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Evidence for a role of CaMKIV in the development of opioid analgesic tolerance

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Abstract

cAMP response-element binding protein (CREB), a transcription factor involved in learning, memory and drug addiction, is phosphorylated by calcium–calmodulin-dependent protein kinase IV (CaMKIV). Here, we show that CaMKIV-knockout (KO) mice developed less analgesic tolerance after chronic morphine administration with no alteration in physical dependence or acute morphine-induced analgesia. The increase in phosphorylated CREB expression observed in wild-type mice after chronic morphine was absent in CaMKIV-KO mice, while there was no difference in the expression or phosphorylation of the μ-opioid receptor between groups. Morphine-treated CaMKIV-KO mice showed less G-protein uncoupling from the μ-opioid receptor than did wild-type mice, while uncoupling was similar in control wild-type and KO mice. In addition, morphine reduced inhibitory transmission to a greater degree in CaMKIV-KO mice than in controls after chronic morphine exposure. Our results provide novel evidence for the role of CaMKIV in the development of opioid analgesic tolerance but not physical dependence.

Introduction

Calcium–calmodulin-dependent protein kinase IV (CaMKIV) activates the cAMP response-element binding protein (CREB) by phosphorylating it at Ser 133 (Deisseroth *et al*., 1996; Deisseroth *et al*., 1998). Phosphorylated CREB recruits the CREB-binding protein, which leads to the activation of CRE (cAMP response-element)-containing promoters and ultimately to gene expression (Matthews *et al*., 1994; Ginty, 1997; Soderling, 1999). CaMKIV is a calcium-dependent protein kinase that is detected in both the nuclei and cytoplasm of neurons, and is the only CREB-phosphorylating protein kinase that is detected predominately in the nuclei of neurons (Jensen *et al*., 1991; Nakamura *et al*., 1995; Kang *et al*., 2001). A recent study reported that CaMKIV-knockout (KO) mice show a defect in fear memory (Wei *et al*., 2002). Learning, memory and drug addiction have certain intracellular signalling cascades in common and depend on CREB (Kandel, 2001; Nestler, 2001b, 2002).

Numerous studies implicate CREB in drug addiction (Guitart *et al*., 1992; Hyman, 1996; Maldonado *et al*., 1996; Blendy & Maldonado, 1998; Walters & Blendy, 2001; Barrot *et al*., 2002). Western blot analysis showed an increase in CREB or phosphorylated CREB (pCREB) expression in morphine-tolerant animals (Li & Clark, 1999; Gao *et al*., 2004), and CREB-mutant mice displayed less severe withdrawal symptoms following cessation of morphine treatment (Maldonado *et al*., 1996). Additionally, the μ-opioid receptor (MOR), a G-protein-coupled receptor that primarily mediates the physiological actions of morphine, contains a CRE element and has been shown to be activated through CREB-mediated pathways (Min *et al*., 1994; Nestler, 1997; Lee & Lee, 2003). CRE-mediated transcription has also been shown to be altered in several brain regions during morphine withdrawal (Shaw-Lutchman *et al*., 2002).

A number of studies report a role for protein kinases in opioid tolerance (Ueda *et al*., 2001; Zeitz *et al*., 2001; Eitan *et al*., 2003; Terman *et al*., 2004). Inhibition of protein kinase C (PKC) blocks the development of opioid tolerance (Narita *et al*., 1995) and PKCγ-mutant mice show reduced analgesic tolerance to morphine (Zeitz *et al*., 2001). Another study has shown that calcium–calmodulin-dependent protein kinase II (CaMKII) activity is increased in the spinal cord of tolerant rats and a CaMKII inhibitor was able to reverse the already established analgesic tolerance (Wang *et al*., 2003).

Several protein kinases, as well as CREB, are known to play roles in opioid analgesic tolerance. However, it is not known whether the protein kinase CaMKIV, which phosphorylates CREB, plays a role in the cellular and behavioural responses to morphine. Dissecting the molecular mechanisms behind the acquisition of opioid analgesic tolerance may lead to the more efficacious use of morphine as a treatment for chronic pain.

Materials and methods

Animals

CaMKIV-KO mice were derived as described (Wei *et al*., 2002) and bred for several generations (F12) on a C57Bl/6 background. Control wild-type (WT) mice were adult male (8–12 weeks old) C57Bl/6 mice from Charles River. At the conclusion of experiments, animals were humanely killed with an overdose of inhaled anaesthetic

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(halothane). The animals were housed on a 12 : 12-h light : dark cycle with food and water available *ad libitum*. All mouse protocols are in accordance with NIH guidelines and were approved by the Animal Care and Use Committee at the University of Toronto. No visual difference between WT and CaMKIV-KO mice is noticeable, and experiments were performed blind.

Open-field activity monitor

To record horizontal locomotor activity, we used the Activity Monitor system from Medical Associates (43.2 × 43.2 × 30.5 cm; MED-associates, St Albans, VT, USA). Briefly, this system uses paired sets of photo beams to detect movement in the open field and movement is recorded as beam breaks. The open field is placed inside an isolation chamber with dim illumination and a fan. Each subject was placed in the centre of the open field and activity was measured for 60 min.

Conditioned place preference

A chamber with two distinct contextual environments (different walls, floor and smell) was used (MED-associates). On the first day of testing, animals were allowed to freely explore both sides of the chamber for 30 min and data were used to separate animals into groups of approximately equal bias. For the next 8 days, each animal was given either 10 mg/kg morphine or an equivalent volume of saline on alternating days in distinct sides of the chamber. The animals were confined to the specific side of the chamber for 30 min. After conditioning, all animals were injected with saline and allowed to freely explore both sides of the chamber for 30 min. Place preference was defined as an increase in the time spent in the morphine-paired side after conditioning as compared to before (preference for drug side = time spent in paired side after treatment − time spent before).

Hotplate and tail-flick tests

The hotplate consisted of a thermally controlled metal plate (55 °C) surrounded by four Plexiglas walls (Columbia Instruments; Columbus, Ohio). The time between placement of the animal on the plate and the licking or lifting of a hindpaw was measured with a digital timer. Mice were removed from the hotplate immediately after the first response and a cut-off time of 30 s was imposed to prevent tissue damage. The spinal tail-flick reflex was evoked by focused, radiant heat from an infra-red lamp (Columbia Instruments; Columbus, OH, USA) applied to the underside of the tail and a cut-off time of 10 s was imposed to prevent tissue damage. Response latencies are reported as a percentage of maximal possible effect [MPE; 100 × (response latency − baseline response latency)/(cutoff latency − baseline response latency)].

Chronic tolerance

To study the development of analgesic tolerance generated by continued morphine administration, mice were injected once a day for 7 days with 10 mg/kg morphine (s.c.). Open-field locomotor activity was recorded for 1 h after injection; this was followed by determination of hotplate and tail-flick response latencies.

