

Calmodulin-Stimulated Adenylyl Cyclase Gene Deletion Affects Morphine Responses

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ABSTRACT

To define the roles of the calmodulin-stimulated adenylyl cyclases (AC1 and AC8) in morphine-induced analgesia, tolerance, physical dependence, and conditioned place preference, we used mice having targeted disruptions of either the AC1 or AC8 genes or both genes [double knockout mice (DKO)]. Mice lacking either AC1 or AC8 genes or DKO did not differ from wild-type mice in short-term antinociceptive responses to morphine measured in the tail-flick analgesia assay. Morphine tolerance that developed immediately within 3 h of morphine administration (10 mg/kg s.c.) was significantly attenuated in DKO mice and AC8 single knockout mice. Tolerance induced continually by daily injections of morphine (10 mg/kg s.c.) was also reduced in DKO mice. In DKO mice continually treated with morphine, there was a significant reduction in withdrawal be-

haviors, including reduced wet-dog shakes and forepaw tremor after naloxone injection (10 mg/kg i.p.). Morphine produced hyperlocomotion and conditioned place preference in wild-type mice, whereas DKO mice displayed significantly less hyperlocomotion and conditioned place preference. Furthermore, the significant increase in phosphorylated cAMP-response element binding protein (CREB) staining in ventral tegmental area induced by long-term morphine treatment was not evident in DKO mice, suggesting that CREB activation by morphine requires cAMP generated by AC1 and AC8. These results support the hypothesis that calmodulin-stimulated adenylyl cyclases are important mediators of the neuronal responses to morphine.

Morphine has been used for centuries to alleviate severe pain; however, the clinical usefulness of morphine is often limited by the development of analgesic tolerance, physical dependence, and addiction. Although the mechanisms underlying opioid tolerance are unclear, early adaptive responses, including β -arrestin-mediated uncoupling from G-protein signaling and receptor internalization, have been suggested to be crucial (Borgland, 2001; Liu and Anand, 2001; Bohn et al., 2002; Kieffer and Evans, 2002). Long-term morphine administration clearly induces behavioral tolerance in animals and humans but fails to strongly desensitize μ -receptors. Instead, long-term morphine use causes adenylyl cyclase (AC) supersensitization, which may underlie opioid tolerance and dependence at the cellular level (Avidor-Reiss et al., 1995, 1996; Harrison et al., 1998; Nestler, 2004a,b).

Adaptations in adenylyl cyclase activities have been noted in several brain regions, including the ventral tegmental area (VTA) and nucleus accumbens (NAc) areas (which are critical for opioid drug reinforcement), and in the locus coeruleus and dorsal raphe nucleus (which are critical for opioid withdrawal) (Nestler and Aghajanian, 1997; Jolas et al., 2000; Williams et al., 2001; Chao and Nestler, 2004).

Although adenylyl cyclases have been suggested to mediate some of the actions of opioids, a lack of specific inhibitors has slowed progress in defining the roles of different AC isozymes. To date, genes for 10 ACs have been cloned, each with a distinct expression pattern in the central nervous system and the peripheral sensory nervous system (Xia and Storm, 1997). Among them, AC1 and AC8 are uniquely stimulated by Ca^{2+} /calmodulin in the brain (Xia et al., 1993; Watson et al., 2000). AC1 and AC8 are widely distributed in the different brain regions including VTA, NAc, locus coeruleus, and dorsal raphe nucleus (Lane-Ladd et al., 1997). Many studies have shown the involvement of Ca^{2+} /calmodulin in morphine action (Nemmani et al., 2005; Tang et al.,

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ABBREVIATIONS: AC, adenylyl cyclase KO, disrupted gene or knockout mouse; DKO, double knockout mouse; CREB, cAMP-response element binding protein; pCREB, phosphorylated cAMP-response element binding protein; CPP, conditioned place preference; VTA, ventral tegmental area; NAc, nucleus accumbens; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s); TBS, Tris-buffered saline; ANOVA, analysis of variance.

2006). Therefore, by using AC1 and AC8 single knockout (KO) mice as well as DKO mice, we assessed short-term tolerance, long-term tolerance, and long-term withdrawal after morphine in both wild-type and KO mice to define the roles of these cyclases in the cellular and behavioral adaptations to opiate. We also measured morphine-induced locomotion and place preference to assess the roles of AC1 and AC8 in morphine reinforcement. We then tested the contributions of AC1 and AC8 to morphine cAMP-response element binding protein (CREB) activation to further characterize the underlying mechanisms.

Materials and Methods

Animals and Housing

Male mutant mice and wild-type C57BL/6 mice were used in these experiments, which were approved by an institutional animal care and use committee and conducted in accordance with the 1996 US National Research Council's Guide for the Care and Use of Laboratory Animals. Mice having specific disruptions of the genes for AC1, AC8, or both were generated as described previously (Wong et al., 1999; Schaefer et al., 2000). Heterozygous breeding pairs, backcrossed >10 generations onto C57BL/6 backgrounds, were used to generate knockout mice (−/−) used for this study. The presence or absence of the AC8 gene was confirmed in genomic DNA isolated from tail tissue samples taken from each mouse using PCR analysis and a procedure described previously (Muglia et al., 1999). In brief, a fraction of isolated DNA was used in PCR assays using the following primers: AC8, exon 1, sense primer: 5'-CTGCCTCTCTATTCTCTGG-3' and antisense primer: 5'-CGCAGATCACACCTCGAT-3'; neomycin, sense primer: 5'-GGTGGATGTGGAATGTGTGC-3' and antisense primer: 5'-GTTTCAGACATCTGTGTCCAC-3'. PCR products were resolved on a 1.5% agarose gel with ethidium bromide and then photographed for analysis. Homologous AC8 KO animals produced PCR products with the neomycin (~300 bp) but not AC8 primer, whereas homologous wild-type and AC1 KO mice produced PCR products with the AC8 (~400 bp) but not neomycin primer (Fig. 1A). The presence or absence of the AC1 gene was confirmed in

