Neurobiology of Disease

Presynaptic and Postsynaptic Amplifications of Neuropathic Pain in the Anterior Cingulate Cortex

Hui Xu,* Long-Jun Wu,* Hansen Wang,* Xuehan Zhang, Kunjumon I. Vadakkan, Susan S. Kim, Hendrik W. Steenland, and Min Zhuo

Department of Physiology, Faculty of Medicine, University of Toronto Centre for the Study of Pain, Toronto, Ontario, Canada M5S1A8

Neuropathic pain is caused by a primary lesion or dysfunction in the nervous system. Investigations have mainly focused on the spinal mechanisms of neuropathic pain, and less is known about cortical changes in neuropathic pain. Here, we report that peripheral nerve injury triggered long-term changes in excitatory synaptic transmission in layer II/III neurons within the anterior cingulate cortex (ACC). Both the presynaptic release probability of glutamate and postsynaptic glutamate AMPA receptor-mediated responses were enhanced after injury using the mouse peripheral nerve injury model. Western blot showed upregulated phosphorylation of GluR1 in the ACC after nerve injury. Finally, we found that both presynaptic and postsynaptic changes after nerve injury were absent in genetic mice lacking calcium-stimulated adenylyl cyclase 1 (AC1). Our studies therefore provide direct integrative evidence for both long-term presynaptic and postsynaptic changes in cortical synapses after nerve injury, and that AC1 is critical for such long-term changes. AC1 thus may serve as a potential therapeutic target for treating neuropathic pain.

Key words: **anterior cingulate cortex; presynaptic release; adenylyl cyclase; AMPA receptor; mice; neuropathic pain**

Introduction

Long-term potentiation (LTP) is believed to be the key mechanism for storing sensory information in the brain (Kaas, 1991; Bliss and Collingridge, 1993; Buonomano and Merzenich, 1998; Kandel, 2001; LeDoux, 2003). For example, noxious or painful foot shocks trigger long-term changes of excitatory glutamate transmission within the amygdala (Davis et al., 1994; Malenka and Nicoll, 1997; McKernan and Shinnick-Gallagher, 1997; Blair et al., 2001; Tsvetkov et al., 2002; Rumpel et al., 2005). It is believed that such changes in synaptic transmission play key roles in fear memory. Similarities between persistent pain and memory have been recently reported (Woolf and Salter, 2000; Wei et al., 2001, 2002; Sandkühler, 2007; Zhuo, 2007). Long-term changes in synaptic transmission, occurring in sensory synapses located along the somatosensory pathway or pain-processing brain regions, contribute to chronic inflammatory and neuropathic pain (Woolf and Salter, 2000; Wei et al., 2001; Sandkühler, 2007; Zhuo, 2007).

Most studies on sensory-related synapses have focused on long-term changes at the periphery and spinal dorsal horn neurons (Boucher and McMahon, 2001; Coull et al., 2003, 2005; Ikeda et al., 2003; Kohno et al., 2003; Balasubramanyan et al., 2006; Ikeda et al., 2006; Nassar et al., 2006), and few studies have examined pain-related cortical synapses in neuropathic pain status. Cumulative evidence from both human and animal studies demonstrates that forebrain neurons, including neurons in the anterior cingulate cortex (ACC) and insular cortex, are important for pain-related perception and chronic pain. Local lesions of the medial frontal cortex, including the ACC, reduced acute nociceptive responses, injury-related aversive behaviors, and chronic pain in patients (Lee et al., 1999; Johansen et al., 2001; Zhuo, 2006). Electrophysiological recordings from ACC neurons showed that ACC cells responded to peripheral noxious stimuli (Sikes and Vogt, 1992; Yamamura et al., 1996; Koyama et al., 1998; Hutchison et al., 1999). Neuroimaging studies in humans have further confirmed these observations and showed that the ACC, together with other cortical structures, were activated by acute noxious stimuli, psychological pain, and social pain (Talbot et al., 1991; Rainville et al., 1997; Casey, 1999; Koyama et al., 2000; Rainville et al., 2001; Eisenberger et al., 2003). Molecular and cellular mechanisms for long-term changes in ACC neurons, however, receive few attentions. For example, in the case of neuropathic pain, it is unknown whether excitatory synaptic transmission undergoes plastic changes. If so, are there presynaptic enhancements of glutamate release or postsynaptic glutamate receptor-mediated responses? Finally, what is the key signaling protein or messenger for triggering such long-term plastic changes in the cortex?

In the present study, we used integrative methods, including electrophysiological, pharmacological, biochemical, behavioral, and genetic approaches to address these questions. We used brain slice preparation to investigate excitatory transmission in the

Received Feb. 21, 2008; revised June 6, 2008; accepted June 9, 2008.

This work was supported by grants from the EJLB-Canadian Institutes of Health Research (CIHR) Michael Smith Chair in Neurosciences and Mental Health, Canada Research Chair, CIHR Grants CIHR84256 and CIHR66975, and National Institutes of Health–National Institute of Neurological Disorders and Stroke Grant NS42722 to M.Z. L.-J.W. was supported by postdoctoral fellowships from the CIHR and Fragile X Foundation of Canada. We would also like to thank Drs. Louis Muglia and Daniel R. Storm for providing *AC1* $^{-/-}$ mice.

*H.X., L.-J.W., and H.W. contributed equally to this work.

Correspondence should be addressed to Dr. Min Zhuo, Department of Physiology, University of Toronto, Faculty of Medicine, Room 3342, Medical Sciences Building, 1 King's College Circle, Toronto, Ontario, Canada M5S1A8. E-mail: min.zhuo@utoronto.ca.

DOI:10.1523/JNEUROSCI.1812-08.2008

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ACC after nerve injury in a mouse model of neuropathic pain (Vadakkan et al., 2005). We found that nerve injury triggered both presynaptic and postsynaptic changes in excitatory synapses within the ACC. In genetic knock-out of calcium-stimulated adenylyl cyclase subtype 1 ($AC1^{-/-}$) mice, we found that both presynaptic and postsynaptic changes were abolished, consistent with the decrease of allodynia in $AC1^{-/-}$ mice in behavioral studies.

Materials and Methods

Animal surgery. Adult (6–8 weeks old) male C57BL/6 mice were purchased from Charles River. Mice were maintained on a 12 h light/dark cycle. Food and water were provided *ad libitum*. $AC1^{-/-}$ mice were bred for several generations on a C57BL/6 background. Experiments were performed under protocols approved by the University of Toronto Animal Care Committee. The $AC1^{-/-}$ male mice at the same age as C57BL/6 male mice were used in this study. A model of neuropathic pain was induced by the ligation of the common peroneal nerve (CPN) as described previously (Vadakkan et al., 2005). Briefly, mice were anesthetized by intraperitoneal injection of a mixture saline of ketamine (0.16 mg/kg; Bimeda-MTC) and xylazine (0.01 mg/kg; Bayer). The CPN was visible between anterior and posterior groups of muscles running almost transversely. The left CPN was ligated with chromic gut suture 5-0 (Ethicon) slowly until contraction of the dorsiflexors of the foot was visible as twitching of the digits. The mechanical allodynia was tested on postsurgical day 7, and the mice were used for electrophysiological studies on postsurgical days 7–14 (Fig. 1*A*).