Morphine withdrawal

Withdrawal behaviours were scored 7 days after receiving twice-daily injections of escalating doses of morphine. The dosing scheme was as follows: Day 1, dose curve (1, 5, 10, 20 and 40 mg/kg, s.c.); Day 2, 20 mg/kg × 2; Day 3, 40 mg/kg × 2; Day 4, 60 mg/kg × 2; Day 5, 80 mg/kg × 2; Day 6, 100 mg/kg × 2; Day 7, 100 mg/kg. Naloxone (1 mg/kg, s.c.) was used to precipitate withdrawal 2 h after the last morphine injection. For the scoring of withdrawal behaviours, mice were placed individually in plastic containers and scored for 30 min. Two researchers blind to the animal's genotype scored wet-dog shakes, paw tremors, jumping, mastication and diarrhea.

Western blot analysis and immunoprecipitation

Spinal cords were dounce-homogenized (18 strokes) in 10 volumes of 10 mM Tris-Cl (pH 7.4) with 320 mM sucrose and 1% SDS. The tissue homogenates were centrifuged at 21 000 ***g*** at 4 °C for 5 min. Equal amounts of protein from tissue samples were separated by 4–12% gradient SDS-PAGE (Invitrogen), followed by Western blotting with anti-MOR-1 (1 : 1000), anti-pCREB (1 : 1000; Santa Cruz Biotechnology) or antiphosphoserine antibody (for immunoprecipitated samples; 1 : 1000; Sigma), followed by revelation with ECL (Amersham). Membranes (BioTrace PVDF; Pall Co.) were routinely stripped and re-probed with a monoclonal antibody for actin (1 : 5000; Sigma) blotting to ensure equal protein loading.

For immunoprecipitation, synaptosomal membrane fractions (LP1) were prepared as previously described (Dunah & Standaert, 2001) and solubilized using 1% SDS in TEVP buffer: Tris-HCl, pH 7.4, 10 mM; EDTA, 1 mM; and EGTA, 1 mM; with 1 × protease inhibitor cocktail (Sigma) and 1 × phosphatase inhibitor cocktail 1 and 2 (Sigma). The solubilized proteins were diluted 1 : 20 with modified RIPA buffer (Tris-HCl, pH 7.4, 50 mM; NP-40, 1%; Na-deoxycholate, 0.25%; NaCl, 150 mM; EDTA, 1 mM; and PMSF, 1 mM), and incubated with 50 μL of protein G–agarose beads precoupled with anti-MOR-1 antibody (Santa Cruz Biotechnology) for 3 h at 4 °C. The reaction mixtures were then washed three times and eluted by boiling in sample loading buffer and subjected to Western blot as described above. Equal amounts of synaptosomal membrane fractions were used for the Western blotting.

Immunostaining

Spinal cord slices at the lumbar region were prepared using standard histological methods. Slices were incubated with rabbit anti-pCREB IgG (1 : 250; Calbiochem) or rabbit anti-MOR IgG (1 : 500; ImmunoStar Incorporated) at 4 °C overnight, followed by donkey antirabbit IgG conjugated with Alexa 488 (1 : 500; Molecular Probes) for 1 hour at room temperature. Measurements were made from at least three randomly selected noncontiguous sections in each mouse (3–5 mice per treatment group), observed from coded slides and averaged so that each animal had a mean value for regional immunoreactivity. The total number and density of positive cells were measured in dorsal horn laminae I and II and ventral horn laminae VI-IX. All data are expressed as a ratio between cell number or density and unit area. Images were obtained with an Olympus microscope. Data were analysed with ImagePro software (Media-Cybernetics).

[^{35}S]GTPγS binding assay

Mouse spinal cord tissue was homogenized by a dounce homogenizer in membrane preparation buffer (in mM: Tris·Cl pH 7.4, 50; EGTA, 1; and $MgCl_2$, 3). The homogenate was centrifuged at 500 ***g*** for 10 min at 4 °C and the supernatant was centrifuged at 48 000 ***g*** for 20 min at 4 °C (Chen & Pan, 2003). The supernatant was discarded and the crude membrane pellet was re-suspended in assay buffer (Tris·Cl

pH 7.4, 50 mM; EGTA, 0.2 mM; $MgCl_2$, 3 mM; NaCl, 100 mM; and GDP, 50 μM). Membrane protein (10 μg) was incubated in 50 pM [^{35}S]GTPγS (1250 Ci/mmol; NEN, Boston, MA, USA) and 10–10 000 nM of the highly selective MOR ligand DAMGO at 30 °C for 1 h (Johnson *et al.*, 2003). Binding was terminated by rapid filtration through a glass microfiber filter using vacuum manifold (Schleicher & Schuell). The filter was washed three times in 50 mM Tris·Cl pH 7.4 with 5 mM $MgCl_2$ and then the bound radioactivity was measured with a liquid scintillation counter (Pharmacia Wallac 1410; Pharmacia Wallac).

Whole-cell patch-clamp recordings in young spinal cord slices

Postnatal mice (12–19 days old) were anaesthetized with 1–2% halothane and the thoracic and lumbar spinal cord were dissected out and put into pre-equilibrated artificial cerebrospinal solution fluid (ACSF) at 1–4 °C. The spinal cord was placed into a shallow groove in an agar block, and several drops of 20% gelatin were placed on the low-thoracic and high-lumbar segment to half-embed the spinal cord. The agar block, together with the half-embedded spinal cord, was glued onto the stage of a vibratome with cyanoacrylate adhesive, and was immersed in ice-cold ACSF. Transverse slices of the lumber spinal cord (350 μm) were prepared. Slices were transferred to a room-temperature submerged recovery chamber with oxygenated (95% O_2 and 5% CO_2) solution containing (in mM): NaCl, 124; $NaHCO_3$, 25; KCl, 4.4; KH_2PO_4, 1; $CaCl_2$, 2; $MgSO_4$, 2; and glucose, 10. After a 1-h recovery, slices were placed in a recording chamber on the stage of an Axioskop 2FS microscope (Zeiss) equipped with infrared differential interference contrast optics for patch-clamp recordings. Substantia gelatiosa (SG) could be identified under the microscope as a translucent band capping the dorsal part of the grey matter. Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded with an Axon 200B amplifier (Axon Instruments, CA, USA). Recording electrodes (3–5 MΩ) contained a pipette solution composed of (in mM): Cs-gluconate, 120; NaCl, 5; $MgCl_2$, 1; EGTA, 0.5; Mg-ATP, 2; Na_3GTP, 0.1; and HEPES, 10; pH 7.2, 280–300 mOsmol. Access resistance was 15–30 MΩ and was monitored throughout the experiment. Data were discarded if access resistance changed > 15% during an experiment. The membrane potential was held at −70 mV throughout the experiment. A holding potential of +10 mV was used to record sIPSCs.

Data analysis and statistics

Statistical comparisons were made using the *t*-test, or one-way or two-way ANOVA (Student–Newmann–Keuls test was used for *post hoc* comparison). All data are represented as mean ± SEM. In all cases, $P < 0.05$ is considered statistically significant.

