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Enhanced Presynaptic Neurotransmitter Release in the Anterior Cingulate Cortex of Mice with Chronic Pain

Ming-Gao Zhao,[1,2]* Shanelle W Ko,[1]* Long-Jun Wu,[1]* Hiroki Toyoda,[1]* Hui Xu,[1] Jessica Quan,[1] Jianguo Li,[3] Yongheng Jia,[1] Ming Ren,[1] Zao C. Xu,[3] and Min Zhuo[1]

[1]Department of Physiology, Faculty of Medicine, and Centre for the Study of Pain, University of Toronto, Toronto, Ontario, Canada M5S 1A8, [2]Department of Pharmacology, Fourth Military Medical University, Xi'an 710032, China, and [3]Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202

The anterior cingulate cortex (ACC) is a forebrain structure known for its roles in learning and memory. Recent studies show that painful stimuli activate the prefrontal cortex and that brain chemistry is altered in this area in patients with chronic pain. Components of the CNS that are involved in pain transmission and modulation, from the spinal cord to the ACC, are very plastic and undergo rapid and long-term changes after injury. Patients suffering from chronic pain often complain of memory and concentration difficulties, but little is known about the neural circuitry underlying these deficits. To address this question, we analyzed synaptic transmission in the ACC from mice with chronic pain induced by hindpaw injection of complete Freund's adjuvant (CFA). *In vitro* whole-cell patch-clamp recordings revealed a significant enhancement in neurotransmitter release probability in ACC synapses from mice with chronic pain. Trace fear memory, which requires sustained attention and the activity of the ACC, was impaired in CFA-injected mice. Using knock-out mice, we found that calmodulin-stimulated adenylyl cyclases, AC1 and/or AC8, were crucial in mediating the long-lasting enhanced presynaptic transmitter release in the ACC of mice with chronic pain. Our findings provide strong evidence that presynaptic alterations caused by peripheral inflammation contribute to memory impairments after injury.

Key words: **trace fear memory; cingulate cortex; attention; calmodulin-stimulated adenylyl cyclase; inflammation; pain**

Introduction

The anterior cingulate cortex (ACC) is involved in learning, memory, attention, and pain (Devinsky et al., 1995; Rainville et al., 1997; Cohen et al., 1999; Zhuo, 2002; Frankland et al., 2004; Buffington et al., 2005; Zhao et al., 2005b). Recent studies using different experimental approaches consistently suggest that the ACC plays important roles in processing pain-related information in humans and in the behavioral responses to noxious stimuli or tissue injury in animals (Talbot et al., 1991; Rainville et al., 1997; Wei et al., 2001, 2002; Davis et al., 2002; Singer et al., 2004; Wager et al., 2004; Wu et al., 2005). Memory loss in today's society can be both an emotional and financial burden. Individuals suffering from persistent pain have a reduced ability to learn (Kewman et al., 1991) and to sustain attention (Eccleston, 1994, 1995; Eccleston et al., 1997). A recent study using functional magnetic resonance imaging suggests that ACC activity is modulated differently during tasks of sustained attention and pain (Buffington et al., 2005), and clinical studies have documented attention deficits in cingulotomy patients (Cohen et al., 1999). Attention is required for certain forms of associative learning (Clark and Squire, 1998) and involves the function of the ACC in rodents (Han et al., 2003; Runyan et al., 2004; Zhao et al., 2005a). However, little is known about how chronic pain-induced changes in the prefrontal cortex affects synaptic transmission and how such changes may affect the formation of attention-demanding associative memory, which requires the function of the ACC (Cohen et al., 1999; Han et al., 2003; Zhao et al., 2005a).

It is believed that cAMP signaling pathways are involved in different forms of memory (Weisskopf et al., 1994; Wong et al., 1999; Wang et al., 2004). Among the 10 members of adenylyl cyclase (AC) family, AC1 and AC8 are the calmodulin-stimulated ACs in the CNS. They couple NMDA receptor-induced cytosolic Ca^{2+} increases to cAMP signaling pathways (Chetkovich and Sweatt, 1993; Wong et al., 1999) and contribute to the behavioral responses to tissue injury and inflammation (Aley and Levine, 1999; Wei et al., 2002, 2006). AC1 and AC8 are both expressed highly in the ACC and play a role in the behavioral responses related to inflammatory pain (Wei et al., 2002), but the synaptic mechanisms underlying this role for AC1 and AC8 within the ACC remains to be investigated.

Here we found that trace fear memory was significantly reduced in mice with inflammatory pain, and whole-cell patch-clamp recordings revealed a significantly enhanced neurotrans-

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*M.-G.Z., S.W.K., L.J.W., and H.T. contributed equally to this work.

Correspondence should be addressed to Dr. Min Zhuo, Department of Physiology, University of Toronto, Faculty of Medicine, Medical Sciences Building, Room 3342, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8. E-mail: min.zhuo@utoronto.ca.

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mitter release probability in the ACC. Calmodulin-stimulated AC1 and AC8 were involved in mediating this enhancement of synaptic transmission. We further showed that AC1 knock-out (KO), AC8 KO, and AC1/AC8 KO mice did not express the complete Freund's adjuvant (CFA)-induced deficit in trace fear memory. This is the first study to correlate a deficit in behavioral memory with an alteration in synaptic transmission in the same brain region and suggests that chronic pain initiates molecular changes that affect the ability to acquire associative memory.

Materials and Methods

Animals. Adult (8–10 weeks) male mice lacking AC1, AC8, and AC1/AC8 (Wong et al., 1999; Wei et al., 2002) were bred for several generations on a C57BL/6 background. To minimize drift of background in a given genotype line, we used several breeding pairs. Both wild-type and mutant mice were well groomed and showed no signs of abnormality or any obvious motor defects. Wild-type and knock-out mice were not physically distinguishable from each other, and all experiments were performed blind to the genotype. Animals were housed under a 12 h light/dark cycle with food and water provided *ad libitum*. The Animal Care and Use Committee of the University of Toronto approved all experimental procedures. To induce inflammatory pain, 10 μl of 50% CFA (Sigma, St. Louis, MO) was injected subcutaneously into the dorsal surface of one hindpaw.

