Published in final edited form as:

J Neurosci Methods. 2011 July 15; 199(1): 62–68. doi:10.1016/j.jneumeth.2011.04.036.

Intrathecal PKA-selective siRNA treatment blocks sustained morphine-mediated pain sensitization and antinociceptive tolerance in rats

S Tumati¹, WR Roeske^{1,2,3}, TM Largent-Milnes¹, TW Vanderah^{1,4}, and EV Varga^{1,3,5,*}

¹Department of Pharmacology, The University of Arizona, Tucson, AZ 85724, USA

²Department of Medicine, The University of Arizona, Tucson, AZ 85724, USA

³The Sarver Heart Center, The University of Arizona, Tucson, AZ 85724, USA

⁴Department of Anesthesiology The University of Arizona, Tucson, AZ 85724, USA

⁵The BIO5 Institute, The University of Arizona, Tucson, AZ 85724, USA

Abstract

Sustained morphine treatment has been shown to produce paradoxical pain sensitization (opioid-induced hyperalgesia) and also causes increase in spinal pain neurotransmitter, such as calcitonin gene related peptide (CGRP), concentration in experimental animals. Studies have also shown that cyclic adenosine-monophosphate (cAMP)-dependent protein kinase (PKA) plays a major role in the regulation of presynaptic neurotransmitter (such as CGRP and substance P) synthesis and release. We have previously shown that in cultured primary sensory dorsal root ganglion (DRG) neurons sustained *in vitro* opioid agonist treatment upregulates cAMP levels (adenylyl cyclase (AC) superactivation) and augments basal and capsaicin evoked CGRP release in a PKA dependent manner. In the present study, we investigated the *in vivo* role of PKA in sustained morphine-mediated pain sensitization. Our data indicates that selective knock-down of spinal PKA activity by intrathecal (i.th) pretreatment of rats with a PKA-selective small interference RNA (siRNA) mixture significantly attenuates sustained morphine-mediated augmentation of spinal CGRP immunoreactivity, thermal hyperalgesia, mechanical allodynia and antinociceptive tolerance. The present findings indicate that sustained morphine-mediated activation of spinal cAMP/PKA-dependent signaling may play an important role in opioid induced hyperalgesia.

Keywords

Opioid; PKA; hyperalgesia; allodynia; analgesic tolerance

Introduction

It was found earlier that long-term opioid treatment leads to antinociceptive tolerance and causes a paradoxical sensitization (opioid-induced hyperalgesia) toward mildly painful (hyperalgesia) and normally innocuous (allodynia) stimuli. Prolonged morphine

*Eva V. Varga, Corresponding author, Department of Pharmacology, College of Medicine, University of Arizona, Tucson, AZ 85724, USA. Tel: +1 520 626 6801, Fax: +1 520 626 2204, evarga@email.arizona.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

administration was also found to upregulate pain neurotransmitter (such as calcitonin gene-related peptide (CGRP)) levels in primary sensory neurons (Ma et al., 2000) and enhances capsaicin-evoked CGRP release in the spinal cord of rats (Gardell et al., 2002). The cellular molecular mechanisms underlying sustained morphine treatment-mediated augmentation of spinal pain neurotransmitter release are currently not entirely clear. It is also not known whether the molecular mechanisms leading to sustained morphine-mediated pain sensitization and antinociceptive tolerance overlap (partially or completely).

Substantial earlier evidence indicates that sustained morphine treatment increases intracellular cAMP (cyclic adenosine-monophosphate) formation (cAMP overshoot) (Crain and Shen, 1998; Malatynska et al., 1996; Rubenzik et al., 2001; Yue et al., 2008; Tumati et al., 2009), leading to augmented cAMP dependent protein kinase A (PKA) activity (Chen et al., 1988) in cultured DRG neurons. It has been suggested that upregulation of cAMP-regulated signaling pathways may have an important role in the excitatory effects of chronic opioids in the central nervous system (CNS) (Sharma et al., 1975, 1977; Nestler and Aghajanian, 1977; Varga et al., 2003). It was also shown earlier that sustained treatment with opioid agonists prolongs action potential duration in primary sensory neurons in a PKA-dependent manner (Chen et al., 1988). Earlier studies have demonstrated that chemical PKA inhibitors attenuate opioid antinociceptive tolerance (Gabra et al., 2008). However, the role of the cAMP/PKA pathway in the development of opioid-induced hyperalgesia has not been established.

Activation of PKA may promote presynaptic pain neurotransmitter release via phosphorylation of neuronal ion channels, transcription factors and synaptic transport proteins (Fraser and Scott, 1999; Leenders and Sheng, 2005; Rathee et al., 2002). Indeed, we have demonstrated recently that chemical PKA inhibitors attenuate sustained morphine-mediated augmentation of both basal (Yue et al., 2008) and capsaicin evoked (Tumati et al., 2009) CGRP release from cultured neonatal rat primary sensory neurons *in vitro*.

Based on our earlier *in vitro* findings we hypothesize that sustained morphine-mediated cAMP overshoot in the primary sensory neurons (Yue et al., 2008; Tumati et al., 2009; Chen et al., 1988) and the consequent activation of PKA (Chen et al., 1988) plays an important role in both opioid antinociceptive tolerance and in sustained morphine-mediated paradoxical pain sensitization *in vivo*. In the present study, we have used a highly selective siRNA (small interfering RNA) knock-down method to reduce PKA protein levels in the spinal cord of rats. Intrathecal pretreatment with a PKA catalytic unit-selective siRNA significantly attenuated PKA activity in the spinal cord of rats, attenuated sustained morphine-mediated augmentation of spinal pain neurotransmitter (CGRP) immunoreactivity and prevented thermal hyperalgesia, mechanical allodynia and antinociceptive tolerance.

Methods

Intrathecal catheter implantation

Adult (200–225 g) male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were kept in a climate-controlled room on a 12 hr light/dark cycle with food and water available *ad libitum*. Handling, care, maintenance and testing of the animals were performed in accordance with the policies and recommendations of the International Association for the Study of Pain and the National Institutes of Health guidelines. The experimental protocols have been approved by the Animal Care and Use Committee of the University of Arizona.