Results

Morphine-induced analgesia in CaMKIV-KO mice

Several bodies of evidence suggest that CaMKIV may play an important role in the behavioural and molecular responses to morphine. CaMKIV is unique in its ability to phosphorylate CREB in the nuclei of neurons and may play a role in the transcriptional modifications following morphine exposure. To determine whether the deletion of CaMKIV alters acute behavioural nociceptive responses to morphine, hotplate responses (55 °C) were measured after escalating doses of morphine in both CaMKIV-KO ($n = 8$) and WT ($n = 8$) mice. Response latencies were similar in CaMKIV-KO and WT mice at each morphine dose tested ($P = 0.80$; Fig. 1A), suggesting that the deletion of CaMKIV did not affect morphine-induced analgesia. Hotplate response latencies were also recorded from CaMKIV-KO ($n = 4$) and WT ($n = 4$) mice after repeated saline injections and were not altered with repeated hotplate exposure ($P = 0.91$).

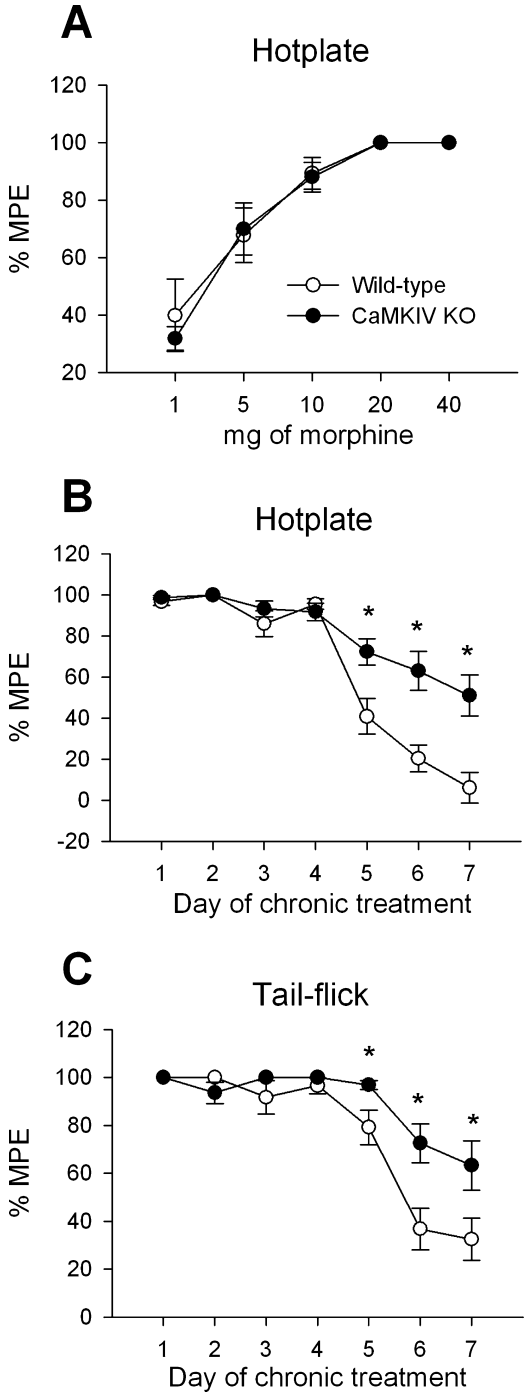

FIG. 1. CaMKIV-KO mice developed less analgesic tolerance after chronic morphine treatment. (A) Behavioural responses on the hotplate were similar in CaMKIV-KO and WT mice after acute, cumulative doses of morphine (s.c.); responses were recorded 30 min after each injection. (B and C) During chronic morphine administration there was a significant effect of genotype over time in both the hotplate and tail-flick tests; $P < 0.001$. *Post hoc* analysis revealed significant decreases in percentage MPE starting 5 days after injection; Student–Newman–Keuls, $P < 0.01$. *Denotes significant difference from wild-type (panel B: days 5, 6 and 7, $P < 0.001$; panel C: day 5, $P < 0.01$; days 6 and 7, $P < 0.001$).

CaMKIV-KO mice developed less analgesic tolerance after chronic morphine

After establishing that morphine-induced analgesia was intact in CaMKIV-KO mice, we next wanted to determine whether CaMKIV plays a role in the analgesic tolerance that develops after chronic morphine treatment. CaMKIV-KO ($n = 13$) and WT ($n = 13$) mice were given daily injections of morphine (10 mg/kg, s.c.) and hotplate and tail-flick response latencies were recorded. Our results show that there was a significant effect of genotype in response latencies after chronic morphine treatment ($P < 0.001$; Fig. 1B and C). By the fifth morphine injection, CaMKIV-KO mice displayed enhanced response latencies compared to WT mice in both the hotplate and tail-flick tests (Fig. 1B and C). While both CaMKIV-KO and WT mice developed analgesic tolerance (WT, $P < 0.001$; KO, $P < 0.001$; Day 1 vs. Day 7), CaMKIV-KO mice displayed significantly higher morphine-induced analgesia than did WT mice on Day 7 of morphine administration ($P < 0.001$). These results suggest that CaMKIV may play a role in the acquisition of opioid analgesic tolerance.

Morphine-induced hyperlocomotion after acute and chronic morphine treatment

To determine whether CaMKIV plays a role in morphine-induced hyperlocomotor activity, locomotor activity was measured in an open field for 60 min following morphine administration. There was no difference in locomotion between CaMKIV-KO ($n = 13$) and WT ($n = 12$) mice after a single injection of 10 mg/kg (s.c.) morphine (Fig. 2A). Additionally, locomotor activity was similar between CaMKIV-KO ($n = 6$) and WT ($n = 6$) mice following chronic morphine administration ($P = 0.22$; 10 mg/kg, s.c., daily for 7 days; Fig. 2B). Taken together, these results suggest that CaMKIV does not play a role in morphine-induced hyperactivity.

