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Glutamatergic activation of anterior cingulate cortex produces an aversive teaching signal

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Noxious stimuli have motivational power and can support associative learning, but the neural circuitry mediating such avoidance learning is poorly understood. The anterior cingulate cortex (ACC) is implicated in the affective response to noxious stimuli and the motivational properties of conditioned stimuli that predict noxious stimulation. Using conditioned place aversion (CPA) in rats, we found that excitatory amino acid microinjection into the ACC during conditioning produces avoidance learning in the absence of a peripheral noxious stimulus. Furthermore, microinjection of an excitatory amino acid antagonist into the ACC during conditioning blocked learning elicited by a noxious stimulus. ACC lesions made after conditioning did not impair expression of CPA. Thus, ACC neuronal activity is necessary and sufficient for noxious stimuli to produce an aversive teaching signal. Our results support the idea that a shared ACC pathway mediates both pain-induced negative affect and a nociceptor-driven aversive teaching signal.

Pairing a stimulus with intrinsic motivational power (unconditioned stimulus, US; *e.g.*, pain, food) with a neutral sensory stimulus produces changes in neural circuitry such that the previously neutral stimulus becomes capable of generating behavioral responses (conditioned responses, CRs). Thus the previously neutral stimulus becomes a conditioned stimulus (CS). We use the term 'teaching signal' to refer to a neural signal that is necessary and sufficient to produce a conditioned response (CR). Accordingly, temporally coincident activation of the pathways transmitting the aversive teaching signal and the initially neutral stimulus produces aversive associative learning by strengthening the connections between the neurons mediating the CS and those whose activity results in the CR. Associative learning using noxious stimuli as the US has been documented behaviorally, and the mediating synaptic change has been elucidated in some systems[1–4]. However, the neural pathways that mediate nociceptor-driven aversive teaching signals in mammals are not well understood.

One candidate pathway for such signals includes projections from the spinal cord dorsal horn to the medial thalamus, and from there to the ACC[5–8]. This pathway was established by functional imaging studies in humans[9] and anatomical and electrophysiological studies in animals[10–15]. In human imaging studies, the degree of ACC activation is positively correlated with the magnitude of unpleasantness in response to a noxious stimulus[16]. In addition, the human, primate, rodent and rabbit ACCs contain neurons that respond to noxious stimuli[13,17–19]. In chronic pain patients, lesions of the ACC or cingulum bundle (an afferent and efferent ACC fiber tract) reduce pain unpleasantness[20,21]. The ACC has extensive direct interconnections with limbic nuclei including the amygdala, hippocampus, posterior cingulate and ventral striatum[22–25], each of which has been implicated in CS-driven aversive behaviors[2,26,27]. The observations that ACC neurons respond to noxious stimulation and that ACC activity is correlated with perceived unpleasantness in humans are consistent with the hypothesis that ACC neurons encode and transmit information related to the aversiveness of noxious stimuli and provide the teaching signal required for the acquisition of conditioned aversion.

We recently found that excitotoxic lesions of the rostral ACC (r-ACC) selectively prevents avoidance learning elicited by tonic noxious stimuli[28]. This is consistent with reports that lesions of frontal cortex or of both anterior and posterior cingulate cortices prior to conditioning reduce avoidance learning[26,29]. Although these studies show that the ACC is required for aversive learning, they do not distinguish between a role for ACC neurons in its acquisition (*i.e.*, in providing an aversive teaching signal) versus expression (*i.e.*, in retrieval). Furthermore, the electrophysiological data are ambiguous on this question. In addition to responding to noxious stimuli (essential if they are to provide an aversive teaching signal and contribute to acquisition of the CR), some ACC neurons respond to pain-predictive sensory stimuli. For example, human imaging and rodent, rabbit and primate electrophysiology studies show activation of ACC neurons in response to a pain-predictive visual CS[18,26,30–32]. This activation supports the idea that ACC neurons encode and transmit information that generates the motivational properties of the CS after conditioning, rather than generating an aversive teaching signal during learning. In other words, this pattern of activity is more consistent with a role for ACC neurons in the expression rather than the acquisition of learned aversive behaviors. Finally, the facts that neurons responding to both nociceptive (US) and aversive CS are found in the ACC and that, after learning, some ACC neurons respond to both types of stimuli[18] raise the intriguing possibility that the ACC is a critical site of plasticity for avoidance learning.

