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Genetic Elimination of Behavioral Sensitization in Mice Lacking Calmodulin-Stimulated Adenylyl Cyclases

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Summary

Adenylyl cyclase types 1 (AC1) and 8 (AC8), the two major calmodulin-stimulated adenylyl cyclases in the brain, couple NMDA receptor activation to cAMP signaling pathways. Cyclic AMP signaling pathways are important for many brain functions, such as learning and memory, drug addiction, and development. Here we show that wild-type, AC1, AC8, or AC1&8 double knockout (DKO) mice were indistinguishable in tests of acute pain, whereas behavioral responses to peripheral injection of two inflammatory stimuli, formalin and complete Freund's adjuvant, were reduced or abolished in AC1&8 DKO mice. AC1 and AC8 are highly expressed in the anterior cingulate cortex (ACC), and contribute to inflammation-induced activation of CREB. Intra-ACC administration of forskolin rescued behavioral allodynia defective in the AC1&8 DKO mice. Our studies suggest that AC1 and AC8 in the ACC selectively contribute to behavioral allodynia.

Introduction

Neurons and synapses in the central nervous systems are very dynamic and plastic, and can undergo changes throughout life (see reviews, Kaas, 1991; Ramachandran, 1993; Gilbert, 1996; Buonomano and Merzenich, 1998; Kandel, 2001). Studies of molecular and cellular mechanisms of such changes not only provide important insight into how we learn and store new knowledge in our brains, but also reveal the mechanisms of pathological changes occurring following an injury. Evidence from numerous studies using different experimental approaches suggest that neuronal mechanisms underlying physiological functions such as learning and memory may share some common signaling pathways with abnormal or injury-related changes in the brain, such as those occurring with drug abuse, tissue injury, or inflammation (see reviews, Kandel, 2001; Nestler, 2001; Hyman and Malenka, 2001; Woolf and Salter, 2000; Zhuo, 2002).

Among many possible candidate molecules, cAMP is an important second messenger for these changes. It is believed that cAMP signaling pathways within the hippocampus, amygdala, and related cortical areas are involved in different forms of memory, such as spatial and emotional memory (Silva et al., 1998; Schafe et al., 2000, 2001). At the synaptic level, cAMP is well known to regulate many neuronal targets, including transmitter receptors, ion channels, and transcription factors that ultimately lead to new gene expression and protein synthesis (see Montminy, 1997 for a review). Many cAMP-mediated regulatory effects directly or indirectly contribute to long-term changes in synaptic transmission known as long-term potentiation (LTP) (see reviews: Nicoll and Malenka, 1995; Kandel, 2001). Pharmacological and genetic manipulation of cAMP signaling pathways affect memory-related hippocampal LTP, including mossy fiber LTP and late-phase LTP in the CA1 region (Weisskopf et al., 1994; Villacres et al., 1998; Wong et al., 1999; Kandel, 2001). At the behavioral level, the role of cAMP in learning and memory has been well demonstrated across different species, from invertebrates to vertebrates, including *Aplysia*, *Drosophila*, mice, and rats. Genetic mutants lacking adenylyl cyclase, cAMP-dependent protein kinase (PKA), or cAMP response element (CRE) binding protein (CREB) exhibit significant defects in different forms of behavioral learning and memory (Livingston et al., 1984; Foster et al., 1984; Bourtchuladze et al., 1994; Yin et al., 1994, 1995; Abel et al., 1997; Bartsch et al., 1995; Wong et al., 1999).

At least nine different isoforms of adenylyl cyclases have been identified, each with a unique pattern of expression within the central nervous system from the peripheral sensory nerves to the frontal cortex (Xia and Storm, 1997). In addition to their distribution in the hippocampus and their involvement in behavioral memory and NMDA receptor-dependent LTP (Wong et al., 1999), specific adenylyl cyclase isoforms are also reported in the spinal cord dorsal horn, thalamus, and cortex, where they may contribute to other functions such as sensory transmission and modulation (Xia et al., 1991, 1993). Among them, AC1 and AC8 are the two calmodulin (CaM)-stimulated adenylyl cyclases in the central nervous system. They couple NMDA receptor-induced cytosolic Ca^{2+} increases to cAMP signaling pathways (Chetkovich and Sweatt, 1993; Wong et al., 1999). In the periphery and spinal cord, it has been demonstrated that cAMP-related signaling pathways contribute to the behavioral responses to tissue injury and inflammation (Taiwo and Levine, 1991; Aley and Levine, 1999; Malmberg et al., 1997; Sluka, 1997), although few studies have addressed potential roles of forebrain cAMP in such changes. In particular, the participation of AC1 and AC8 in behavioral sensitization within the anterior cingulate cortex (ACC), an area mediating the emotional component of pain, have not been investigated.

Here we used AC1 and AC8 single knockout mice as well as AC1&8 DKO mice to test the roles of these ACs

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in chronic pain. We show that behavioral nociceptive responses to subcutaneous formalin injection or nerve injury, but not acute nociceptive responses to noxious heat or mechanical pressure, were significantly reduced in mice lacking AC1 or AC8 and more profoundly compromised in AC1&8 DKO mice. Allodynia, the inflammation-related behavioral sensitization to a non-noxious stimulus, was significantly reduced in AC1 knockout mice and absent in AC1&8 DKO mice. AC1 and AC8 were both expressed at high levels in two pain-related forebrain areas, the ACC and the insular cortex, and at a low level in the spinal cord. In support of the involvement of forebrain adenylyl cyclases, local injection of forskolin into the ACC rescued the behavioral lack of allodynia of AC1&8 DKO mice. Our studies suggest that AC1 and AC8 are important in the genesis of behavioral responses related to inflammation or nerve injury.

Results

Behavioral Responses to Acute Noxious Stimuli and Inflammation

We first examined the potential contributions of AC1 and AC8 to the animals' behavioral responses to acute noxious stimuli and tissue inflammation. To test the behavioral responses to acute noxious stimuli, we performed the hot-plate test at 52.5°C and 55.0°C and the tail-flick reflex test. We found that behavioral responses to noxious thermal stimuli in both tests were identical in wild-type, AC1, AC8, or AC1&8 DKO mice (hot-plate test: wild-type, n = 10 mice; AC1, n = 6 mice; AC8, n = 7 mice; AC1&8, n = 6 mice; Figure 1A; tail-flick test: wild-type, n = 6 mice; AC1, n = 10 mice; AC8, n = 7 mice; AC1&8, n = 10; data now shown for the tail-flick test), indicating that AC1 and AC8 do not significantly contribute to acute sensory responses to noxious stimuli. We also measured hindpaw withdrawal to mechanical pressure and found that mechanical withdrawal thresholds were not affected in mice lacking AC1, AC8, and AC1&8 DKO (n = 4 mice for each group, Figure 1B). Together, these results consistently demonstrate that the behavioral responses to acute noxious thermal and mechanical stimuli do not require the activity of AC1 and AC8.

AC1 and AC8 act downstream from NMDA receptors in neurons and contribute to NMDA receptor-dependent synaptic potentiation lasting several hours (Wong et al., 1999). Thus, it is possible that AC1 and AC8 contribute to behavioral responses to tissue injury and inflammation, a long-lasting form of behavioral sensitization. The formalin test is a common test of tissue injury and inflammation on a timescale of hours (Dubuisson and Dennis, 1977; Haley et al., 1990; Wei et al., 2001). Formalin-induced behavioral responses consist of three phases and depend on NMDA receptors at different levels of the brain (Haley et al., 1990; Wei et al., 2001). We next tested formalin-induced nociceptive responses in wild-type, AC1, AC8, and AC1&8 DKO mice. Whereas phase 1 responses did not differ between the mutant strains and wild-type (n = 13 mice) mice, in mice lacking AC1 (n = 5 mice) or AC8 (n = 7 mice), phase 2 responses were significantly decreased relative to wild-type mice. A greater reduction in phase 2 responses was observed in AC1&8 DKO mice as compared with that in AC8 but not AC1 (n = 5 mice, Figures 1C–1F). Phase 3 responses were not affected in AC1 or AC8 knockout mice, but were significantly reduced in AC1&8 DKO mice relative to wild-type.

