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The role of spinal neuropeptides and prostaglandins in opioid physical dependence

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1 This study examined the role of spinal calcitonin gene-related peptide (CGRP), substance P, and prostaglandins in the development and expression of opioid physical dependence.

2 Administration of escalating doses (5–100 mg kg^{-1}, i.p.) of morphine for 7 days markedly elevated CGRP and substance P^{-} immunoreactivity in the dorsal horn of the rat spinal cord. Naloxone (2 mg kg^{-1}, i.p.) challenge decreased both CGRP and substance P immunoreactivity and precipitated a robust withdrawal syndrome.

3 Acute intrathecal pre-treatment with a CGRP receptor antagonist, $CGRP_{8-37}$ (4, 8 μg), a substance P receptor antagonist, SR 140333 (1.4, 2.8 μg), a cyclo-oxygenase (COX) inhibitor, ketorolac (30, 45 μg), and COX-2 selective inhibitors, DuP 697 (10, 30 μg) and nimesulide (30 μg), 30 min before naloxone challenge, partially attenuated the symptoms of morphine withdrawal. $CGRP_{8-37}$ (8 μg), but no other agents, inhibited the decrease in CGRP immunoreactivity.

4 Chronic intrathecal treatment with $CGRP_{8-37}$ (4, 8 μg), SR 140333 (1.4 μg), ketorolac (15, 30 μg), DuP 697 (10, 30 μg), and nimesulide (30 μg), delivered with daily morphine injection significantly attenuated both the symptoms of withdrawal and the decrease in CGRP but not substance P immunoreactivity.

5 The results of this study suggest that activation of CGRP and substance P receptors, at the spinal level, contributes to the induction and expression of opioid physical dependence and that this activity may be partially expressed through the intermediary actions of prostaglandins.
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Abbreviations: CGRP, calcitonin gene-related peptide; COX, cyclo-oxygenase; NMDA, N-methyl-D-Aspartate

Introduction

Morphine and related opioid drugs are widely used as analgesics in the management of severe pain. However, repeated administration of these agents leads to the development of opioid tolerance and physical dependence, factors that limit their therapeutic usefulness. Opioid tolerance manifests as a loss in analgesic potency, whereas physical dependence is indicated by the onset of a characteristic withdrawal syndrome precipitated by cessation of opioid drug treatment or a challenge with an opioid receptor antagonist such as naloxone. The mechanisms underlying the development and expression of opioid tolerance-dependence are not completely understood, however recent evidence suggests that increased activity of spinal excitatory amino acid (L-glutamate/L-aspartate) and neuropeptide transmitters (calcitonin gene-related peptide (CGRP), substance P) may play an important role in these phenomena (see Jhamandas *et al*., 2000).

Considerable attention has been focused on the role of excitatory amino acids and the activity of the NMDA receptor (an excitatory amino acid receptor sub-type) in the development of opioid tolerance and physical dependence. Several studies have shown that blockade of spinal NMDA receptors effectively inhibits development of tolerance to the antinociceptive actions of morphine (Trujillo & Akil, 1991; Mao *et al*., 1994; Dunbar & Yaksh, 1996; Shimoyama *et al*., 1996). Additionally, blockade of this receptor also inhibits the expression of naloxone-precipitated morphine withdrawal (Trujillo & Akil, 1991; Dunbar & Yaksh, 1996; Dambisya & Lee, 1996). These and other findings have led to the proposal that chronic exposure to opioid drugs induces a latent increase in NMDA receptor activity that physiologically antagonizes the inhibitory effects of these agents and compromises the analgesic response (Mao *et al*., 1994; Mao, 1999). Cessation of drug treatment unmasks this increased NMDA receptor activity and gives rise to the autonomic and behavioural hyperactivity that constitutes the opioid withdrawal syndrome.