genomic DNA isolated from tail tissue samples taken from each mouse using Southern blotting (Wu et al., 1995). In brief, tail DNA from each mouse was digested with restriction enzyme BglII and electrophoresed on a 0.8 to 1.0% agarose gel. The probe DNA was a 600-bp *Ava*I/*Xba*I fragment of AC1 gene locus [α -³²P]dCTP by using random primers and a DNA labeling system. Homologous AC1 KO animals showed up as ~6 kb bands, whereas homologous wild-type mice showed up as ~5 kb bands (Fig. 1B). The DKO mice were confirmed by combining two analyses. Mice used were 10 to 14 weeks of age and weighed 20 to 25 g at the time of the start of the procedures. Both wild-type and mutant mice were well-groomed and did not show any obvious motor deficits. No indication of tremor, seizure, or ataxia was observed. Mice were housed two to four per cage within the animal care facility at the University of Washington with access to food and water ad libitum. Mice were transferred 1 week before training into a colony room adjacent to the testing room to acclimatize to environment. Housing rooms were maintained on a 12-h light/dark cycle (lights on from 7:00 AM to 7:00 PM) under standard conditions (22 ± 1°C room temperature, 33% humidity). Each animal was used only once.

Nociceptive Assays

A digital hotplate (IITC Life Sciences, Woodland Hills, CA) set at 53.5 ± 0.1°C and a water bath (Precision Scientific, Chicago, IL) set at 55.0 ± 0.5°C were chosen in this study. In each assay, latencies for the mouse to lick a hind paw or jump off the plate (hotplate) and to move the tail from the water (tail withdrawal) were taken as the endpoint. Cutoff values were 50 s (hotplate) and 15 s (tail withdrawal) to prevent tissue damage. Antinociception (maximal possible effect) was calculated by the following equation: % MPE = 100 × (test latency – control latency)/(cutoff – control latency).

Development of Tolerance

Short-Term Tolerance. Short-term antinociceptive tolerance was assessed by a second subcutaneous injection of morphine (10 mg/kg) 3 h after an initial injection (10 mg/kg s.c.). Previous studies have shown that a decrease in latency was not due to repeated injection, handling, or testing of the mice, because mice receiving saline in place of drug in the first injection displayed a normal

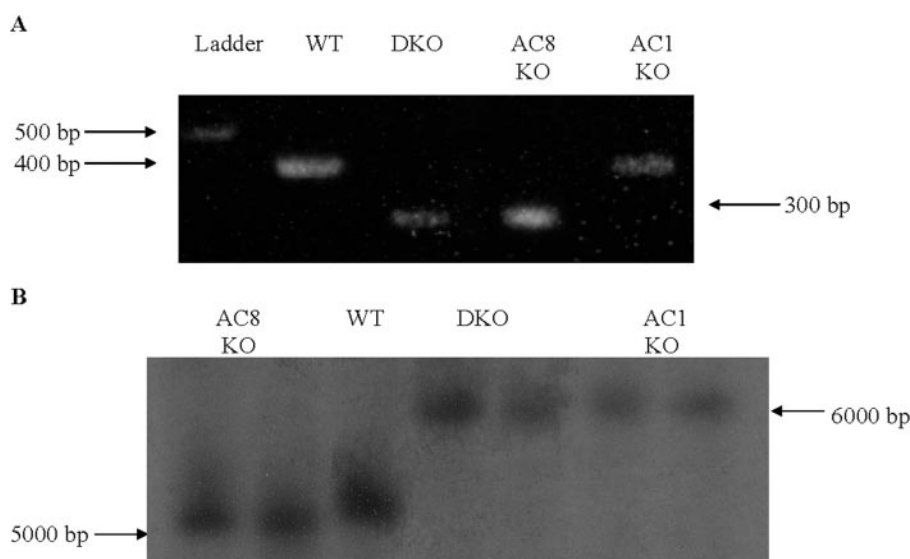


Fig. 1. Absence of the AC8 mRNA in AC8 KO mice and DKO mice. A, the presence or absence of the AC8 gene was confirmed in genomic DNA isolated from tail tissue samples taken from each mouse using PCR analysis as described previously. Shown is an ethidium bromide-stained agarose gel demonstrating the homologous AC8 KO and DKO mice produce PCR products with the neomycin (~300 bp) but not AC8 primer, whereas homologous wild-type and AC1 KO mice produce PCR products with the AC8 (~400 bp) but not neomycin primer. B, absence of the AC1 gene in the AC1 KO mice and DKO mice. The presence or absence of the AC1 gene was confirmed in genomic DNA isolated from tail tissue samples taken from each mouse using Southern blotting and a procedure described previously. Shown is a film exposed to radiolabeled membrane demonstrating the expected size of genomic DNA digested by restriction enzyme BglII in wild-type, AC8 KO at 5 kb but not in the AC1 KO, DKO mice at 6 kb.

antinociceptive response to a subsequent administration of morphine (data not shown).

Long-Term Tolerance. Wild-type and transgenic mice were treated daily (between 3:00 PM and 4:00 PM) with morphine (10 mg/kg s.c.), and antinociception was assessed 30 min later on each day for the first 5 days (Bohn et al., 2002).

