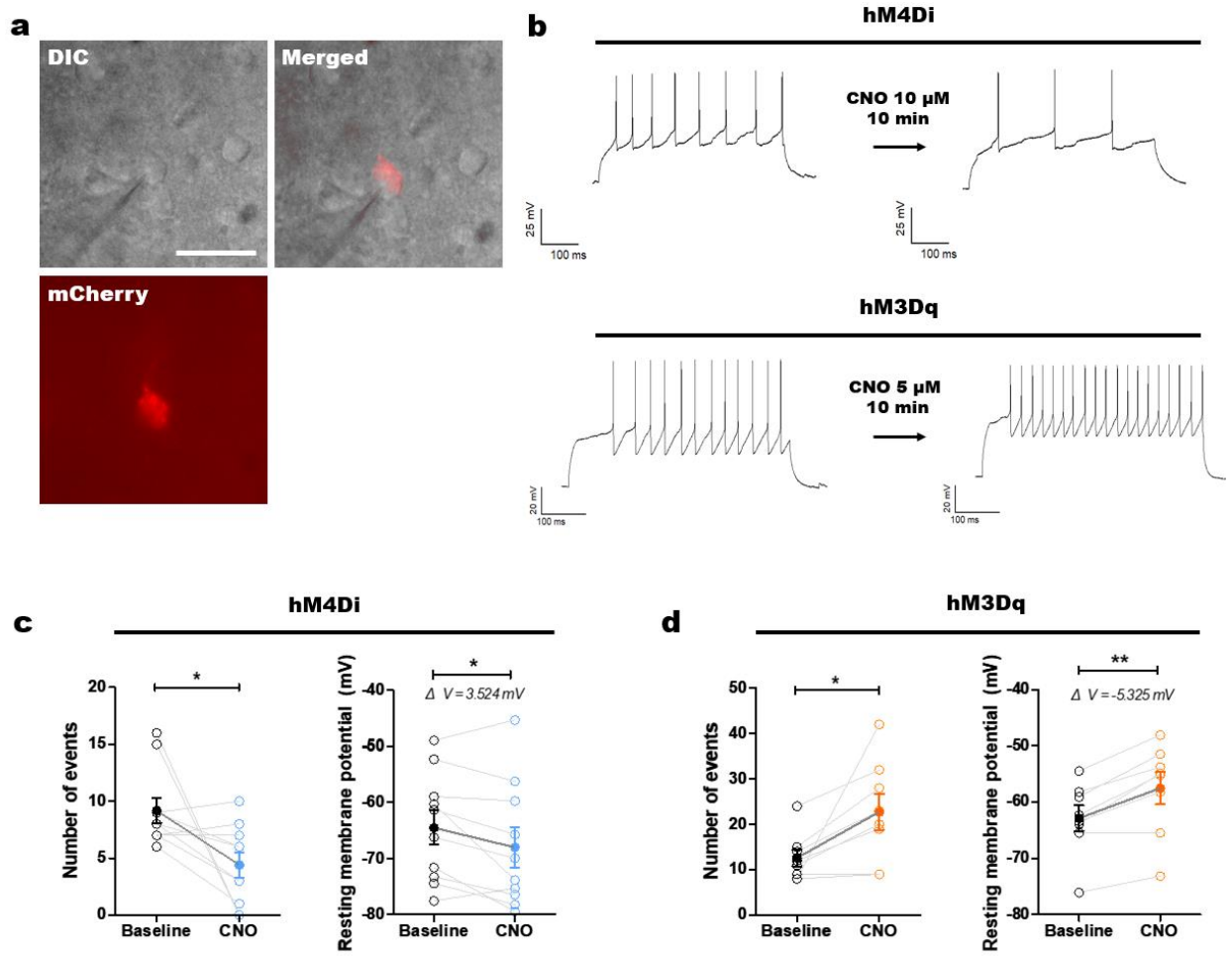
Supplementary Fig. 7. ROC (Receiver Operating Characteristic) curve for the machine learning classification. The ROC curves for the itch-activated neuron are treated as the positive class (**a**: SGD, **b**: SMO, **c**: RF). The black dotted line represents the level of statistical chance. The pain class ROC curve is symmetrical about the line y = -x + 1.