Trace fear memory. Trace fear conditioning was performed in an isolated shock chamber (Med Associates, St. Albans, VT). The conditioned stimulus (CS) used was an 80 dB white noise, delivered for 15 s, and the unconditioned stimulus (US) was a 0.75 mA scrambled footshock for 0.5 s. Mice were acclimated for 60 s, and were presented with 10 CS–trace–US–intertrial interval trials (trace of 30 s, ITI of 210 s). One day after training, mice were acclimated for 60 s and subjected to 10 CS–ITI trials (ITI of 210 s) in a novel chamber to test for trace fear memory (Huerta et al., 2000). For delay conditioning, a single CS–US pairing was used (Zhao et al., 2005b). In this experiment, the footshock coterminated with the tone, and freezing behavior was recorded 1 d later in the contextual environment and in a novel chamber before and during the presentation of the CS (cued conditioning). All data were recorded using the video-based FreezeFrame fear conditioning system and analyzed by Actimetrics Software (Coulbourn Instruments, Wilmette, IL). Average freezing for the baseline and for each ITI during the training and testing sessions were analyzed. Bouts of 1.00 s were used to define freezing (the absence of movement aside from respiration).

ACC cannula implantation and microinjection. Under ketamine and xylazine anesthesia, 24 gauge guide cannulas were implanted bilaterally into the ACC (0.7 mm anterior to bregma, ±0.4 mm lateral from the midline, 1.7 mm beneath the surface of the skull). Mice were given at least 2 weeks to recover after cannula implantation. For intra-ACC injections, mice were anesthetized with 2–3% isoflurane anesthesia in a gas mixture of 30% O_2 balanced with nitrogen and placed in a David Kopf Instruments (Tujunga, CA) stereotaxic instrument. The 30 gauge injection cannula was 0.1 mm lower than the guide. The microinjection apparatus consisted of a Hamilton syringe (5 ml), connected to an injector needle (30 gauge) by a thin polyethylene tube, and motorized syringe pump. Forskolin (12 nmol) was infused into each side of the ACC at a rate of 0.05 μl/min, and vehicle injections were used as a control. After injection, the microinjection needle was left in place for at least 2 min. Mice were tested for trace fear memory 15 min after injection.

Slice preparation. Coronal brain slices (300 μm) containing the ACC were prepared as described previously (Zhao et al., 2005b). Slices were transferred to submerged recovery chamber with oxygenated (95% O_2 and 5% CO_2) artificial CSF containing the following (in mM): 124 NaCl, 2.5 KCl, 2 CaCl2, 2 $MgSO_4$, 25 $NaHCO_3$, 1 NaH_2PO_4, and 10 glucose at room temperature for at least 1 h.

Whole-cell patch-clamp recordings. Experiments were performed in a recording chamber on the stage of an Axioskop 2FS microscope (Zeiss, Oberkochen, Germany) with infrared differential interference contrast optics for visualization of whole-cell patch-clamp recording. EPSCs were recorded from layer II–III neurons with an Axopatch 200B amplifier (Molecular Devices, Palo Alto, CA), and the stimulations were delivered by a bipolar tungsten stimulating electrode placed in layer V of the ACC. AMPA receptor-mediated EPSCs were induced by repetitive stimulations at 0.02 Hz, and neurons were voltage clamped at −70 mV. NMDA receptor-mediated EPSCs were recorded in the presence of AMPA receptor antagonist CNQX (20 μM) at holding potential. For MK-801 [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo [a,d] cyclohepten-5,10-imine maleate] blocking experiments, 0.1 Hz to stimulation was used and NMDA EPSCs were recorded for 20 min in MK-801 (35 μM). The recording pipettes (3–5 MΩ) were filled with solution containing the following (in mM): 145 K-gluconate, 5 NaCl, 1 $MgCl_2$, 0.2 EGTA, 10 HEPES, 2 Mg-ATP, and 0.1 Na_3-GTP, adjusted to pH 7.2 with KOH. For miniature EPSC (mEPSC) recording, 0.5 μM TTX was added in the perfusion solution. Picrotoxin (100 μM) was always present to block $GABA_A$ receptor-mediated inhibitory synaptic currents. Access resistance was 15–30 MΩ and monitored throughout the experiment. Data were discarded if access resistance changed >15% during an experiment.

Data analysis. Results were expressed as mean ± SEM. Statistical comparisons were performed using a *t* test, one-way ANOVA, two-way ANOVA, or two-way repeated-measures ANOVA with Student's *t* test for *post hoc* analysis. In all cases, $p < 0.05$ was considered statistically significant.

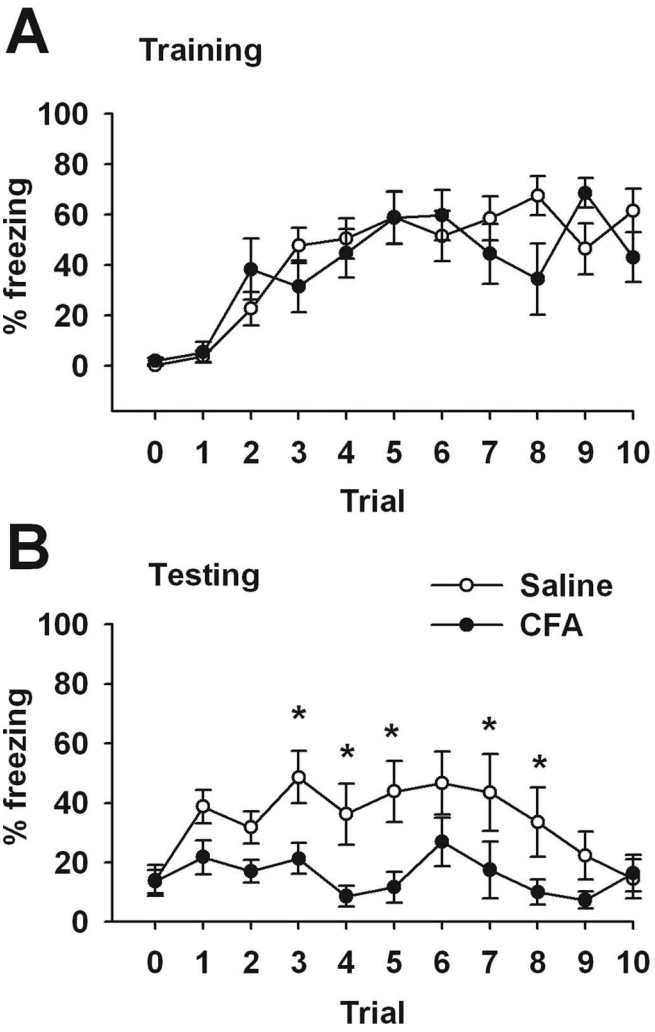

Figure 1. Trace fear memory deficit in CFA-injected mice. ***A***, ***B***, Freezing during trace training (***A***) and testing (***B***) over 10 trials 3 d after injection of CFA ($n = 8$) or saline ($n = 7$) in wild-type mice. The asterisks indicate significantly different from saline-injected mice; in all cases, $p < 0.05$ is considered significant.