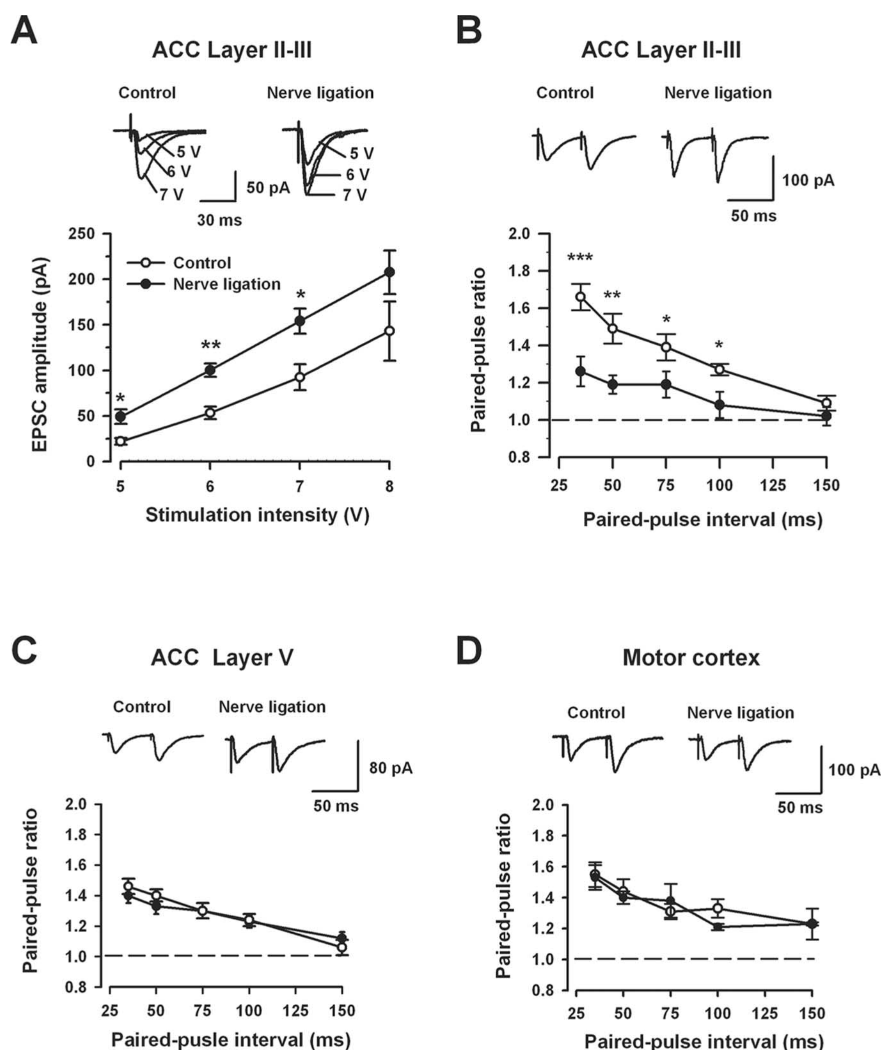

Figure 1. Increased synaptic transmission in layer II/III in the ACC after peripheral nerve ligation. ***A***, Synaptic input–output curves in slices from control ($n = 6$ neurons) and nerve-ligated ($n = 7$ neurons) mice. *$p < 0.05$ and **$p < 0.01$ compared with those of control group. Open circles, Neurons from control mice; filled circles, neurons from mice with nerve ligation. ***B***, Representative traces with an interval of 50 ms recorded in layer II/III of the ACC. Paired-pulse ratio (the ratio of EPSC2/EPSC1) was recorded at intervals of 35, 50, 75, 100, and 150 ms from control and nerve-ligated mice. Open circles, Neurons from control mice ($n = 17$ neurons); filled circles, neurons from mice with nerve ligation ($n = 19$ neurons). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. ***C***, PPF in layer V of the ACC from control and nerve-ligated mice. Open circles, Neurons from control mice ($n = 9$ neurons); filled circles, neurons from mice with nerve ligation ($n = 15$ neurons). ***D***, PPF in motor cortex neurons from control and nerve-ligated mice. Open circles, Neurons from control mice ($n = 5$ neurons); filled circles, neurons from mice with nerve ligation ($n = 5$ neurons).

Whole-cell patch-clamp recordings. Coronal brain slices (300 μm) at the level of the ACC were prepared using standard methods (Wu et al., 2005; Zhao et al., 2006). Slices were transferred to submerged recovery chamber with oxygenated (95% O_2 and 5% CO_2) artificial CSF containing (in mM) 124 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 25 $NaHCO_3$, 1 NaH_2PO_4, and 10 glucose at room temperature for at least 1 h. Experiments were performed in a recording chamber on the stage of a BX51W1 microscope equipped with infrared differential interference contrast optics for visualization. EPSCs were recorded from layer II/III neurons with an Axon 200B amplifier (Molecular Devices), 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 0.05 Hz, and neurons were voltage clamped at −70 mV in the presence of AP5 (50 μM). The recording pipettes (3–5 MΩ) were filled with a solution containing (in mM) 145 K-gluconate, 5 NaCl, 1 $MgCl_2$, 0.2 EGTA, 10 HEPES, 2 Mg-ATP, 0.1 Na_3-GTP, and 10 phosphocreatine disodium (adjusted to pH 7.2 with KOH). The internal solution (in mM) 140 cesium methanesulfonate, 5 NaCl, 0.5 EGTA, 10 HEPES, 2 MgATP, 0.1 Na_3GTP, 0.1 spermine, 2 QX-314 bromide, and 10 phosphocreatine disodium (adjusted to pH 7.2 with CsOH) was used in the rectification of AMPA receptor-mediated transmission experiment. For calculating the rectification of AMPA receptor-mediated EPSCs, we recorded the currents at holding potentials of −65, −5, and +35 mV. Then the ratio of peak EPSC amplitude at negative (−65 mV) and positive (+35 mV) holding potentials was used as a measure of the rectification index. 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 in all experiments. Access resistance was 15–30 MΩ and monitored throughout the experiment. Data were discarded if access resistance changed >15% during an experiment. Data were filtered at 1 kHz, and digitized at 10 kHz.

Western blot. Bilateral ACCs from the mice were dissected and homogenized in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% SDS, 1× protease inhibitor mixture (Sigma), and 1× phosphatase inhibitor mixture I and II (Sigma). The analysis was performed according to the procedures as reported previously (Wang et al., 2007). Briefly, the protein samples were quantified by Bradford assay. Electrophoresis of equal amounts of total protein was performed on SDS-polyacrylamide gels. Separated proteins were transferred overnight to polyvinylidene fluoride membranes at 4°C. Membranes were probed with 1:3000 dilution of anti-GluR1 (Millipore), anti-GluR2/3 (Millipore Bioscience Research Reagents), and 1:1000 dilution of anti-phospho-GluR1 Ser845 (Millipore). The membranes were incubated in the appropriate horse-

radish peroxidase-coupled secondary antibody diluted at 1:3000 for 1 h followed by enhanced chemiluminescence detection of the proteins with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). To ensure equal loading, membranes were also probed with 1:3000 dilution of anti-actin antibody (Sigma). The density of immunoblots of proteins was quantified with NIH ImageJ software.