Development of Withdrawal Behaviors

Morphine dependence was induced by repeated drug injection at escalating doses from 10 to 80 mg/kg s.c. over a period of 8 days. The numbers inside the parenthesis represent the doses of morphine (in milligrams per kilogram) injected at 8:00 AM and 6:00 PM, respectively: 1st and 2nd day (10, 10); 3rd and 4th day (20, 20); 5th and 6th day (40, 40); and 7th and 8th day (80, 80). On the 9th day, mice were injected with morphine (40 mg/kg) in the morning. Morphine withdrawal behavior was precipitated by intraperitoneal naloxone injection (10 mg/kg) 3 h after the last morphine administration. Mouse behavior was observed for 20 min immediately after naloxone administration, and the total number of wet-dog shakes, total number of jumps, and the number of minutes during which paw tremor and teeth chattering behaviors occurred was recorded (Terman et al., 2004). After the 20-min observation period, the mouse and filter paper on which the mouse was standing during the test were weighed again to quantify body weight change and amount of urination/defecation (both were converted to the percentage of body weight). No stereotypic opioid withdrawal behaviors were observed from either morphine-pretreated/saline-treated wild-type and DKO mice or saline-pretreated/naloxone-treated wild-type and DKO mice (data not shown).

Conditioned Place Preference

Mice were used in place-conditioning studies using a three-compartment box as described previously (McLaughlin et al., 2003). The compartmentalized box was divided into two equal-sized outer sections (25 × 25 × 25 cm) joined by a small central compartment (8.5 × 25 × 25 cm) accessed through a single doorway (3 × 3 cm). The compartments differ in wall striping (vertical versus horizontal alternating black and white lines, 1.5 cm in width) and floor texture [wood chips (The Andersons, Maumee, OH) versus Bed-o-Cob (Northeastern Products Corporation, Warrensburg, NY)]. Mice were screened for initial preferences on the morning of day 1 by placing mice in the small central compartment and allowing them to move freely throughout the entire apparatus for 30 min while recording time spent in the two outer compartments. The biased design was used. On day 2, mice were injected (subcutaneously) with saline immediately placed in the compartment in which they had spent more time in the preconditioning test for 30 min. Four hours later, mice received morphine (2.5 or 5 mg/kg s.c.) and were immediately confined for 30 min to the opposite outer compartment. The morphine doses were chosen based on previous studies (Langroudi et al., 2005; Rezaïyof et al., 2006) in which the doses of 1 to 10 mg/kg morphine have been shown to induce place preference, and the maximal response was obtained with 5.0 mg/kg morphine. On days 3 and 4, mice again were conditioned first with saline followed by morphine 4 h later in the appropriate compartments. On day 5, mice were placed in the small central compartment and were allowed to roam freely between the two outer compartments for 30 min, and movement through the chambers was detected by infrared beam sensors (LabLinc; Coulbourn Instruments, Allentown, PA). Time spent in the two outer compartments was then calculated by GraphicState 2 software (Coulbourn Instruments). Data were plotted as the difference in the times spent on the drug-paired side before training subtracted from the times spent on the drug-paired side after training.

Locomotor Activity

Activity of each mouse was evaluated in the test chamber using video tracking and motion analysis, which was performed via a

personal computer-based data acquisition system (Ethovision version 3.0; Noldus, Wageningen, The Netherlands) that received video input from a digital camera (ZR60; Canon, Tokyo, Japan). The camera was placed 3 m above the chamber, and the acquisition program collected five images per second for each recording episode. To evaluate the effects of morphine on locomotor behavior, mice were placed in test chamber for 60 min of environmental habituation. Then, immediately after the habituation period, mice were injected with morphine (10 mg/kg s.c.) or saline (s.c.) and then placed back into the test chamber. Locomotor activity was recorded during the subsequent 180 min.

Brain Dissections

Mice were killed by cervical dislocation, and the brains were rapidly removed and sliced into 1-mm thick sections using a brain matrix (Zivic Laboratories Inc., Pittsburgh, PA). VTA and NAc areas were identified according to a mouse brain stereotaxic atlas (Franklin and Paxinos, 1997). The areas were then dissected from the appropriate slices and frozen in liquid nitrogen.

Immunoblotting

Tissue samples were homogenized in ice-cold lysis buffer containing the following: 50 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 1 mM NaF, 10% glycerol, 1% Nonidet P-40, 1:100 of Phosphatase Inhibitor Cocktail Set 1 (Calbiochem, La Jolla, CA), and 1:100 of Protease Inhibitor Cocktail Set 1 (Calbiochem). Lysates were sonicated for 20 s and then centrifuged for 15 min (14,000g, 4°C), the pellet was discarded, and sample supernatants were stored at -20°C. Protein concentration was determined by Pierce bicinchoninic assay with bovine serum albumin as the standard before loading 50 µg onto nondenaturing 10% bisacrylamide precast gels (Invitrogen, Carlsbad, CA) and running at 150 V for 1.5 h. For the determination of molecular weight, Benchmark prestained standards (Invitrogen) were loaded alongside protein samples. Blots were transferred to nitrocellulose for 1.5 h at 30 mV, blocked in 1× TBS/5% milk for 1 h, incubated overnight at 4°C with a 1:1000 dilution of goat-anti-rabbit phospho-CREB antibody which detects endogenous levels of CREB only when phosphorylated at serine 133 (pCREB; Cell Signaling Technologies, Danvers, MA). After overnight incubation, membranes were washed 4 × 15 min in Tris-buffered saline/1% Tween 20 and then incubated with the IRDye 800-conjugated affinity-purified anti-rabbit IgG (Rockland, Gilbertsville, PA) at a dilution of 1:10,000 in a 1:1 mixture of 5% milk/TBS and Li-Cor Blocking Buffer (Li-Cor Biosciences, Lincoln, NE) for 1 h at room temperature. Membranes were then washed 4 × 15 min in Tris-buffered saline/1% Tween 20 and 1 × 10 min in TBS to remove Tween 20, which can cause high background on the Odyssey Imaging System, and analyzed as described below. Membranes were reprobed with rabbit anti-β-actin (3 h at room temperature, 5% milk/TBS) and secondary antibody (as above) to confirm equal protein loading in each lane. Immunoblots were scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences); pCREB bands were detected at 43 kDa, and β-actin bands were detected at 46 kDa. Band pixel intensity was measured using the Odyssey software, which subtracts background and calculates band density in pixels. Data were normalized to a percentage of control samples.