In addition to the excitatory amino acid activity, the activity of sensory neuropeptide transmitters also contributes to the genesis of the opioid tolerant-dependent state (Menard *et al*., 1995; 1996; Powell *et al*., 1999; 2000). In nociceptive primary afferents that terminate in the superficial laminae of the spinal dorsal horn, L-glutamate is co-localized with CGRP and substance P (Merighi *et al*., 1991). In recent studies, we reported that repeated daily intrathecal administration of morphine significantly increased CGRP and substance P immunoreactivity in the rat spinal cord (Menard

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et al., 1996; Powell *et al*., 2000) and in dorsal root ganglion neurons which gives rise to neuropeptide expressing primary afferent fibres (Ma *et al*., 2000). Co-treatment with a CGRP receptor antagonist consistently blocked this effect and prevented the development of morphine tolerance (Powell *et al*., 2000). Preliminary findings indicated that treatment with an NK-1 receptor antagonist can also inhibit and reverse spinal morphine tolerance (Powell *et al*., 2000). These findings suggest that the activity of spinal CGRP and substance P contribute to the induction and expression of opioid analgesic tolerance, however their role in the development of opioid physical dependence is relatively unknown. A recent study demonstrating that morphine withdrawal is attenuated in CGRP deficient transgenic mice supports the involvement of this neuropeptide in the genesis of opioid physical dependence (Salmon *et al*., 2001). Previous studies have also shown that blockade of the NK-1 receptor reduces the magnitude of opioid withdrawal associated contractions in isolated guinea-pig ileum (Johnston & Chahl, 1991) and inhibits some signs of morphine withdrawal in the rat (Buccafusco & Shuster, 1997; Maldonado *et al*., 1993). Thus, these studies suggest the involvement of CGRP and substance P in the development of opioid physical dependence. The present study examined this possibility by determining the changes in CGRP and substance P immunoreactivity that accompany precipitated morphine withdrawal, and by assessing the effects of spinally administered CGRP and NK-1 receptor antagonists on physiological and behavioural manifestations of the withdrawal syndrome.

The mechanisms by which increased neuropeptide activity contributes to the development of opioid withdrawal are not known, but evidence from tolerance studies suggests an intermediary role of prostaglandins (Powell *et al*., 1999). Activation of neuropeptide and amino acid receptor activity in the spinal cord results in prostaglandin release (Malmberg & Yaksh, 1992; Hua *et al*., 1999; Marriott *et al*., 1991a, b). Prostaglandins in turn act on terminals of primary afferents to further release CGRP, substance P, and glutamate, initiating a positive feedback loop (Vasko *et al*., 1994; Malmberg & Yaksh, 1992). Thus, to investigate the potential role of spinal prostaglandins in opioid withdrawal, we examined the effects of inhibitors of cyclo-oxygenase (COX), the enzyme catalysing prostaglandin synthesis, on the morphine withdrawal associated responses.

Methods

Intrathecal catheterization and drug injection

All experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care using protocols approved by the University Animal Care Committee. Male Sprague Dawley rats (Charles River, Quebec, Canada) weighing 250–300 g were housed in separate cages and maintained on a 12 h light/12 h dark cycle with access to food and water *ad libitum*. Animals were implanted with indwelling intrathecal catheters using the method previously described by Yaksh & Rudy (1976). Briefly, the animal was anaesthetized with halothane (4%), placed in a stereotaxic frame and the cisterna magna exposed for insertion of the catheter. A small puncture was made in the atlanto-occipital membrane and a polythene catheter (PE-10; 7.5 cm long) was inserted caudally into the subarachnoid space such that the tip rested on the lumber enlargement of the spinal cord and the rostral end was exteriorized through the skin to facilitate drug administration. Surgical wounds were closed with sutures and the animal allowed to recover for 1 week. Animals showing signs of motor dysfunction (forelimb or hindlimb paralysis) were excluded from experiments. Drugs were injected intrathecally in a 10 μl volume followed by 10 μl of 0.9% saline to flush the catheter.

Induction of morphine dependence and nociceptive testing

Morphine dependence was induced using a dosage protocol modified from Gray (1996). The agonist was administered intraperitoneally twice daily, separated by an 8 h interval, in escalating doses for a period of 7 days as follows; day 1: 5 and 10 mg kg^{-1}; day 2: 20, 30 mg kg^{-1}; day 3: 30, 40 mg kg^{-1}; day 4: 40, 50 mg kg^{-1}; day 5: 50, 60 mg kg^{-1}; and day 6: 60, 80 mg kg^{-1}. On day 7, animals received a morning injection of 100 mg kg^{-1} and 3 h later a single injection of the opioid antagonist, naloxone (2 mg kg^{-1}; i.p.), was administered to produce morphine withdrawal. The efficacy of morphine was determined by assessing its antinociceptive action in the tail flick test (D'Amour & Smith, 1941) 30 min after the first daily injection. The stimulus strength in the tailflick test was adjusted to yield a baseline latency of 2–3 s and a 10-s cutoff was employed to prevent tissue damage.