We next tested the possible roles of AC1 and AC8 in allodynia induced by a hindpaw injection of complete Freund's adjuvant (CFA, 50%, 10 μl). Application of a 0.4 mN von Frey fiber to the dorsum of a hindpaw elicited no response in untreated mice, but at 1 and 3 days after CFA injection into the dorsum of a single hindpaw, mice withdrew their hindpaw in response to stimulation of the ipsilateral or, to a lesser extent, the contralateral hindpaw. This mechanical allodynia was significantly reduced in AC1 knockout mice relative to wild-type mice (n = 5 mice for each group; Figure 2A). No significant changes were seen in AC8 knockout mice (n = 5 mice), indicating that ablation of AC8 alone is not sufficient to affect allodynia. Interestingly, allodynia was completely abolished in AC1&8 DKO mice (n = 5 mice). Similar results were observed at the contralateral hindpaw. We also evaluated hindpaw edema by measuring hindpaw diameter. A similar degree of inflammation was found in wild-type, AC1, AC8, and DKO mice (n = 5 mice for each group, Figure 2B), indicating that the peripheral responses to inflammation are likely identical in these mice.

To further determine whether peripheral neurogenic plasma extravasation was altered in mice lacking AC1, AC8, and AC1&8, we measured Evans blue dye extravasation evoked by capsaicin (1 μg/10 μl) injection in the hindpaw, after intravenous administration of the dye (see Malmberg et al., 1997). Capsaicin induced similar increases in dye extravasation in AC1 (5.73 ± 0.75 μg, n = 4 mice), AC8 (5.56 ± 0.47 μg, n = 4 mice), or AC1&8 DKO mice (6.33 ± 1.05 μg, n = 5 mice; see Figure 2), as compared to that in wild-type mice (5.44 ± 0.95 μg, n = 4 mice). No statistical difference was observed between the four groups of mice. These results are consistent with the fact that AC1 is neuron specific (Xia and Storm, 1997) and AC8 is mostly expressed in the brain and lung (Muglia et al., 1999).

Changes in mechanical nociceptive threshold were also examined in wild-type and AC1&8 DKO mice after the nerve injury. In wild-type mice, the injury to the sciatic nerve produced a significant reduction in the mechanical sensitivity of the ipsilateral hindpaw 1–22 days after the injury (n = 9 mice). Changes in the mechanical sensitivity at the contralateral hindpaw were also observed 13–22 days after the injury. In AC1&8 DKO mice, the reduction in the mechanical sensitivity following the nerve injury was significantly reduced or completely blocked at the ipsilateral hindpaw (n = 4 mice). At the contralateral hindpaw, the reduction in the mechanical sensitivity was also blocked in AC1&8 DKO mice (see Figure 2D).

Distribution in Pain-Related Areas and cAMP Signaling Pathways

Recent studies using different experimental approaches consistently suggest that the ACC and/or the insular cortex play important roles in processing pain-related information in humans and in the behavioral responses

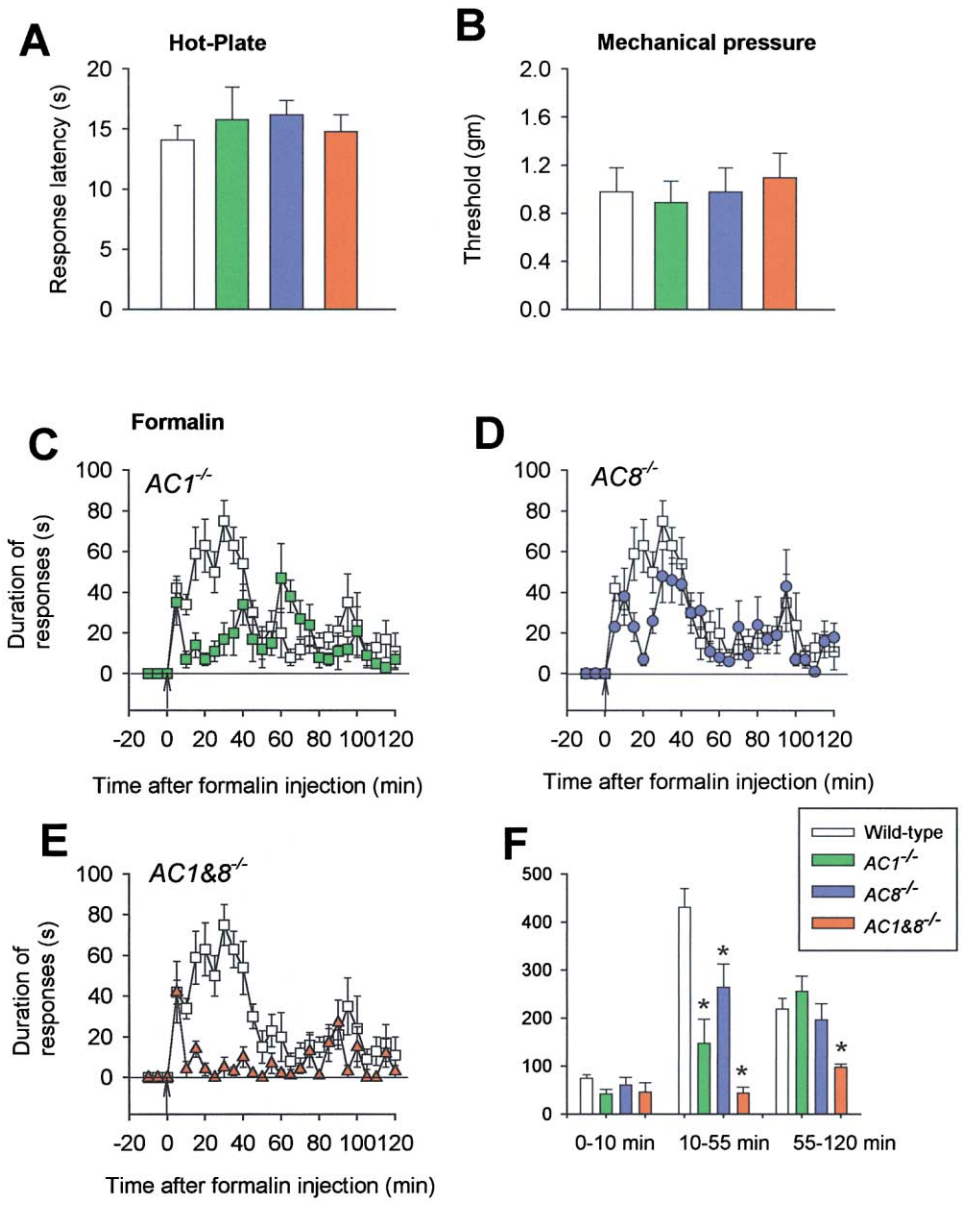

Figure 1. Reduced Behavioral Responses to Formalin Injection but Normal Acute Nociception in AC1, AC8, and AC1&8 Double Knockout Mice

(A and B) Behavioral nociceptive responses to noxious heating (A) or mechanical pressure applied to the hindpaw (B).

(C–E) Behavioral nociceptive responses to hindpaw formalin injection, plotted in 5 min intervals, in wild-type mice (open squares) as compared to AC1, AC8, and AC1&8 DKO mice.

(F) Data from experiments as in (C)–(E) were grouped into three phases. *Significant difference from wild-type mice.

to noxious stimuli or tissue injury and information in animals (Casey, 1999; Talbot et al., 1991; Rainville et al., 1997; Hutchison et al., 1999; Bushnell et al., 1999; Wei et al., 2001). To examine the roles of forebrain AC1 and AC8, we first examined the morphology of the forebrains of the mutant mice, and performed in situ hybridization for AC1 and AC8 in the ACC and insular cortex. First, the gross anatomy of the brain in mutant mice was observed by Cresyl violet staining to examine whether the development of several sensory-related areas was affected in mutant mice. Analysis of serial coronal sections, examined by light microscopy, showed no detectable morphological differences in the ACC, somatosensory cortex, insular cortex, thalamus, periaqueductal gray (PAG), locus coeruleus, raphe nucleus, spinal dorsal horn, and dorsal root ganglia. Higher magnification of the stained sections further demonstrated no apparent differences in the number and distribution of cells in these areas (Figure 3). Second, we studied the pattern of expression of AC1 and AC8 in wild-type mice using in situ hybridization, focusing on the hippocampus, ACC, insular cortex, and spinal cord. Consistent with previous reports (Xia et al., 1991; Schaefer et al., 2000), AC1 and AC8 mRNA exhibited a complementary expression pat-