The procedure for catheter implantation has been described earlier (Tumati et al., 2008, 2010). Briefly, rats (4–6 animals in each treatment group) were anesthetized by ketamine-

xylazine (100 mg/kg, i.p) and were implanted with 8cm polyethylene-10 tubing so that the catheter terminated in the lumbar region of the spinal cord (Yaksh and Rudy, 1976). The rats were allowed to recover for 7 days. Animals with motor weakness or paralysis were excluded from further experiments. Prior to drug treatment, baseline nociceptive latencies were determined and catheter patency was tested using physiological saline for each animal. The timeline of the full experimental protocol is shown in Fig 2.

Intrathecal siRNA administration

A rat PKA α subunit-specific siRNA mixture (Smart Pool # L-093299-01; Dharmacon Inc; Chicago, IL,) was dissolved in double distilled RNase-free water to obtain a 100 μ M stock solution and stored in aliquots at -80°C . We have chosen an siRNA Smart Pool mixture, since earlier studies indicated that pooling of multiple rationally designed siRNAs can significantly improve the degree of silencing (Karpilow et al., 2004). We have targeted the PKA α catalytic subunit since earlier investigations have demonstrated its presence in the spinal cord (Distler et al., 2003). On the day of the experiment, stock solution aliquots were mixed with the transfection reagent (i-Fect; Neuromics, Edina, MN) to achieve a final concentration of 2 μ g/10 μ l and were intrathecally administered to the appropriate rat groups once daily for 3 days. We selected 2 μ g/10 μ l siRNA dose as this dose was found to knock-down targeted protein levels without causing any noticeable behavioral toxicity (Tumati et al., 2010; Luo et al., 2005). In the vehicle control group the rats received 10 μ l of i-Fect reagent. Since in earlier investigations we have found that after cessation of the i.th. siRNA treatment, the targeted protein levels started to recover in the spinal cord from the 3rd day (Tumati et al., 2010), we continued the siRNA treatment on alternate days during the whole experimental protocol (see Fig 1).

Sustained morphine administration

After 3 day siRNA (PKA siRNA group) or transfection reagent (vehicle control group) pretreatment, the animals have been implanted with subcutaneous (s.c.) osmotic minipumps (Alza, Mountain view, CA) and received continuous s.c. morphine (45 nmol/ μ l/h) or saline (1 μ l/h) infusions for 7 days.

Testing for Thermal hyperalgesia

The method developed by Hargreaves (Hargreaves et al., 1988) was used to assess sensitivity of rats to a mildly noxious thermal stimulus (radiant heat) as described (Tumati et al., 2008). Paw withdrawal latencies were measured before (baseline), during and after drug (morphine or saline) administration (see Fig 1 for experimental design). The heat source was automatically turned off after 33 s in order to prevent tissue damage in the animals. All the treatment groups were indicated in Fig 1. Six individual animals were included under each treatment group.

Testing for Mechanical allodynia

Paw withdrawal thresholds in response to normally innocuous tactile stimuli were determined by applying von Frey filaments (0.4 –15.1 g) to the plantar surface of the right hind paw of the rats (Chaplan et al., 1994; Vanderah et al., 2000). Paw withdrawal thresholds (g) were measured using the “up-and-down” method before, during and after drug treatments (refer to Fig 1 for experimental design). The data were analyzed using a Dixon nonparametric test (Dixon, 1980). All the treatment groups were indicated in Fig 1. Six individual animals were included under each treatment group.

Testing for Antinociceptive tolerance

Nociceptive latencies were measured using the warm (52°C) water tail withdrawal assay, as described earlier (Vanderah et al., 2000). A cutoff time of 10 sec was used to prevent tissue injury. Morphine antinociceptive dose range (1, 3, 10 µg/5µl / 225 g) was selected based on earlier observations (Vanderah et al., 2000). In order to assess the development of antinociceptive tolerance, morphine dose response curves were measured before any drug administration (naïve control), after i.th. PKA-selective siRNA or transfection reagent pretreatment (siRNA- and vehicle-treated baselines) and after sustained (6 days) s.c. morphine or saline (saline control) infusions. Data were converted to % maximal possible effect (MPE), using the formula: %MPE = (response latency – baseline latency) / (cutoff – baseline latency) × 100. The animals were considered tolerant if they exhibited significant ($p < 0.05$) reduction in their tail-withdrawal latency in response to the naïve A₉₀ dose (the morphine dose (10 µg/5 µl) that produced 90% MPE in the naïve animals). The tolerant animals would show a considerable rightward shift in the acute (30 min) morphine dose-antinociceptive response. The A₉₀ dose was calculated in all the treatment groups by extrapolation of the dose-response curves. All the treatment groups were indicated in Fig 1. Five individual animals were included under each treatment group.

Spinal cord tissue preparation

After behavioral tests, the animals were deeply anesthetized with halothane and sacrificed by decapitation. The spinal columns were hydraulically extruded with ice-cold saline and the dorsal half of the lumbar cord was dissected on ice as described in (Gardell et al., 2002). The tissue samples were immediately frozen in liquid nitrogen and stored at –80°C until the time of the *in vitro* assays.

Measurement of spinal active PKA activity

Frozen lumbar spinal cord tissues from control and PKA siRNA-treated rats were thawed and homogenized in homogenization buffer as previously described (Gardell et al., 2002). After centrifugation (10,000 g, 30 min, 4°C), the supernatants were collected and total protein concentrations were determined by the Bradford method. A PKA kinase activity assay kit (Assay Designs, Ann Arbor, MI), that utilizes a synthetic peptide as a PKA substrate, and a polyclonal antibody that recognizes the phosphorylated form of the substrate was used to measure active PKA concentrations in the supernatants. The active PKA concentration in the samples was assessed according to the manufacturer's instructions, by measuring the absorbance of the final colorimetric reaction product at $\lambda=450$ nm. Standard PKA dose-absorbance curves (range 10–1000 pg/ml) were constructed using serial dilutions of purified PKA supplied in the kit. Active PKA content in the samples was calculated using the Graph Pad Prism 4.0 software (San Diego, CA).

Measurement of spinal CGRP immunoreactivity

Lumbar spinal cord sections were thawed, homogenized in a homogenization buffer and centrifuged, as previously described (Gardell et al., 2002). Protein concentration in the supernatants was determined by the Bradford method. A CGRP enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI) was used as described in (Tumati et al., 2009, 2010) to measure CGRP immunoreactivity in the samples. Standard curves were constructed using synthetic CGRP solutions and supernatant CGRP concentrations (pg/ml) were calculated using the Graph Pad Prism 4.0 software (San Diego, CA). Pair-wise comparisons between treatment groups were performed using the Student's *t*-test. Differences were considered significant at $p < 0.05$. Data are represented as mean±S.E.M, unless otherwise indicated.