Supplementary Fig. 8. Validation of the thalamic regions presynaptic to the ACC. a, Schematic illustration of the virus combination and the injection site used for Ai14 reporter mouse (above), as well as a representative image of virus expression (below). Blue: DAPI, Green: eYFP, Red: tdTomato, Scale bar, 100 μm. **b,** Representative images showing monosynaptically connected tdTomato neurons in the thalamic regions pre-synaptic to the ACC. Blue: DAPI, Red: tdTomato. Scale bars, 300 μm (above), 100 μm (below). PVA; Paraventricular thalamic nucleus, anterior part. VL; Ventrolateral thalamic nucleus. AM; Anteromedial thalamic nucleus. RE: Reuniens thalamic nucleus. PVN; Paraventricular hypothalamic nucleus. VM; Ventromedial thalamic nucleus. D3V; 3rd ventricle. PV; Paraventricular thalamic nucleus. Hb; Habenula. DG; Dentate gyrus. LP; Lateral posterior thalamic nucleus. CL; Claustrum. MDL; Mediodorsal thalamic nucleus, lateral part. MDC; Mediodorsal thalamic nucleus, central part. MDM; Mediodorsal thalamic nucleus, medial part. ZI; Zona incerta.

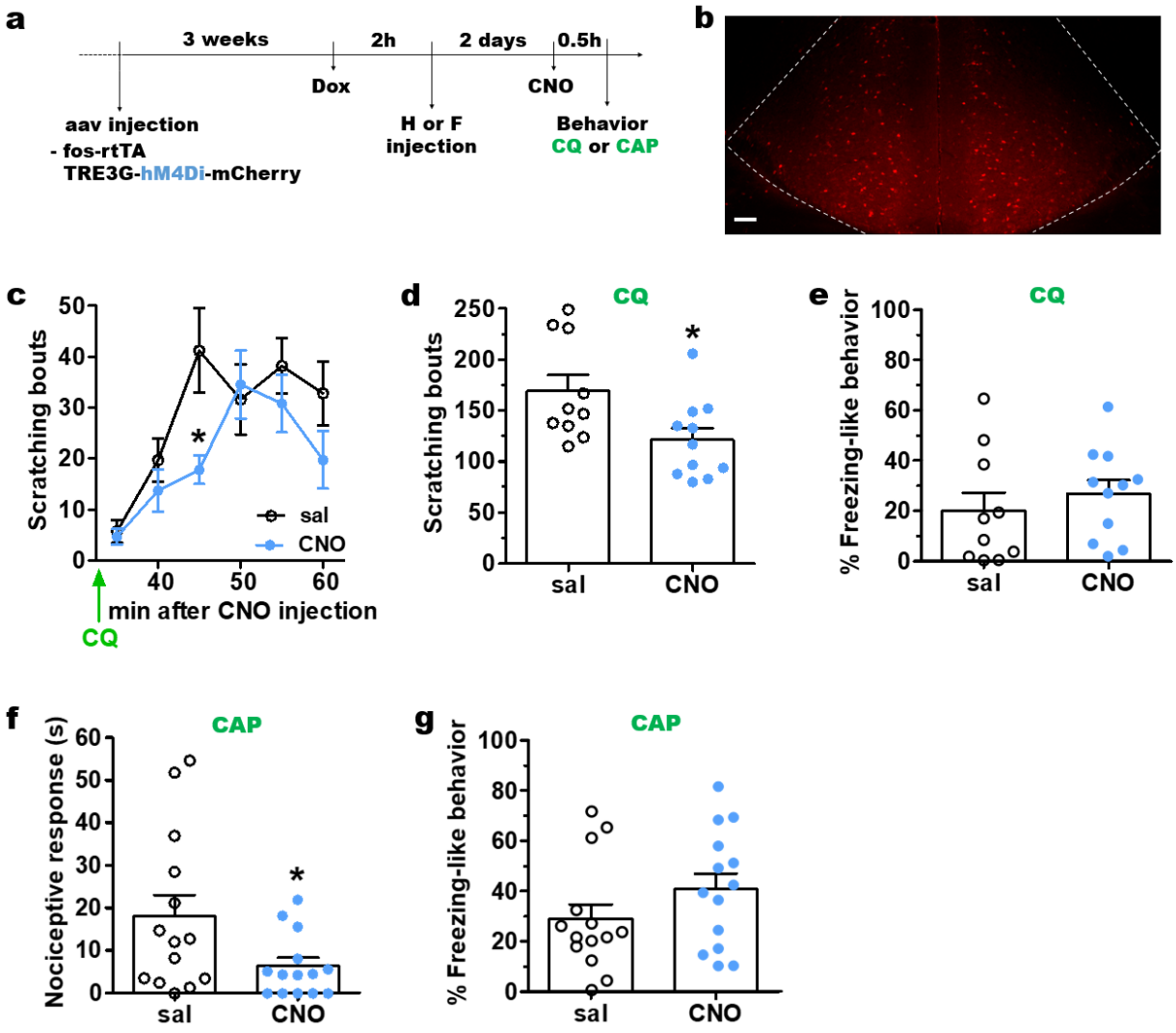


Supplementary Fig. 9. Labeling of neurons responding to itch and pain stimuli in the ACC and MD using the AAV-based TetTag system and TRAP2 mice. a, Schematic illustration of the virus combination and the injection sites used for TRAP2 mice. **b,** Experimental scheme. **c,** Confocal images of the ACC (above) and MD (below) showing itch stimulus-activated neurons expressing mCherry and pain stimulus-activated neurons expressing mEmeraldNuc. Scale