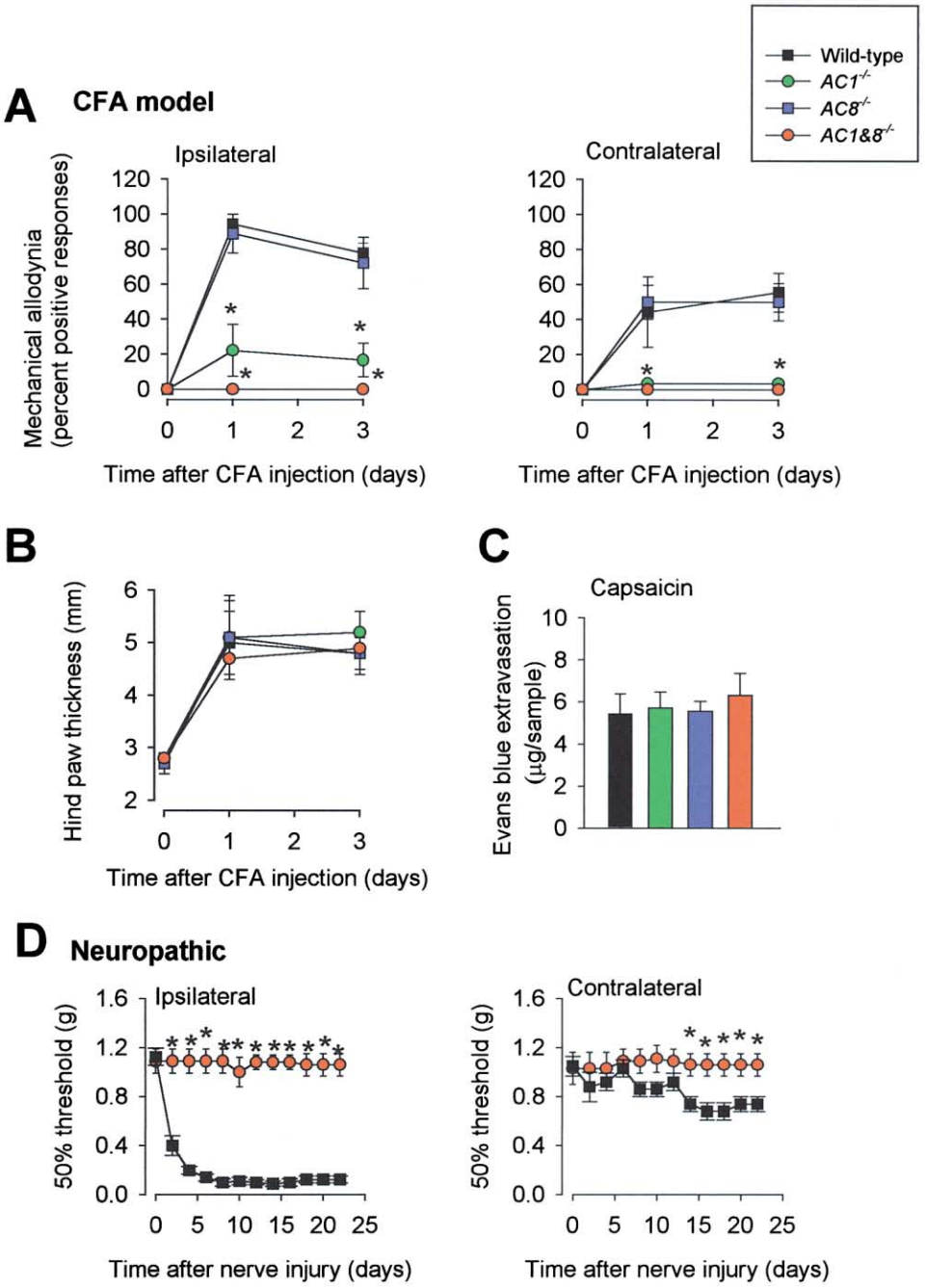

Figure 2. Elimination of Behavioral Sensitization (Allodynia) to CFA Injection and Nerve Injury in AC1&8 Double Knockout Mice

(A) The behavioral responses of animals to a non-noxious mechanical stimulus (a 0.4 mN von Frey fiber), which elicited no responses before a dorsal hindpaw CFA injection, were recorded 1 and 3 days after the injection. The data were plotted as percentage-positive responses to stimulation of the ipsilateral or contralateral hindpaw of wild-type, AC1, AC8, and AC1&8 DKO mice.

(B) Hindpaw edema was measured with a fine caliper in wild-type and AC1, AC8, AC1&8 DKO mice.

(C) Evans blue dye extravasation evoked by capsaicin (1 μg/10 μl) injection in the hindpaw in wild-type, AC1, AC8, and AC1&8 DKO mice. No significant difference was detected among these four groups.

(D) The mechanical sensitivity (presented as 50% threshold) of the ipsi- and contralateral hindpaw before and after nerve injury in wild-type (filled squares) and AC1&8 DKO mice (red circles). *Significant difference from wild-type mice.

tern in the hippocampus. AC1 mRNA was highly expressed in the granular cells of the dentate gyrus and the pyramidal cells of CA2 field, with moderate expression of AC1 in the pyramidal cells of CA1 and weak expression found in CA3 (Figure 4C). AC8 expression was strong in pyramidal cells of CA1 and weak in CA3 and the dentate gyrus (Figure 4C). In the ACC, strong and homogeneous patterns of AC1 and AC8 expression were ob-

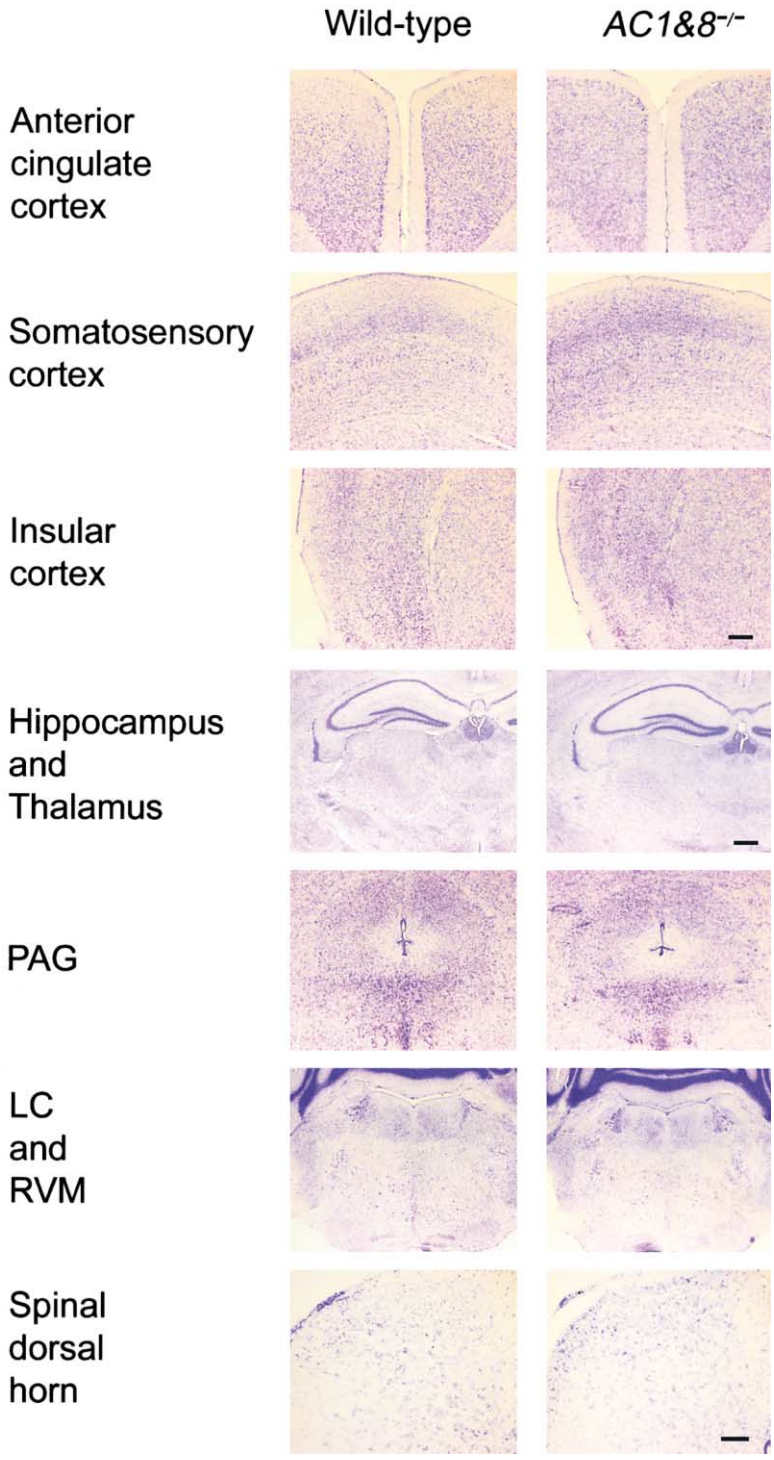

Figure 3. Brain Morphology of Wild-Type and AC1&8 DKO Mice

Coronal sections showed no detectable morphological differences in the ACC, somatosensory cortex, insular cortex, hippocampus, thalamus, periaqueductal gray (PAG), locus coeruleus (LC), rostroventral medulla (RVM), and adjacent nuclei, as well as spinal cord dorsal horn. Scale bar: 250 μm (ACC, somatosensory cortex, insular cortex, PAG, LC, and RVM), 500 μm (hippocampus and thalamus), 100 μm (spinal dorsal horn).

served in all cell layers (Figure 4A). In the insular cortex, more intense and widespread AC8 reactivity was found as compared with AC1 mRNA (Figure 4B). In the spinal dorsal horn, weak expression of AC8, and minimal AC1 expression, was found (Figure 4D). As expected, we found no expression of AC1 or AC8 in AC1&8 DKO mice. In mice lacking AC1 or AC8, we did not see significant changes in the expression of AC8 or AC1 mRNA by the in situ hybridization using the same probes or radiolabeled probes in these brain areas (data not shown), suggesting that the remaining adenylyl cyclase did not undergo compensatory upregulation at the mRNA level. This conclusion is also supported by results from the analysis of Ca^{2+}-stimulated adenylyl cyclase activity in wild-type, AC1, AC8, or AC1&8 DKO mice (see Wong et al., 1999; Schaefer et al., 2000).