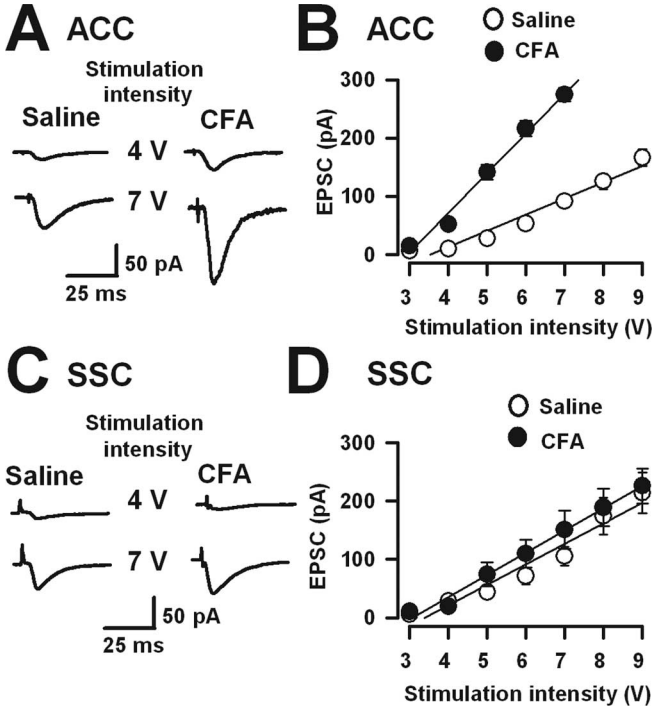

Figure 2. Potentiation of input–output relationship in CFA-injected mice. ***A***, Representative traces show averages of five EPSCs at 4 and 7 V stimulation intensity in the ACC. ***B***, Plot of input–output curves shows significant enhancement of synaptic transmission in the ACC of CFA-injected mice. Open circles, From saline mice, $n = 10$; filled circles, from CFA-injected mice, $n = 16$. ***C***, Representative traces show averages of five EPSCs at 4 and 7 V stimulation intensity in the SSC. ***D***, Plot of input–output curves shows no difference in synaptic transmission in the SSC between saline- and CFA-injected mice. Open circles, From saline control mice, $n = 6$; filled circles, from CFA-injected mice, $n = 8$.

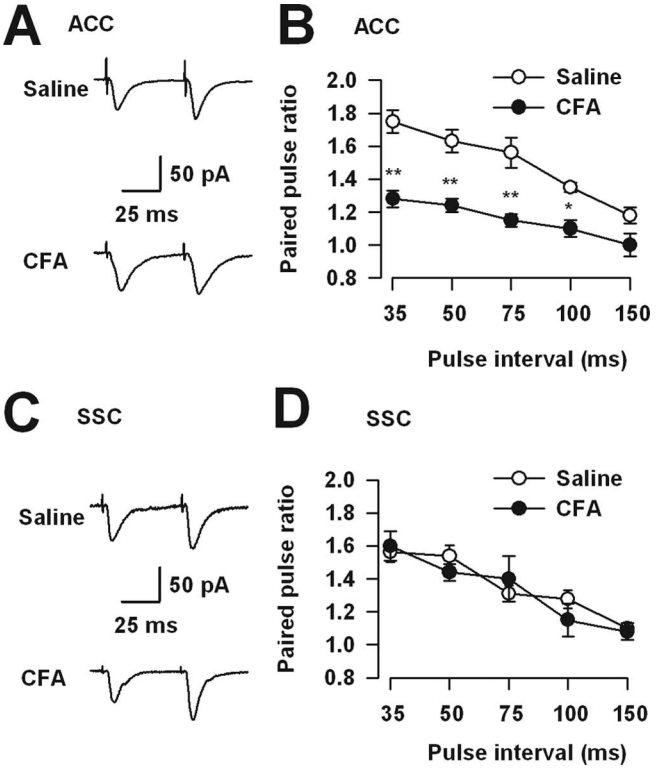

Figure 3. Paired-pulse facilitation in mice with chronic pain. PPF (the ratio of EPSC2/EPSC1) was recorded with intervals of 35, 50, 75, 100, and 150 ms. ***A***, Representative traces of PPF with an interval of 50 ms recorded in the ACC. ***B***, PPF was significantly reduced at each interval in CFA-injected mice. Open circles, From saline control mice, $n = 10$; filled circles, from CFA-injected mice, $n = 17$. $^*p < 0.05$; $^{**}p < 0.01$. ***C***, Representative traces of PPF with intervals of 50 ms recorded in the SSC. ***D***, PPF was not altered in the SSC of CFA-injected mice. Open circles, From saline mice, $n = 6$; filled circles, from CFA-injected mice, $n = 8$.

Results

Inflammatory pain interferes with the expression of trace fear memory

To test whether CFA-induced inflammatory pain influences the acquisition of attention-demanding associative memory, we tested CFA- and saline-injected mice in the trace fear conditioning paradigm. Mice were injected with either saline ($n = 7$) or CFA ($n = 8$) in one hindpaw 3 d before trace fear conditioning. After a 1 min baseline (ITI-0), mice received 10 CS–US pairings. Trace fear memory was tested the following day with the presentation of the CS in a novel chamber. There was not a significant affect of CFA on freezing behavior during the training session ($p = 0.65$), and freezing significantly increased in both saline- and CFA-injected mice throughout training (comparing percentage freezing between ITI-1 and ITI-10: saline, $p < 0.01$; CFA, $p < 0.001$) (Fig. 1*A*). When tested the next day, however, there was a significant affect of CFA treatment on freezing behavior ($p < 0.01$) (Fig. 1*B*). CFA-injected mice froze significantly less during ITI-3 ($p < 0.05$), ITI-4 ($p < 0.05$), ITI-5 ($p < 0.01$), ITI-7 ($p < 0.05$), and ITI-8 ($p < 0.05$) compared with mice injected with saline. This difference was not attributable to a change in the sensitivity to the footshock because shock reactivity was not significantly altered between CFA-injected ($n = 4$) and saline-injected ($n = 3$) mice ($p = 0.69$). To show that the CFA-induced fear memory deficit was specific for trace fear memory, CFA-injected ($n = 7$) and saline-injected ($n = 6$) mice were tested in the traditional delay conditioning paradigm. Mice were trained with a single CS–US pairing and tested for contextual and cued fear memory the following day. There was no difference in the freezing behavior between CFA- and saline-injected mice for either contextual ($p = 0.46$) or cued ($p = 0.15$) conditioning. These results suggest that CFA-induced peripheral inflammation selectively interferes with the expression of trace fear memory.