bars, 100 μm. **d,** The ratio of mCherry-positive to mEmeraldNuc-positive neurons in the ACC (above) and MD (below). PV; Paraventricular thalamic nucleus. LHb; Lateral habenula. MDL; Mediodorsal thalamic nucleus, lateral part. MDC; Mediodorsal thalamic nucleus, central part. MDM; Mediodorsal thalamic nucleus, medial part. Source data are provided as a Source Data file.



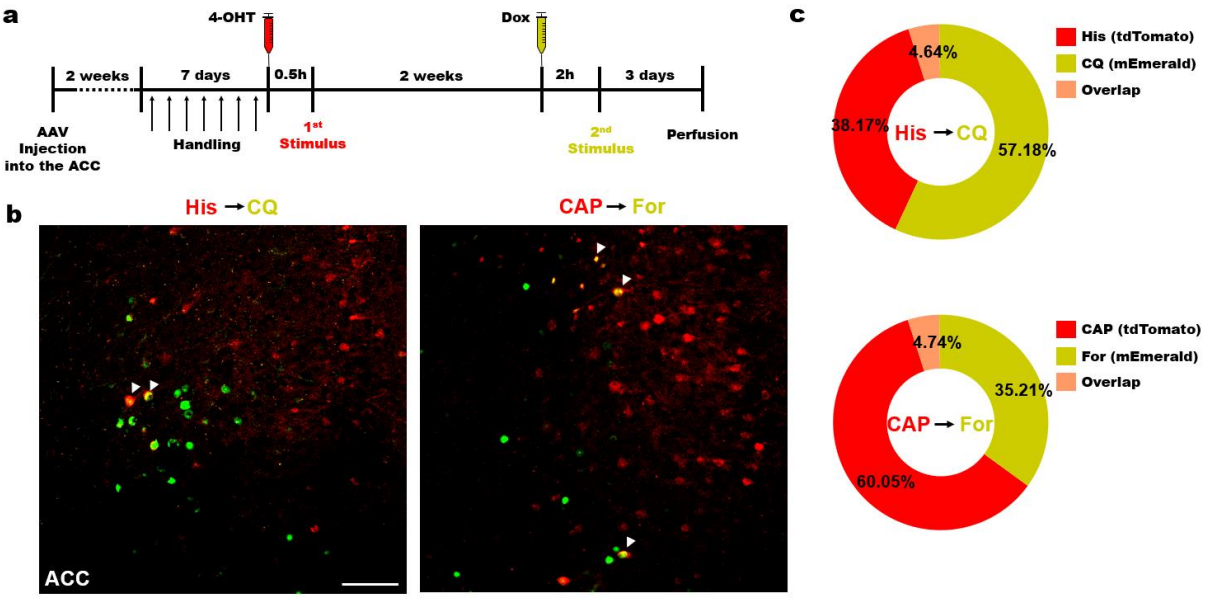
Supplementary Fig. 10. Bath application of CNO effectively modulates the excitability of ACC neurons expressing TRE3G-hM4Di/hM3Dq-mCherry. a, Representative images of whole-cell recording of mCherry (+) neurons. Scale bar, 10 μm. **b,** Representative traces of current injection (500 ms) before and after the CNO application (5 ~ 10 μM, 10 min). **c,** ACC neurons with hM4Di expression showed significantly decreased excitability following the CNO application, as evidenced by a reduced number of action potentials (n = 10 cells; two tailed paired t-test, $t_9 = 2.501$, * p = 0.0338) and hyperpolarized resting membrane potential (n = 10 cells; two tailed paired t-test, $t_9 = 2.305$, * p = 0.0466). **d,** Conversely, hM3Dq-mCherry (+) ACC neurons demonstrated increased excitability upon the CNO application, as shown by an increased number of action potentials (n = 8 cells; two tailed paired t-test, $t_7 = 2.711$, * p = 0.0301) and a depolarized resting membrane potential (n = 8 cells; two tailed paired t-test, t_7 =

5.228, ** p = 0.0012). Data presented as mean ± SEM. Source data are provided as a Source Data file.