Data Analysis

The results were expressed as mean ± S.E.M. Differences in withdrawal behaviors and locomotor activities between groups were assessed by the Student's *t* test. Differences in tolerance, conditioned place preference (CPP) behavioral test, and pCREB immunoblotting staining between groups were analyzed by one-way or two-way analyses of variance (ANOVAs); post hoc pairwise comparisons were made with the Bonferroni test. ED₅₀ value was calculated using the WinNonlin software (Pharsight, Mountain View, CA). In all analyses, the null hypothesis was rejected at the 0.05 level.

Results

Morphine-Induced Antinociception. Morphine-induced antinociception was assessed in the hotplate (Fig. 2A) and tail-withdrawal assays (Fig. 3A). The short-term analgesic response to morphine (10 mg/kg s.c.) was not significantly different in the DKO mice in the hotplate assay at the different time point compared with wild-type mice (Kruskal-Wallis one-way ANOVA followed by Bonferroni post hoc test, $P > 0.05$, Fig. 2A). Likewise, the morphine dose-response curves using the tail-withdrawal (Fig. 2B) and hotplate (Fig. 2C) antinociception assays were not significantly different for wild-type and DKO mice ($P > 0.05$, one-way ANOVA followed by Bonferroni post hoc test, $P > 0.05$). The morphine ED_{50} values were 8.3 (wild-type) and 7.5 (DKO) in the tail-withdrawal assay and were 6.3 (wild-type) and 5.9 (DKO) in the hot-plate assay. There were no significant differences in the ED_{50} value between wild-type and DKO in either assay.

Morphine-Induced Antinociceptive Tolerance. The basal responses to noxious thermal stimuli in tail-flick test were identical in wild-type, AC1, AC8, or DKO mice, which were consistent with the previous findings (Wei et al., 2002). In addition, mice lacking either AC1 or AC8 genes or both displayed morphine antinociceptive responses that were not different from wild-type mice (Fig. 3A). Two-way repeated-measure ANOVA (factor genotype + factor time) showed there were significant effects of time but no significant effects of genotype and interaction ($F_{\text{genotype}/3, 234} = 1.88$, $P > 0.05$; $F_{\text{time}/5, 234} = 42.55$, $P < 0.001$; $F_{\text{genotype} \times \text{time}/15, 234} = 0.26$, $P > 0.05$). Morphine tolerance that developed immediately within 3 h after an initial morphine administration (10 mg/kg s.c.) was reduced in the DKO mice and AC8 single knockout mice. Two-way repeated-measure ANOVA (factor genotype + factor time) showed there were significant effects of both genotype and time ($F_{\text{genotype}/3, 234} = 13.4$, $P < 0.001$; $F_{\text{time}/5, 234} = 23.57$, $P < 0.001$). It is noteworthy that a decrease in latency in the wild-type mice was not due to repeated injection, handling, or testing of the mice, because mice receiving one injection of saline in the place of morphine did not display changes in tail-withdrawal latency when measured every 30 min (data not shown).

To assess the roles of AC1 and AC8 in the development of long-term morphine tolerance, we treated animals with morphine on a daily basis and monitored their responsiveness in the tail-withdrawal assay. Both wild-type and KO mice began to lose their sensitivity to morphine on the second day of treatment. However, the DKO mice displayed significantly less morphine tolerance than wild-type mice. Two-way repeated-measure ANOVA (factor genotype + factor day) showed there were significant effects of both genotype and days ($F_{\text{genotype}/3, 224} = 12.34$, $P < 0.001$; $F_{\text{time}/4, 224} = 86.81$, $P < 0.001$; Fig. 3B). However, by the fourth day of treatment, the DKO mice were not different from the wild-type mice in their responsiveness to morphine, and both genotypes of mice seemed to be completely tolerant to morphine challenge (10 mg/kg s.c.) in the tail-withdrawal assay.

Morphine-Induced Withdrawal Behaviors. Consistent with a reduced morphine tolerance in the DKO mice, the severity of withdrawal behaviors precipitated by injection of the opioid antagonist naloxone (10 mg/kg i.p.) after 8-day repeated morphine treatment was also reduced in the DKO mice. There were significantly attenuated appearance of wet