Conditioned place preference to morphine

The conditioned place-preference paradigm was used to show that CREB-mutant mice have a decreased response to the rewarding properties of morphine (Walters & Blendy, 2001). To determine

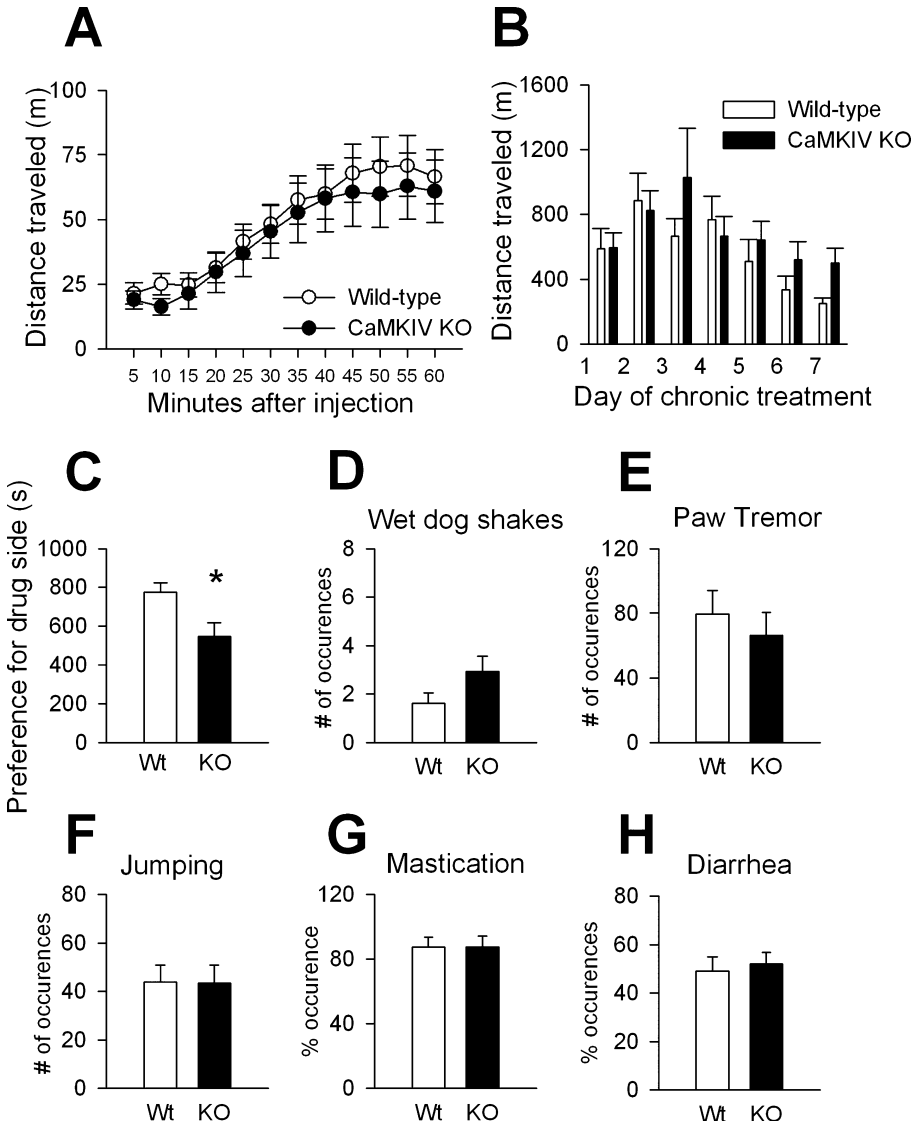

FIG. 2. No difference in withdrawal behaviours and locomotor activity after morphine but a decrease in conditioned place preference between CaMKIV-KO and WT mice. (A) Locomotor activity after an acute dose of 10 mg/kg morphine. (B) Locomotor activity over 7 days of chronic morphine treatment (10 mg/kg). (C) CaMKIV-KO mice showed a reduced preference for the morphine-paired side in the conditioned place-preference paradigm. (D–H) No difference in withdrawal behaviours between CaMKIV-KO and WT mice; behaviours were scored for 30 min after injection of 1 mg/kg naloxone. *$P < 0.02$, compared with wild-type.

whether CaMKIV plays a role in this form of psychological dependence, we tested CaMKIV-KO ($n = 6$) and WT ($n = 6$) mice in the conditioned place-preference paradigm. CaMKIV-KO mice spent significantly less time exploring the morphine-paired side of the chamber than did WT mice, suggesting that CaMKIV may play a role in the reinforcing properties of morphine ($P < 0.02$, Fig. 2C). CaMKIV-KO and WT mice did not differ in their initial preference for either side of the chamber.

Withdrawal behaviours in CaMKIV-KO mice

Prolonged morphine use produces a state of physical dependence in addition to analgesic tolerance. To determine whether CaMKIV plays a role in the development of opioid dependence, withdrawal behaviours were scored following chronic administration of morphine. Both WT and KO animals were injected with escalating doses of morphine for 7 days, after which physical withdrawal was precipitated with naloxone (1 mg/kg, s.c.). Stereotypic withdrawal behaviours (e.g. wet-dog shakes, paw tremors, jumping, mastication and diarrhea) were scored for 30 min in both groups. There were no differences between CaMKIV-KO ($n = 8$) and WT ($n = 8$) mice in any of the behaviours induced by the opioid receptor antagonist (Fig. 2D–H). Although CaMKIV-KO mice developed less analgesic tolerance to chronic administration of morphine, it appears that CaMKIV is not involved in morphine-induced physical dependence.

pCREB and MOR expression in CaMKIV-KO and WT mice

Studies have shown that chronic morphine treatment increases levels of pCREB expression in the spinal cord (Li & Clark, 1999). Here, we measured pCREB expression in the spinal cord of CaMKIV-KO and WT mice before and after chronic morphine treatment. There was no difference in pCREB expression between CaMKIV-KO ($n = 4$) and WT ($n = 4$) mice treated with saline. Chronic morphine administration significantly increased pCREB expression in WT mice compared to mice receiving saline ($n = 4$, $P < 0.001$). However, morphine failed to induce an increase in pCREB expression in CaMKIV-KO mice ($n = 3$, $P = 0.15$; Fig. 3A and B). Additionally, there was significantly more pCREB expression in morphine-treated WT mice than in KO mice ($P < 0.001$). These results suggest that CaMKIV plays a role in the morphine-induced up-regulation of pCREB in the spinal cord.

As the MOR was shown to be activated through CREB-mediated pathways (Lee & Lee, 2003), we measured the expression of the MOR in CaMKIV-KO mice before and after chronic morphine. There was no difference in MOR expression between CaMKIV-KO ($n = 4$) and WT ($n = 4$) mice treated with saline ($P = 0.88$, Fig. 3A and C). Additionally, expression levels of the MOR were not significantly changed in either CaMKIV-KO ($n = 3$) or WT ($n = 4$) mice after morphine treatment compared to mice receiving saline (WT, $P = 0.06$; KO, $P = 0.12$; Fig. 3A and C). There was also no difference in MOR expression between KO and WT mice treated with morphine ($P = 0.41$). Taken together, our results suggest the deletion of CaMKIV did not alter MOR expression and that chronic morphine treatment did not alter the expression of the MOR in either WT or KO mice.