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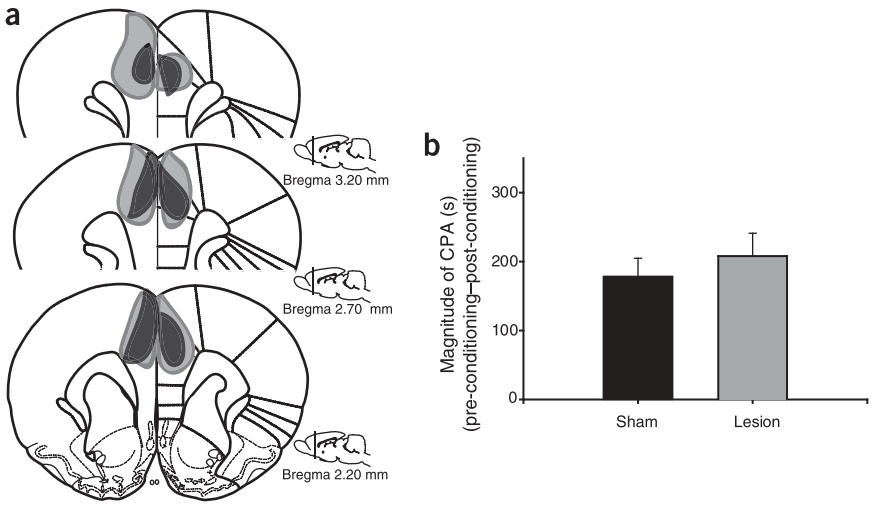

Figure 1 ACC lesions after training do not affect expression of place aversion. (**a**) Examples of the largest (gray) and smallest (black) lesions among animals in the group. Sections are in the coronal plane, numbers in mm anterior to Bregma in this and subsequent figures. (**b**) Rats with post-training lesions ($n = 7$) did not differ from those with sham lesions ($n = 10$). F-CPA scores are shown as mean ± s.e.m.

While not mutually exclusive, these hypotheses lead to clearly different predictions of the effect of ACC manipulations on the acquisition and expression of avoidance learning. Thus, if ACC neurons are required to mediate the motivational effect of aversive conditioned stimuli, then lesions after conditioning should block the expression of avoidance learning. In contrast, if ACC neurons are necessary to provide a nociceptive aversive teaching signal, then ACC lesions before conditioning (or reversible inactivation during conditioning) should block acquisition, but lesions made after conditioning should not affect expression of CPA after it has been learned. Furthermore, if ACC neuronal activity is sufficient to provide an aversive teaching signal, direct activation of these ACC neurons during conditioning, in the absence of a peripheral noxious stimulus, should produce an aversive teaching signal. Finally, if the aversive learning is associated with requisite synaptic plasticity within the ACC, lesions before conditioning should block acquisition, and lesions after conditioning should block expression of avoidance learning.

We previously examined the functional significance of the ACC using a nociceptor-driven, associative avoidance-learning assay: formalin-induced conditioned place aversion (F-CPA)[28]. However, because the lesions in our earlier study were irreversible and made before conditioning, one could not distinguish an effect on acquisition from one on expression. In the current study, to address this question, we inactivated or lesioned the ACC in a temporally specific manner. In addition, by activating ACC neurons directly, in the absence of a peripheral nociceptive input, we explored whether activity of ACC neurons is sufficient to provide an aversive teaching signal. Our results provide direct evidence that ACC neuronal activity is sufficient to produce avoidance learning and necessary for noxious stimuli to elicit an aversive teaching signal.

RESULTS

r-ACC lesions do not affect the expression of avoidance learning

Excitotoxin-induced r-ACC lesions were made after acquisition of the conditioned response to test whether the r-ACC is necessary for the expression of previously learned avoidance behavior.