Preparation of cytosolic and membrane fractions. The cytosolic and membrane fractions were prepared as described previously (Morozov et al., 2003) 1 week after nerve ligation. Briefly, the ACC samples were homogenized in the lysis buffer (10 mM Tris, pH 7.4, 300 mM sucrose, and 1 mM EDTA) containing protease inhibitor mixture and then centrifuged at $8000 \times g$ for 5 min. The pellet (P1) containing nuclei and debris was discarded. The supernatant (S1) was then centrifuged at $40,000 \times g$ for 30 min to obtain the cytosol extract in the supernatant (S2) and crude membrane in the pellet (P2). This pellet (P2), which includes cellular membranes, was resuspended in lysis buffer with protease inhibitor mixture. Protein concentrations were determined using Bio-Rad protein reagent. The expression of GluR1 and GluR2/3 subunits was characterized by Western blot. The expression of actin and cadherin was detected for equal loading of cytosolic and membrane extracts, respectively.

Cannulation surgery and microinjection. Mice were anesthetized with isoflurane (1–3%, as needed) inhalation with 30% oxygen balanced with nitrogen. The scalp was shaved and then cleaned with iodine (Triadine) and alcohol. The head of the mouse was fixed into a stereotaxic adapter mounted on a stereotaxic frame (Kopf model 962) and lubricant (Artificial Tears) was applied to the eyes. An incision was made over the skull and the surface was exposed. Two small holes were drilled above the ACC, and the dura was gently reflected. Guide cannulas were placed so that the final coordinates of the microinjection would be 0.7 mm anterior to bregma, 0.3 mm lateral to the midline, and 1.75 mm ventral to the surface of the skull (Wei et al., 2002). For microinjection, mice were restrained in a plastic cone (Braintree Scientific), and a small hole was cut in the plastic overlying the microinjection guides. The dummy cannulas were removed, and the microinjection cannula was inserted into the guide. A 30 gauge injection cannula was used, which was 0.8 mm lower than the guide. Microinjection was conducted using a motorized syringe pump (Razel Scientific Instruments) and a Hamilton syringe. CNQX (1 mM) dissolved in saline was delivered to left and right ACC (500 nL in 1 min) through the cannula. The volume delivered was confirmed by watching the movement of the meniscus down a length of calibrated polyethylene (PE10) tubing. After delivery to each side of the brain, the injection cannula was left in place for 1 min to help prevent any solution from flowing back up the guide. The cannula was then retracted and inserted into the opposite side of the brain. After microinjection, the animals were immediately delivered to a clear plastic cylinder for behavioral observation.

Mechanical allodynia test. Mice were placed in a round container and allowed to acclimate for 30 min before testing. Mechanical allodynia was assessed based on the responsiveness of the hindpaw to the application of von Frey filaments (Stoelting) to the point of bending. Positive responses include licking, biting, and sudden withdrawal of the hindpaw. Experiments were performed to characterize the threshold stimulus. Mechanical pressure from a 1.65 filament (force, 0.008 g) was found to be innocuous in normal mice. This filament was then used to test the mechanical allodynia after nerve ligation. Mechanical allodynia was tested five times with an intertrial interval of 10 min. Animals were then permitted a rest period for 20 min, after which mechanical allodynia was again tested. A total of three mechanical allodynia test sets were performed as baseline (day 5), and two test sets (day 6) were performed after drug infusion. The results are expressed as a percentage of positive responses.

Data analysis. Results are presented as means ± SEM. Statistical comparisons were performed using two-way ANOVA and the Student's *t* test. Analysis of mEPSCs was performed with cumulative probability plots. The level of significance was set at $p < 0.05$.

Results

Enhanced excitatory synaptic transmission

To explore whether there is any change in basal synaptic transmission within the ACC after nerve injury, we recorded AMPA receptor-mediated EPSCs in pyramidal neurons in the layer II/III of the ACC in mice with peripheral nerve ligation (Vadakkan et al., 2005). Recorded neurons were identified as pyramidal neurons based on their ability to show spike frequency adaptation in response to the prolonged depolarizing-current injection (Tsvetkov et al., 2004). Input (stimulation intensity)–output (EPSC amplitude) curve of AMPA receptor-mediated current was significantly shifted to the left after peripheral nerve injury ($n = 7$ neurons/5 mice), compared with that in control group ($n = 6$ neurons/4 mice, two-way ANOVA, $p < 0.05$) (Fig. 1*A*). These results suggest that excitatory synaptic transmission was increased in the ACC after peripheral nerve injury.

Altered paired-pulse facilitation

To examine whether presynaptic or postsynaptic mechanisms mediate the enhanced excitatory synaptic transmission in the ACC in mice with neuropathic pain, paired-pulse facilitation (PPF) was examined in ACC neurons in the present study. PPF is a transient form of plasticity commonly used as a measure of presynaptic function, in which the response to the second stimulus is enhanced as a result of residual calcium in the presynaptic terminal after the first stimulus (Foster and McNaughton, 1991). In control mice, PPF was observed at different stimulus intervals of 35, 50, 75, 100, and 150 ms. After nerve ligation, there was a significant reduction in PPF in ACC neurons ($n = 19$ neurons/7 mice) compared with those from control mice ($n = 17$ neurons/7 mice, two-way ANOVA, $p < 0.05$) (Fig. 1*B*). To test whether the change of PPF is region specific for the ACC after nerve injury, we tested the ratios of PPF layer V in the ACC and in the motor cortex. The result showed that there was no difference in the ratio of PPF either in layer V of the ACC (nerve-ligated group: $n = 15/4$ mice; control group: $n = 9$ neurons/5 mice; two-way ANOVA, $p > 0.05$) (Fig. 1*C*) or in the motor cortex between nerve-ligated ($n = 5$) and control ($n = 5$, two-way ANOVA, $p > 0.05$) (Fig. 1*D*) groups. These results indicate that presynaptic enhancement of the excitatory synaptic transmission selectively occurs in the layer II/III of the ACC after nerve injury.

Enhanced presynaptic transmitter release probability

To further determine whether decreased PPF in the ACC after peripheral nerve injury might be attributable to the increased presynaptic transmitter release probability, we recorded AMPA receptor-mediated mEPSCs in ACC neurons in the presence of 0.5 μM tetrodotoxin. After peripheral nerve injury, there was an obvious increase of mEPSC frequency in ACC neurons compared with that of control group (control: 0.9 ± 0.1 Hz, $n = 9$ neurons/5 mice; nerve ligation: 2.3 ± 0.5 Hz, $n = 13$ neurons/5 mice; $p < 0.05$) (Fig. 2*B*,*C*). Furthermore, there was significant difference in the amplitude of mEPSCs between the two groups (control: 11.2 ± 0.8 pA, $n = 9$ neurons/5 mice; nerve ligation: 14.1 ± 0.5 pA, $n = 13$ neurons/5 mice; $p < 0.01$) (Fig. 2*B*,*C*).