Enhanced synaptic transmission in the ACC from CFA-injected mice

One possible mechanism underlying the deficit in trace fear memory in CFA-injected mice is an alteration in the neuronal circuitry in the ACC, a region required for the expression of trace fear memory (Han et al., 2003; Zhao et al., 2005a). To test this hypothesis, synaptic alterations induced by peripheral inflammation were studied in mice receiving saline or CFA in one hindpaw 3–7 d previously. EPSCs recorded from layer II–III neurons were elicited by a bipolar tungsten stimulating electrode placed in layer V of the ACC. We found that EPSCs were potentiated in neurons from CFA-injected mice. The input–output relationships, measuring EPSCs amplitude (output) as a function of the afferent stimulus intensity (input), were compared. The slope of the curves was significantly greater in neurons from CFA-injected mice (79.3 ± 4.2 pA/V; $n = 16$) compared with saline controls (30.7 ± 3.2 pA/V; $n = 10$; $p < 0.001$) (Fig. 2*A*,*B*). These data suggest an increase in synaptic efficacy in ACC neurons from CFA-injected mice. To rule out the possibility that potentiation is a nonspecific result of chronic pain, not limited to changes in cingulate cortex circuitry, we tested synaptic transmission in the somatosensory cortex (SSC) of the same animals. As shown in Figure 2, *C* and *D*, the slope of input–output curves did not differ

between the CFA-injected animals (44.5 ± 6.1 pA/V; $n = 8$) and saline controls (46.3 ± 9.5 pA/V; $n = 6$; $p = 0.88$) (Fig. 2*C*,*D*). These results suggest that enhancement in transmitter release was regionally specific to the ACC.

Altered paired-pulse facilitation in the ACC of CFA-injected animals

To determine whether the enhanced synaptic transmission observed in CFA-injected mice was attributable to presynaptic or postsynaptic mechanisms, we measured paired-pulse facilitation (PPF) in neurons from the ACC. PPF is a phenomenon by which a second synaptic stimulation of equal magnitude evokes a larger synaptic response than the first and has been used as a tool to implicate presynaptic probability (p) of transmitter release (Creager et al., 1980; Schulz et al., 1994). PPF was significantly decreased in ACC neurons from CFA-injected mice ($n = 17$) when compared with saline controls ($n = 10$; $p < 0.05$) (Fig. 3*A*,*B*), indicating an increased presynaptic probability of transmitter release in ACC synapses of CFA-injected mice. However, in the SSC, the ratio of PPF did not differ between the CFA-injected ($n = 8$) and saline control ($n = 6$) mice (Fig. 3*C*,*D*). These results suggest that synaptic transmission is selectively enhanced in the ACC of mice with chronic pain induced by CFA inflammation.

Enhanced presynaptic transmitter release probability in the ACC of CFA-injected mice

To determine whether the reduced PPF observed in the synapses of CFA-injected mice might be associated with an increase of the release probability, two independent experimental approaches were used. First, we recorded AMPA receptor-mediated mEPSCs (Fig. 4*A*), reflecting the release of a single quanta of neurotransmitter. A significant increase in mEPSC frequency was detected in CFA-injected mice compared with saline controls (saline, 1.1 ± 0.1 Hz, $n = 14$; CFA, 1.5 ± 0.2 Hz, $n = 11$; $p < 0.05$) (Fig. 4*C*,*D*), but there was no difference in the amplitude of mEPSCs between groups (saline, 7.6 ± 0.3 pA, $n = 14$; CFA, 7.6 ± 0.5 pA, $n = 11$; $p > 0.05$) (Fig. 4*C*,*D*). Analysis of the mEPSC kinetics showed no difference between the two treatment groups (rising time: saline, 2.1 ± 0.2 ms, $n = 13$; CFA, 2.2 ± 0.3 ms, $n = 10$; decay time: saline, 12.3 ± 2.0 ms, $n = 13$; CFA, 12.8 ± 3.2 ms, $n = 10$) (Fig. 4*B*). In addition, analysis of the *I–V* relationship of AMPA receptor-mediated mEPSCs, with peak mEPSC currents recorded at holding potentials of −70 to +50 mV, showed no difference between the two groups (Fig. 4*E*). The reversal potentials of the mEPSCs were −1.9 ± 1.0 mV in control mice ($n = 6$) and 1.9 ± 1.6 mV in CFA-injected mice ($n = 7$; $p = 0.10$).