Bilateral infusions of the excitotoxin ibotenic acid (IBO) made into r-ACC produced neuronal cell loss and proliferation of small glial cells (data not shown; see ref. 28). All animals included in our analyses met lesion inclusion criterion as described in Methods (**Fig. 1a**). Mean percent damage calculations for each hemisphere and an overall bilateral mean are as follows: left hemisphere, 66 ± 11%; right hemisphere, 58 ± 11%; mean, 62 ± 9%. Importantly, the lesion extents in this experiment were not different from those in our previous study[28].

When hindpaw formalin injections were paired with a particular compartment in the place-conditioning apparatus, rats with post-training r-ACC sham lesions spent less time in the formalin-paired room (*i.e.*, CPA was produced; 389.8 ± 54.8 s pre-conditioning vs. 211.6 ± 90.2 s post-conditioning; Student's *t*-test, $P < 0.05$). Hindpaw formalin also produced CPA in post-training r-ACC lesioned rats (392 ± 131.6 s pre-conditioning vs. 184 ± 94.9 s post-conditioning; Student's *t*-test, $P < 0.05$). Group comparisons revealed no significant difference between sham and lesion groups (**Fig. 1b**; Student's *t*-test, $P > 0.05$). Thus, r-ACC lesions made after training have no effect on the expression of F-CPA. Two critical conclusions can be drawn from this result. First, the r-ACC is not a significant site of plasticity for F-CPA learning and, second, it is not required for retrieval of information related to the prediction of aversive stimuli by contextual cues.

r-ACC glutamate receptor blockade prevents F-CPA acquisition

The fact that lesions made before[28] but not after conditioning block F-CPA learning strongly supports the hypothesis that the r-ACC is necessary specifically during the acquisition of F-CPA. The existence of a significant spino-thalamo-cingulate nociceptive projection pathway[10–15] is also consistent with a major role for the r-ACC in afferent nociceptive processing. Assuming that the thalamo-cingulate projection is glutamatergic[33], it is likely that glutamatergic activation of r-ACC neurons by a prolonged noxious stimulus is necessary for the acquisition of CPA. To address this question, we made glutamate receptor antagonist microinjections into the r-ACC during formalin conditioning sessions.

Microinjections of kynurenic acid (KyA) into the r-ACC before F-CPA conditioning blocked the acquisition of F-CPA learning (**Fig. 2**). There was no difference in the amount of time the r-ACC KyA animals spent in the formalin-paired context before versus after conditioning (357.5 ± 50.6 s before, 320.6 ± 83.2 s after; Student's *t*-test, $P > 0.05$). In contrast, microinjections of vehicle into the r-ACC during conditioning had no effect on F-CPA acquisition (388.3 ± 79.4 s before, 234.8 ± 106.5 s after conditioning; Student's *t*-test, $P < 0.01$). For group comparisons, see **Figure 2a**. Notably, in a separate group of animals, KyA alone did not produce motivational effects. KyA microinjected into the r-ACC in the absence of hindpaw formalin had no effect on room preference (336.6 ± 86.3 s before vs. 321.3 ± 137.1 s after conditioning; Student's *t*-test, $P > 0.05$; **Fig. 2b**). Furthermore, the reduction of F-CPA by KyA injected into the r-ACC is unlikely to be due to a sedating effect since it did not alter motor activity (data not shown). In further support of this conclusion, our previous study showed that similarly located IBO-induced