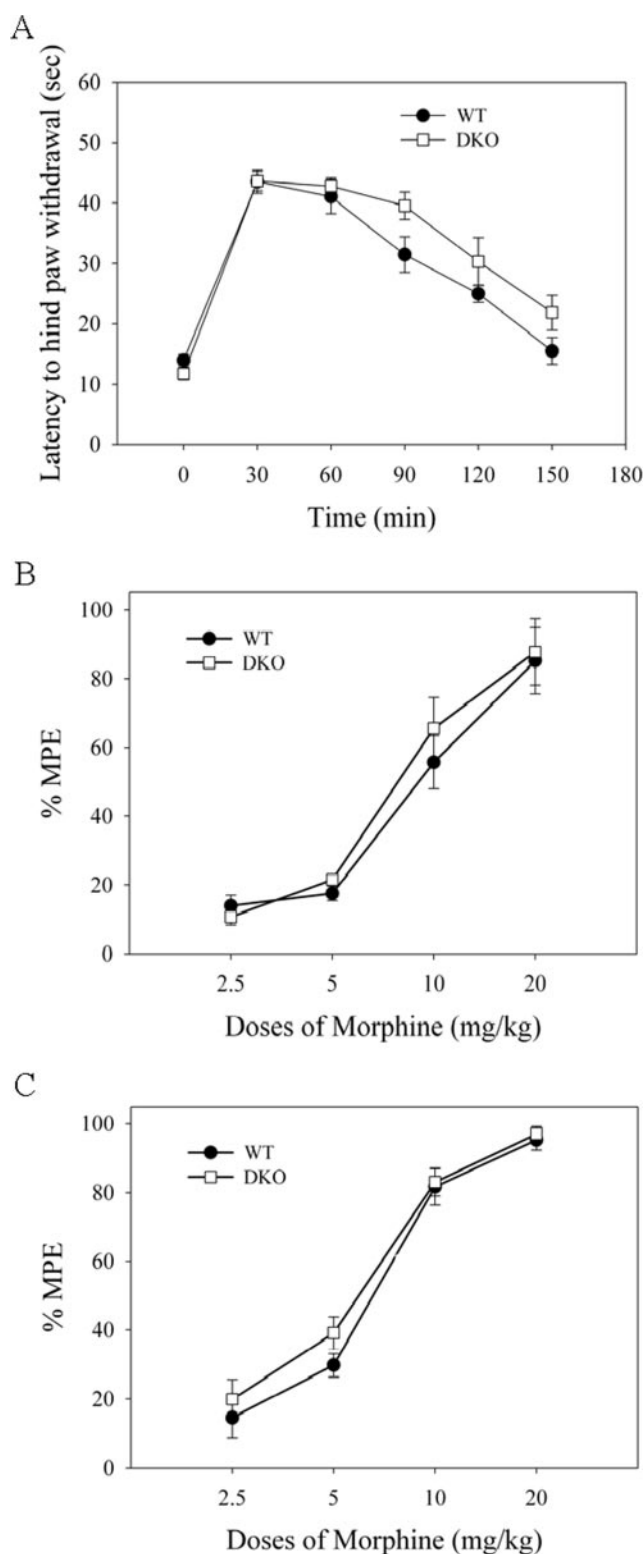


Fig. 2. A, the time course of morphine-induced antinociception was obtained by measuring hind paw withdrawal latencies before and every 30 min for 150 min after the wild-type ($n = 9$) and DKO mice ($n = 10$) were treated with morphine (10 mg/kg s.c.). The dose response of morphine antinociception was measured 30 min after morphine treatment in the tail withdrawal (B; 2.5 mg/kg, wild-type, $n = 6$ and DKO $n = 6$; 5 mg/kg, wild-type $n = 6$ and DKO $n = 6$; 10 mg/kg, wild-type $n = 11$ and DKO $n = 10$; 20 mg/kg, wild-type $n = 7$ and DKO $n = 7$) and in the hotplate assay (C; 2.5 mg/kg, wild-type $n = 6$ and DKO $n = 6$; 5 mg/kg, wild-type $n = 6$ and DKO $n = 6$; 10 mg/kg, wild-type $n = 9$ and DKO $n = 10$; 20 mg/kg, wild-type $n = 7$ and DKO $n = 7$).

dog shakes and forepaw tremor in the DKO mice compared with the wild-type mice (Student's *t* test, $P < 0.05$; Fig. 4). In contrast, other withdrawal behaviors (e.g., jumping, teeth chatter, diarrhea, and weight loss) were not different in the wild-type and DKO mice (Fig. 4).

Morphine-Induced Hyperlocomotor Activity and CPP. To examine the effects of the deletion of both AC1 and AC8 genes on reinforcing and psychomotor properties of morphine, we assessed morphine induced hyperlocomotor activity and conditioned place preference in the KO mice and wild-type mice. Morphine (10 mg/kg s.c.) produced marked increases in locomotor activity in both genotypes during a

120-min period compared with saline-treated group. It is interesting that the wild-type mice seemed to be significantly more active than the DKO (two-way ANOVA, time: $F_{\text{time}/23,528} = 8.25$, $P < 0.001$; genotype: $F_{\text{genotype}/1,528} = 31.84$, $P < 0.001$; interaction: $F_{\text{genotype} \times \text{time}/23,528} = 1.78$, $P < 0.05$, Fig. 5A). Compared with wild-type mice, DKO mice displayed significantly less total distance traveled as accumulated during 30- to 60-min and 60- to 90-min periods after morphine treatment (Student's *t* test, $P < 0.05$; Fig. 5B).

Morphine CPP was induced in both wild-type and DKO mice. Wild-type mice showed robust morphine conditioned place preference that was significant at the doses of 2.5 and 5 mg/kg (one-way ANOVA, $F_{5,49} = 6.86$, $P < 0.001$, Bonferroni post hoc test, $P < 0.05$ versus saline controls), whereas DKO mice showed a significant place preference only at the higher dose of 5 mg/kg morphine and not at the lower dose of 2.5 mg/kg morphine. There was a significant decrease in time spent in drug-paired compartment in DKO mice compared with wild-type mice when mice were trained by 2.5 mg/kg morphine (Bonferroni post hoc test, $P < 0.05$, Fig. 6).

Contribution to Long-Term Morphine Treatment-Induced CREB Activation. CREB is a transcription factor that has been suggested to mediate the opiate-induced up-regulation of the cAMP pathway. To determine the contribution of Ca^{2+} -stimulated cAMP to morphine-induced phosphorylation of CREB in the brain, we tested whether pCREB induction after long-term morphine treatment was dependent on AC1 and AC8. The average pCREB staining in morphine pretreated wild-type mice adjusted by saline-pretreated wild-type mice exhibited a mean of $146.6 \pm 10.2\%$ (mean \pm S.E.M.). Five days of repeated morphine treatment significantly increased pCREB staining in VTA compared with saline pretreatment controls ($P < 0.05$, Fig. 7). The increased pCREB induced by long-term morphine was blocked in DKO mice. The basal levels of pCREB in both wild-type and DKO saline control were not different. In NAc, we found that pCREB staining was moderately elevated; however, this increase was not statistically significant (data not shown).