We further tested the blocking rate of an irreversible NMDA receptor blocker, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo-[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801), in both control mice and mice with neuropathic pain. The blocking rate of NMDA receptor-mediated synaptic current by MK-801 is used to estimate the transmitter-release probability (Hessler et al., 1993; Weisskopf and Nicoll, 1995). At a holding potential of −10 mV, NMDA receptor-mediated EPSCs were recorded in the presence of CNQX (20 μM) and picrotoxin (100 μM) at 0.1 Hz. MK-801 (35 μM) was perfused after obtaining stable NMDA receptor-mediated EPSCs. Our results showed that MK-801 progressively blocked NMDA receptor-mediated EPSCs and completely inhib-

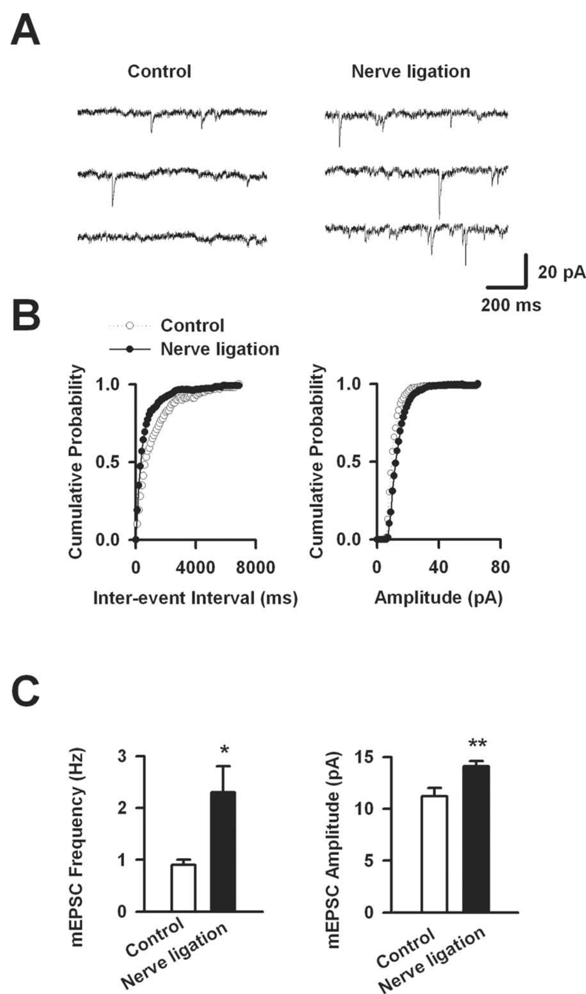

Figure 2. mEPSCs recorded in the ACC in mice after peripheral nerve ligation. ***A***, Representative mEPSCs recorded in the ACC neuron in slices from control mice (left) and mice with nerve ligation (right) at a holding potential of −70 mV. ***B***, Cumulative interevent interval (left) and amplitude (right) histograms of mEPSCs recorded in slices from control mice (open circles; n = 9 neurons) and mice with nerve ligation (filled circles; n = 13 neurons). ***C***, Summary plots of mEPSC data. Averaged values of mEPSC parameters: mean peak frequency (left) and amplitude (right). *$p < 0.05$; **$p < 0.01$.

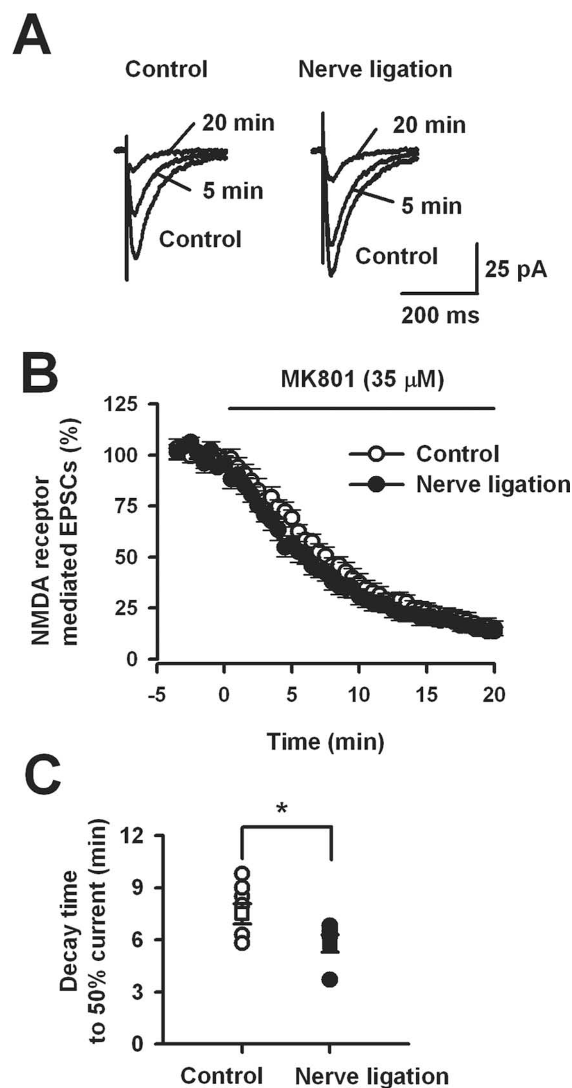

Figure 3. Faster MK-801 blockade of NMDA receptor-mediated EPSCs in mice with nerve ligation. ***A***, Representative traces show NMDA receptor-mediated EPSCs at 0, 5, and 20 min in the presence of MK-801 (35 μM) in control and nerve-ligated mice. ***B***, Plot of time course of MK-801 blockade of NMDA receptor-mediated EPSCs in control mice and mice with nerve ligation. Open circles, From control mice (n = 7 neurons); filled circles, from mice with nerve ligation (n = 8 neurons). ***C***, Individual and statistical data showed the decay time required for the peak amplitude of NMDA receptor-mediated EPSC to decrease to 50% of initial value in the presence of MK-801. Significantly faster time was observed in mice with nerve ligation (n = 8 neurons) compared with control mice (n = 7 neurons). *$p < 0.05$.

ited the current in 25 min (Fig. 3*A*,*B*). The blocking rate of the inhibition of NMDA receptor-mediated EPSCs by MK-801 in mice with neuropathic pain was considerably faster than that of the control mice (Fig. 3*A*,*B*). We tested the time required for peak amplitude of NMDA receptor-mediated EPSCs to decay to 50% of initial value in the presence of MK-801. Significantly faster decay time was observed in mice with nerve ligation (5.8 ± 0.5 min; n = 8 neurons/6 mice) than control mice (7.5 ± 0.6 min; n = 7 neurons/2 mice; $p < 0.05$) (Fig. 3*C*). Together, these results indicate that the enhanced excitatory synaptic transmission in the ACC after nerve injury is attributable to an increase in probability of presynaptic neurotransmitter release and an increase of postsynaptic responsiveness as well.