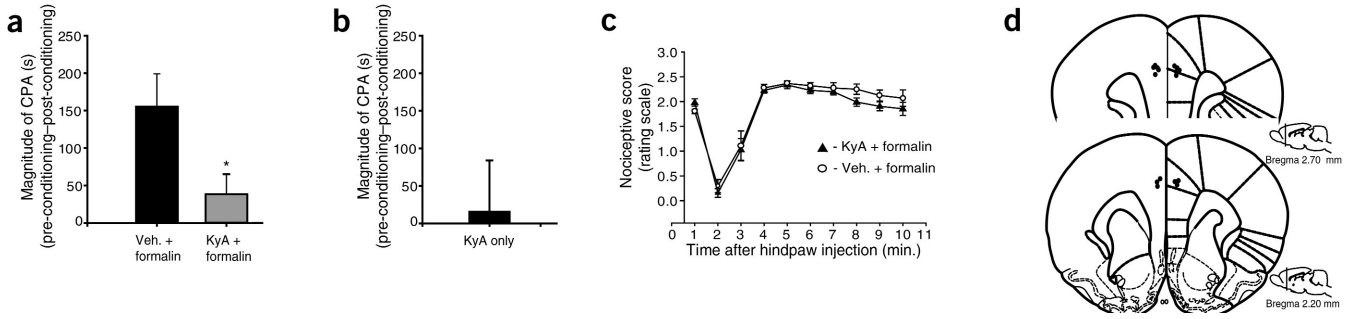

Figure 2 Intra-r-ACC microinjection of the ionotropic glutamate receptor antagonist kynurenic acid (KyA) blocks F-CPA. (**a**–**c**) Data are represented as mean ± s.e.m. (**a**) The effect of bilateral intra-r-ACC vehicle ($n = 10$) or KyA ($n = 8$) on the magnitude of F-CPA scores. (**b**) The magnitude of CPA scores for intra-r-ACC KyA in the absence of hindpaw formalin ($n = 8$). (**c**) Acute formalin-induced nociceptive scores (rating scale). (**d**) Injection sites for 50 mM KyA-treated rats. $^*P < 0.05$, Student's *t*-test as compared with vehicle-injected rats.

r-ACC lesions had no effect on the place aversion elicited by systemic injection of the kappa opioid agonist U69,593 (ref. 28). As in our earlier lesion study, we found no significant main effect of intracerebral treatment (vehicle vs. KyA) on acute formalin rating scale scores ($F_{1,12} = 0.97$; $P > 0.05$) and no significant interaction between intracerebral treatment and time ($F_{9,108} = 0.72$, $P > 0.05$; **Fig. 2c**), indicating that KyA reduction of F-CPA is not due to a general decrease in nociceptive processing.

Glutamatergic r-ACC stimulation produces avoidance learning

The results of experiments 1 and 2 indicate that activation of r-ACC neurons is necessary for acquisition, but not expression, of F-CPA. However, they do not rule out the possibility that nociceptive activation of r-ACC neurons serves a permissive role during conditioning and that activation of r-ACC neurons alone is not sufficient to produce F-CPA learning. To test this possibility, we directly stimulated the r-ACC by microinjecting an ionotropic glutamate receptor agonist into the r-ACC in the absence of a peripheral nociceptive stimulus.

Homocysteic acid microinjected into the r-ACC produced significant, dose-dependent CPA learning. Rats spent significantly less time in the treatment-paired context (366.8 ± 44.8 before vs. 251.2 ± 59.4 after conditioning; Student's *t*-test, $P < 0.01$). Neither intra-r-ACC vehicle nor low-dose homocysteic acid produced CPA (vehicle, 336.1 ± 58.3 s before vs. 308.4 ± 92.2 s after conditioning; Student's *t*-test, $P > 0.05$; low-dose, 352.1 ± 34.6 s before vs. 365.75 ± 69.8 s after conditioning; Student's *t*-test, $P > 0.05$). Group comparisons of magnitude of CPA scores analyzed using a one-way ANOVA revealed a significant effect of treatment ($F_{2,27} = 6.46$; $P < 0.01$). Further analysis revealed significantly higher CPA scores for the 100 mM HCA treatment group compared to the vehicle group, but no significant difference between vehicle and 5 mM HCA (Newman-Keuls test; $P < 0.05$ and $P > 0.05$, respectively; **Fig. 3a**). To establish the anatomical specificity of our r-ACC microinjections, we used off-site controls (**Fig. 3b**). High-dose HCA had no motivational effects when injected into a cortical control site lateral to our target r-ACC injections ($n = 8$; 347.4 ± 43.8 s before vs. 356.5 ± 157.6 s after conditioning; Student's *t*-test, $P > 0.05$).