AC1 and AC8 are the only CaM-stimulated adenylyl cyclases (Wong et al., 1999). In extracts from the whole

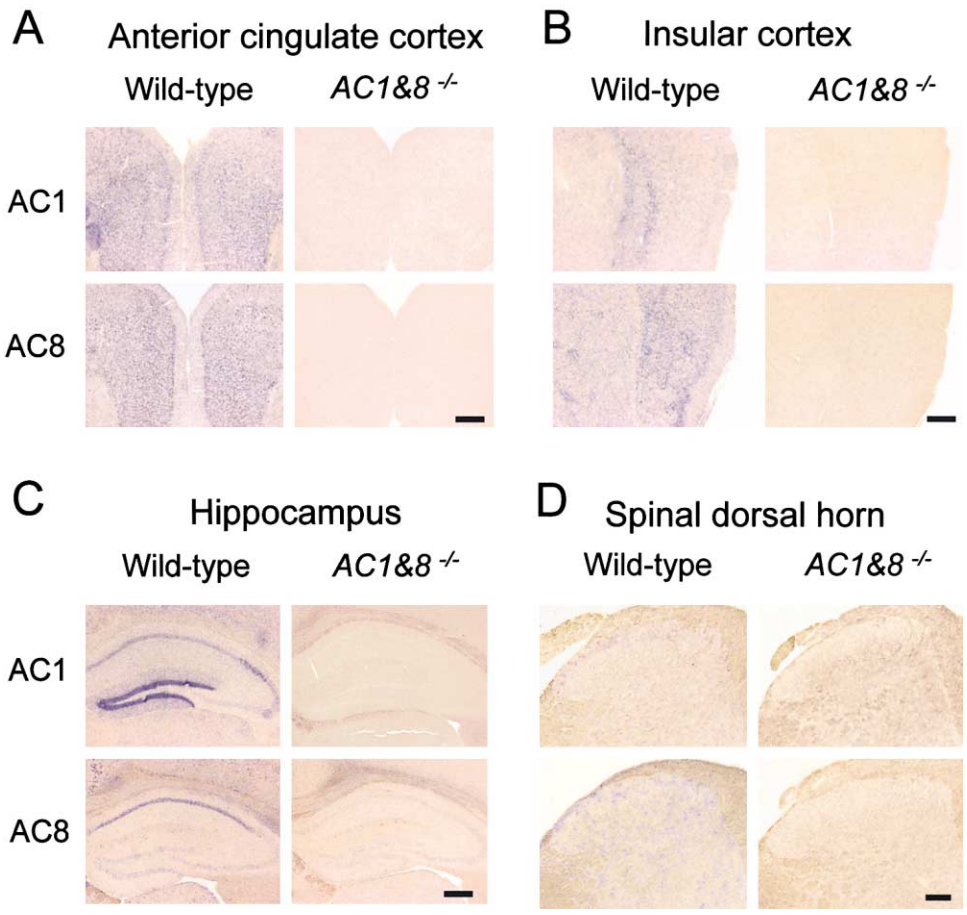

Figure 4. AC1 and AC8 Are Highly Expressed in the ACC, Insular Cortex, and Hippocampus but Not the Spinal Cord

The expression of AC1 and AC8 mRNA was examined by in situ hybridization in the ACC (A), insular cortex (B), hippocampus (C), and spinal dorsal horn (D) of wild-type and AC1&8 DKO mice. High levels of AC1 and AC8 were found in the ACC and insular cortex of wild-type mice (see Results). Weak staining was seen in the spinal cord dorsal horn of wild-type mice. Scale bar: top, 300 μm; bottom, 100 μm.

brain or from specific regions such as the hippocampus or cerebellum, the genetic deletion of AC1 or AC8 significantly reduced Ca^{2+}-stimulated adenylyl cyclase activity, such that no measurable Ca^{2+}-stimulated adenylyl cyclase activity was found in AC1&8 DKO mice (see Wong et al., 1999). We measured Ca^{2+}-stimulated AC activity in the spinal cord of wild-type ($n = 4$ mice), AC1, AC8, and AC1&8 DKO mice ($n = 5$ mice for each group). Whereas no significant changes were seen in AC1 knockout mice, a significant reduction in Ca^{2+}-stimulated AC activity was seen in AC8 knockout mice. Furthermore, Ca^{2+}-stimulated adenylyl cyclase activity was completely blocked in AC1&8 DKO mice (Figure 5). These results indicate that AC8 and AC1 contribute to Ca^{2+}-stimulated adenylyl cyclase activity in the spinal cord.

Contribution to Injury-Induced CREB Activation

The cyclic AMP-responsive element binding protein (CREB) is a transcription factor that plays an important role in the formation of long-term memory (see Silva et al., 1998; Tully, 1998; West et al., 2001). In the spinal cord and forebrain, neuronal CREB is activated after tissue injury (Ji and Rupp, 1997; Wei et al., 1999). Although the exact link between activation of CREB and persistent pain remains unclear, phosphorylated CREB (pCREB) can be used as a marker for activation of AC1 and AC8 in the central nervous system. In addition to cAMP, the calcium/CaM-dependent protein kinase pathway also activates CREB (Bito et al., 1996; Soderling, 2000). Our recent data from CaMKIV knockout mice revealed that the behavioral responses to acute noxious stimuli, subcutaneous formalin injection, and CFA injection were normal, indicating that the CaMKIV signaling does not significantly contribute to the behavioral response to injury (Wei et al., 2002). To determine the contribution of Ca^{2+}-stimulated cAMP to injury-activated CREB in the brain, we tested whether pCREB induction by hindpaw formalin injection depends on AC1 or AC8. In support of a role for forebrain areas in inflammatory pain (Wei et al., 2001), we found that formalin activated CREB in the ACC and insular cortex (Figure 6). Interestingly, pCREB immunoreactivity was reduced to a similar extent in AC1, AC8, or AC1&8 DKO mice; the presence of significant residual pCREB in formalin-injected AC1&8 DKO mice indicates that Ca^{2+}-stimulated AC1 and AC8 are not the only pathways linked to CREB activation during inflammation. Future studies

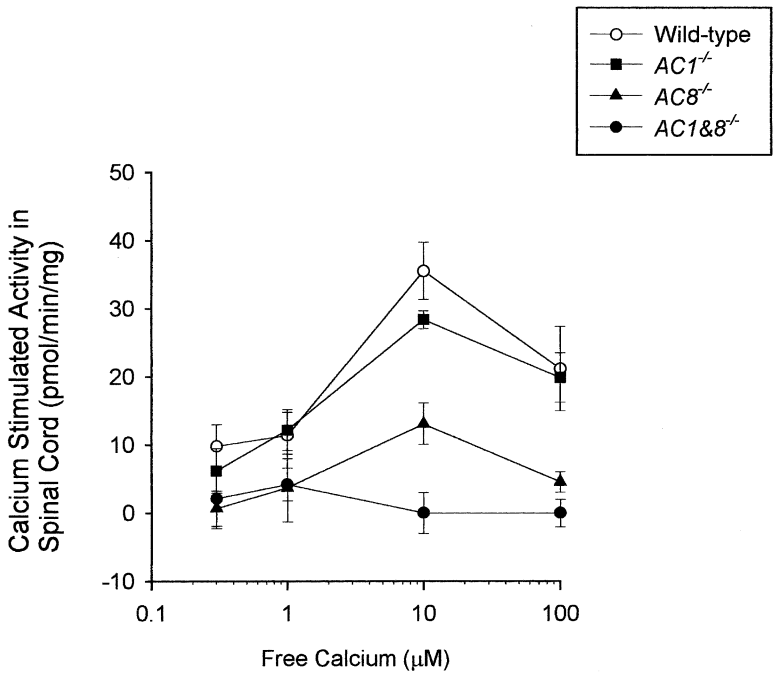

Figure 5. Ca^{2+}-Stimulated Adenylyl Cyclase Activity Is Abolished in the Spinal Cord of AC1&8 Double Knockout Mice

Ca^{2+}-stimulated adenylyl cyclase activity of membrane preparations from the spinal cord of wild-type, AC1, AC8, or AC1&8 DKO mice.

using mice with mutation in CREB signaling pathway may help us to understand the exact roles of CREB activation in the behavioral sensitization after the injury.