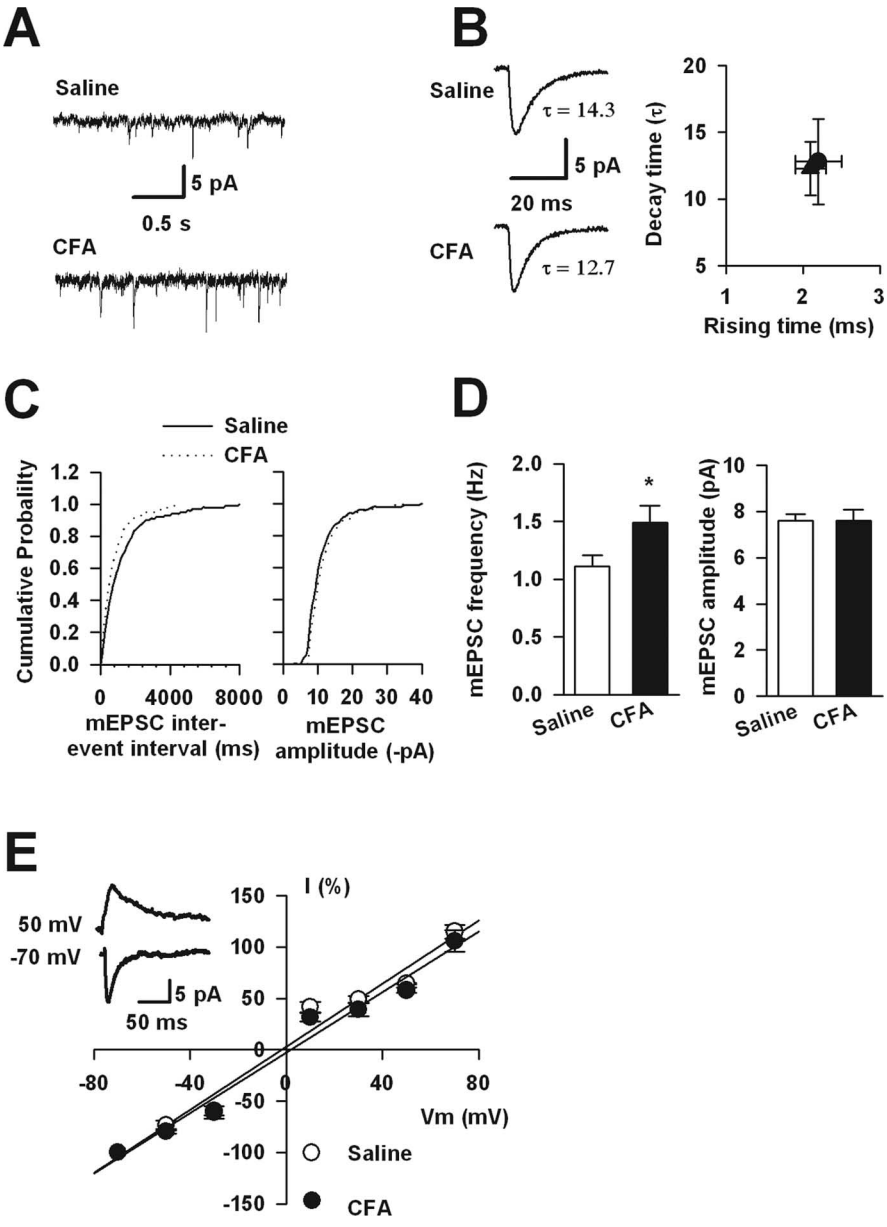

Figure 4. Miniature EPSCs in ACC neurons. ***A***, Representative mEPSCs recorded in pyramidal neurons at a holding potential of −70 mV from saline-injected (top) and CFA-injected (bottom) mice. ***B***, Top left, Average mEPSC of 50 events from a saline mouse; bottom left, average mEPSC of 53 events from a CFA-injected mouse. Right, Time constant of mEPSC decay (τ) versus the rising time (10–90%) in the recordings from saline-injected (triangle; $n = 13$) and CFA-injected (circle; $n = 10$) mice. ***C***, Cumulative frequency (left) and amplitude (right) histogram of the mEPSCs from the cells in ***A***. Solid line, Recording from a saline mouse; dashed line, recording from a CFA-injected mouse. ***D***, mEPSCs frequency (left) and amplitude (right) in neurons from saline-injected ($n = 14$) and CFA-injected ($n = 11$) mice. *$p < 0.05$. ***E***, Current–voltage plots of peak mEPSCs recorded at holding potentials of −70 to +70 mV. Open circle, Recording from saline control mice ($n = 6$); filled circles, recording from CFA-injected mice ($n = 7$). Values were obtained by normalizing the mean peak currents at each holding potential to a holding potential of −70 mV. Inset shows the mEPSCs recording at +50 and −70 mV.

Next, we tested the rate of blockade of the whole-cell NMDA receptor-mediated EPSCs by an irreversible NMDA receptor blocker, MK-801, in control and CFA-injected mice. In the presence of MK-801, the rate of NMDA EPSC decline is correlated with release probability (Hessler et al., 1993; Rosenmund et al., 1993). At holding potential of −30 mV, NMDA EPSCs were recorded in the presence of CNQX (20 μM) and picrotoxin (100 μM) at 0.1 Hz (Wu et al., 2005). MK-801 (35 μM) was perfused after obtaining stable NMDA EPSCs. We found that MK-801 could progressively block NMDA EPSCs and completely inhib-

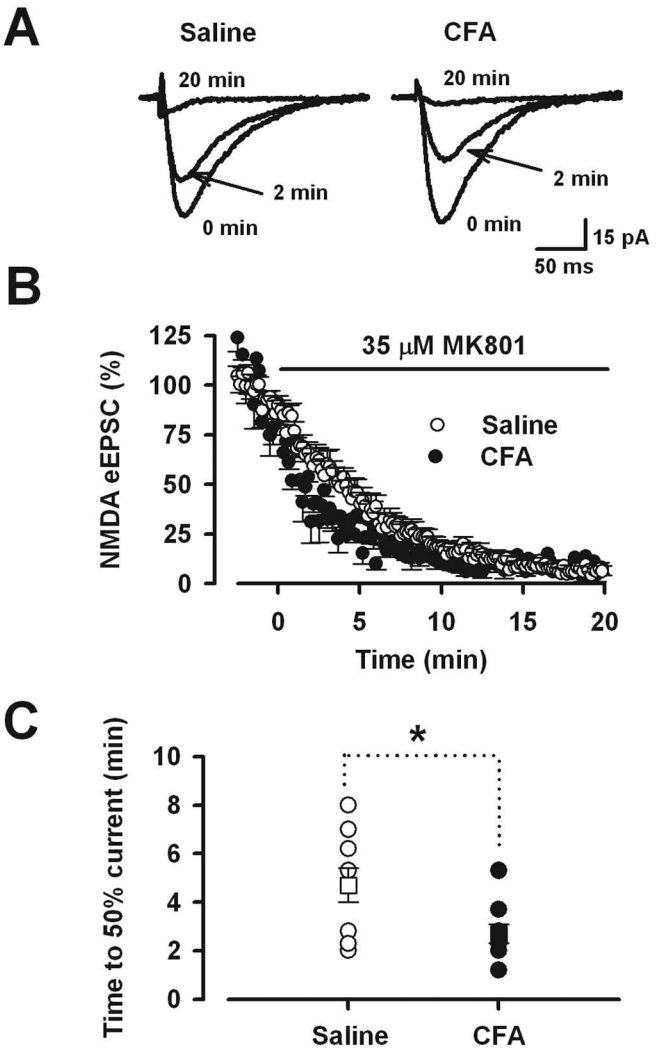

Figure 5. Faster MK-801 blockade of NMDA EPSCs in CFA-injected mice. ***A***, Representative traces show NMDA EPSCs at 0, 2, and 20 min in the presence of MK-801 (35 μM) in saline- and CFA-injected mice. ***B***, Plot of time course of MK-801 blockade of NMDA EPSCs in saline- and CFA-injected mice. Open circles, From saline mice, $n = 9$; filled circles, from CFA-injected mice, $n = 8$. ***C***, Individual and statistical results showed the decay time required for the peak amplitude of NMDA EPSC to decrease to 50% of initial value in MK-801. Significantly faster time was found in CFA-injected mice ($n = 8$) than control mice ($n = 9$; $p < 0.05$).