DISCUSSION

Previously we demonstrated that excitotoxic lesions of the r-ACC before conditioning abolish nociceptor-driven learned avoidance behavior (F-CPA) without affecting acute nociceptive behaviors or non-nociceptive avoidance behavior[28]. Our current study extends those findings by showing that activation of r-ACC neurons is required specifically for the acquisition of F-CPA, as lesions made after conditioning have no effect on the expression of F-CPA. In addition, our data implicate r-ACC excitatory neurotransmission specifically in the acquisition of F-CPA, as r-ACC microinjection of a glutamate receptor antagonist during acquisition blocks F-CPA conditioning.

Importantly, our data provide critical evidence supporting the hypothesis that r-ACC neuronal activity is sufficient to generate an aversive teaching signal. Thus, microinjection of a glutamate receptor agonist into the r-ACC, but not into an adjacent cortical site, during conditioning produces robust CPA in the absence of a concomitant peripheral noxious stimulus. That selective activation of r-ACC neurons is sufficient to produce avoidance learning in the absence of input from primary afferent nociceptors is direct evidence that ACC neuronal activity is causal rather than permissive for avoidance learning.

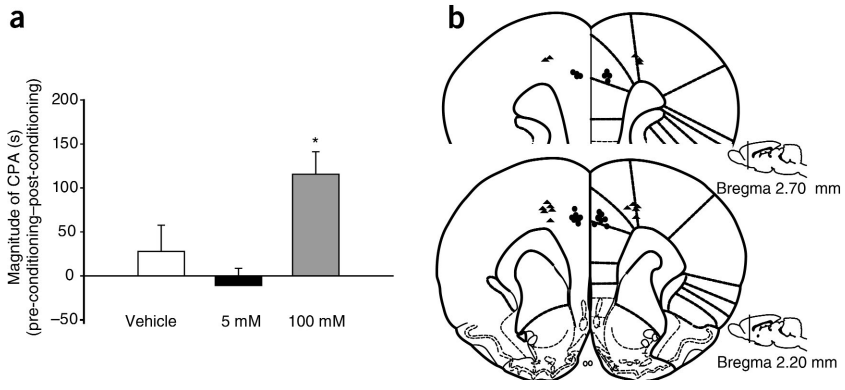

Figure 3 CPA is produced by glutamatergic stimulation of the r-ACC. (**a**) Magnitude of CPA scores in animals given intra-r-ACC microinjection of vehicle ($n = 9$), 5 mM ($n = 8$) or 100 mM HCA ($n = 11$). Data are represented as mean ± s.e.m. $^*P < 0.05$, as compared with vehicle injected rats. (**b**) Injection sites for 100 mM HCA r-ACC (circles) and off-site injection sites (triangles).

Although the evidence is not conclusive, a parsimonious explanation of these results is that formalin injection produces an aversive teaching signal through activation of r-ACC neurons during CPA conditioning.

A model: r-ACC pathway encodes an aversive teaching signal

Because dilute intradermal formalin selectively activates nociceptive Aδ and C-fiber primary afferent nociceptors[34] and is painful in humans[35], F-CPA is, by definition, a nociceptor-driven learned behavior. Nociceptive stimuli reliably activate neurons in the ACC[13,17–19]. Furthermore, together with the fact that inactivation of the medial thalamus[13]—an area that receives direct and indirect spinal cord projections and projects to the r-ACC[10–12,14,15]—reduces this activation, the current results strongly support the idea that the ACC is a major terminus or relay site for a nociceptive afferent pathway. Consistent with the idea that this region of the r-ACC contributes selectively to nociceptor-driven aversive processing, we previously showed that CPA produced by the systemic administration of the kappa opioid agonist U69,593 is unaffected by lesions of the r-ACC[28]. Importantly, this result implies that r-ACC lesions do not produce a general disruption of associative learning. In addition, we and others have shown that lesions of the rostral[28] or caudal ACC[36,37] (but see also ref. 38) spare other unconditioned behavioral responses (UR) elicited by noxious stimuli. On the other hand, caudal ACC lesions appear to reduce acute escape responses to noxious heat[36].