Phosphorylation of the μ-opioid receptor

As CaMKIV is detected in both the nuclei and cytoplasm of neurons (Jensen *et al.*, 1991; Nakamura *et al.*, 1995), its role in the development of opioid tolerance may extend beyond interactions with CREB. For example, phosphorylation of the MOR by several protein kinases (PKA, PKC and CaMKII) can lead to desensitization,

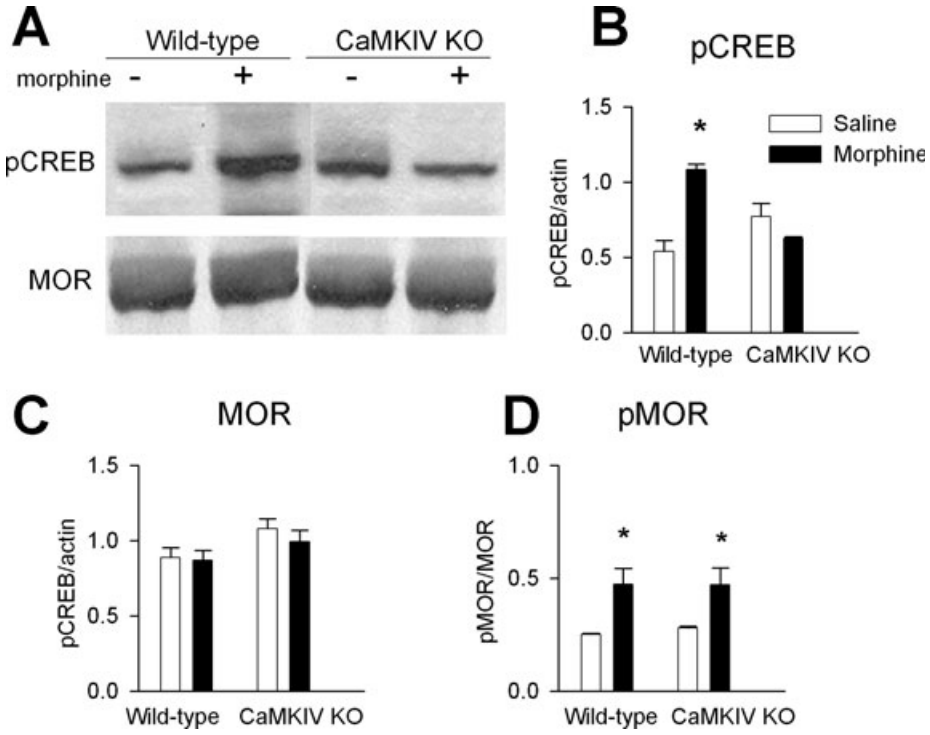

FIG. 3. Chronic morphine-induced increase in pCREB expression is absent in CaMKIV-KO mice while levels of MOR and phosphorylated MOR are similar to WT mice. (A) Representative Western blot showing the expression of pCREB and MOR in the spinal cords of CaMKIV-KO and WT mice with or without morphine. (B) Summary of pCREB data shown in (A); pCREB was significantly increased in WT mice after chronic morphine treatment. (C) Summary of MOR data shown in (A). (D) Morphine significantly increased pMOR in both WT and CaMKIV-KO mice compared to mice receiving saline. In panel B, $*P < 0.001$, compared with saline-treated animals; in panel D, $*P < 0.05$, compared with saline-treated animals.

which is one of the proposed mechanisms for the development of opioid tolerance and dependence (Nestler, 1997; Liu & Anand, 2001). To determine whether CaMKIV may play a role in this process, the serine phosphorylation state of the MOR was evaluated. The MOR was immunoprecipitated from spinal cord samples of CaMKIV-KO ($n = 3$) and WT ($n = 3$) mice treated with either saline or chronic morphine. The deletion of CaMKIV did not affect serine phosphorylation of the MOR in saline-treated mice ($P = 0.79$, WT vs. KO) or in mice receiving chronic morphine treatment ($P = 0.99$, WT vs. KO; Fig. 3D). However, morphine treatment significantly increased the phosphorylation of the MOR in both CaMKIV-KO and WT mice ($P < 0.05$). These results suggest that the decrease in analgesic tolerance is not due to an alteration in the phosphorylation state of the MOR in CaMKIV-KO mice.

Expression of pCREB and MOR in the spinal cord of CaMKIV-KO mice

A previous study reported that the morphine-induced up-regulation of pCREB was predominantly in laminae I and II of the dorsal horn (Li & Clark, 1999). To determine whether CaMKIV plays a role in this anatomically specific up-regulation of pCREB, dorsal horn samples of saline- and morphine-treated KO and WT mice were examined for changes in pCREB expression. Consistently, immunohistochemical staining for pCREB revealed a significant role for CaMKIV in the morphine-induced up-regulation of pCREB in laminae I and II of the dorsal horn (Fig. 4A and B). As before, there was no significant difference in pCREB expression between KO and WT mice treated with saline ($P = 0.06$). Compared to saline, morphine significantly increased pCREB in laminae I and II of WT mice ($P < 0.001$) while such an increase was absent in KO mice ($P = 0.122$). In addition, pCREB levels were significantly lower in morphine-treated CaMKIV-KO mice than in WT mice ($P < 0.001$). Notably, there was no effect of genotype ($P = 0.22$) or treatment ($P = 0.28$) on the expression of pCREB in the ventral horn (Fig. 4B). Taken together, these results suggest that CaMKIV plays a role in the selective up-regulation of pCREB in laminae I and II of the dorsal horn after chronic morphine administration.

To determine whether the expression of the MOR may be altered in the dorsal horn after chronic morphine treatment, samples of