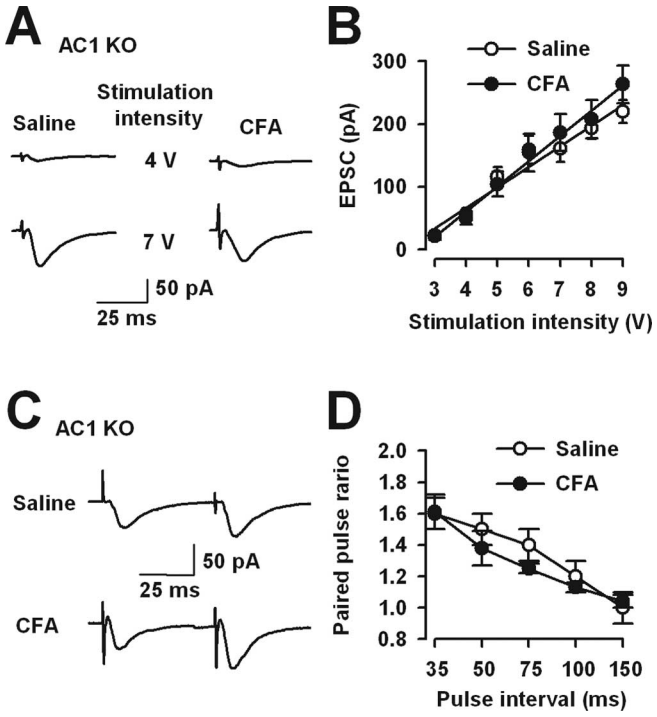

Figure 6. ACC synaptic transmission in AC1 KO mice. ***A***, Representative traces show averages of five EPSCs at 4 and 7 V stimulation intensity in the ACC. ***B***, Plot of input–output curves shows no difference of synaptic transmission in the ACC between the saline- and CFA-injected AC1 KO mice. Open circles, From saline mice, $n = 6$; filled circles, from CFA-injected mice, $n = 7$. ***C***, Representative traces of PPF with interval of 50 ms recorded in the ACC. ***D***, PPFs were no different at each interval between the saline- and CFA-injected AC1 KO mice. Open circles, From saline mice, $n = 7$; filled circles, from CFA-injected mice, $n = 6$.

ited the current in 10 min (Fig. 5*A*,*B*). When blocking rates were compared in control and CFA-injected mice, NMDA EPSC of CFA-injected mice decays considerably faster than that of the control mice (Fig. 5*A*,*B*). We analyzed the time required for peak amplitude of NMDA EPSC to decay to 50% of initial value in MK-801. Significant faster time was found in CFA-injected mice (2.7 ± 0.4 min; $n = 8$) than control mice (4.7 ± 0.7 min; $n = 9$; $p < 0.05$) (Fig. 5*C*). Together, these results indicate that the enhanced synaptic transmission is attributable to an increase in presynaptic probability of neurotransmitter release and are not likely attributable to postsynaptic AMPA receptor modifications in ACC synapses of CFA-injected mice.

We also measured synaptic LTP induced by pairing presynaptic activity with postsynaptic depolarization in cingulate neurons. We found that the ability of neurons to undergo LTP was significantly reduced ($113.5 \pm 6.5\%$; $n = 15$; $p < 0.01$ compared with control mice) compared with animals receiving saline injections (mean of $168.2 \pm 12.7\%$ of baseline; $n = 7$; $p < 0.01$ compared with baseline responses before the pairing training). These results show that LTP may be at least partially occluded in CFA-injected mice.

Synaptic transmission in the ACC of adenylyl cyclase knock-out mice

Because AC1 and AC8 are both highly expressed in the ACC and are involved in inflammation-related behavioral sensitization (Wei et al., 2002), CFA-induced alterations in synaptic transmission were examined in AC KO mice. First, we tested synaptic transmission in AC1 KO mice. In contrast to the enhanced synaptic transmission found in wild-type mice, input–output relationships and PPF were not altered in CFA-injected AC1 KO mice compared with the saline control AC1 KO mice. The slope of the curves was not different in neurons from CFA-injected mice (40.8 ± 4.4 pA/V; $n = 7$) compared with control AC1 KO mice (34.2 ± 2.4 pA/V; $n = 6$; $p = 0.232$) (Fig. 6*A*,*B*). PPF between saline- and CFA-injected groups also showed no difference in AC1 KO mice (saline, $n = 7$; CFA, $n = 6$) (Fig. 6*C*,*D*). The unaltered synaptic transmission in the ACC of CFA-injected AC1 KO mice suggests that AC1 plays a crucial role in the CFA-induced synaptic modifications within this region.

In the hippocampus, late-phase LTP was lost in AC1/AC8 double knock-out mice but not in the single AC1 or AC8 mutants, suggesting that either AC1 or AC8 can generate the necessary cAMP signal (Wong et al., 1999). Next, we tested synaptic transmission in the ACC of AC1/AC8 KO mice after CFA injection. Similar to AC1 KO mice, both the slope of the input–output relationship (saline, 33.0 ± 1.3 pA/V, $n = 6$; CFA, 39.7 ± 1.6 pA/V, $n = 6$; $p = 0.396$) (Fig. 7*A*,*B*) and PPF (saline, $n = 9$; CFA,

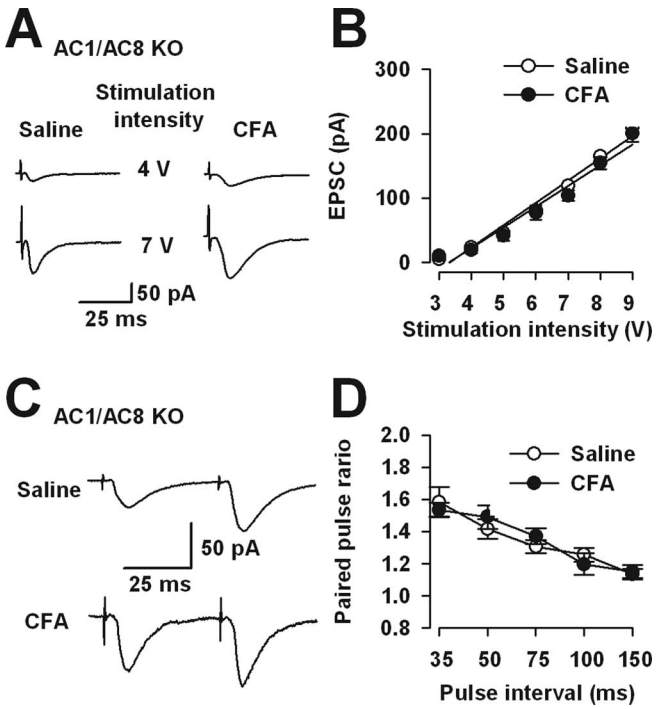

Figure 7. ACC synaptic transmission in AC1 and AC8 KO mice. ***A***, Representative traces show averages of five EPSCs at 4 and 7 V stimulation intensity in the ACC. ***B***, Plot of input–output curves shows no difference of synaptic transmission in the ACC between the saline- and CFA-injected AC1/AC8 KO mice. Open circles, From saline mice, $n = 6$; filled circles, from CFA-injected mice, $n = 6$. ***C***, Representative traces of PPF with interval of 50 ms recorded in the ACC. ***D***, PPFs were no different at each interval between the saline- and CFA-injected AC1/AC8 KO mice. Open circles, From saline mice, $n = 9$; filled circles, from CFA-injected mice, $n = 13$.