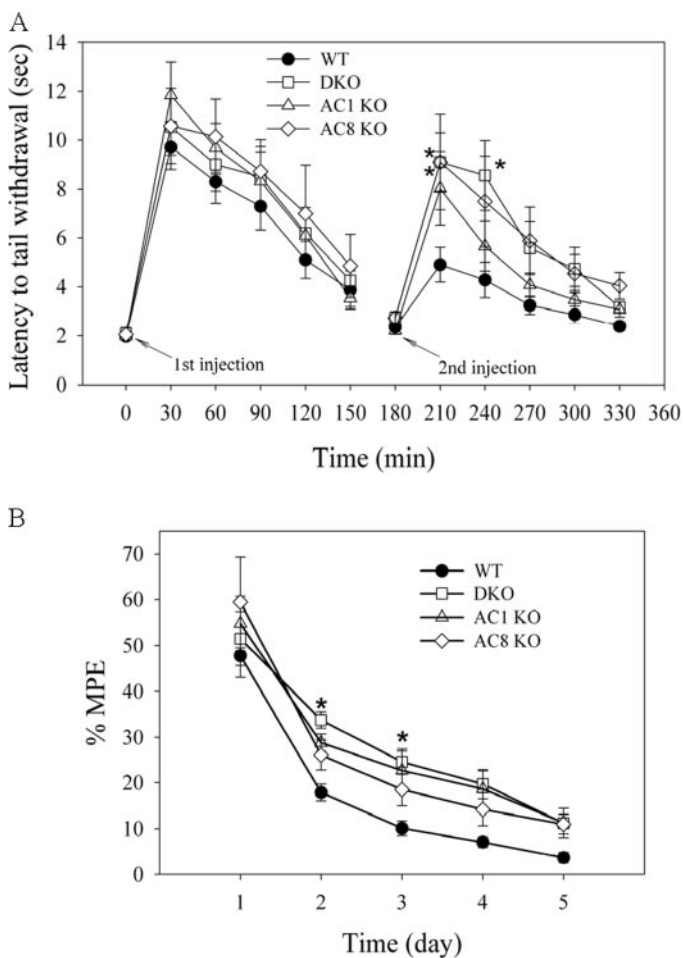


Fig. 3. Morphine antinociceptive response and morphine tolerance in wild-type mice and AC1, AC8 single KO mice, and DKO mice. **A**, the time course of short-term morphine-induced antinociception was obtained by measuring tail withdrawal latencies before and every 30 min for 150 min after the wild-type ($n = 15$) AC1 single KO ($n = 9$), AC8 single KO ($n = 7$), and DKO mice ($n = 10$) were treated with morphine (10 mg/kg s.c.). Short-term tolerance to morphine antinociceptive effects was demonstrated by administering the second injection of morphine (10 mg/kg s.c.) to mice 180 min after the initial injection (arrow) and then measuring tail withdrawal latencies every 30 min for another 150 min. Data are presented as the mean \pm S.E.M., with *, $P < 0.05$ compared with wild-type mice, two-way ANOVA followed by Bonferroni multiple comparison post hoc test. **B**, long-term morphine tolerance in the wild-type mice and AC1, AC8, single KO mice, and DKO mice. Wild-type ($n = 17$) AC1 single KO ($n = 8$), AC8 single KO ($n = 9$), and DKO mice ($n = 11$) were treated daily with morphine (10 mg/kg s.c.). Antinociceptive effect of morphine was assessed 30 min after the injection on the days indicated. Data are presented as the mean \pm S.E.M.; *, $P < 0.05$ compared with wild-type mice, two-way ANOVA followed by Bonferroni multiple comparison post hoc test.

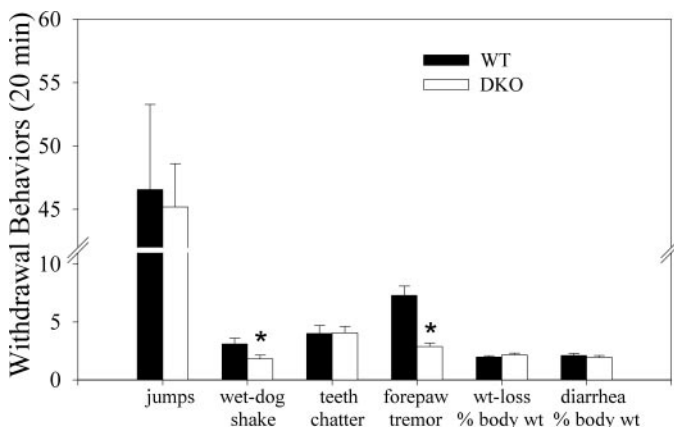


Fig. 4. Morphine withdrawal in wild-type and DKO mice. Wild-type ($n = 18$) and DKO mice ($n = 16$) were treated daily with increasing dose of morphine (10–80 mg/kg s.c.) for 8 days. Withdrawal behavior was observed for 20 min immediately after the naloxone injection (10 mg/kg, i.p.) on day 9. Data are presented as the mean \pm S.E.M., *, $P < 0.05$ compared with wild-type mice, Student's *t* test.

Discussion

The current study provided pharmacological and behavioral evidence that AC1 and AC8 contribute to morphine tolerance, withdrawal, and reinforcing properties. Deletion of AC1 and AC8 genes reduced both short- and long-term morphine tolerance and reduced the degree of morphine dependence. The severity of morphine withdrawal behaviors evoked by naloxone was significantly reduced by AC gene deletion. Moreover, the removal of AC1 and AC8 reduced the hyperlocomotor activities and conditioned place preference induced by morphine.

The findings are consistent with prior studies showing that AC1 and AC8, but not other types of ACs, were up-regulated by long-term exposure to morphine (Lane-Ladd et al., 1997; Nestler and Aghajanian, 1997; Jolas et al., 2000). The obser-

variations that the deletion of AC1 and AC8 genes strongly reduced the development of morphine short-term tolerance and partially reduced morphine long-term tolerance only at the first 2 days suggest that the AC system contributes the morphine antinociceptive tolerance at the initial stages and that an alternative mechanism may mediate response at the late course of long-term morphine antinociceptive tolerance.