GluR1 modulation

AMPA receptors are heteromultimers assembled from GluR1, GluR2, GluR3, and GluR4 subunits (Sommer et al., 1991; Hollmann and Heinemann, 1994). To further determine the role of AMPA receptors in the enhanced synaptic transmission in the ACC after nerve injury, we examined the expression levels of GluR1 and GluR2/3, the dominant subunits of AMPA receptors at central synapses (Geiger et al., 1995; Lambolez et al., 1996) after nerve injury. By Western blot, we found that there was no difference in the expression levels of both GluR1 and GluR2/3 receptors in ACC between the control mice and mice with nerve ligation ($p > 0.05$; n = 6) (Fig. 4*A*,*B*). These results indicate that the nerve injury has not altered the total protein expression levels of GluR1 and GluR2/3 subunits of AMPA receptors, and the enhanced synaptic transmission in the ACC may not be caused by the changes in the total protein levels of AMPA receptors.

The phosphorylation of GluR1 subunit of AMPA receptors is critical for synaptic expression of the receptors, their channel properties, and synaptic plasticity (Esteban et al., 2003; Lee et al., 2003; Vanhoose et al., 2006). We next tested the phosphorylation levels of GluR1 subunit at the PKA phosphorylation site (Ser 845)

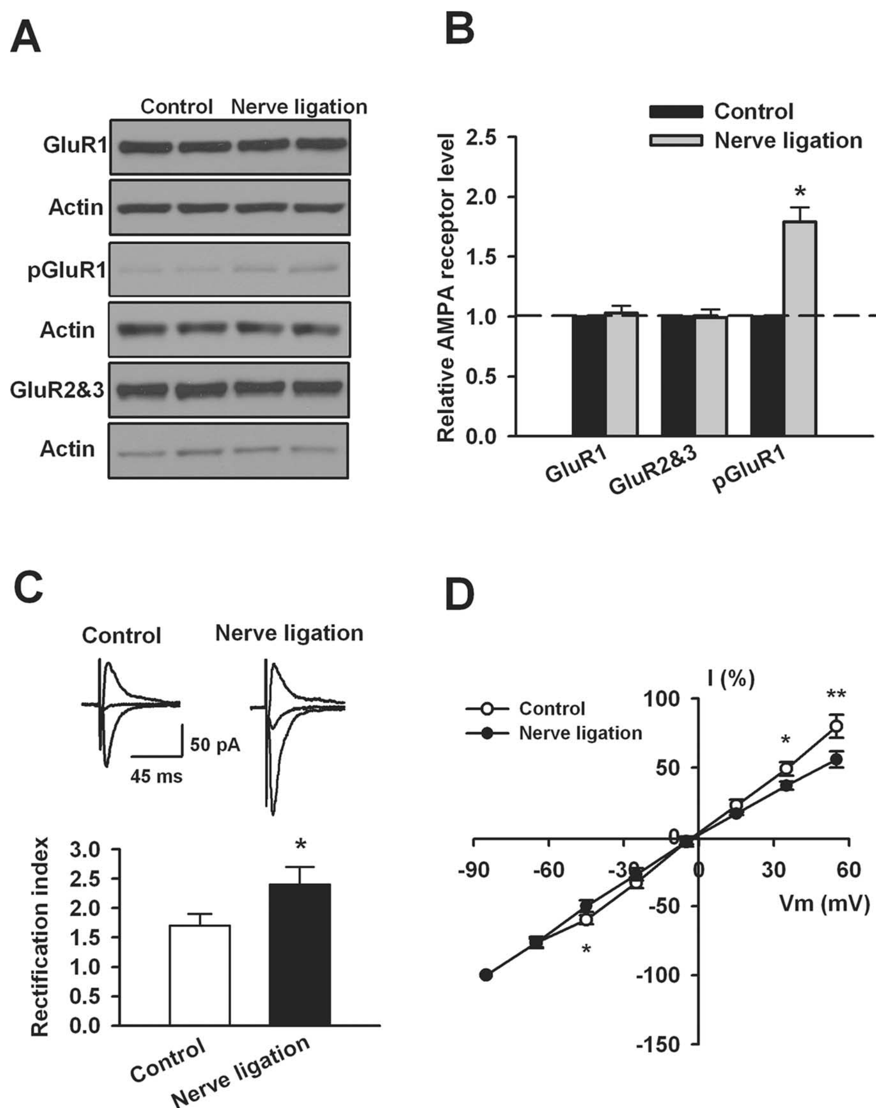

Figure 4. Altered phosphorylation of GluR1 and rectification index of AMPA receptor-mediated current in the ACC after nerve injury. ***A***, Representative expression of GluR1 and phosphorylation of GluR1 and GluR2/3 by Western blot in the ACC from control and nerve-ligated mice. ***B***, Pooled data showing that phosphorylation of GluR1 was upregulated in mice with nerve ligation. ***C***, Representative traces of evoked AMPA receptor-mediated postsynaptic currents at −65, −5, and +35 mV holding potentials were recorded from one ACC neuron in control and nerve-ligated groups, respectively. Pooled data of rectification of AMPA receptor-mediated peak current show significant differences (*$p < 0.05$). Open bars, Neurons from control mice ($n = 12$ neurons); filled bars, neurons from mice with nerve ligation ($n = 18$ neurons). Rectification index = (amplitude at −65 mV holding potential)/(amplitude at +35 mV holding potential). ***D***, Mean *I–V* curve of AMPA EPSCs in ACC neurons from control and nerve-ligated mice. *$p < 0.05$; **$p < 0.01$.

in the ACC of the mice with nerve ligation. We found that the phosphorylation levels of GluR1 were significantly increased in the ACC after nerve injury (1.79 ± 0.12 times the control value; $p < 0.01$; $n = 6$ mice) (Fig. 4*A*,*B*). The data indicate that the nerve injury can increase the phosphorylation levels of GluR1 through the PKA signaling pathway.

GluR1-mediated rectification

AMPA receptors without GluR2 are Ca^{2+} permeable and inwardly rectifying (Geiger et al., 1995; Gu et al., 1996; Washburn et al., 1997). Inward rectification occurs by voltage-dependent blockade by polyamines (Washburn and Dingledine, 1996). We tried to enhance inward rectification by increasing intracellular polyamines concentration. To identify whether there are inwardly rectifying properties of AMPA receptors as a result of an alteration of their subunit composition in ACC neurons after nerve injury, we examined evoked AMPA receptor-mediated EPSCs at the holding potentials of −65, −5, and +35 mV in ACC neurons. We found that there was significant difference in the rectification of AMPA receptor-mediated transmission in the ACC between control ($n = 12$ neurons/5 mice) and nerve-ligated ($n = 18$ neurons/6 mice; *t* test, $p < 0.05$) (Fig. 4*C*) mice. Consistently, when the mean current–voltage (*I–V*) relationship was plotted, less inward currents were found in ACC neurons from mice with nerve injury ($n = 18$ neurons/6 mice) compared with control mice ($n = 12$ neurons/5 mice) (Fig. 4*D*). These results display that AMPA receptors in ACC neurons had an inward rectification property in neuropathic pain.