We also found that formalin injection activated pCREB in the spinal dorsal horn neurons of wild-type mice (n = 8 mice). In AC1, AC8, or AC1&8 DKO mice (n = 5 mice for each group), no increase in pCREB in superficial dorsal horn neurons was seen after formalin injection (Figure 6). In deep dorsal horn neurons, formalin-induced pCREB expression was significantly reduced in AC1 or AC8 knockout mice (n = 5 mice for each group). The magnitude of pCREB reduction in AC8 knockout mice is significantly greater than that in AC1 knockout mice. No significant difference was found in the magnitude of pCREB reduction between AC8 knockout mice and AC1&8 DKO mice. These findings suggest that both AC8 and AC1 contribute to CREB activation following the injury.

Pharmacological Rescue by Local Injection of Forskolin in the ACC

It has been reported that other forms of adenylyl cyclases are also present in the ACC (Xia and Storm, 1997). While the preceding data implicate forebrain expression of AC1 and AC8 as the critical component mediating allodynia, we sought to further prove that cAMP produced in this area was responsible for the observed phenotype. Furthermore, we wished to determine whether the lack of allodynia was due to an acute requirement for AC1 and AC8 or developmental changes in cortical function due to chronic deficiency of these isoforms. To attempt an acute behavioral rescue, forskolin was microinjected into the ACC to activate adenylyl cyclases. The dose of forskolin was selected based on previous studies in the hippocampus (see Wong et al., 1999). We performed microinjection of forskolin (120 nmoles, 0.5 μl) into both sides of ACC of AC1&8 DKO mice before CFA injection. The time interval between forskolin pretreatment and CFA injection was 30 min. At 1 day after CFA injection, AC1&8 DKO mice (n = 5 mice) showed reliable allodynia (Figure 7A). The forskolin-rescued allodynia was still present at 3 and 5 days after CFA treatment. In other AC1&8 DKO mice receiving CFA injection (n = 5 mice), microinjection of the vehicle solution into bilateral ACC did not cause allodynia (Figure 7A). In animals without CFA injection, the microinjection of forskolin did not cause behavioral sensitization in either wild-type or AC1&8 DKO mice (n = 3 mice for each group). To determine if the effect of forskolin is region selective, we injected the same dose of forskolin into the adjacent motor cortex (n = 4 mice) or the visual cortex (n = 4 mice) of AC1&8 DKO mice. No significant recovery of allodynia was observed at 1, 3, and 5 days after the injection in both cases (see Figure 7A for the visual cortex data), indicating that the effect of forskolin is region selective.

To examine if cAMP production in the ACC contributes to the expression or maintenance of behavioral allodynia, forskolin was injected into the ACC at 1 day after CFA injection. To our surprise, AC1&8 DKO mice showed hypersensitive responses to a non-noxious stimulus following the forskolin injection (n = 5 mice, Figure 7B). In contrast to forskolin, the vehicle injection did not cause any significant effect (n = 4 mice). We also examined if the same treatment with forskolin may rescue changes in the mechanical sensitivity of AC1&8 DKO mice after the nerve injury. At 30 days after the nerve injury, no significant changes in the mechanical sensitivity were found in AC1&8 DKO mice (n = 4). Following the bilateral microinjection of forskolin into the ACC, a significant reduction in the mechanical sensitivity was found at the ipsilateral hindpaw. The magnitude of the reduction is comparable to that in wild-type mice (see Figure 2). A significant reduction was also detected at the contralateral hindpaw (Figure 7C).

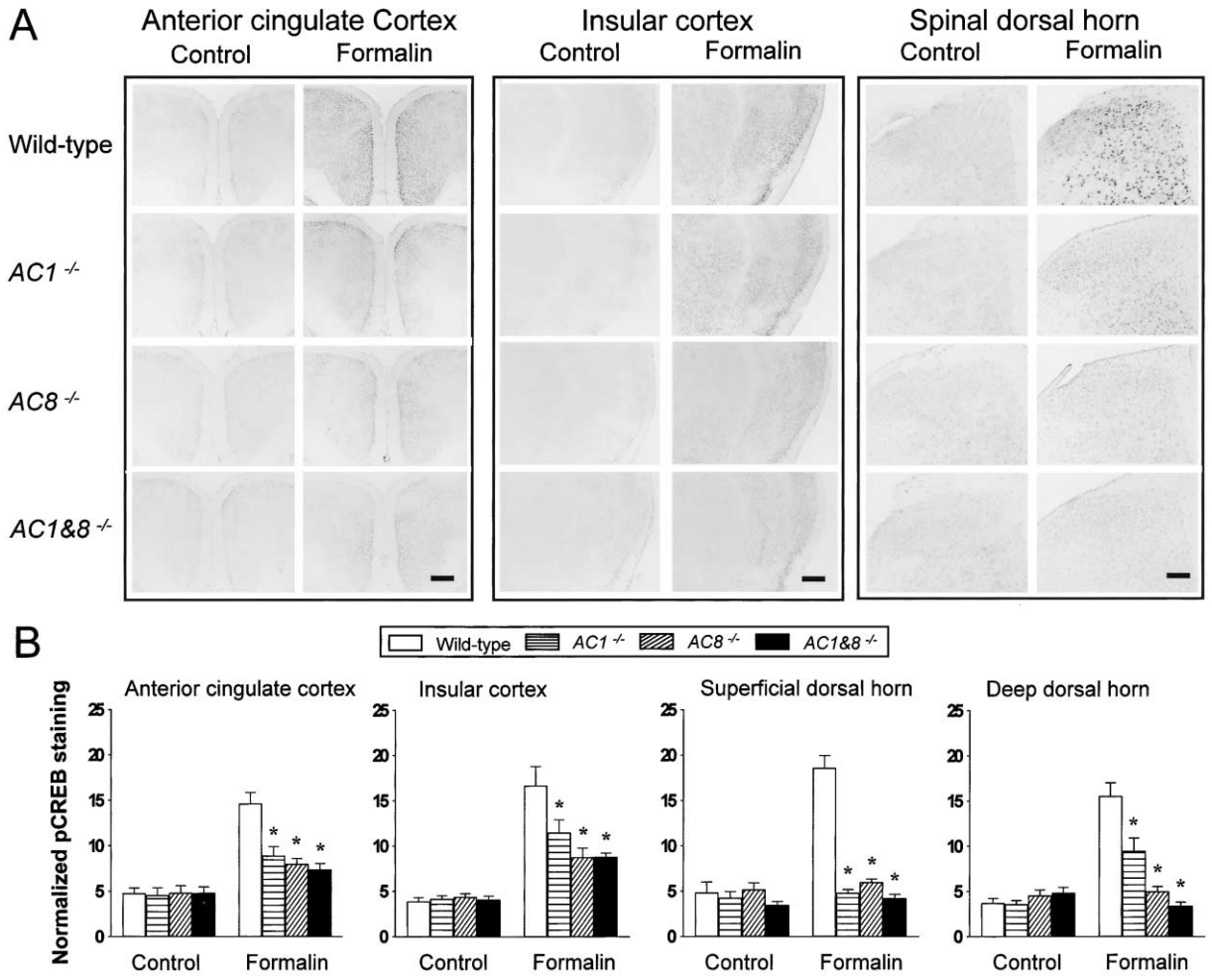

Figure 6. AC1 and AC8 Contributed to CREB Activation following Formalin Injection

(A) Immunohistochemical staining for pCREB in the ACC, insular cortex, and spinal dorsal horn of wild-type AC1, AC8, and AC1&8 DKO mice after hindpaw injection of saline (control) or formalin.

(B) Quantification of pCREB staining in the ACC, insular cortex, superficial (lamina I–II), and deep (V-VI) dorsal horn of lumbar spinal cord. *Significant difference from wild-type mice.

Scale bar: cortex, 300 μm; spinal cord, 100 μm.