Data analysis

Thermal and tactile hypersensitivity data were analyzed by two-way repeated measures ANOVA and/or two-way ANOVA (post hoc, Newman-Keuls) while, the measurement of CGRP content data and antinociceptive tolerance data were analyzed by nonparametric one-way ANOVA (post hoc, Newman-Keuls) using the GraphPad Prism 4.0 software. A nonparametric repeated measures one-way ANOVA (post hoc, Newman-Keuls) was also performed to compare the baselines to the entire time course within each treatment group (within subjects comparison for variation over time). Statistical differences were considered significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). Data were represented as mean $\pm$ S.E.M, unless otherwise indicated.

Results

The effect of intrathecal PKA-selective siRNA treatment on spinal PKA activity of rats

The PKA activity in the lumbar dorsal horn of control animals was 426 $\pm$ 43 pg/mg protein. Intrathecal pretreatment with a PKA-selective siRNA mixture (2 μ g/day for 3 consecutive days) reduced PKA activity by 75% (131 $\pm$ 10 pg/mg protein; *** $p < 0.001$ relative to control group; $n = 4$) (Fig 2) indicating that the PKA α subunit-selective siRNA treatment significantly reduced active PKA protein activity in the lumbar spinal cord of rats. On the contrary, the non-targeting siRNA has not produced any reduction in the PKA activity (unpublished observations) indicating that the PKA-selective siRNA mixture has not been structurally damaged or acted through a non-selective mechanism.

Intrathecal PKA-selective siRNA administration prevents sustained morphine-mediated augmentation of CGRP levels in the dorsal horn of the lumbar spinal cord

CGRP concentration in lumbar spinal cord dorsal horn homogenates was measured by enzyme immunoassay. Basal CGRP content in the lumbar dorsal horn tissue of vehicle saline-infused rats was 1770 $\pm$ 100 pg/mg protein. Sustained (6 d) morphine treatment augmented CGRP content by 130% (4080 $\pm$ 170 pg/mg, ** $p < 0.01$ relative to saline control group, one-way ANOVA, $n = 4$) (Fig 3). Intrathecal pre-treatment with a PKA-selective siRNA mixture significantly attenuated sustained (6 d) morphine-mediated augmentation of CGRP immunoreactivity in the dorsal horn of the lumbar spinal cord. CGRP immunoreactivity in PKA-selective siRNA-treated group that received morphine was 1970 $\pm$ 90 pg/mg protein ($p > 0.05$ relative to saline-treated control; $n = 4$) (Fig 3). The CGRP content in animals pretreated with PKA-selective siRNA and receiving saline infusion (1870 $\pm$ 90 pg/mg) was similar to the basal CGRP value in the vehicle-pre-treated saline-infused animals ($p > 0.05$, oneway ANOVA, $n=4$). Our earlier experiments have shown that intrathecal pretreatment of rats with a non-targeting dsRNA has no effect on spinal CGRP content (Tumati et al., 2010). These data indicate the role of PKA in the regulation of pain neurotransmitter synthesis and/or release in the dorsal horn of the lumbar spinal cord of rats.

Intrathecal PKA-selective siRNA pretreatment attenuates sustained morphine-induced thermal hyperalgesia

Intrathecal catheter implantation caused no significant change in the sensitivity of the animals toward mildly noxious heat stimulus (post-surgery (pre-infusion) baseline paw withdrawal latencies (22.3 $\pm$ 0.3 s) were similar to the pre-surgery baseline of 23.5 $\pm$ 1 s ($p > 0.05$, $n=6$) (Fig 4). Intrathecal pre-treatments with either the vehicle (22.2 $\pm$ 1 s), or the PKA-selective siRNA (21.5 $\pm$ 1 s) alone did not alter response thresholds in rats receiving sustained saline-infusions for 7 days ($p > 0.05$ relative to pre-infusion baseline, two-way ANOVA, $n=6$). Paw withdrawal latencies remained similar in the above groups throughout the 7-day time period ($p > 0.05$ within each group, one-way repeated measures ANOVA, $n=6$) (Fig 4).

Acute (6 h) morphine (45 nmol/ μ l/h) administration significantly increased radiant heat paw-withdrawal latencies in vehicle-pre-treated rats (31.2 ± 1 s; $**p < 0.01$ relative to the pre-infusion baseline of 22.3 ± 0.3 s, one-way repeated measures ANOVA, $n=6$). PKA-selective siRNA pre-treatment had no effect on the acute antinociceptive effectiveness of morphine (paw withdrawal latencies after 6 h of s.c. morphine infusion was 32 ± 2 s; $**p < 0.01$ relative to the vehicle saline-infused group, one-way repeated measures ANOVA, $n=6$) (Fig 4). Sustained morphine administration led to a progressive decline in the apparent antinociceptive effect of morphine in vehicle pre-treated animals (17.4 ± 2.0 s; $*p < 0.05$ in each group relative to paw withdrawal latency in the vehicle saline-infused group of 22 ± 1 s, two-way ANOVA, $n=6$) (Fig 4). Finally, longer (>2 days) sustained s.c. morphine infusion led to a gradual development of thermal hypersensitivity in vehicle pre-treated animals ($p < 0.05$ within each group, one-way repeated measures ANOVA, $n=6$). Thermal hyperalgesia in these animals was detectable after 3 days of morphine treatment (Fig 4) and reached maximal level (10 ± 2 s respectively; $**p < 0.01$ relative to the vehicle saline-infused group, two-way ANOVA) on day 6 of morphine infusion (Fig 4). Interestingly, intrathecal pre-treatment of the rats with the PKA catalytic unit (PKA α)-selective siRNA mixture completely blocked the development of thermal hypersensitivity upon sustained morphine treatment. Thus, paw withdrawal latencies in the PKA siRNA pre-treated animals were not significantly different from the pre-infusion baseline even after 6 days of sustained morphine infusion (23.1 ± 1 s, $p > 0.05$ relative to the pre-infusion baseline of 22.3 ± 0.3 s, two-way ANOVA, $n=6$) (Fig 4).