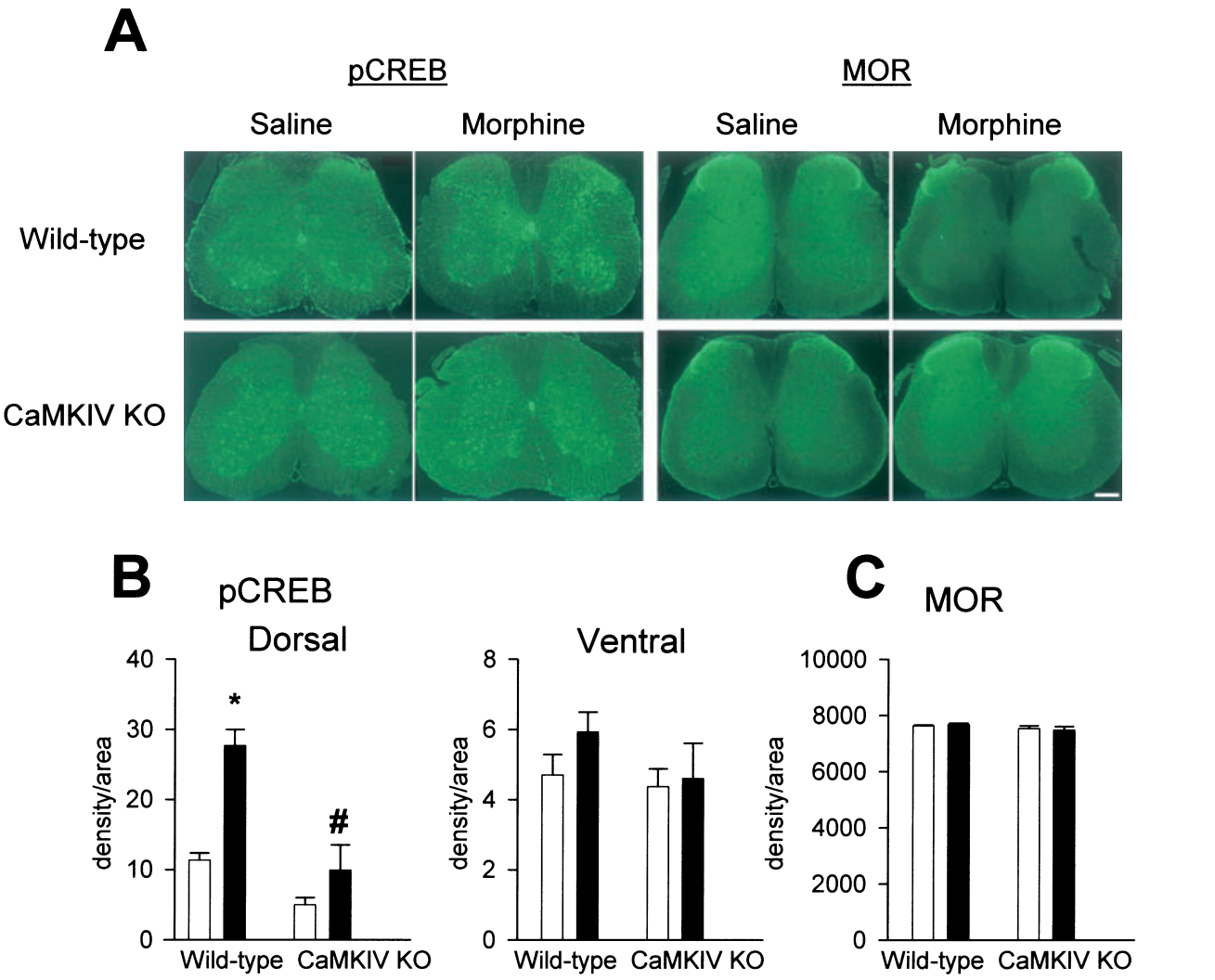

FIG. 4. Immunohistochemical analysis of pCREB and MOR expression in the lumbar enlargement of the spinal cord in saline- and morphine-treated CaMKIV-KO and WT mice. (A and B) Morphine significantly increased pCREB in laminae I and II of the dorsal horn in WT mice, but pCREB was significantly lower in KO mice than in WT with morphine. There were no differences in pCREB expression in lamina IV of the ventral horn. *Significantly different from saline; #significantly different from WT. (A and C) There was no difference in MOR expression between saline- and morphine-treated CaMKIV-KO and WT mice in laminae I and II of the dorsal horn. Scale bar, 500 μm.

CaMKIV-KO and WT mice treated with morphine were compared to saline-treated animals. Neither the genotype ($P = 0.21$) nor the treatment ($P = 0.68$) affected MOR expression in laminae I and II of the dorsal horn (Fig. 4A and C).

μ-Opioid receptor coupling in CaMKIV-KO mice

Uncoupling of G-proteins from the MOR is a hallmark of the cellular adaptations of opioid tolerance (Hyman, 1996; Nestler, 1997). G-protein binding was measured following activation by the highly selective MOR ligand DAMGO in the spinal cords of CaMKIV-KO and WT mice after chronic morphine (10 mg/kg, s.c., daily for 7 days). There was no difference in DAMGO-induced GTPγS binding between samples taken from CaMKIV-KO and WT mice without morphine ($n = 2$ for both; Fig. 5A). However, GTPγS binding was significantly reduced in WT mice compared to CaMKIV-KO mice chronically treated with morphine ($P < 0.02$, $n = 3$ for both; Fig. 5B). This cellular adaptation in response to chronic morphine treatment appears to be decreased in the absence of CaMKIV.

CaMKIV played a role in morphine-induced reduction of inhibitory transmission after prolonged morphine exposure

The analgesic effects of opioids are thought to be mediated by inhibiting GABAergic synaptic transmission in the periaqueductal grey and locus coeruleus (Vaughan *et al*., 1997; Pan *et al*., 2002) and the activation of MORs has been shown to suppress inhibitory transmission between spinal cord dorsal horn neurons (Kerchner & Zhuo, 2002). While we have shown that CaMKIV-KO mice displayed less analgesic tolerance after chronic morphine, any morphine-induced electrophysiological changes in inhibitory transmission are unknown. To determine whether CaMKIV plays a role in morphine-induced changes in inhibitory synaptic transmission, sIPSCs were recorded from SG neurons in lamina II of the dorsal horn. Acutely applied morphine dramatically decreased the frequency of sIPSCs in all SG neurons tested (WT, 68.9 ± 10.4%, $n = 7$, $P < 0.05$; CaMKIV-KO, 66.2 ± 4.0%, $n = 6$, $P < 0.05$; Fig. 6A–C). To determine whether a prolonged pre-exposure to morphine (mimicking chronic morphine administration) would alter sIPSCs upon morphine application, slices were incubated in 10 μM morphine for at least 1 h before testing. After slices were pre-exposed to morphine, acutely applied morphine dramatically decreased the frequency of sIPSCs in CaMKIV-KO mice (by 55.7 ± 6.9%; $n = 9$, $P < 0.05$), but did not decrease sIPSCs in WT mice ($n = 8$, $P > 0.05$; Fig. 6D). Taken together, these results suggest that pre-exposure to morphine is able to abolish the morphine-induced decreased in sIPSCs in WT mice but this effect is blocked in CaMKIV-KO mice.

Discussion

In recent years, a number of studies have implicated CREB and several protein kinases in opioid tolerance and dependence (Blendy & Maldonado, 1998; Liu & Anand, 2001; Nestler, 2001a). Here, we present evidence for the role of a nuclear protein kinase, which can phosphorylate CREB, in opioid tolerance but not physical dependence. While CaMKIV-KO mice developed less analgesic tolerance after chronic morphine treatment, there was no change in morphine-induced analgesia or physical dependence. The increase in pCREB seen in WT mice after chronic morphine administration was absent in CaMKIV-KO mice. Additionally, G-protein uncoupling to the MOR, a hallmark of cellular opioid tolerance (Nestler, 2001a), was significantly less in CaMKIV-KO than in WT mice after morphine treatment, while uncoupling was similar in saline-treated controls. Morphine significantly decreased the frequency of sIPSCs in morphine pretreated CaMKIV-KO SG neurons, while sIPSCs were not inhibited in morphine-pretreated WT slices. These results demonstrate for the first time the importance of a nuclear protein kinase in both the behavioural and cellular adaptations following chronic morphine treatment.