The DKO mice also displayed significantly attenuated withdrawal behaviors (e.g., wet dog shakes and forepaw tremor). The cellular basis for these effects is not clear, but long-term opiate exposure in the NAc and VTA brain regions up-regulates the cAMP pathway within γ -aminobutyric acid-containing GABAergic neurons that innervate the dopaminergic and serotonergic cells (Bonci and Williams, 1996, 1997; Tolliver et al., 1996). A reduced serotonergic tone in the neuraxis may contribute to both somatic and motivational aspects of withdrawal (Koob and LeMoal, 1997; Nestler and Aghajanian, 1997). We note that the removal of both AC1 and AC8 genes only attenuated some but not all of the withdrawal behaviors. This finding is consistent with other reports in which the CREB antisense oligonucleotide infusion significantly attenuated the appearance of withdrawal behaviors (teeth chatter, wet dog shakes, ptosis, vacuous chewing, and irritability) but not other withdrawal behaviors (lacrimation, salivation, piloerection, stereotypy, weight loss, and diarrhea) (Lane-Ladd et al., 1997), and CREB knockout mice displayed attenuated withdrawal behaviors including piloerection, teeth chattering, and paw tremor but had no effect on jumping, ptosis, and body tremor (Valverde et al., 2004). The detailed mechanisms and circuitry underlying different morphine withdrawal behaviors are incompletely understood.

Several studies have also examined the involvement of CREB activation in morphine response (Lane-Ladd et al., 1997). For example, a single morphine injection was reported

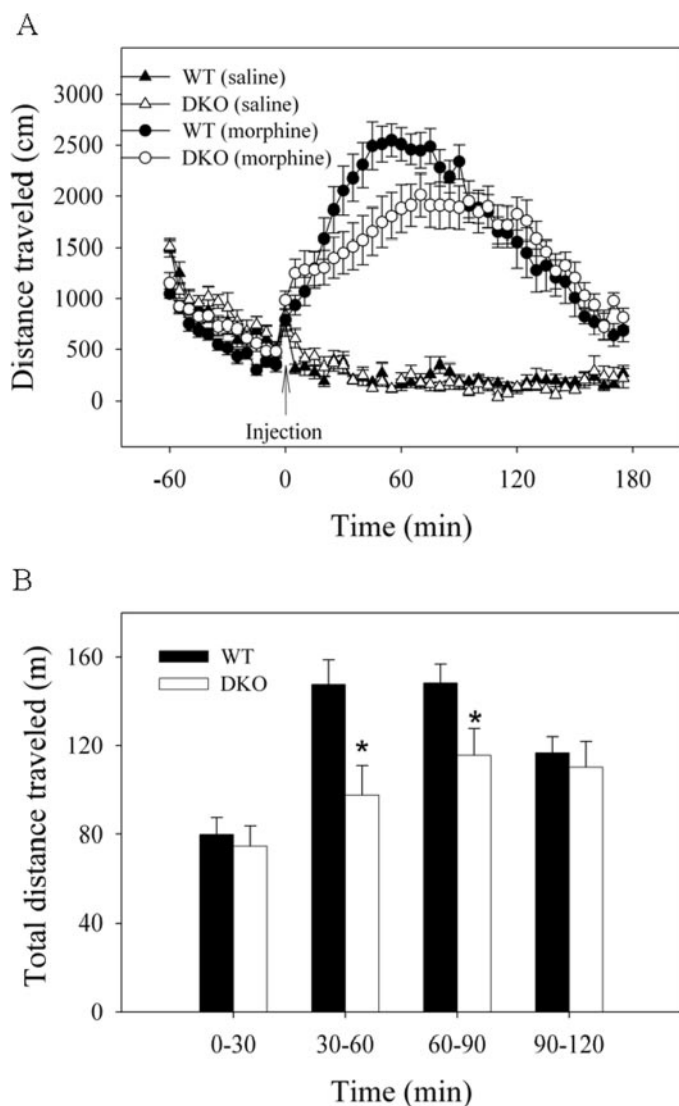


Fig. 5. Locomotor activity after short-term morphine treatment. A, effects of a short-term dose of morphine. Morphine (10 mg/kg s.c.) or saline was administered to the wild-type mice (morphine, $n = 12$; saline, $n = 5$) and DKO mice (morphine, $n = 12$; saline, $n = 5$) after a 1-h habituation period. Locomotor activity was measured during the 1-h habituation period and for 3 h after injection. Data are presented as the mean \pm S.E.M. B, total distance traveled was collected every 30 min after morphine injection for 2 h in the wild-type mice ($n = 12$) and DKO ($n = 12$). *, $P < 0.05$ compared with wild-type, Student's t test.

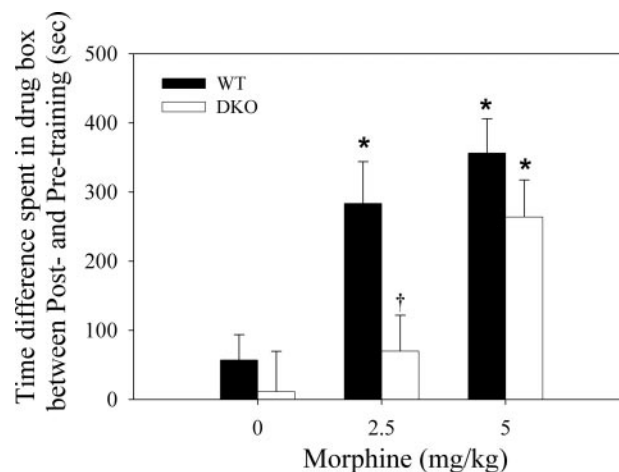


Fig. 6. Morphine-conditioned place preference in wild-type and DKO mice. Time scores shown represent differences between postconditioning (Post) and preconditioning (Pre) time spent in the morphine-paired compartment. *, $P < 0.05$ compared with saline-injected group; †, $P < 0.05$ compared with wild-type mice by ANOVA followed by Bonferroni post hoc test. The following data show the real mean time scores \pm S.E.M. (in seconds) of preconditioning (Pre) and postconditioning (Post) sessions on initially nonpreferred compartment in each genotype. Wild-type mice [Pre/Post, n], DKO mice [Pre/Post]: saline ([531.0 \pm 26.7/588.0 \pm 51.0, 6], [517.7 \pm 69.9/529.0 \pm 40.0, 7), morphine 2.5 mg/kg ([504.8 \pm 26.2/788.1 \pm 50.1, 11], [581.5 \pm 25.2/651.3 \pm 49.9, 10]), and morphine 5 mg/kg ([574.9 \pm 28.4/931.2 \pm 53.6, 12], [637.7 \pm 22.5/901.5 \pm 44.4, 9]).