Intrathecal PKA-selective siRNA pretreatment blocks sustained morphine-induced mechanical allodynia

Sustained administration of morphine by subcutaneous infusion through an osmotic pump (45 nmol/ μ l/h) produced tactile allodynia in vehicle-pre-treated rats. Baseline paw withdrawal thresholds (14.7 ± 0.3 g; $p > 0.05$) were similar to the pre-infusion baseline (14.4 ± 1 g; $p > 0.05$) (Fig 5). Intrathecal pre-treatments with either the vehicle (12 ± 2 g), or the PKA-selective siRNA (12 ± 1 g) alone did not alter response thresholds in rats receiving sustained saline-infusions for 6 days ($p > 0.05$ relative to pre-infusion baseline, two-way ANOVA) (Fig 5). In addition, the paw withdrawal thresholds remained similar within the above groups throughout the 7-day time period (unpublished observations).

Sustained (6 d) continuous s.c. morphine (45 nmol/ μ l/h) administration to vehicle pretreated rats also led to a gradual increase in their sensitivity to innocuous mechanical stimuli (tactile allodynia). Thus, on day 6 of continued morphine infusion, paw withdrawal threshold in the von Frey filament test was significantly lower (4 ± 1 g) in vehicle pretreated/morphine infused animals as compared to 12 ± 2 g in the vehicle pretreated/saline-infused animals ($**p < 0.01$, one-way repeated measures ANOVA, $n=6$) (Fig 5).

Intrathecal PKA-selective siRNA pretreatment significantly attenuated sustained morphine-mediated tactile allodynia (paw withdrawal threshold in the siRNA pretreated group was 10 ± 1 g after 6 days of morphine treatment; $**p < 0.01$ relative to the morphine group, one-way repeated measures ANOVA, $n=6$). Intrathecal administration of PKA-selective siRNA did not produce a significant alteration in the response threshold in saline-infused animals (11 ± 2 g on day 6 of saline infusion) (Fig 5). These data indicate an important role for PKA in the development of sustained morphine-mediated tactile allodynia.

Intrathecal PKA-selective siRNA treatment blocks the development of morphine antinociceptive tolerance

Acute morphine dose-antinociceptive response curves were measured after 7 days of continuous systemic morphine or saline infusions in vehicle and PKA siRNA pretreated rats

using the warm (52°C) water tail-flick nociceptive test. Acute intrathecal morphine injections (1 µg/5 µl, 3 µg/5 µl, 10 µg/5 µl) produced antinociception in a dose dependent manner with an A₉₀ value of 10 µg (10 µg dose produced 90% antinociception) in vehicle-pretreated saline-infused animals). Sustained (6 days) systemic morphine (45 nmol/µl/h) infusion caused a rightward shift in the dose-response curve, with the previous A₉₀ dose causing only 20±1% MPE (***p* < 0.01 relative to vehicle-pretreated saline-infused rats, one-way ANOVA, n=5) (Fig 6), indicating that a tolerance developed to the antinociceptive effect of morphine.

Intrathecal pre-treatment of PKA- selective siRNA had no effect on the antinociceptive efficacy of acute i.th morphine in saline infused animals (89±2% of MPE at the naive A₉₀ dose) (Fig 6). Interestingly however, the intrathecal PKAselective siRNA pre-treatment greatly attenuated sustained morphine-mediated rightward shift in the acute i.th morphine dose-response curve. Thus, re-challenge with the naive A₉₀ dose (10 µg/5µl) produced 93±2% antinociception in the PKA-selective siRNA pre-treated rats (***p* < 0.01 relative to vehicle pre-treated-morphine infused rats, one-way ANOVA, n=5). Thus, Intrathecal pre-treatment with PKA-selective siRNA significantly attenuated the development of antinociceptive tolerance in the morphine-infused group (Fig 6).

Discussion

In the present study, we have used a highly selective PKA siRNA knockdown method to show that sustained opioid-mediated pain hypersensitivity and antinociceptive tolerance share a common, PKA-dependent molecular mechanism. We demonstrate that intrathecal pretreatment with a PKAα subunit-selective siRNA mixture significantly reduces PKA activity in the lumbar spinal cord of rats (Fig 2) and attenuates sustained morphine-mediated a/augmentation of CGRP immunoreactivity in the lumbar dorsal horn of the spinal cord (Fig 3); b/ thermal hyperalgesia, c/ tactile allodynia (Figs 4–5) and d/ antinociceptive tolerance (Fig 6).

Sustained morphine treatment produces antinociceptive tolerance and paradoxical pain sensitivity (thermal hyperalgesia and mechanical allodynia) in experimental animals as well as in human patients (Gardell et al., 2006; Ossipov et al., 2005, 2003). Previous studies have suggested that the long-term opioid agonist-mediated antinociceptive tolerance might be the result of augmented pain sensitivity. However, it is presently not clear whether these processes are mediated (entirely or partially) by the same molecular mechanism(s). Studies have also shown that sustained morphine treatment significantly increases pain neurotransmitter (such as Substance P and CGRP) immunoreactivity in the spinal dorsal horn of rats (Gardell et al., 2002; Ossipov et al., 2003; Ma et al., 2001). Such augmentation of spinal pain neurotransmitter concentration may underlie sustained opioid-induced paradoxical pain sensitization (Gardell et al., 2002). In the present study, we tested the role of sustained morphine-mediated intracellular compensatory adaptations in the upregulation of spinal pain neurotransmission.