Membrane GluR1 expression

The trafficking of AMPA receptor subunits has been proposed to contribute to synaptic plasticity underlying hyperalgesia (Woolf and Salter, 2000). It has been reported that painful stimuli can recruit AMPA receptor GluR1 subunits to neuronal plasma membranes in the lumbar spinal cord (Galan et al., 2004). We next investigated the distribution of AMPA receptor subunits in the ACC after nerve ligation. We found that induction of neuropathic pain by nerve ligation was associated with an increase in the abundance of the GluR1 subunits in the membrane fraction and a corresponding decrease in the levels in the cytosolic fraction ($p < 0.05$, compared with the control; $n = 4$ mice) (Fig. 5*A*). In contrast, nerve ligation had no effect on the intracellular distribution of GluR2/3 subunits in ACC neurons ($p > 0.05$, compared with the control; $n = 4$ mice) (Fig. 5*B*). The data show that AMPA receptor GluR1 subunit is redistributed in ACC neurons as a result of nerve injury.

ACC AMPA receptor and allodynia

We have shown that peripheral nerve injury caused increased distribution of GluR1 and AMPA receptor-mediated synaptic transmission in the ACC. To directly address the critical role of AMPA receptor in neuropathic pain, we next performed experiment of intra-ACC infusion of CNQX (1 mM), a non-NMDA receptor antagonist, in mice after nerve injury and then tested for allodynia. The mechanical allodynia was tested on postsurgical days 1, 3, 5, and 6. On day 6, mice were microinjected with CNQX ($n = 6$) (Fig. 5*C*). We found that bilateral microinjection of CNQX significantly reduced the allodynia in both contralateral and ipsilateral sides, and the effect of CNQX disappeared when tested 2 h after injection ($n = 6$) (Fig. 5*C*). As a control, we found that CNQX did not affect the 50% paw-withdrawal threshold

when microinjected into the ACC of mice that had not undergone nerve ligation ($n = 3$ mice; $p > 0.05$ compared with baseline responses).

Role of AC1 in presynaptic changes

We have previously shown that AC1 is critical for chronic pain. For example, in $AC1^{-/-}$ mice, reduced chronic pain sensitization was found in inflammatory pain and neuropathic pain model (Wei et al., 2002). We want to know whether AC1 is also involved in behavioral and synaptic changes in the current neuropathic pain model. We found that the mechanical allodynia is significantly reduced in both ipsilateral ($p < 0.05$; $n = 4$ mice) (Fig. 6*A*) and contralateral ($p < 0.05$; $n = 4$ mice) (Fig. 6*A*) sides of nerve injury in the knock-out mice. Moreover, after nerve injury, the $AC1^{-/-}$ mice did not exhibit reduction in PPF of AMPA receptor-mediated EPSCs as shown by the wild-type mice after the nerve injury (control $AC1^{-/-}$, $n = 7$ neurons; $AC1^{-/-}$ mice with nerve ligation, $n = 9$ neurons; $p > 0.05$) (Fig. 6*B*). We then tested the frequency and amplitude of mEPSCs in $AC1^{-/-}$ mice after nerve injury. Although both frequency and amplitude of mEPSCs in ACC neurons were consistently increased in wild-type mice after nerve injury, no increase of mEPSC frequency or amplitude was observed in $AC1^{-/-}$ mice [frequency in $AC1^{-/-}$ control mice: 1.5 ± 0.3 Hz, $n = 5$ neurons/2 mice; frequency in $AC1^{-/-}$ mice with nerve ligation: 1.9 ± 0.5 Hz, $n = 11$ neurons/4 mice; $p > 0.05$ (Fig. 6*C*,*D*); amplitude in $AC1^{-/-}$control mice: 16.9 ± 0.5 pA, $n = 5$ neurons/2 mice; $AC1^{-/-}$ amplitude in mice with nerve ligation: 15.4 ± 1.7 pA, $n = 11$ neurons/4 mice; $p > 0.05$ (Fig. 6*C*,*D*)]. These results indicate that both presynaptic and postsynaptic enhancement of excitatory synaptic transmission in the ACC depend on AC1 in neuropathic pain.

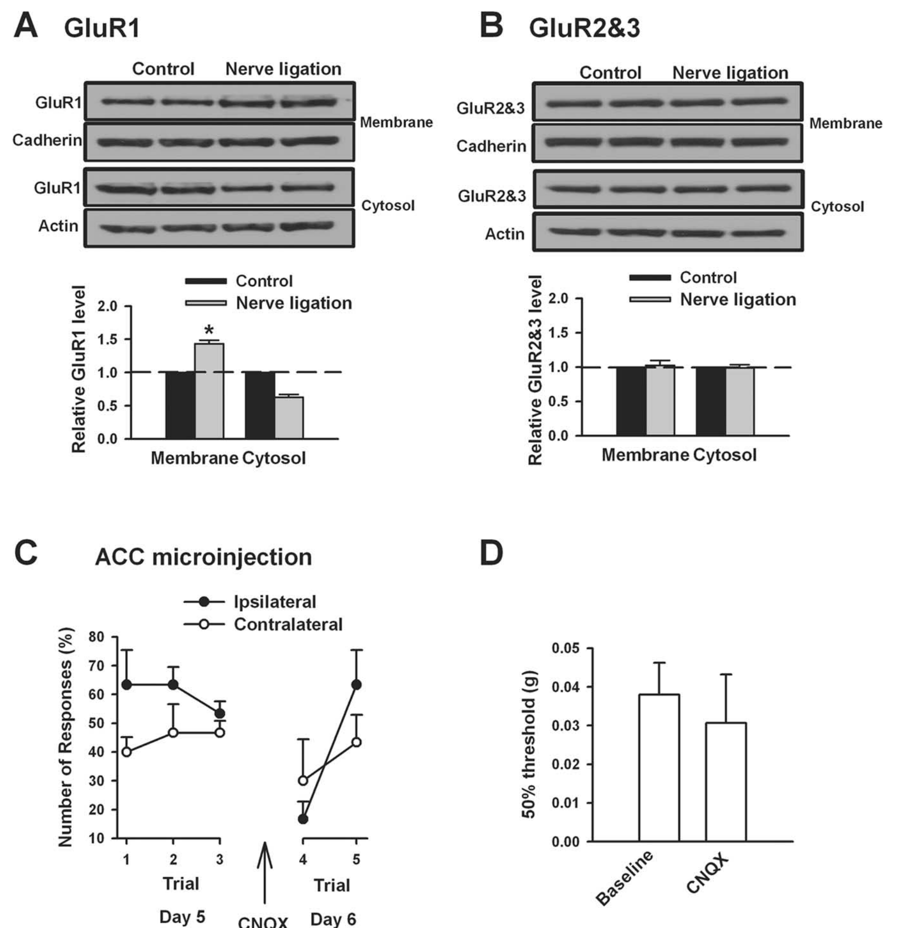

Figure 5. The redistribution of AMPA receptor GluR1 subunits in ACC neurons after nerve injury. ***A***, The subcellular distribution of GluR1 subunits in the membrane and cytosolic fraction of ACC neurons. The abundance of GluR1 subunits increased in the membrane fraction, whereas it decreased correspondingly in the cytosolic fraction after nerve injury. ***B***, The subcellular distribution of GluR2/3 subunits was not affected in ACC neurons by nerve injury. The membrane and cytosolic fractions were prepared 1 week after nerve ligation. $*p < 0.05$; $n = 4$ mice for each group. ***C***, Bilateral microinjection of CNQX (1 mM) in the ACC reduced the mechanical allodynia ($n = 6$ mice). ***D***, Microinjection of CNQX did not affect the 50% paw-withdrawal threshold in control mice ($n = 3$ mice).