to increase the number of pCREB-positive cells in VTA (Walters et al., 2003). Moreover, long-term morphine exposure increases cAMP response element-mediated transcription in the both VTA and the locus coeruleus (Widnell et al., 1994; Olson et al., 2005). The mechanisms linking opioid receptor activation to pCREB are not clear. CREB phosphorylation may involve cAMP-activated protein kinase A, calcium/calmodulin-dependent protein kinase, or mitogen-activated protein kinase pathways (Bito et al., 1996; Soderling, 2000; Licata and Pierce, 2003; Thomas and Huganir, 2004). In this study, we found that an increase of pCREB induction in VTA by long-term morphine treatment was significantly reduced in the DKO mice, suggesting that AC1 and AC8 mediate the CREB activation in these neurons. However, the presence of a significant residual pCREB in morphine-injected DKO mice indicates that Ca^{2+} -stimulated AC1 and AC8 are not solely responsible. This conclusion is consistent with a recent report showing that a Ca^{2+} -independent form of adenylyl cyclase, AC5, has also been implicated in morphine actions within the striatum (Kim et al., 2006).

The removal of AC1 and AC8 genes reduced both the hyperlocomotor response and conditioned place preference, indicating that AC1 and AC8 mediate morphine reinforcement. This observation is consistent with the results using CREB^Δ mutant mice that did not show morphine CPP (Walters and Blendy, 2001). CPP is a complex learning paradigm (Bardo

and Bevins, 2000), and cAMP-CREB signaling is critical for memory formation and synaptic plasticity (Wong et al., 1999; Wang et al., 2004). These results suggest that the impairment of memory and learning caused by the removal of both AC1 and AC8 signaling may contribute to the reduction in morphine CPP. However, this effect may be specific for morphine CPP because CREB-deficient mice displayed an augmented cocaine CPP paradigm (Walters and Blendy, 2001). The cellular mechanisms underlying morphine effects are not completely clear, but it is known that morphine disinhibits VTA dopaminergic cell firing by inhibiting neighboring GABAergic neurons. A reduction of cAMP/CREB signaling in these GABAergic neurons might prevent morphine's effects in the nucleus accumbens and reduce the reinforcing properties of morphine (Walters and Blendy, 2001).

In conclusion, the studies here support a fundamental reconsideration of the roles of calmodulin-stimulated adenylyl cyclases in the morphine response. We demonstrated that AC1 and AC8 contribute to the initial stages of morphine tolerance. We found that AC1 and AC8 contribute to the expression of the somatic signs of opiate withdrawal and severity of morphine physical dependence. Finally, we found that AC1, AC8, and CREB contribute to the reinforcing properties of morphine. Further work is needed to identify the downstream targets of these enzymes and cellular pathways contributing to the morphine response.

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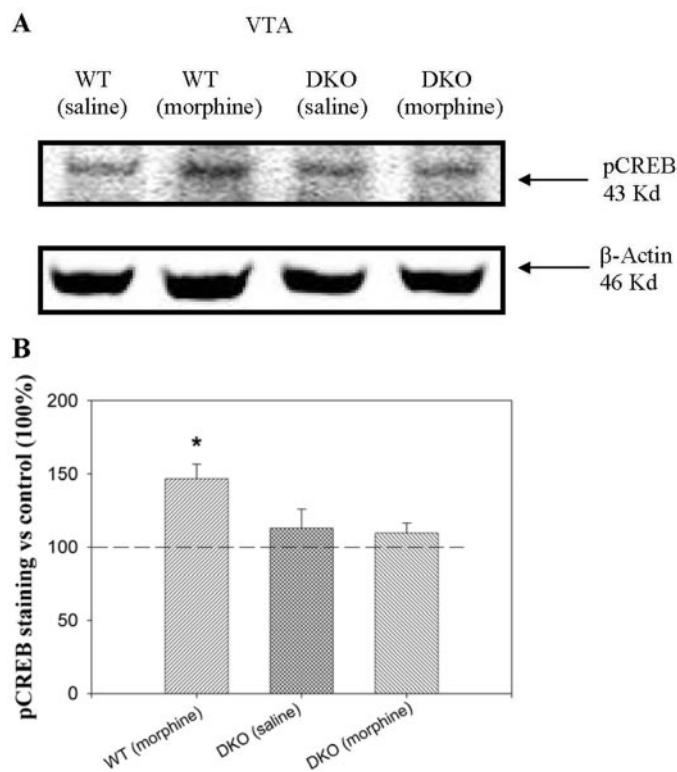


Fig. 7. A, representative Western blot of isolated mouse brain VTA membrane protein (50 $\mu\text{g}/\text{lane}$) taken from either morphine-treated (daily 10 mg/kg s.c. over 5 days: wild-type = 10, DKO = 10) or saline-treated (daily, s.c. over 5 days: wild-type = 10, DKO = 4) mice and incubated with either pCREB antibody detecting endogenous levels of CREB only when phosphorylated at serine 133 (top) or β -actin antibody (bottom). B, morphine-induced CREB activation in the wild-type mice (wild-type morphine = 10, wild-type saline = 10) and DKO mice (DKO morphine = 10, DKO saline = 4). Broken line indicates wild-type saline-injected group by one-way ANOVA followed by Bonferroni post hoc test.

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