$n = 13$) (Fig. 7*C*,*D*) was unchanged in CFA-injected AC1/AC8 KO mice compared with saline control mice.

Trace fear memory in adenylyl cyclase knock-out mice treated with CFA

After establishing that AC1 and AC8 play a key role in the CFA-induced changes in ACC synaptic transmission, we asked whether the deletion of AC1 and/or AC8 would affect the acquisition of trace fear memory and whether this memory would be affected by CFA injection. In AC1 KO mice, there was not a significant affect of treatment on freezing during trace training ($p = 0.49$), and both saline- and CFA-injected mice froze significantly more throughout the training trails (comparing percentage freezing between ITI-1 and ITI-10: saline, $n = 7$, $p < 0.001$; CFA, $n = 6$, $p < 0.001$). In contrast to the freezing deficit induced by CFA in wild-type mice, there was not a significant affect of treatment on freezing during testing in AC1 KO mice ($p = 0.84$) (Fig. 8*A*). Similarly, in AC8 KO mice, there was not a significant affect of treatment on freezing during trace training ($p = 0.46$), and both saline- and CFA-injected mice froze significantly more throughout the training trails (comparing percentage freezing between ITI-1 and ITI-10: saline, $n = 4$, $p < 0.01$; CFA, $n = 5$, $p < 0.01$). The CFA-induced trace fear memory deficit was also absent in AC8 KO mice, because there was not a significant affect of treatment on freezing during testing ($p = 0.78$) (Fig. 8*B*). Freezing behavior in AC1/AC8 KO mice was also unaffected by CFA during trace testing ($p = 0.98$) (Fig. 8*C*). Together, it appears that adenylyl cyclases may play a role in the CFA-induced impairment of trace fear memory.

Interestingly, although AC1/AC8 KO mice froze significantly more throughout the training trails (comparing percentage freezing between ITI-1 and ITI-10: saline, $n = 6$, $p < 0.001$; CFA, $n = 6$, $p < 0.001$), they displayed significantly less freezing behavior compared with wild-type ($p < 0.001$), AC1 KO ($p < 0.01$), and AC8 KO ($p < 0.01$) mice (comparing average freezing over 10 trials) (Fig. 8*E*). The deficit in trace fear memory in AC1/AC8 KO mice could be reversed by intra-ACC injection of forskolin ($n = 4$; 12 nmol) before testing (vehicle, $n = 3$; $p < 0.05$) (Fig. 8*D*). Interestingly, the significant increase in freezing behavior only lasted from ITI-1 through ITI-4 (ITI-4, $p = 0.02$ vs ITI-5. $p = 0.06$), possibly representing the time it takes for forskolin to diffuse away from the injection site. During training, there was a significant affect of genotype ($p < 0.001$) but not treatment ($p = 0.27$) (Fig. 8*E*) between groups, suggesting that CFA did not interfere with the acquisition of trace fear memory. When comparing average freezing during testing, wild-type mice were the only group to be significantly affected by CFA treatment (wild type, $p < 0.01$; AC1 KO, $p = 0.81$; AC8 KO, $p = 0.70$; AC1/AC8 KO, $p = 0.99$) (Fig. 7*F*). In mice treated with CFA, freezing was significantly lower in wild-type mice compared with AC1 KO ($p < 0.05$) and AC 8 KO ($p < 0.05$) mice but not AC1/AC8 KO mice ($p = 0.88$). Importantly, there was not a significant affect of genotype ($p = 0.21$) or treatment ($p = 0.51$) on baseline freezing behavior during trace fear training or testing (genotype, $p = 0.10$; treatment, $p = 0.71$) between wild-type, AC1, AC8, and AC1/AC8 KO mice. Together, these results indicate that, although CFA-induced inflammation can interfere with the expression of trace fear memory in wild-type mice, this interference is absent in AC1, AC8, and AC1/AC8 KO mice. Additionally, the deletion of AC1 or AC8 did not affect either the acquisition or expression of trace memory, although both seemed to be impaired in mice lacking the expression of both AC1 and AC8.

Discussion

In the present study, we showed that trace fear memory was impaired in mice suffering from inflammatory pain, indicating that chronic pain can interfere with attention-demanding associative learning. Furthermore, we showed that there was a significant enhancement in neurotransmitter release probability in ACC synapses of mice with chronic pain and that Ca^{2+}-stimulated adenylyl cyclases were crucial in mediating this long-lasting enhanced presynaptic transmitter release. This is the first study, to our knowledge, to correlate a deficit in behavioral learning in animals experiencing chronic pain with alterations in *in vitro* synaptic plasticity within the ACC.

Attention, chronic pain, and the ACC

Patients with chronic pain often complain of memory and concentration difficulties. The ACC plays important roles in both attention and pain perception (Cohen et al., 1999; Eccleston and Crombez, 1999; Hutchison et al., 1999; Buffington et al., 2005). The ACC becomes more active as the length and intensity of painful stimuli increases (Rainville et al., 1997). The negative affect of persistent pain on an individual's ability to sustain attention has been well documented (Eccleston, 1994, 1995; Eccleston et al., 1997). Using the trace fear conditioning paradigm, which differs from the classic delay paradigm in that the animal must sustain attention during the trace interval to learn the CS–US association (Huerta et al., 2000; Han et al., 2003), we show that mice with chronic pain have less trace fear memory, suggesting that chronic pain affects the ability to sustain attention in a manner that is needed to learn the trace conditioning. This defect is consistent with deficits in attention reported in patients with