Role of AC1 in postsynaptic changes

Next, Western blot analysis of basal levels of GluR1 and GluR2/3 proteins was performed on the ACC of control and nerve-ligated $AC1^{-/-}$ mice. We found that the expression of GluR1 and GluR2/3 was not altered in the ACC of $AC1^{-/-}$ mice compared with wild-type mice ($p > 0.05$; $n = 4$ mice) (Fig. 7*A*). The data indicate that genetic deletion of *AC1* has not affected the expression of AMPA receptors in the ACC. We then tested the phosphorylation levels of GluR1 subunit in the ACC of $AC1^{-/-}$ mice with neuropathic pain. We found that the basal levels of phosphorylated GluR1 were not changed in the ACC of $AC1^{-/-}$ mice compared with wild-type mice. However, the increase of the phosphorylation levels of GluR1 induced by nerve injury was blocked in the ACC of $AC1^{-/-}$ mice compared with wild-type mice (1.06 ± 0.08 times increase in $AC1^{-/-}$ mice vs 1.76 ± 0.10 times increase in wild-type mice; $p < 0.01$; $n = 4$ mice) (Fig. 7*B*). These results indicate that genetic deletion of *AC1* has not altered the basal levels of phosphorylated GluR1, and AC1 is involved in the phosphorylation of GluR1 receptors in the ACC during neuropathic pain.

In addition, we also examined the rectification of AMPA receptors in the ACC in $AC1^{-/-}$ mice with internal spermine included in the pipette at holding potentials of −65, −5, and +35 mV. There was no obvious difference in the rectification of AMPA receptor-mediated transmission in the ACC between $AC1^{-/-}$ control ($n = 7$ neurons) and $AC1^{-/-}$ mice with nerve ligation ($n = 9$ neurons) (two-way ANOVA, $p > 0.05$) (Fig. 7*C*). This result indicates that postsynaptic GluR1 composition was not altered in ACC neurons in $AC1^{-/-}$ mice in neuropathic pain. Together, these results suggest that both presynaptic and postsynaptic AC1 might play key roles in regulating the increased excitatory synaptic transmission in the ACC after chronic nerve injury.

Discussion

The present study is built on our two previous observations. First, peripheral injuries trigger plastic changes in the ACC, including an array of activity-dependent immediate early genes, and enhanced synaptic responses (Wei et al., 1999; Wu et al., 2005; Zhuo, 2007). No identification of presynaptic versus postsynaptic mechanisms have been performed in the ACC in case of nerve

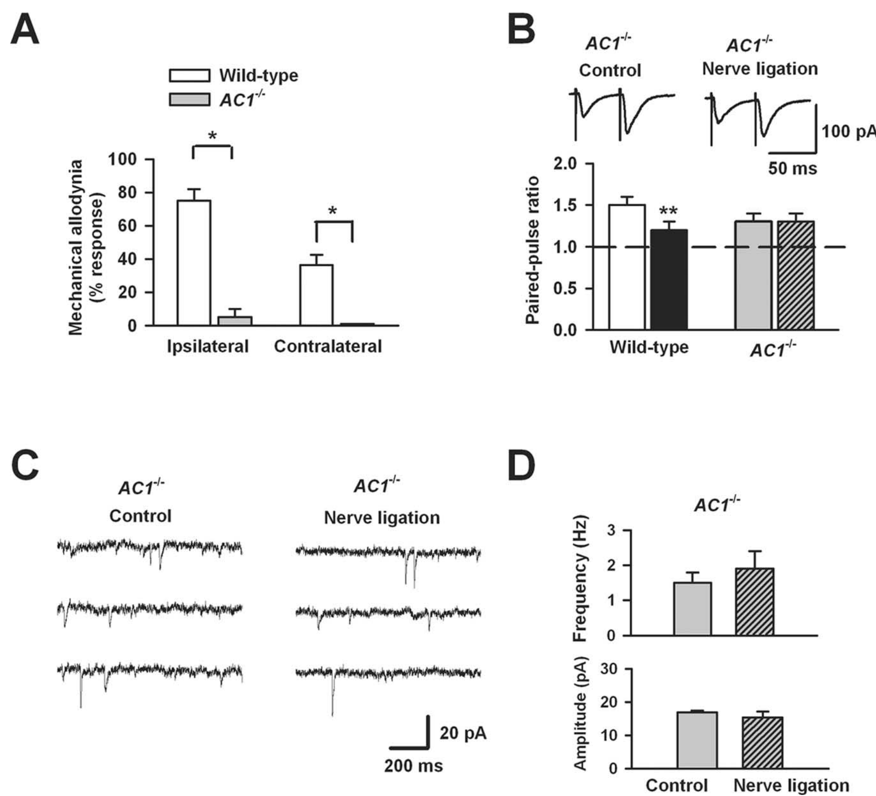

Figure 6. Paired-pulse facilitation in ACC neurons in *AC1* $^{-/-}$ mice with nerve ligation. ***A***, Reduced mechanical allodynia in *AC1* $^{-/-}$ mice with nerve ligation ($n = 4$ mice). *$p < 0.05$. ***B***, The reduction in PPF of AMPA receptor-mediated EPSCs as shown by the wild-type mice after nerve injury was abolished in *AC1* $^{-/-}$ mice after nerve injury. **$p < 0.01$. Insets, Representative traces of PPF with an interval of 50 ms recorded in the ACC. ***C***, mEPSCs recorded in ACC neurons from *AC1* $^{-/-}$ mice with nerve ligation. Shown are representative mEPSCs recorded in the ACC neuron in slices from *AC1* $^{-/-}$ control mice (left) and mice with nerve injury (right) at a holding potential of −70 mV. ***D***, Summary plots of mEPSC data. Average values of mEPSC parameters are shown. Shown are mean peak frequency (top) and amplitude (bottom) in *AC1* $^{-/-}$ control mice ($n = 5$ neurons) and *AC1* $^{-/-}$ mice with nerve ligation ($n = 11$ neurons; $p > 0.05$).

injury (Wu et al., 2005; Zhao et al., 2006). Second, AC1 and AC8, two major forms of calcium-stimulated ACs, are critical for chronic pain in animal models including neuropathic pain (Wei et al., 2002; Vadakkan et al., 2006). No selective or relatively selective AC1 inhibitor has been reported, to our knowledge. Our current findings thus provide the evidence of synaptic mechanism for neuronal plastic changes in the ACC after nerve injury, namely presynaptic enhancement of glutamate release probability, as well as postsynaptic enhancement of AMPA receptor-mediated responses.