The results of the current experiment and previous work thus demonstrate that a nociceptive pathway through the r-ACC is necessary and sufficient for peripheral noxious stimuli to produce aversive teaching signals. The r-ACC is not necessary for other URs to nociceptive stimuli (e.g., acute formalin behaviors). Thus at some point afferent to the r-ACC, the afferent pathway mediating the aversive teaching signal diverges from that mediating many of the acute behavioral responses elicited by noxious stimuli. Our data also indicate that the neural plasticity underlying the development of avoidance learning occurs in areas of the brain that receive convergent input from the r-ACC neurons encoding the aversive teaching signal and from other sensory pathways whose neurons encode information about initially neutral conditioned stimuli. Working within this model, glutamatergic activation of r-ACC neurons by noxious stimuli is necessary to produce F-CPA, and direct activation of r-ACC neurons is sufficient to serve as a teaching signal for this type of avoidance learning.

A teaching signal, in this model, serves to strengthen the CS inputs onto neurons that receive convergent input from both nociceptive (teaching input) and other sensory CS pathways. The strengthening of the CS input such that it becomes capable of eliciting the CR is manifest in the current study as the acquisition of CPA. This type of plasticity elicited by a noxious US has been reported at the synaptic level in other neural systems. For example, using *in-vivo* intracellular recordings, one study demonstrated enhanced synaptic strength of an olfactory input to an amygdala neuron by temporally coincident activation of a noxious stimulus input onto the same neuron[4]. Interestingly, a recent report suggests that ACC stimulation is also necessary and sufficient to produce amygdala-dependent aversive conditioning (Tang, T. & Zhuo, M. *Soc. Neurosci. Abstr.* 293.4, 2003), suggesting that an aversive teaching signal generated by ACC neuronal activity is involved in other forms of aversive learning. Although we have shown that activation of r-ACC neurons is necessary and sufficient to produce an aversive teaching signal, future studies in regions that receive ACC input and convergent contextual sensory inputs are necessary to determine the site and mechanisms of the synaptic plasticity that underlies such learning.

CS-responsive neurons in ACC

Although there are r-ACC neurons that respond to stimuli (CS) that predict a nociceptive stimulus (in the current experiments, contextual sensory cues in the chamber where the rats received either formalin or intra-ACC HCA), our results do not bear on the function of such CS responses. Although our work does not preclude a role for r-ACC neurons with pain-predictive responses in F-CPA conditioning, it is clear that they are not required for the expression of F-CPA under the conditions of our experiment. One possibility is that different forms of aversive learning recruit the ACC differentially[39–41]. Another possibility is that r-ACC CS-responsive neurons are involved in a process other than aversive learning. Some studies have implicated the ACC in nociceptive modulation[42–45] and also in learned hormonal responses to pain-predictive cues[46]. Further experiments are necessary to explore these questions and to define other functional roles for CS-responsive neurons in the r-ACC.

Implications for chronic pain syndromes

Because psychological and emotional dysfunctions are characteristic of chronic pain syndromes, it may be of great clinical importance to understand how the nociceptive pathway through the r-ACC contributes to the long-term behavioral and subjective effects of chronic conditions associated with recurrent and/or prolonged nociceptor activation. Indeed, animal studies report persistent activity[47] and plastic changes within the ACC after nerve injury[48,49], suggesting that persistent noxious input can lead to local ACC plasticity (sensitization). If the ACC nociceptive system is tonically sensitized under chronic pain conditions, an understanding of the processes that lead to this change and its consequences in downstream projection targets would be of significant clinical importance.

In summary, the ACC is part of a nociceptor-activated circuit that, when paired with a contextual CS, can produce a teaching signal resulting in avoidance learning. Since lesions or glutamate receptor blockade of r-ACC neurons reduce acquisition, but post-conditioning lesions do not affect expression of such learning, the aversive teaching signal must act on other areas of the brain where nociceptive US and contextual information (CS) converge to produce the synaptic changes underlying the learned avoidance response (CR). Consistent with this idea, excitatory amino acid stimulation of the r-ACC without peripheral noxious stimulation is sufficient to produce CPA learning. Whereas human studies suggest that the ACC processes information relating to the unpleasantness of the stimulus, our data indicate that this signal is necessary to produce avoidance learning. Together, the human and animal studies support the hypothesis that a circuit through the ACC encodes the negative affective quality elicited by noxious stimuli and concomitantly provides an aversive teaching signal.