Learning, memory and drug addiction share certain intracellular signalling cascades in common and depend on the transcription factor CREB (see Nestler, 2002 for review). Morphine exposure increases CREB-mediated transcription and may up-regulate genes involved in drug addiction (Hyman & Malenka, 2001; Lee & Lee, 2003). Previous studies have demonstrated that the increase in pCREB in the spinal cord after chronic morphine was primarily localized to neuronal nuclei (Li & Clark, 1999). A recent study showed that chronic morphine treatment was able to increase the expression levels of both CaMKIV and pCREB in the hippocampus and that the two molecules were colocalized in the nucleus (Gao *et al*., 2004). Another study confirms the up-regulation of CaMKIV and pCREB in the hippocampus of

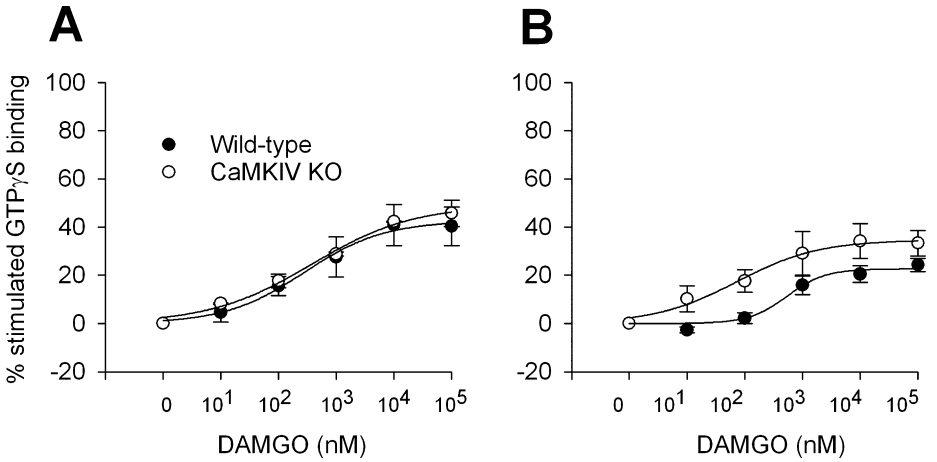

FIG. 5. Significantly less MOR uncoupling in CaMKIV-KO mice than in WT mice after chronic morphine. (A) No difference in GTPγS binding between CaMKIV-KO and WT mice treated with saline. (B) GTPγS binding was significantly decreased in WT mice compared to CaMKIV-KO mice after 7 days of morphine treatment ($P < 0.02$).

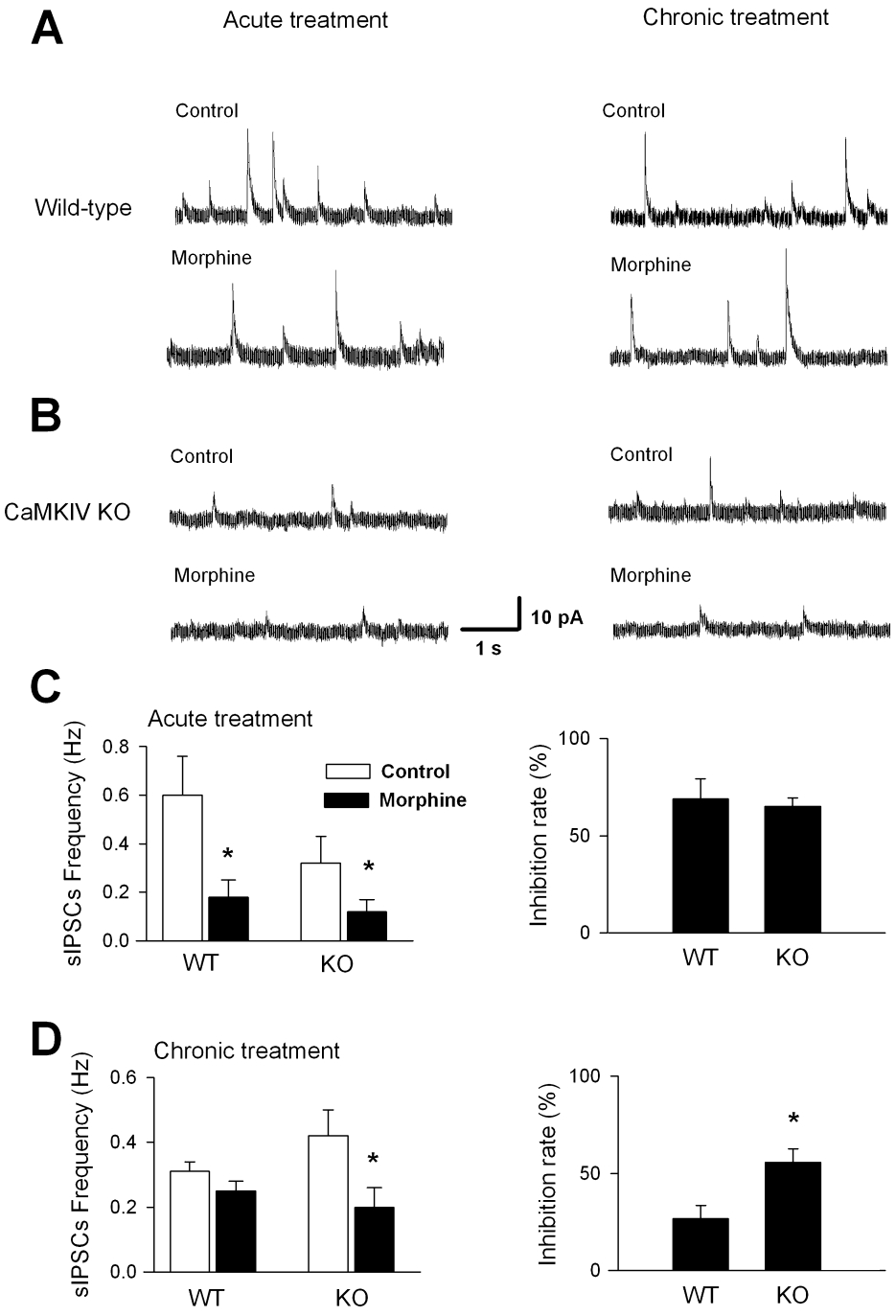

FIG. 6. Increased morphine-induced inhibition of IPSCs in chronically-treated CaMKIV-KO compared to control. (A and B) Representative examples of sIPSCs in SG neurons in dorsal horn from control and morphine-treated (A) WT and (B) CaMKIV-KO mice. (C, left) sIPSC frequency after acute morphine treatment; (C, right) average inhibition rates after acute morphine. (D, left) sIPSC frequency after chronic morphine treatment; (D, right) average inhibition rates after chronic morphine. In panel C, $*P < 0.05$, compared with controls; in panel D, $*P < 0.05$, compared with controls (left) and wild-type (right).

morphine-treated mice (Nemmani *et al*., 2005). Together with the present results, this suggests that CaMKIV may play an important role in the morphine-induced transcriptional changes that are required for the acquisition of opioid tolerance. Indeed, the increase in pCREB seen in WT mice treated with morphine was absent or reduced in CaMKIV-KO mice (Figs 3 and 4).