We also wanted to examine if the same treatment with forskolin also rescued CREB activation in AC1&8 DKO mice through other forms of adenylyl cyclases. As shown in Figure 7E, forskolin significantly increased pCREB within the ACC areas (n = 4 mice). The effects of forskolin are relatively region selective as no significant change in pCREB was seen in the somatosensory cortex of the same animals (Figure 7F).

Pharmacological Inhibition in the ACC

To determine potential up and downstream signaling pathways related to AC1 and AC8, we carried out microinjection of pharmacological inhibitors of NMDA receptor, calmodulin, and cAMP-dependent protein kinase (PKA). Consistent with the involvement of calcium-stimulated AC1 and AC8 in CFA-induced allodynia, bilateral microinjections of the NMDA receptor antagonist AP-5 (25 nmoles/0.5 μl per side; n = 6 mice) or the calmodulin inhibitor calmidazolium (125 nmoles/0.5 μl per side; n = 5) produced a significant attenuation of behavioral responses (Figure 8). Electrophysiological and behavioral studies have implicated that PKA is a major protein kinase contributing to CAMP-induced synaptic plasticity and behavioral effects. We also wanted to test if the activity of PKA is required for CFA-induced allodynia. As shown in Figure 8, bilateral microinjection of Rp-cAMPs, a cAMP analog, which inhibits PKA activation, produced significant attenuation of the allodynia (1 nmoles/0.5 μl per side; n = 8 mice; $p < 0.01$ as compared with responses before the injection). Similar inhibitory effects were found with H89 (25 nmoles/0.5 μl per side, n = 6 mice), another PKA inhibitor.

Discussion

Our results provide genetic, pharmacological, and behavioral evidence that forebrain AC1 and AC8, two Ca^{2+} CaM-stimulated adenylyl cyclases, are important for synaptic potentiation and behavioral sensitization after tissue injury and inflammation. These studies demon-

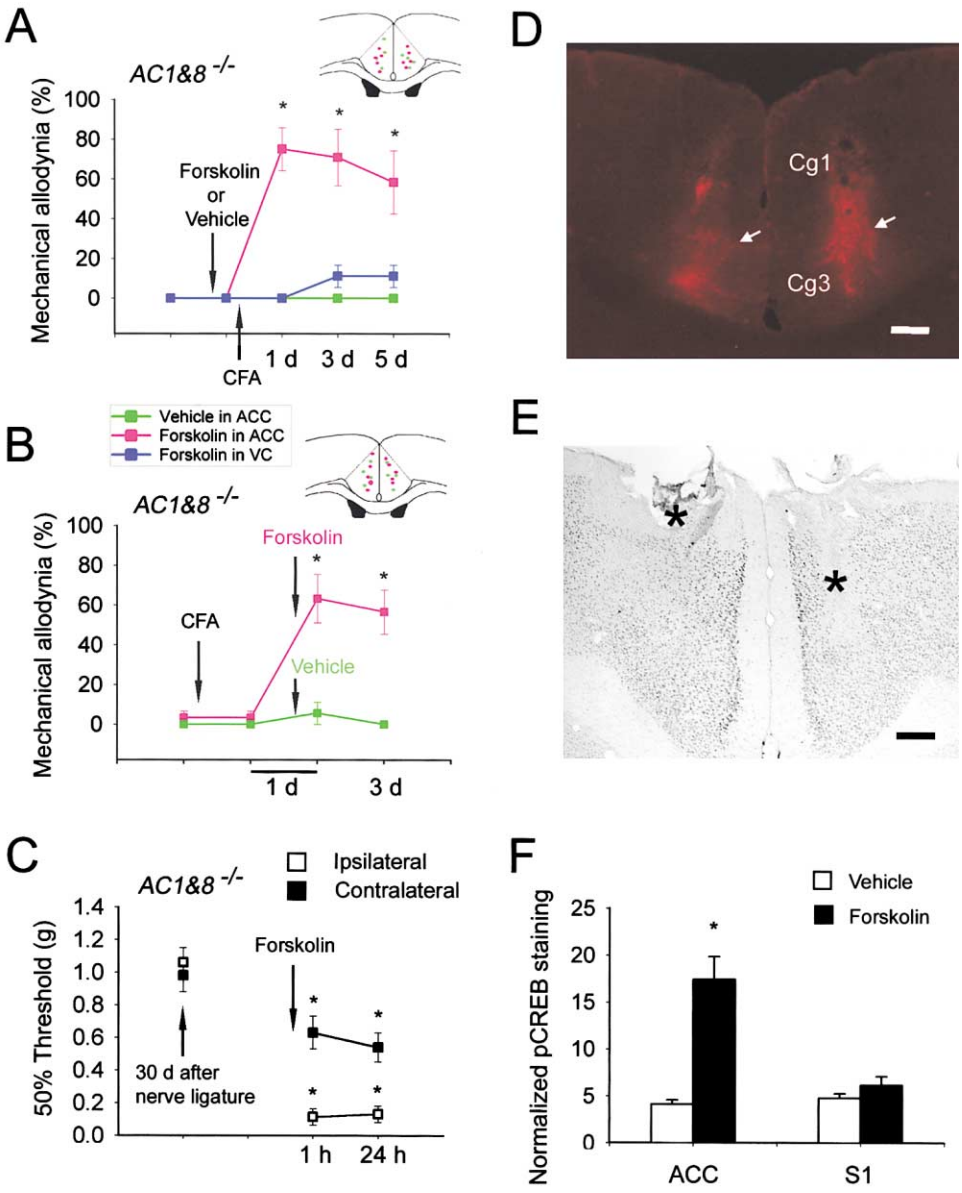

Figure 7. Pharmacological Rescue of Behavioral Allodynia in AC1&8 DKO Mice

(A) Effects of intra-ACC forskolin (120 nmoles in 0.5 μl per side) pretreatment (magenta line) on behavioral allodynia induced by CFA injection in AC1&8 DKO mice. Vehicle pretreatment (green line) did not produce any effect. Injection of the same amount of forskolin into the visual cortex (VC, blue line) also did not produce significant effect. Inset: sites of microinjection of forskolin (magenta circles) or vehicle (green circles) in the ACC.

(B) Effects of intra-ACC forskolin 1 day after hindpaw CFA injection. Behavioral measurements were performed at 30 min after forskolin injection.

Inset: sites of microinjection of forskolin (magenta circles) or vehicle (green circles) in the ACC.

(C) Effects of intra-ACC forskolin injection 30 days after the nerve injury. Behavioral measurements were performed at 1 and 24 hr after forskolin injection. (A–C) *Significant difference from baseline.

(D) Representative coronal section showing the extent of BODIPY forskolin diffusion in the ACC 2 hr after bilateral microinjections. Cg1, anterior cingulate cortex area1; Cg3, anterior cingulate cortex area3. Scale bar: 300 μm.

(E) Rescued pCREB staining in AC1&8 DKO mice by intra-ACC forskolin injection. The injection sites are indicated by *.

(F) Summary data of pCREB staining in the ACC with vehicle or forskolin treatment. *Significant difference from baseline.

strate the important role of the cAMP-related signaling pathways in the ACC in behavioral sensitization to tissue injury and inflammation. Lesion experiments and local chemical injection experiments fail to dissociate behavioral responses to acute noxious stimuli from long-term sensitization to the injury as these manipulations alone significantly affect behavioral responses to acute stimuli (Pastoriza et al., 1996; Calejesan et al., 2000; Lee et al., 1999). Despite cumulative evidence from human brain functional imaging and electrophysiology that the neu-