Presynaptic and postsynaptic changes in LTP and chronic pain

Both presynaptic and postsynaptic changes have been found to contribute to LTP (Malgaroli and Tsien, 1992; Bolshakov and Siegelbaum, 1995; Nicoll and Malenka, 1995; Kandel, 2001; Malinow and Malenka, 2002; Nicoll and Schmitz, 2005). Depending on the central regions of the brain and experimental conditions, presynaptic enhancement of glutamate release or postsynaptic enhancement of AMPA receptor-mediated responses can be responsible for the expression of LTP. For example, in the CA3 region of hippocampus, it has been reported that LTP is presynaptically expressed and requires the activity of AC1 (Nicoll and Schmitz, 2005). In the CA1 region of the hippocampus, presynaptic and postsynaptic mechanisms for LTP have been hotly debated, including the postsynaptic AMPA receptor trafficking and insertion, and may also require retrograde messengers under certain conditions (see reviews above). Hippocampus and hippocampus-related limbic structures are proposed to be critical for spatial learning and memory (Kandel, 2001). Unlike the hippocampus, the ACC is critical for affective cognitive functions, including decision making, trace memory, attention, and persistent pain (Zhuo, 2007). ACC LTP can be induced by different induction protocols. Our recent pharmacological and genetic approaches have found that AMPA GluR1 receptor is critical for the expression of LTP (Toyoda et al., 2007). In the present study, in ACC slices of neuropathic pain mice, we found that both presynaptic and postsynaptic glutamate transmission are undergoing long-term potentiation. Presynaptic enhancement, as evaluated by three different methods, was observed after the injury, indicating the presynaptic enhancement of glutamate releases. At postsynaptic site, both electrophysiological and biochemical data suggest that AMPA receptor GluR1-mediated responses are enhanced. Although our studies cannot draw any conclusion of possible insertion for GluR1 receptors, electrophysiological data do suggest this possibility. Our results thus provide the evidence for pain-related both presynaptic and postsynaptic enhancement in the ACC, and stimulation-induced LTP in normal brain slices can be used to mimic or model postsynaptic mechanisms of injury-induced changes (Zhao et al., 2005).

Calcium-stimulated AC1 in synaptic plasticity and pain

Previous studies have shown that AC1 mRNA was highly expressed in the ACC and that AC1 contributed to behavioral nociceptive responses to nerve injury and inflammation (Wei et al., 2002). In mice lacking AC1, we found that chronic pain, including neuropathic pain and inflammation-related allodynia, is significantly reduced, whereas acute pain in response to noxious thermal or mechanical stimuli remain intact (Wei et al., 2002). These results suggest that AC1 is selectively involved in persistent or chronic pain. In neurons, it has been proposed that AC1 couples NMDA receptor-induced cytosolic Ca^{2+} elevation to cAMP signaling pathways (Chetkovich and Sweatt, 1993; Wong et al., 1999). Because activation of NMDA receptor contributes to persistent/chronic pain (Wu et al., 2005), it is likely that AC1 can act downstream from the NMDA receptor, and thus contribute to chronic pain (Zhuo, 2008). The molecular mechanisms may involve the activation of PKA pathway after AC1 activation, and the following phosphorylation and the recruitment of GluR1 to membrane. The similar mechanism for GluR1 trafficking has been reported in hippocampus (Lee et al., 2003; Derkach et al., 2007). Therefore, AC1 in the ACC is an attractive candidate serving for the initiation of GluR1 trafficking and chronic pain.

In the present study, we found that both presynaptic enhancements and postsynaptic changes in AMPA receptor-mediated responses were blocked in AC1 knock-out mice, indicating that AC1 may contribute both presynaptically and postsynaptically to

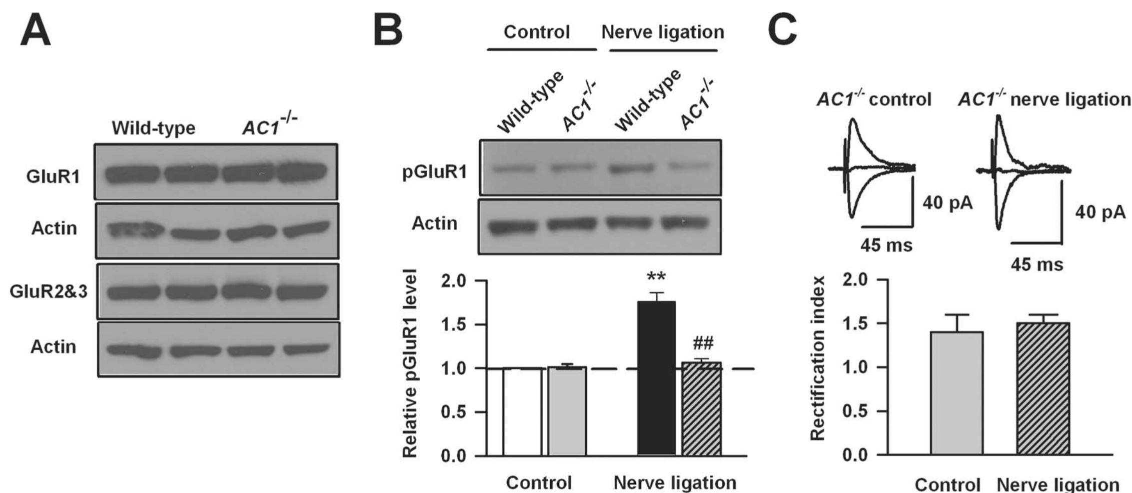

Figure 7. Expression of GluR1 and rectification index of AMPA receptor-mediated currents in ACC neurons from *AC1* $^{-/-}$ mice with nerve ligation. ***A***, Representative Western blot of GluR1 and GluR2/3 in the ACC from wild-type and *AC1* $^{-/-}$ mice with nerve ligation. ***B***, Representative Western blot and corresponding quantitation of the phosphorylation of GluR1 at Ser845 in the ACC from wild-type mice ($n = 6$), wild-type mice with nerve ligation ($n = 6$), *AC1* $^{-/-}$ control mice ($n = 4$), and *AC1* $^{-/-}$ mice with nerve ligation ($n = 4$). The phosphorylation levels of GluR1 were significantly increased in the ACC after nerve injury (**$p < 0.01$; $n = 6$) in wild-type mice. The increase of the phosphorylation levels of GluR1 induced by nerve injury was blocked in the ACC of *AC1* $^{-/-}$ mice compared with wild-type mice ($^{\#\#}p < 0.01$; $n = 4$). ***C***, Representative traces and pooled data of rectification index of AMPA receptor-mediated current in the ACC from *AC1* $^{-/-}$ control and *AC1* $^{-/-}$ mice with nerve ligation.

injury-induced plastic changes in the ACC. In fact, the stimulation of AC1 is critical for mossy fiber LTP (Bolshakov et al., 1997; Villacres et al., 1998). Because basic properties of synapses are not affected, including paired-pulse facilitation, AMPA receptor-mediated responses, and NMDA receptor-mediated responses in AC1 knock-out mice, we believe that the involvement of AC1 is activity dependent, rather than other nonselective side effects. In addition to cortical region, AC1 is also likely involved in spinal cord plasticity. In previous studies, we have demonstrated that genetic deletion of AC1 inhibits spinal cord LTP induced by pairing training protocol and synaptic facilitation induced by coapplication of forskolin and serotonin (Wang and Zhuo, 2002; Wei et al., 2006). Considering the increasing evidence of spinal LTP and descending facilitation in chronic pain, it is conceivable that spinal AC1 mechanisms may also contribute to allodynia.

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