CREB-mutant mice displayed less severe withdrawal symptoms after chronic morphine (Maldonado *et al*., 1996) but CaMKIV-KO mice displayed normal morphine withdrawal. This demonstrates a selective role for CaMKIV in the cellular adaptations related to tolerance and suggests a separate mechanism for physical dependence. Other studies have shown a genetic dissociation between tolerance and dependence (Bohn *et al*., 2000; Nitsche *et al*., 2002). β-Arrestin 2-KO mice showed enhanced morphine analgesia and a reduction in chronic morphine tolerance while physical withdrawal symptoms were similar to those of WT mice (Bohn *et al*., 1999; Bohn *et al*., 2000). Here, we report a reduction in analgesic tolerance that is independent of changes in acute morphine sensitivity. As both opioid tolerance and dependence were decreased in CREB-mutant mice (Maldonado *et al*., 1996), CaMKIV may play an important role in the dissociation of

these two processes. CaMKIV-KO mice may provide a useful animal model for comparing the different molecular changes that occur with the development of tolerance and dependence. CREB also plays a role in the reinforcing properties of morphine (Walters & Blendy, 2001; Barrot *et al*., 2002; Olson *et al*., 2005). Our results indicate that CaMKIV also plays a role in this response and future studies will elucidate the precise nature of CaMKIV's involvement in the reinforcing properties of morphine.

As CaMKIV is detected in both the nuclei and cytoplasm of neurons (Jensen *et al*., 1991; Nakamura *et al*., 1995), its role in the development of opioid tolerance may extend beyond interactions with CREB. For example, phosphorylation of the MOR by several protein kinases (PKA, PKC and CaMKII) can lead to desensitization, which is one of the proposed mechanisms for the development of opioid tolerance and dependence (Nestler, 1997; Liu & Anand, 2001). As we found no difference in the phosphorylation of the MOR, CaMKIV's role in the acquisition of analgesic tolerance may be through interaction with other molecules or protein kinase signalling cascades. CaMKIV interacts with many other molecules and signalling cascades: PKA, MAPK and adenylyl cyclases for example (Soderling, 1999).

Immunohistochemical and Western blot analysis of spinal cord samples from saline- and morphine-treated CaMKIV-KO and WT mice show that the increase in pCREB seen after chronic morphine administration is, at least in part, CaMKIV-dependent. In addition, the increase in pCREB was localized to laminae I and II of the dorsal horn while expression levels in the ventral horn were similar between morphine- and saline-treated WT and KO mice (Fig. 4). This agrees with a previous study which reports that the increase in pCREB was localized to laminae I and II of the dorsal horn while the ventral horn contained less pCREB (Li & Clark, 1999). The MOR gene contains a CRE element (Wang *et al*., 1993; Min *et al*., 1994) and may be regulated through CREB-mediated pathways. As a recent study showed that expression of the MOR was not altered in CREB-mutant mice (Walters *et al*., 2005), it is not surprising that we did not see a difference in MOR expression in CaMKIV-KO mice. This result suggests that the reduction in opioid tolerance is not due to MOR down-regulation. The role of MOR down-regulation in the development of opioid tolerance remains unclear as some studies report down-regulation of MOR after morphine treatment (Bernstein & Welch, 1998; Tao *et al*., 1998), while others report no change (Werling *et al*., 1989; De Vries *et al*., 1993) or even an increase (Brady *et al*., 1989; Rothman *et al*., 1991; Ray *et al*., 2004). A recent study reported that CaMKIV expression was strongly localized with MOR after chronic morphine treatment in many brain areas implicated in morphine tolerance and dependence (Nemmani *et al*., 2005); this colocalization suggests that CaMKIV may be an important member in a pathway connecting that activation of the MOR to CREB.

Uncoupling of G-proteins from the MOR is a hallmark of the cellular adaptations of opioid tolerance (Hyman, 1996; Nestler, 1997). After morphine treatment, CaMKIV-KO mice showed significantly more G-protein coupling than did WT mice, suggesting that the MOR may be more functional in CaMKIV-KO mice after chronic morphine than in WT mice. This may explain why morphine pretreatment abolished the morphine-induced decrease in sIPSCs in WT but not CaMKIV-KO slices. The activation of MORs in the spinal cord dorsal horn has been shown to suppress inhibitory transmission (Kerchner & Zhuo, 2002). Superficial laminae of the dorsal horn, particularly the substantia gelatinosa (SG), receives nociceptive information from fine myelinated Aδ and unmyelinated C primary afferent fibres (Willis & Coggeshall, 1991). The SG also contains high densities of inhibitory interneurons (Furue *et al*., 2004). Whole-cell patch-clamp recordings from the SG of morphine-naïve CaMKIV-KO and WT mice showed no difference in the reduction of IPSCs upon morphine application. However, when dorsal horn slices where pretreated with morphine, the application of morphine during recording failed to induce a reduction in IPSCs in WT mice. A significant reduction still occurred in morphine-pretreated CaMKIV-KO mice, suggesting a role for CaMKIV in this form of synaptic opioid tolerance. These results further suggest that CaMKIV plays a role in the cellular adaptations that occur upon prolonged morphine exposure. Our results suggest that a presynaptic reduction in GABAergic transmission may modulate local inhibitory circuits in the spinal cord, which would then contribute to the analgesic affect of morphine.

Morphine increases the conductance of potassium channels through coupling with the $G_{i/o}$ family of G-proteins (North & Williams, 1985; Christie *et al*., 1987; Williams *et al*., 1988; Nestler, 1996). Increases in intracellular calcium due to this morphine-induced activation of potassium channels could also potentially lead to the activation of CaMKIV, which in turn phosphorylates CREB, leading to changes in gene expression. Future experiments are clearly needed to explore this possibility.

CaMKIV is expressed throughout the central nervous system and is unique in its ability to phosphorylate CREB in the nuclei of neurons (Soderling, 1999). Here, CaMKIV-KO mice are used to show the selective role of CaMKIV in the development of opioid tolerance but not dependence. Future work must elucidate the molecular signalling cascades that may be altered in CaMKIV-KO mice following morphine administration, and the role that CaMKIV plays in the CREB-mediated transcriptional changes after chronic morphine. These results suggest that the selective modulation of CaMKIV activity may increase the efficacy of morphine for the treatment of chronic pain.

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Abbreviations

ACSF, artificial cerebrospinal fluid; CaMKII, calcium–calmodulin-dependent protein kinase II; CaMKIV, calcium–calmodulin-dependent protein kinase IV; CRE, cAMP response-element; CREB, cAMP response-element binding protein; DAMGO, [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin; KO, knockout; MOR, μ-opioid receptor; MPE, maximal possible effect; pCREB, phosphorylated CREB; PKC, protein kinase C; SG, substantia gelatiosa; sIPSCs, spontaneous inhibitory postsynaptic currents; WT, wild-type.

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