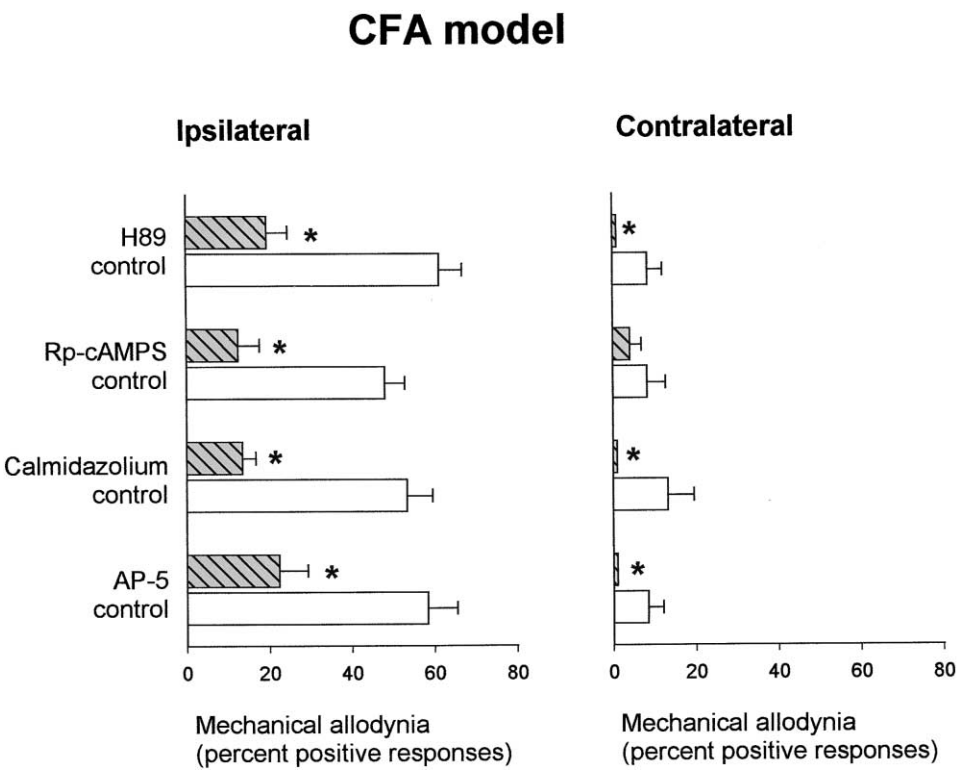

Figure 8. Pharmacological Inhibition of Behavioral Allodynia in Wild-Type Mice

Summary data for mechanical allodynia in wild-type mice (1 day after CFA injection) before (open columns) and after bilateral intra-ACC microinjection of different inhibitors including AP-5 for NMDA receptors, calmidazolium for calmodulin, Rp-cAMPs and H89 for PKA (filled columns). *Significant difference from the response before the injection.

rons in the ACC are important for coding injury-induced pain and discomfort, previous studies of behavioral allodynia have mainly focused on spinal mechanisms. Genetic deletion of AC1 and AC8 completely abolished behavioral allodynia in adult mice, and microinjection of forskolin into the ACC rescued behavioral allodynia, suggesting an important role of cAMP signaling pathways in behavioral allodynia. We would like to point out that our studies do not rule out the possible contribution of AC1 and AC8 to persistent pain in other sensory-related areas, such as the dorsal root ganglion cells, spinal dorsal horn neurons, thalamus, and somatosensory cortex. It is most likely that changes happening in sensory-related areas after the injury lead to enhanced neuronal excitability in higher brain structures including the ACC. Due to ACC's important roles in pain perception, it is likely that changes in the ACC contribute at least in part to chronic pain in humans and to behavioral sensitization in animals.

ACC as a Key Structure for Coding Emotional Responses to Pain and Injury

The ACC plays important roles in the cognitive, motor, and emotional functions of the brain (D'Esposito et al., 1995; Botvinick et al., 1999; Davidson et al., 2000; Price, 2000; Johansen et al., 2001; Paus, 2001). It has been suggested to contribute to the perception of pain, to the learning processes associated with the prediction and avoidance of noxious sensory stimuli, as well as to pathological phantom pain (see Devinsky et al., 1995 for a review). 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. In humans, results from electrophysiological recordings from the ACC and functional imaging studies show that the ACC neurons respond to noxious stimuli (Talbot et al., 1991; Vogt et al., 1996; Craig et al., 1996; Derbyshire et al., 1998). In animals, ACC neurons respond to peripheral noxious stimuli or electrical shocks at high intensities (Sikes and Vogt, 1992; Koyama et al., 1998; Wei and Zhuo, 2001). It is proposed that activity in the ACC may underlie the unpleasantness or discomfort associated with some somatosensory stimuli. Consistently, lesions in the ACC can reduce chronic pain in patients (Yarnitsky et al., 1988). In animal models of acute pain and persistent pain, lesions of the ACC produce antinociceptive effects (Pastoriza et al., 1996; Lee et al., 1999). In freely moving animals, local administration of various opioid receptor agonists in the ACC produces powerful antinociceptive effects (Lee et al., 1999). In the present studies, we found that AC1 and AC8 both were highly expressed in the ACC neurons and genetic deletion of AC1 and AC8 led to a complete abolishment of the behavioral allodynia caused by tissue injury and inflammation. Consistent with these findings, behavioral nociceptive responses in the formalin test were also significantly reduced. These results support a role of the ACC in the

processing of pain-related information. Behavioral responses to acute noxious stimuli were normal in these mutant mice, which strongly suggests that AC1 and AC8 are selectively involved in mediating the behavioral responses to injury. Our results are consistent with findings from in vitro brain slices that the activity of adenylyl cyclases is primarily required for plastic changes, while basal synaptic transmission is unaffected by deletion of AC1 and AC8 (for example, see Wong et al., 1999). The pharmacological rescue of behavioral allodynia by local forskolin microinjection into the ACC provides further evidence for an important role of the ACC in persistent pain.

Possible Synaptic Potentiation and CREB Activation in the ACC

What is the possible synaptic mechanism for the action of AC1 and AC8 within the ACC? We think that activation of adenylyl cyclases in the ACC may lead to long-lasting changes in synaptic transmission. Glutamate is a major fast excitatory transmitter within the ACC (Sah and Nicoll, 1991; Tanaka and North, 1994; Wei et al., 1999). In our recent studies, we found that theta burst stimulation caused long-term potentiation of synaptic responses in ACC slices from adult mice. The potentiation was completely absent in mice lacking both AC1 and AC8, suggesting that Ca^{2+}-CaM sensitive adenylyl cyclases are important for synaptic potentiation (F.W. et al., unpublished data). As reported in many other regions of the brain (Rosenmund et al., 1994; Moss et al., 1992; Trudeau et al., 1996), cAMP clearly contributes to the synaptic potentiation observed 5–40 min after the induction. Our results suggest that the enhancement of synaptic responses within the ACC may serve as a synaptic mechanism contributing to injury-related behavioral sensitization. We cannot rule out the possible presynaptic effects of forskolin within the ACC. It is quite possible that both pre- and postsynaptic changes occur within the ACC during forskolin treatment. Future studies are clearly needed to dissect the detailed synaptic mechanisms within the ACC.

In the present study, we provide evidence that AC1 and AC8 are important for CREB activation following tissue injury and inflammation in the ACC and insular cortex. Deletion of AC1 or AC8 caused significant reduction of CREB activated by inflammation. Interestingly, no further reduction was found in the AC1&8 DKO mice. Furthermore, injury-triggered CREB activation is not completely blocked in any mice, suggesting that other signaling pathways also contribute to CREB activation in the forebrain. These findings are slightly different from those in the spinal cord. In spinal cord dorsal horn, injury-induced activation of CREB was completely blocked in AC1&8 DKO mice. Future studies are needed to identify other signaling molecules for injury-related CREB activation in the ACC and insular cortex. In both the spinal cord and ACC, signaling molecules downstream of activated CREB remain to be identified.

AC1 versus AC8: Two Coincidence Detectors with Different Sensitivity

Regarding behavioral responses to a non-noxious stimulus, AC1 and AC8 contribute differently to allodynia. AC1 deletion caused significant reduction in allodynia while AC8 deletion alone had no effect. This difference may result from AC1 and AC8 exhibiting different affinities for Ca^{2+} (Xia and Storm, 1997). AC1 may contribute more to cAMP production after the injury than AC8 does since AC1 is four to five times more sensitive to Ca^{2+} than AC8 (Cali et al., 1996). Elimination of behavioral allodynia in DKO mice suggests that both AC1 and AC8 are important. Although AC1 and AC8 are located along the somatosensory pathways, pharmacological rescue of behavioral allodynia by microinjection of forskolin into the ACC suggests that cAMP levels in the forebrain ACC are critical for the behavioral allodynia. Unlike learning-related synaptic potentiation paradigms, we found that application of forskolin after the CFA injection is also sufficient to rescue behavioral allodynia. Our results suggest that AC1 and AC8 are not necessarily required when the injury occurs. One possible explanation is that inflammation and nerve injury leads to long-lasting changes in the activity of peripheral afferent fibers, which is different from the instantaneous behavioral conditioning in vivo or strong activation used to induce synaptic potentiation in in vitro brain slices. Therefore, increasing cAMP levels during long-lasting inflammation by CFA can still lead to behavioral allodynia in the absence of AC1 and AC8.

We believe that it is unlikely that forskolin rescue experiments suggest that other AC isoforms are important for pain-enhancing effects. Forskolin is a nonselective AC activator and was used to *experimentally* raise cAMP levels by activating other forms of ACs not usually activated in association with persistent pain. The rescue effect of forskolin does not at all imply that *other isoforms of ACs can play roles in persistent pain*. Instead, it serves as an important test to assess possible developmental changes that could occur as a consequence of chronic loss of AC1 and AC8.