Sustained opioid exposure was found to upregulate cAMP formation in primary sensory neurons (Crain and Shen, 1998; Yue et al., 2008; Tumati et al., 2009). The main cellular effector of cAMP is cAMP-dependent protein kinase A (PKA). Activated PKA was shown to augment presynaptic neurotransmitter synthesis and vesicular transport by phosphorylating key transcription factors (such as CREB) and synaptic vesicle proteins (such as snapin) (Koppert, 2004; King et al., 2005). In addition, activated PKA also regulates neuronal excitability by phosphorylation of sodium channels and voltage-sensitive as well as ligand-gated calcium channels (Fraser and Scott, 1999; Rathee et al., 2002; Alberts et al., 2007; Blackstone et al., 2004) leading to facilitation of presynaptic

neurotransmitter release. Indeed, we have found recently that chemical PKA inhibitors (H-89 and PKI (14–22) amide) attenuate sustained morphine-mediated augmentation of capsaicin-evoked (Tumati et al., 2009) CGRP release from cultured primary sensory neurons *in vitro*. Liou et al. (2007) has shown that pretreatment with PKA inhibitors attenuated hyperalgesia and allodynia induced by spinal capsaicin injection in mice. In addition, pretreatment with PKA inhibitor also prevented the development of morphine tolerance in mice (Gabra et al., 2008). Based on these data, we hypothesized that sustained morphine-mediated cAMP overshoot and the resulting PKA activation may have a role *in vivo*, in the development of thermal hypersensitivity after sustained morphine treatment. As the use of siRNA knock-down methods in pain research has been successful as described by Röhl and Kurreck (2006), we have chosen the highly selective siRNA knockdown method to reduce PKA protein levels in the spinal cord of rats. Indeed, our current data demonstrates that selective knock-down of spinal PKA activity by intrathecal PKA-selective siRNA pretreatment completely attenuated sustained morphine-mediated thermal hyperalgesia (Fig 4).

Inflammation and tissue damage also leads to a sensitization toward innocuous mechanical stimuli (tactile allodynia). Peripheral sensitization at the primary sensory neuron level may also contribute to the development of tactile allodynia (De Petrocellis et al., 2001). Interestingly, it was found earlier that the mechanical allodynia produced by intraplantar injection of the inflammatory mediator, PGE₂, is antagonized by both adenylyl cyclase (Lopshire and Nicol, 1998) and PKA (Lopshire and Nicol, 1998; Mohapatra and Nau, 2005) inhibitors. It was also shown earlier that PKA activation in the primary sensory neurons is especially important in the introductory phase of inflammatory hypernociception - whereas in the maintenance phase other protein kinases (such as PKC ϵ) also play a role (Lopshire and Nicol, 1998). Since it was shown that inflammation and sustained morphine-treatment may lead to similar neuronal adaptations (Gardell et al., 2006), in the present work, we tested the role of PKA in sustained morphine treatment-mediated mechanical allodynia. Our data show that intrathecal pretreatment with a PKA-selective siRNA significantly attenuates sustained morphine-mediated mechanical allodynia (Fig 5). Finally, since our earlier data indicate that sustained morphine-treatment of cultured neonatal rat primary sensory neurons also upregulates basal CGRP release from these cells in a PKA-dependent manner (Yue et al., 2008), we hypothesize that PKA may also play a role in chronic morphine-mediated spontaneous pain sensations (Gardell et al., 2006; Ossipov et al., 2005, 2003) in human patients.

It was suggested that Opioid-induced hyperalgesia may contribute to the development of antinociceptive tolerance (Gardell et al., 2006; Ossipov et al., 2005; Ma et al., 2001; Yang and Gereau, 2002). However presently it is not entirely clear whether opioid-induced hyperalgesia and antinociceptive tolerance share the same molecular mechanism(s). It has been established earlier that chemical PKA and PKC inhibitors attenuate the development of morphine tolerance in mice (Gabra et al., 2008). Our current data also indicates that intrathecal pretreatment of the rats with a PKA-selective siRNA mixture completely abolishes long-term morphine-mediated rightward shift of the acute morphine dose-nociceptive response curves (Fig 6). Therefore, we propose that sustained morphine-mediated AC superactivation and the ensuing PKA activation in primary sensory neurons plays a trigger role in both sustained morphine mediated paradoxical pain sensitization and in antinociceptive tolerance.

Previously, we have found sustained morphine-mediated AC superactivation is dependent on Raf-1 activation in cultured primary sensory neurons. Accordingly, we have shown recently (Tumati et al., 2010) that knock-down of spinal Raf-1 protein levels by intrathecal Raf-1-specific siRNA treatment significantly attenuates both sustained morphine-mediated

paradoxical pain sensitization and antinociceptive tolerance in rats. Taken together, our previous and present data indicate that sustained morphine-mediated pain sensitization and antinociceptive tolerance may share a common Raf-1/cAMP/PKA-dependent molecular mechanism (see Fig.7). Consequently, we conclude that selective inhibition of either sustained morphine mediated cAMP overshoot (Raf-1 inhibitors) or the resulting PKA activation (PKA inhibitors) in the spinal cord may prevent the development of both paradoxical pain sensitization and antinociceptive tolerance during sustained morphine treatment and thus, increase the efficacy and duration of morphine antinociception in chronic pain treatment.

Acknowledgments

Grants: This work was supported by grants from the National Institute of Health (DA 06284, GM 065465).

References

- Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, JD. Cell signaling. In: Chapman, Hall, editor. *Molecular Biology of the Cell*. second ed.. Garland Publishing Inc.; New York: 2007. p. 681-726.
- Blackstone C, Murphy TH, Moss SJ, Baraban JM, Huganir RL. Cyclic AMP and synaptic activity dependent phosphorylation of AMP preferring glutamate receptors. *J Neurosci*. 2004; 14:7585–7593. [PubMed: 7527845]
- Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods*. 1994; 53:55–63. [PubMed: 7990513]
- Chen GG, Chalazonitis A, Shen KF, Crain SM. Inhibitor of cyclic AMP-dependent protein kinase blocks opioid-induced prolongation of the action potential of mouse sensory ganglion neurons in dissociated cell cultures. *Brain Res*. 1988; 462:372–377. [PubMed: 2847853]
- Crain SM, Shen KF. Modulation of opioid analgesia, tolerance and dependence by Gs-coupled, GM1 ganglioside-regulated opioid receptor functions. *Trends in Pharm Sci*. 1998; 19:358–365. [PubMed: 9786023]
- De Petrocellis L, Harrison S, Bisogno T, Tognetto M, Brandi I, Smith GD, Creminon C, Davis JB, Geppetti P, Di Marzo V. The vanilloid receptor (VR1)-mediated effects of anandamide are potently enhanced by the cAMP-dependent protein kinase. *J Neurochem*. 2001; 77:1660–1663. [PubMed: 11413249]
- Distler C, Rathee PK, Lips KS, Obreja O, Neuhuber W, Kress M. Fast Ca²⁺-induced potentiation of heat-activated ionic currents requires cAMP/PKA signaling and functional AKAP anchoring. *J Neurophysiol*. 2003; 89:2499–2505. [PubMed: 12740405]
- Dixon WJ. Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol*. 1980; 20:441–462. [PubMed: 7387124]
- Fraser IDC, Scott JD. Modulation of Ion Channels: A Current View of AKAPs. *Neuron*. 1999; 23:423–426. [PubMed: 10433254]
- Gabra BH, Bailey CP, Kelly E, Smith FL, Henderson G, Dewey WL. Pre-treatment with a PKC or PKA inhibitor prevents the development of morphine tolerance but not physical dependence in mice. *Brain Res*. 2008; 1217:70–77. [PubMed: 18501877]
- Gardell LR, Wang R, Burgess SE, Ossipov MH, Vanderah TW, Malan TP Jr, Lai J, Porreca F. Sustained morphine exposure induces a spinal dynorphin-dependent enhancement of excitatory transmitter release from primary afferent fibers. *J. Neurosci*. 2002; 22:6747–6755. [PubMed: 12151554]
- Gardell LR, King T, Ossipov MH, Rice KC, Lai J, Vanderah TW, Porreca F. Opioid receptor-mediated hyperalgesia and antinociceptive tolerance induced by sustained opiate delivery. *Neuroscience Letters*. 2006; 396:44–49. [PubMed: 16343768]
- Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain*. 1988; 32:77–88. [PubMed: 3340425]