METHODS

Subjects. Subjects were male Long Evans rats (Simonsen Laboratories) weighing 300–350 g at the start of the experiments. Rats were group-housed on a 12-h light-dark schedule with food and water available *ad libitum*. All experiments were carried out with the approval of the Institutional Animal Care and Use Committee at the University of California, San Francisco. All efforts were made to minimize animal suffering and reduce the numbers of animals used.

Drugs. Ibotenic acid (IBO, 1.9 M) was dissolved in 0.1 M PBS and adjusted to pH 7.2–7.4 using 1.0 M NaOH. Stock formaldehyde solution (37% formaldehyde or 100% formalin) was diluted to 2.5% formalin in isotonic saline. The glutamate agonist, homocysteic acid (HCA, 5 or 100 mM) was dissolved in isotonic saline and adjusted to pH 7.2–7.4 using 1.0 M NaOH. The glutamate antagonist

kynurenic acid (KyA, 50 mM) was dissolved in a vehicle solution (60% isotonic saline/40% 0.1 M NaOH) and adjusted to pH 7.2–7.4 using 1.0 M HCl.

Surgery. Animals were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg). Surgery was performed using a Kopf stereotaxic apparatus. For lesion experiments, an injection cannula (30-gauge stainless steel tubing) filled with IBO or 0.1 M PBS was connected to a microinfusion pump (Razel Scientific Instruments) via PE 10 tubing. Surgery details and coordinates for lesion procedures are as previously reported[28].

For microinjection studies, chronic guide cannulae (33-gauge, Small Parts) were implanted using the stereotaxic procedure described above. Double (1.2 mm spacing between barrels) stainless steel guide cannulae were implanted 1 mm above the ACC injection site (coordinates from Bregma: anterior/posterior (AP), +2.6; dorsal/ventral (DV), –1.6; medial/lateral (ML), 0.6 mm on each side). Single barreled stainless steel guide cannulae were implanted lateral to the ACC injection site for off-site control experiments (coordinates from Bregma: AP, +2.6; DV, –1.5; ML, 2.5 on each side). For both on and off-site experiments, injectors were inserted into the guide cannulae and extended 1 mm beyond the guide tip (see below for microinjection details). Stainless steel dummy cannulae extending to the tip of the guide cannulae were inserted and kept the guide free of debris during the recovery period. All animals (lesion, sham and microinjection) recovered normally from surgery as evidenced by a weight gain on the first test day.

Behavioral training and microinjections. All experiments were done as described previously[28] using a counterbalanced, unbiased CPA design. The apparatus was exactly as described[28]: a box with three distinct compartments (a neutral room and two conditioning rooms with distinct olfactory and visual cues) with a removable door to allow room isolation when necessary and photo beams along the floor to record the animal's position and motor activity. All animals were handled for 3 d prior to testing and habituated to the injection chamber (for microinjection studies). The amount of time the animal spent in the treatment-paired room before vs. after testing was recorded and used for analysis (see below). No initial preferences for any of the compartments in the place-conditioning apparatus were detected before conditioning, indicating that the rats did not prefer any one compartment to the others before conditioning.

Lesion study of F-CPA expression. Briefly, experiments began with a pre-test day, during which the animal was allowed to roam freely around all the rooms, and we recorded the amount of time spent in each. This was followed by four conditioning days where the animals were confined to one of the conditioning rooms and received, on alternating days, either nothing in one context or a formalin injection (alternating hindpaws) in the other context (2 UCS pairings total). Conditioning was followed on day 6 by a first post-test day on which the animals were again given free access to all three rooms, and again we recorded the amount of time spent in each room. Surgeries were performed the day after the first post-test, and testing began at least 6 d after surgery. After recovery, the animals were given a second post-test that was identical to the first.