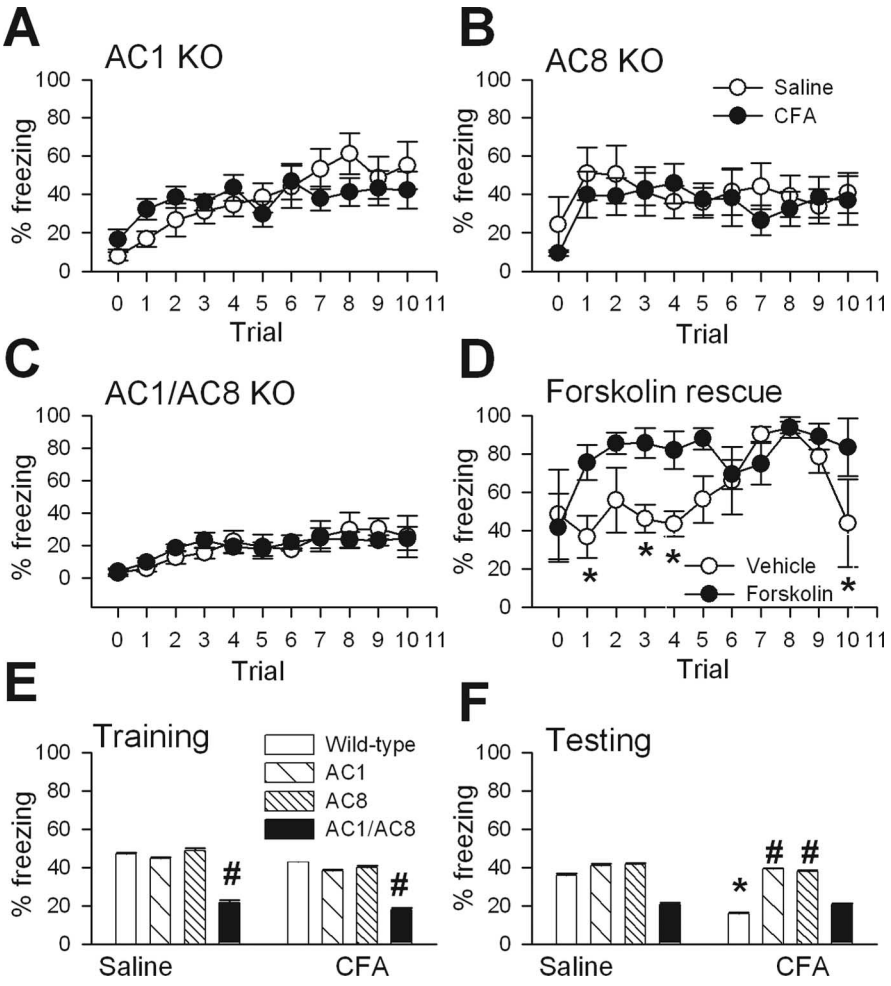

Figure 8. CFA and trace fear memory in adenylyl cyclase knock-out mice. ***A–C***, Freezing during trace testing over 10 trials, 3 d after injection of CFA or saline in AC1 KO (***A***), AC8 KO (***B***), or AC1/AC8 KO (***C***) mice. ***D***, Freezing during trace testing over 10 trials after intra-ACC forskolin or vehicle injection in AC1/AC8 KO mice. ***E***, ***F***, Average freezing over the 10 trials of the training and testing sessions. $^{*}p < 0.05$, significantly different from saline-injected mice. $^{\#}p < 0.05$, significantly different from wild-type mice.

chronic pain (Eccleston and Crombez, 1999). To our knowledge, this is the first study to demonstrate the deleterious effect of chronic pain on the retention of associative memory in mice.

Altered synaptic transmission in the ACC of mice with inflammatory pain

Our study is the first to examine presynaptic transmitter release in the ACC of mice suffering from chronic pain. The ACC plays important roles in the cognitive and emotional functions of the brain (Devinsky et al., 1995; Botvinick et al., 1999; Price, 2000; Frankland et al., 2004). Recent studies from animals and humans demonstrate that ACC neurons play key roles in behavioral nociceptive responses to injury in animals and pain perception or unpleasantness in humans (Talbot et al., 1991; Craig et al., 1996; Rainville et al., 1997; Wei et al., 2001; Wu et al., 2005). Our previous work shows that amputation caused long-lasting potentiation of ACC responses to peripheral electrical stimulation and suggests that enhanced neuronal responses to subsequent somatosensory stimuli may contribute to phantom-limb pain (Wei and Zhuo, 2001). The present study demonstrates an enhancement of synaptic transmission in the ACC of animals suffering from chronic inflammatory pain. This is demonstrated by the increased input–output curves and decreased paired-pulsed facilitation in ACC slice recordings. Furthermore, we show that the enhancement in synaptic transmission is the result of an increased presynaptic probability of neurotransmitter release in ACC synapses, as demonstrated by the increased mEPSC frequency as well as faster blocking rate of NMDA EPSCs by MK-801. In addition, analysis of the *I–V* relationship and reversal potential of AMPA receptor-mediated mEPSCs showed no difference between control and CFA-injected mice. Together, these results suggest that the enhanced synaptic transmission results from the increased probability of presynaptic neurotransmitter release rather than a possible postsynaptic modification of functional AMPA receptors. Our recent study showed that postsynaptic NR2B-containing NMDA receptors were upregulated in the ACC of CFA-injected mice (Wu et al., 2005). Thus, we cannot completely rule out the involvement of postsynaptic mechanisms involved in the alteration of synaptic transmission after inflammatory pain.

AC1 and AC8 in ACC synaptic transmission and trace fear memory

Our previous studies showed that synaptic potentiation was completely absent in mice lacking both AC1 and AC8, suggesting that Ca^{2+}-activated adenylyl cyclases are important for synaptic potentiation (Liauw et al., 2005). AC1 and AC8 are both highly expressed in ACC neurons, and genetic deletion of AC1 and AC8 led to a complete abolishment of the behavioral allodynia caused by tissue injury and inflammation (Wei et al., 2002). Behavioral responses to acute noxious stimuli were normal in these mice, which strongly suggests that AC1 and AC8 are selectively involved in mediating the behavioral responses to chronic inflammation (Wei et al., 2002). Our results are consistent with findings from *in vitro* brain slices that report that basal synaptic transmission is unaffected by the deletion of AC1 and AC8 (Wong et al., 1999). In slice recording, deletion of AC1 alone is sufficient to block the inflammation-induced synaptic changes in the ACC. However, the deletion of AC1 or AC8 did not affect either the acquisition or expression of trace memory, although both seemed to be impaired in mice lacking the expression of both AC1 and AC8, suggesting that either AC1 or AC8 can generate the necessary cAMP signal (Wong et al., 1999) and that both AC1 and AC8 are important in the processing of nociceptive information and fear memory in the ACC.

The present study provides strong evidence that peripheral inflammation can induce changes in synaptic transmission within the ACC and interfere with the acquisition of attention-based associative learning. In addition, we provide evidence for the roles of Ca^{2+}-stimulated AC1 and AC8 in the processing of nociceptive information in the ACC.

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