- Karpilow J, Leake D, Marshall B. siRNA: Enhanced functionality through rational design and chemical modification. *PharmaGenomics*. 2004;32–40.
- King T, Ossipov MH, Vanderah TW, Porreca F, Lai F. Is Paradoxical Pain Induced by Sustained Opioid Exposure an Underlying Mechanism of Opioid Antinociceptive Tolerance? *Neurosignals*. 2005; 14:194–205. [PubMed: 16215302]
- Koppert W. Opioid-induced hyperalgesia. Pathophysiology and clinical relevance. *Anaesthesist*. 2004; 53:455–466. [PubMed: 15034638]
- Leenders AG, Sheng ZH. Modulation of neurotransmitter release by the second messenger-activated protein kinases: implications for presynaptic plasticity. *Pharmacol Ther*. 2005; 105:69–84. [PubMed: 15626456]
- Liou J, Liu F, Hsin S, Yang C, Lui P. Inhibition of the Cyclic Adenosine Monophosphate Pathway Attenuates Neuropathic Pain and Reduces Phosphorylation of Cyclic Adenosine Monophosphate Response Element-Binding in the Spinal Cord After Partial Sciatic Nerve Ligation in Rats. *Anesth Analg*. 2007; 105:1830–1837. [PubMed: 18042889]
- Lopshire JC, Nicol GD. The cAMP transduction cascade mediates the prostaglandin E2 enhancement of the capsaicin-elicited current in rat sensory neurons: whole-cell and single-channel studies. *J Neurosci*. 1998; 18:6081–6092. [PubMed: 9698303]
- Luo MC, Zhang DQ, Ma SW, Huang YY, Shuster SJ, Porreca F, Lai J. An efficient intrathecal delivery of small interfering RNA to the spinal cord and peripheral neurons. *Mol Pain*. 2005; 28:1–29.
- Ma W, Zheng WH, Kar S, Quirion R. Morphine treatment induced calcitonin gene-related peptide and substance P increases in cultured dorsal root ganglion neurons. *Neuroscience*. 2000; 99:529–539. [PubMed: 11029544]
- Ma W, Zheng WH, Powell K, Jhamandas K, Quirion R. Chronic morphine exposure increases the phosphorylation of MAP kinases and the transcription factor CREB in dorsal root ganglion neurons: an in vitro and in vivo study. *Eur J Neurosci*. 2001; 14:1091–1104. [PubMed: 11683901]
- Malatynska E, Wang Y, Knapp RJ, Waite S, Calderon S, Rice K, Hruby VJ, Yamamura HI, Roeske WR. Human delta opioid receptor: functional studies on stably transfected Chinese hamster ovary cells after acute and chronic treatment with the selective nonpeptidic agonist SNC-80. *J Pharmacol Exp Ther*. 1996; 278:1083–1089. [PubMed: 8819489]
- Mohapatra DP, Nau C. Regulation of Ca²⁺-dependent desensitization in the vanilloid receptor TRPV1 by calcineurin and cAMP-dependent protein kinase. *J Biol Chem*. 2005; 280:13424–13432. [PubMed: 15691846]
- Nestler E, Aghajanian GK. Molecular and cellular basis of addiction. *Science*. 1977; 278:58–63. [PubMed: 9311927]
- Ossipov MH, Lai J, Vanderah TW, Porreca F. Induction of pain facilitation by sustained opioid exposure: relationship to opioid antinociceptive tolerance. *Life Sci*. 2003; 73:783–800. [PubMed: 12801599]
- Ossipov MH, Lai J, King T, Vanderah TW, Porreca F. Underlying mechanisms of pronociceptive consequences of prolonged morphine exposure. *Biopolymers*. 2005; 80:319–324. [PubMed: 15795927]
- Rathee PK, Distler C, Obreja O, Neuhuber W, Wang GK, Wang SY, Nau C, Kress M. PKA/AKAP/VR-1 module: A common link of Gs-mediated signaling to thermal hyperalgesia. *J Neurosci*. 2002; 22:4740–4745. [PubMed: 12040081]
- Richardson JD, Vasko MR. Cellular mechanisms of neurogenic inflammation. *J Pharmacol Exp Ther*. 2002; 302:839–845. [PubMed: 12183638]
- Röhl T, Kurreck J. RNA interference in pain research. *J Neurochem*. 2006; 99:371–380. [PubMed: 17029593]
- Rubenzik M, Varga E, Stropova D, Roeske WR, Yamamura HI. Expression of alpha-transducin in Chinese hamster ovary cells stably transfected with the human delta-opioid receptor attenuates chronic opioid agonist-induced adenylyl cyclase superactivation. *Mol Pharmacol*. 2001; 60:1076–1082. [PubMed: 11641436]
- Sharma SK, Klee WA, Nirenberg M. Dual regulation of adenylyl cyclase accounts for narcotic dependence and tolerance. *Proc Natl Acad Sci USA*. 1975; 72:3092–3096. [PubMed: 1059094]