Microinjection experiments. For all experiments, injectors were inserted into the guide cannulae after removal of the dummy cannulae, and animals were placed in an injection chamber (injectors protruded 1 mm beyond the guide tip for on- and off-site experiments, so add 1 mm to coordinates given above in "surgery" section for correct DV coordinates). The injectors were attached to a microinfusion pump (Razel Scientific Instruments) via PE 10 tubing. Microinjections of drug or vehicle were made at a rate of 0.5 µl/1.5 min (0.5 µl total volume/side), and the microinjection cannula was left in place for 2 min before and after microinjection.

For the glutamate antagonist experiments, microinjection of KyA was made 5 min before the animal received a hindpaw formalin injection and was placed in the box for 50 min. Conditioning was accomplished in 2 d, not 4 d as in the lesion experiment, and included a pre-test and a post-test (4 d total). Thus, all animals received treatment (drug/formalin or just drug) and vehicle context pairings on the same day (counterbalanced by morning or afternoon) and not separated by 1 d as in the lesion experiments. They still received the same number of formalin pairings (2) as in the lesion experiments and no difference in the magnitude of F-CPA was detected between the 4-d and 2-d conditioning regimens (data not shown). Formalin behaviors were also scored using the rating scale method[50] on the first or second pairing day (counterbalanced).

For experiment 3, intra-ACC or off-site microinjections of a glutamate receptor agonist (HCA) were given without hindpaw formalin injections, and the animals were placed in the conditioning context 5 min after microinjection for 30 min. Experiment 3 was done using the same conditioning regimen as in experiment 2, but three pairings of treatment (drug) and context were made instead of two (5 d total). Pre- and post-tests were identical to the first two experiments.

Histology. After completion of the experiments, animals were given a lethal dose of sodium pentobarbital and perfused transcardially with isotonic saline followed by 10% formalin. For microinjection experiments, microinjections of dilute methylene blue were made into the r-ACC just before perfusion. The brains were then removed and fixed first in formalin for 24 h, then in 30% sucrose 24–72 h before slicing. The brains were cut on a sledge microtome at a thickness of 50 µm, stained with cresyl violet and analyzed to assess the extent of the lesion (or injection site) using a light microscope. Using a camera lucida (Nikon), lesions were traced and analyzed using an unbiased stereological method[28]. Intra-ACC microinjection of IBO produced lesions with clearly definable borders of neuronal cell loss and gliosis as compared with intra-r-ACC microinjection of PBS. Based on past studies, areas of the rodent ACC rich in nociceptive input were targeted (see ref. 28 for detailed region-of-interest). Lesions meeting inclusion criteria had a minimum 'percentage bilateral damage' of 50% and at least 30% damage in the least damaged hemisphere within the region of interest.

Statistical analyses. For the CPA data, the amount of time spent in the conditioning compartment (*i.e.*, compartment paired with formalin, drug/formalin or drug) on the post-conditioning day (*i.e.*, final test day) was subtracted from the amount of time spent in the same compartment on the pre-conditioning day. This resulted in a 'magnitude of CPA score' for each rat. Magnitude of CPA scores between groups were compared using a Student's *t*-test when comparing two groups (experiments 1 and 2) or a one-factor ANOVA (intracerebral treatment) followed by a Newman-Keuls *post-hoc* test when comparing more than two groups (experiment 3). In addition, the absolute amount of time spent in the conditioning compartment on the pre-conditioning day versus the post-conditioning day was compared in sham lesion, lesion, vehicle or drug treated animals using correlated Student's *t*-tests.

For analysis of formalin behaviors in experiment 2, rating scale nociceptive scores were collected either on day 1 or day 2 (counterbalanced) from formalin-treated rats during each 5-min time bin. The data then were analyzed in separate two-factor ANOVAs (intracerebral treatment × time), with time analyzed as a repeated measure. *Post-hoc* analyses were performed using the Newman-Keuls test. The accepted level of statistical significance for all experiments was $P < 0.05$.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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