Supplementary Fig. 11. Itch- or pain-specific neurons in the ACC contribute to pruriception or nociception, respectively, regardless of the specific subtype of stimulus within each modality. a. Experimental scheme involved using the Tet-On system to investigate whether activating itch- or pain-specific neurons in the ACC enhances pruriception or nociception, respectively. The AAV mixture, capable of selectively expressing inhibitory hM4Di only in activated neurons upon Dox injection, was microinjected into the ACC. hM4Di expression was assessed by measuring mCherry expression. **b,** Representative images of mCherry expression in the ACC. Scale bar, 100 μm. **c and d,** Scratching bouts significantly decreased after CQ injection when neurons previously activated by histamine were suppressed using CNO (**c**: n = 10 mice in sal, n =11 mice in CNO; two-way repeated measures ANOVA followed by Bonferroni post-test: effect of time, $F_{(5, 95)} = 9.272$, $p < 0.0001$; effect of CNO, $F_{(1,}$

$_{95)}$ = 6.154, p = 0.0226; interaction effect, $F_{(5, 95)}$ = 1.585, p = 0.1717; posttest, * p < 0.05. **d**: two tailed unpaired t-test, t_{19} = 2.481, * p = 0.0226). **e,** No change in freezing-like behavior was observed with CQ injection when neurons previously activated by histamine were inhibited using CNO (n = 10 mice in sal, n =11 mice in CNO; unpaired t-test, t_{19} = 0.7316, p = 0.4733). **f,** Nocifensive responses significantly decreased after CAP injection when neurons previously activated by formalin were suppressed using CNO (n = 14 mice/group; two tailed unpaired t-test, t_{26} = 2.217, * p = 0.0356). **g,** No change in freezing-like behavior was observed with CAP injection when neurons previously activated by formalin were inhibited using CNO (n = 14 mice/group; two tailed unpaired t-test, t_{26} = 1.388, p = 0.1771). sal; Saline. H; Histamine. F; Formalin. CQ; Chloroquine. CAP; Capsaicin. Data presented as mean ± SEM. Source data are provided as a Source Data file.



Supplementary Fig. 12. Labeling of neurons in the ACC responding to various types of pruritogen or algogen. a, Experimental scheme. The TRAP2 mice combined with the AAV-based TetTag system employed in Supplementary Fig. 9 were used in this experiment, with the substitution of EF1α::DIO-mCherry by CaMKII::DIO-tdTomato. **b,** Left: Confocal image of the ACC showing neurons activated by histamine (Red; tdTomato) and those activated by CQ (Green; mEmeraldNuc). Right: Confocal image of the ACC showing neurons activated by CAP (Red; tdTomato) and those activated by formalin (Green; mEmeraldNuc). Scale bars, 100 μm. **c,** The ratio of tdTomato (+) to mEmeraldNuc (+) neurons. (above; His → CQ, below; CAP → For). His; Histamine. CQ; Chloroquine. CAP; Capsaicin. For; Formalin. Source data are provided as a Source Data file.

Algorithm	Precision	Recall	F-measure	MCC[1]	ROC[2] area
SGD[3]	0.772	0.772	0.770	0.501	0.746
SMO[4]	0.759	0.761	0.750	0.466	0.712
RF[5]	0.676	0.664	0.582	0.195	0.741

Supplementary Table. 1. Evaluation of machine learning models for itch- or pain-specific neuron classification. Statistical evaluation of machine learning classifiers for itch- or pain-specific neurons. The presented evaluation parameters represent the weighted average of both classes (itch and pain).

MCC[1]: Matthews Correlation Coefficient

ROC[2]: Receiver Operating Characteristics

SGD[3]: Stochastic Gradient Descent

SMO[4]: Sequential Minimal Optimization

RF[5]: Random Forest