- Sharma SK, Klee WA, Nirenberg M. Opiate-dependent modulation of adenylate cyclase. *Proc Natl Acad Sci USA*. 1977; 74:3365–3369. [PubMed: 269396]
- Tan CM, Kelvin DJ, Litchfield DW, Ferguson SSG, Feldman RD. Tyrosine kinase-mediated serine phosphorylation of adenylyl cyclase. *Biochemistry*. 2001; 40:1702–1709. [PubMed: 11327830]
- Tumati S, Largent-Milnes T, Yamamura HI, Vanderah TW, Roeske WR, Varga EV. Intrathecal Raf-1-selective siRNA attenuates sustained morphine-mediated thermal hyperalgesia. *Eur J Pharmacol*. 2008; 601:207–208. [PubMed: 18976650]
- Tumati S, Yamamura HI, Vanderah TW, Roeske WR, Varga EV. Sustained morphine treatment augments capsaicin evoked CGRP release in a PKA and Raf-1 dependent manner. *J Pharmacol Exp Ther*. 2009; 330:810–817. [PubMed: 19491327]
- Tumati S, Roeske WR, Largent-Milnes T, Wang R, Vanderah TW, Varga EV. Sustained morphine-mediated pain sensitization and antinociceptive tolerance are blocked by intrathecal treatment with Raf-1-selective siRNA. *Br J Pharmacol*. 2010; 161:51–64. [PubMed: 20718739]
- Vanderah TW, Gardell LR, Burgess SE, Ibrahim M, Dogrul A, Zhong CM, Zhang ET, Malan TP Jr, Ossipov MH, Lai J, Porreca F. Dynorphin promotes abnormal pain and spinal opioid antinociceptive tolerance. *J Neurosci*. 2000; 20:7074–7079. [PubMed: 10995854]
- Varga EV, Yamamura HI, Rubenzik MK, Stropova D, Navratilova E, Roeske WR. Molecular mechanisms of excitatory signaling upon chronic opioid agonist treatment. *Life Sci*. 2003; 74:299–311. [PubMed: 14607258]
- Yaksh TL, Rudy TA. Chronic catheterization of the spinal subarachnoid space. *Physiol Behav*. 1976; 17:1031–1036. [PubMed: 14677603]
- Yang D, Gereau RW. Peripheral group II metabotropic glutamate receptors (mGluR2/3) regulate prostaglandin E2-mediated sensitization of capsaicin responses and thermal nociception. *J Neurosci*. 2002; 22:6388–6393. [PubMed: 12151517]
- Yue X, Tumati S, Navratilova E, Stropova D, St John PA, Roeske WR, Vanderah TW, Yamamura HI, Varga EV. Sustained morphine treatment augments basal CGRP release from cultured primary sensory neurons in a Raf-1 dependent manner. *Eur J Pharmacol*. 2008; 584:272–277. [PubMed: 18328477]

Research Highlights

In the present study, we demonstrate that

- Intrathecal pretreatment with a PKA α subunit-selective siRNA mixture significantly reduces PKA activity in the lumbar spinal cord of rats.
- Intrathecal pretreatment with a PKA α subunit-selective siRNA mixture significantly attenuates sustained morphine-mediated augmentation of CGRP immunoreactivity in the lumbar dorsal horn of the spinal cord in rats.
- Intrathecal pretreatment with a PKA α subunit-selective siRNA mixture significantly attenuates sustained morphine-mediated thermal hyperalgesia, tactile allodynia and antinociceptive tolerance in rats.

Fig. 1. Experimental design

A) All the rats used for the study underwent intrathecal catheterization, followed by a 7-day recovery period. B) On the eighth day, represented as Day -3 (D -3), all the animals were pre-baselined for paw withdrawal latencies (Radiant heat test), paw withdrawal thresholds (VonFrey filament test) and tail flick latencies (Tail Flick test). C) After pre-baselining, the animals received once-daily injections (intrathecal) of vehicle (transfection reagent, 10 μ l) or PKA siRNA (2 μ g/10 μ l) for 3 days until Day -1. D) On Day 0, all the animals were post-baselined followed by osmotic minipump implantation into the subcutaneous space closer to the thoracic region on the dorsal side. Saline (1 μ l/h) [vehicle-saline and PKA siRNA-saline groups] or morphine (45 nmol/ μ l/h) [vehicle-morphine and PKA siRNA-morphine groups] was delivered for 7 days through the minipumps. E) All of the animals were checked for paw withdrawal latency and threshold 6 hours (one-fourth day) after saline or morphine pump implantation. Acute antinociception was measured after 6h. F) From Day 1 to Day 6, the animals received vehicle or PKA siRNA every other day. Paw withdrawal latencies and thresholds, tail flick latencies were measured once daily. Finally, on Day 6, the animals were challenged with three doses of morphine (1, 3, 10 μ g per 10 μ l) and acute nociception was recorded using tail flick test. The remaining animals (n=4 per group) were sacrificed for measurement of CGRP content in their spinal cords. None of the animals died through out the experimental procedure.

Fig 2. Intrathecal PKA-selective siRNA treatment significantly reduces PKA activity in the lumbar spinal cord of rats

Male Sprague-Dawley rats received intrathecal injections of 2 µg / 10 µl PKA specific siRNA once for 3 consecutive days. After 3 days of vehicle or siRNA treatment, lumbar spinal cord sections were harvested and PKA activity was determined by measuring the phosphorylated PKA substrate immunoreactivity in tissue homogenates by ELISA (Assay Designs, Ann Arbor, MI). Phosphorylated PKA substrate immunoreactivity was 426±43 pg/mg of soluble protein in the lumbar dorsal horn tissue of control, vehicle treated animals. Intrathecal pretreatment with a PKA-selective siRNA mixture (2 µg/day for 3 consecutive days) reduced PKA substrate phosphorylation by 75% in rat lumbar spinal cord homogenates (131±10 pg/mg protein; *** $p < 0.001$ relative to control group; $n = 4$).

Fig 3. Intrathecal PKA selective siRNA treatment attenuates sustained morphine-mediated augmentation of CGRP immunoreactivity in the lumbar dorsal horn

After i.th. pretreatment with vehicle (control) or PKA-selective siRNA mixture, male Sprague Dawley rats received continuous subcutaneous (s.c.) morphine (45 nmol/μl/h) (dark bars) or saline (open bars) infusion for 6 days. CGRP content in the supernatants of lumbar dorsal horn homogenates was measured using a rat CGRP EIA kit. Basal CGRP content in the lumbar dorsal horn tissue of vehicle saline-infused rats was 1770 ± 100 pg/mg protein. Sustained (6 d) morphine treatment augmented CGRP content by 130% ($**p < 0.01$ relative to control group, one-way ANOVA, $n = 4$). CGRP immunoreactivity in PKA-selective siRNA-treated group on the other hand, was not significantly different from control ($p > 0.05$; $n = 4$). The CGRP content in animals pretreated with PKA-selective siRNA was similar to the basal value ($p > 0.05$, one-way ANOVA, $n=4$).