Our pharmacological data suggest that the behavioral defects in allodynia are not due to developmental changes in AC1&8 DKO mice; the acute attenuation of cAMP levels within the ACC play an important role in the hypersensitive behavioral responses related to tissue injury and inflammation. Our previous studies show that forebrain NR2B overexpression selectively enhances behavioral responses to peripheral injection of formalin and CFA in similar tests without any changes in tests of acute pain (Wei et al., 2001). Because AC1 and AC8 couple NMDA receptor activation to cAMP production in postsynaptic cells, these findings suggest that the NMDA receptor-AC1 and AC8-cAMP pathway in ACC neurons is important in processing information regarding prolonged behavioral sensitization to inflammation and nerve injury.

In summary, we provide strong evidence by different approaches that the CaM-stimulated AC1 and AC8 are important for behavioral nociceptive responses to nerve injury and inflammation. The cAMP signaling pathways within the ACC, a central region known to encode the unpleasantness of pain in humans, mediate the hypersensitive responses of animals after the injury. Our findings suggest that AC1 and AC8 play important roles in the processing of nociceptive information in the ACC.

Experimental Procedures

Mice

Adult (8–12 weeks), male mice lacking AC1, AC8, or AC1&8 and wild-type mice were of mixed 129 X Black Swiss background (see Wong et al., 1999; Schaefer et al., 2000). To most closely match mice for background yet efficiently generate the large number of mice used in these studies, we generated WT/WT, AC1 KO/AC8 WT, AC8 KO/AC1 WT, and AC1 KO/AC8 KO breeders from AC1 het/AC8 het matings, and used the offspring from these breeders for the described studies. To minimize drift of background in a given genotype line, we used several breeding pairs. Both WT and mutant mice were well groomed and showed no signs of abnormality or any obvious motor defects. No indication of tremor, seizure, or ataxia was observed. As it was impossible visually to distinguish mutant mice from wild-type mice, experimenters were blind to genotype. The experimental protocol was approved by the Animal Studies Committee at Washington University.

In Situ Hybridization

In situ hybridization experiments were performed as previously described (Wei et al., 2001). Briefly, the AC1and AC8 plasmids were digested with HindIII and reverse-transcribed using T7 RNA polymerase (Promega). Brain and spinal slices taken from wild-type, AC1, AC8, and AC1&8 DKO mice were fixed and stained.

Adenylyl Cyclase Assay

Adenylyl cyclase activity of the spinal cord was determined as previously described (see Wong et al., 1999). Adenylyl cyclase activity levels are the means of triplicate measurements.

Behavioral Experiments

Behavioral allodynia was induced by CFA (50% in saline, 10 μl; Sigma) injection into the dorsal surface of the left hindpaw under halothane anesthesia as previously described (Wei et al., 2001). Mechanical sensitivity was assessed with a set of von Frey filaments (Stoelting; Wood Dale, Illinois). Based on preliminary experiments that characterized the threshold stimulus in untreated animals, the innocuous 0.4 mN (No. 2.44) filament was used to detect mechanical allodynia. The filament was applied to the point of bending six times each to the dorsal surfaces of the left and right hindpaws. Positive responses consisted of prolonged hindpaw withdrawal followed by licking or scratching. For each time point, the percent response frequency of hindpaw withdrawal was expressed as (number of positive responses)/6 × 100 per hindpaw. Hindpaw edema was evaluated with a fine caliper 3 days after CFA injection.

Formalin (5%, 10 μl) was injected subcutaneously into the dorsal side of a hindpaw. The total time spent licking or biting the injected hindpaw was recorded for each 5 min interval over the course of 2 hr. The spinal tail-flick reflex was evoked by focused, radiant heat applied to underside of the tail. The latency to reflexive removal of the tail away from the heat was measured by a photocell timer. In the hot-plate test, mice were placed on a thermally controlled metal plate (Columbia Instruments; Columbus, Ohio). The time between placements of a mouse on the plate and licking or lifting of a hindpaw was measured with a digital timer. Two different temperatures were used, 52.5°C and 55.0°C. Mice were removed from the hot plate immediately after the first response. In all three tests, the mean response latency was calculated as the average of 3–4 measurements performed at 10 min intervals. Nerve injury was induced by tying a tight ligature around one-third to one-half of the diameter of the sciatic nerve as previously described in rats by Seltzer et al. (1990) and mice by Malmberg et al. (1997). Briefly, the sciatic nerve was exposed at one hindlimb under halothane anesthesia (2%–3%). A silk suture was used to tightly ligate the nerve. Experiments were performed blind, and different individuals were responsible for the surgery and the measurements of the mechanical sensitivity of mice.

Neurogenic Plasma Extravasation

Under deep halothane anesthesia, the femoral vein of the wild-type and mutant mice was exposed unilaterally for intravenous injection of Evans blue dye (50 mg/kg, Sigma). Five minutes later, capsaicin (1 μg/10 μl, Sigma) or vehicle (10% ethanol, 10% Tween 80, and 80% saline) was injected intradermally into dorsal part of the hindpaw. Punches of skin from the base of the heel to the tip of the all digits were sampled 30 min later and placed in 2 ml of formamide for 3 days. The dye concentration was then determined spectrophometrically at a wavelength of 620 nm. The total amount of Evans blue extracted from the hindpaw skin was then calculated from standard curve of dye concentration.

Brain Local Injection and Histological Identification

Adult mice were anesthetized with 2%–3% halothane anesthesia in a gas mixture of 30% O_2 balanced with nitrogen. After the mouse was placed in a Kopf sterotaxic instrument for mouse and incised at midline, bilateral openings were made in the skull to allow the insertion of a microinjection needle into the ACC. The coordinates (Franklin and Paxinos, 1997) were 0.7 mm anterior to Bregma, 0.3 mm lateral to the midline, and 1.75 mm ventral of the surface to the skull. 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 (Razel Scientific Instruments Inc., Stamford, Connecticut). A 0.5 μl forskolin (120 nmoles) or vehicle (20% DMSO in filter-sterilized phosphate-buffered saline, pH 7.4) was infused into each side of the ACC at a rate of 0.05 μl/min. The needle was withdrawn 15 min after completion of the injection and the incision sutured and covered with a local anesthetic ointment (Nupercainal, Rugby Laboratories, Inc., Norcross, Georgia). For identification of injection site, on completion of experiment, all animals were deeply anesthetized and perfused transcardially with saline, followed by 4% paraformaldehyde. Serial cryostat coronal sections (30 μm) of the ACC were mounted on glass slides and counterstained with cresyl violet. To access the infused extent in the ACC, we replaced forskolin by BODIPY forskolin (240 μM, Molecular Probes, Eugene, Oregon) for injection in some experiments. The fluorescence-labeled ACC sections were visualized by using Olympus Fluoview laser scanning confocal microscopy. Drugs were all purchased from Sigma-RBI (St. Louis, Missouri).

Immunocytochemistry

Experiments were performed blind to the genotypes of mice. Tissue sections from wild-type and knockout mice were processed simultaneously to allow the same condition and time for DAB staining. Mice were deeply anesthetized with 3%–4% halothane and perfused through the ascending aorta with 50 ml of saline, followed by 200 ml of cold 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde. Cryostat-cut brain sections (30 μm) were processed with rabbit anti-pCREB antibody (1:5000; Upstate Bio). We used the avidin-biotin protocol as described (Wei et al., 1999), with nickel-intensified diaminobenzidine and glucose oxidase to localize the reaction product. Anatomical terminology is based on the atlas of Franklin and Paxinos (1997). The rostrocaudal levels of each section corresponded to 0.98 to 0.5 mm (ACC), and 1.10 to 0.5 mm (insular cortex) anterior to Bregma. The lumbar spinal cord (L4–L5) was selected. Images were collected on Olympus BX60 microscopy and analyzed using NIH imaging software (Scion Image). The integrated intensity for the selected regions was normalized to the corresponding integrated intensity in the adjacent white matter as described previously to reduce variations between slices (Impey et al., 1998; Wei et al., 2002). For each nucleus, ten measurements were made per mouse from three randomly selected noncontiguous sections observed from coded slides and averaged so that each animal had a mean value for regional pCREB immunoreactivity.

Data Analysis

Results were expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed with the use of one- or two-way analysis of variance (ANOVA) with the post-hoc Scheffe F-test in immunocytochemical experiments, or the post-hoc Turkey or Student-Newmann-Keuls test in behavioral experiments, to identify significant differences. In all cases, $p < 0.05$ was considered statistically significant.

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