Fig 4. Sustained morphine-induced thermal hypersensitivity is blocked by intrathecal PKA-selective siRNA pretreatment

After intrathecal pretreatment with vehicle or PKA-selective siRNA mixture, male Sprague Dawley rats received continuous s.c. saline or morphine (45 nmol/ μ l/h) infusion for 6 d. Paw withdrawal latencies (PWL) in response to radiant heat applied to the plantar surface of the hindpaw of the rats were determined 6 h after osmotic minipump implantation (acute antinociception) and afterwards, once daily until day 6. Post-surgery baseline paw withdrawal latencies (22.3 ± 0.3 s) of the animals were similar to the pre-surgery baseline ($p > 0.05$, $n=6$). Intrathecal pre-treatments with PKA-selective siRNA alone did not alter PWL ($p > 0.05$ relative to pre-infusion baseline, two-way ANOVA, $n=6$). Acute (6 h) morphine (45 nmol/ μ l/h) administration significantly increased radiant heat paw-withdrawal latencies in vehicle-pre-treated rats (31.2 ± 1 s; $**p < 0.01$ relative to the pre-infusion baseline of 22.3 ± 0.3 s, one-way repeated measures ANOVA, $n=6$). PKA-selective siRNA pre-treatment had no effect on the acute antinociceptive effectiveness of morphine ($**p < 0.01$ relative to the vehicle saline-infused group, one-way repeated measures ANOVA, $n=6$). Sustained morphine administration led to a progressive decline in the apparent antinociceptive effect of morphine. Thermal hyperalgesia was detectable after 3 days of morphine treatment and reached maximal level (10 ± 2 s; $**p < 0.01$ relative to the vehicle saline-infused group, two-way ANOVA) on day 6 of morphine infusion. The paw withdrawal latencies in the PKA siRNA pre-treated animals were not significantly different from baseline (23.1 ± 1 , day 6; $p > 0.05$ relative to baseline, two-way ANOVA, $n=6$).

Fig 5. Intrathecal PKA-selective siRNA pretreatment blocks sustained morphine-mediated mechanical allodynia

Male Sprague Dawley rats received intrathecal vehicle or PKA-selective siRNA injections for 3 days. After the pretreatment the animals received continuous saline (open bars) or morphine (45 nmol/ μ l/h) (dark bars) infusion for 6 days (refer to Fig 2 for drug treatment schedule). Paw withdrawal thresholds (g) were measured for 6 days after continuous s.c. morphine or saline infusion. Baseline paw withdrawal thresholds (14.7 ± 0.3 g; $n=24$) were similar to the pre-infusion baseline ($p > 0.05$, $n=24$). Intrathecal pre-treatments with vehicle or PKA-selective siRNA (12 ± 1 g) alone did not alter response threshold ($p > 0.05$ relative to pre-infusion baseline, two-way ANOVA, $n=6$ per group). Sustained s.c.morphine (45 nmol/ μ l/h) administration led to a gradual increase in their paw withdrawal threshold. Thus, on day 6 of continued morphine infusion, paw withdrawal threshold was (4 ± 1 g; $**p < 0.01$ relative to saline treated control, one-way repeated measures ANOVA, $n=6$). Intrathecal PKA-selective siRNA pretreatment significantly attenuated sustained morphine-mediated tactile allodynia (10 ± 1 g after 6 days of morphine treatment; $**p < 0.01$ relative to the morphine group, one-way repeated measures ANOVA, $n=6$). Intrathecal administration of PKA-selective siRNA alone did not produce a significant alteration in the response threshold (11 ± 2 g on day 6 of saline infusion; $p > 0.05$ relative to the morphine group, one-way repeated measures ANOVA, $n=6$).

Fig 6. Intrathecal PKA-selective siRNA treatment blocks the development of morphine antinociceptive tolerance

Male Sprague Dawley rats were pretreated i.th. with vehicle (inverted triangle) or PKA-selective siRNA (circle) for 3 days. After the pretreatments, the animals received continuous saline (open symbols, error bars within the symbol) or morphine (45 nmol/μl/h) (closed symbols, error bars within the symbol) infusion for 6 days, with continued i.th. siRNA or vehicle injections on alternate days. a rightward shift in the dose-response curve, with the previous A₉₀ dose causing only 20±1% MPE (***p* < 0.01 relative to control, one-way ANOVA, n=5). Intrathecal PKAselective siRNA pre-treatment greatly attenuated sustained morphine-mediated rightward shift in the morphine dose-response curve. Thus, re-challenge with the naive A₉₀ dose (10 μg/5μl) produced 93±2% antinociception in the PKA-siRNA pre-treated rats (***p* < 0.01 relative to vehicle pre-treated morphine-infused rats, one-way ANOVA, n=5).

Fig 7. The putative molecular mechanism underlying sustained morphine mediated pain sensitization

Stimulation of the opioid receptor by an opioid agonist (morphine) liberates G protein $\beta\gamma$ subunits in the primary sensory DRG neurons. The free G protein $\beta\gamma$ subunits interact with multiple effectors, leading to the activation of Raf-1 through multiple parallel pathways (Varga et al., 2003). Activated Raf-1 phosphorylates and sensitizes adenylyl cyclase(s) (Tan et al., 2001), leading to the upregulation of cellular cAMP (cAMP overshoot) concentration. Increased cAMP formation leads to activation of cAMP-dependent protein kinase (PKA). PKA, by means of phosphorylation of sodium channels, calcium channels, TRPV1 channels, transcription regulatory proteins and vesicular trafficking proteins (Leenders and Sheng, 2005; Richardson and Vasko, 2002; Rathee et al., 2002) augments CGRP synthesis and release in primary sensory neurons. CGRP released from the central termini of the sensory neurons activates secondary dorsal horn neurons that in turn, convey pain sensation to the brain.