Assessment of naloxone-precipitated morphine withdrawal

One hour prior to the naloxone (2 mg kg^{-1}; i.p.) injection, animals were placed in a plexiglass cylinder for habituation to the test environment. Following injection of naloxone, signs of withdrawal were scored at 10 min intervals for a total of 50 min. Scored signs included allodynia to hair deflection or light touch, chewing and licking, piloerection, and teeth chattering, and were assigned a standardized score ranging from 0–3 (0 = absent, 1 = mild, 2 = moderate, 3 = severe). The incidence of headshakes, jumping and wet dog shakes was counted over the 50 min observation period and then assigned a standardized score of 0–3 (0 = absent; 1 = 1–3 episodes; 2 = 4–6 episodes, and 3 = 7 episodes and greater). Animals were also weighed before and after naloxone challenge and weight loss (an indicator of urination and defecation) was calculated. Assessments of withdrawal signs were made in blind fashion by two investigators, and the scores were averaged.

Study 1: Acute drug effect on the expression of opioid physical dependence

To examine the role of spinal substance P, CGRP and prostaglandins in the expression of opioid physical dependence, animals were given morphine according to the 7 day dosing paradigm described above. On the last day of treatment, animals were administered a single intrathecal injection of one of the following agents: SR 140333 (1.4, 2.8 μg), $CGRP_{8-37}$ (4, 8 μg), ketorolac (30, 45 μg), DuP 697 (10, 30 μg) or nimesulide (30 μg), 30 min prior to the

naloxone challenge. Control groups received a 10 μl injection of the vehicle. Morphine withdrawal was assessed for 50 min according to the guidelines outlined above. The ability of acute drug treatment to influence naloxone-induced morphine withdrawal was assessed by observing changes in the cumulative withdrawal scores.

Study 2: Chronic drug effect on the induction of opioid physical dependence

To examine the role of spinal substance P, CGRP and prostaglandins in the induction of morphine physical dependence, spinal injections of SR 140333 (1.4 μg), $CGRP_{8\text{-}37}$ (4, 8 μg), ketorolac (15, 30 μg), DuP697 (10, 30 μg), or nimesulide (30 μg) were administered on a daily basis in conjunction with the morning injection of systemic morphine (see above). The control group received a spinal injection of the vehicle in conjunction with systemic morphine. At the end of the treatment period, animals were challenged with naloxone and withdrawal was assessed for 50 min according to the protocol described above. The ability of these drugs to influence the induction of morphine physical dependence was assessed by observing their effect on cumulative withdrawal scores.

Study 3: Relative changes in sensory neuropeptide immunoreactivity associated with physical dependence

Following naloxone-induced withdrawal, the spinal cords from animals in studies 1 and 2 were isolated and stained for CGRP or substance P immunoreactivity using the method described by Powell *et al*. (2000). Briefly, animals were anaesthetized with urethane and perfused intracardially with cold phosphate buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The L4–L5 segments of the spinal cord were removed and postfixed overnight in 4% paraformaldehyde. Samples were then transferred to 30% sucrose for cryoprotection and sliced into 40 μm sections using a cryostat. Sections were incubated with 0.3% H_2O_2 for 30 min prior to incubation with 10% NGS for 1 h and then incubated with rabbit polyclonal anti-CGRP antibody (1 : 4000) or anti-substance P antibody (1 : 8000) diluted in PBS containing 0.3% Triton-X and 3% NGS for 36 h at 4°C. Following incubation with biotinylated anti-rabbit secondary antibody (1 : 200), sections were processed with Vecastain ABC kit (Vector, Burlingame, CA, U.S.A.) according to the manufacturer's instructions and developed using 3,-3 diaminobenzedine (Vector, Burlingame, CA, U.S.A.).

Relative optical density (OD) of CGRP and substance P immunoreactivity in tissue sections was measured using image analysis software (Imaging Research Inc., St. Catherine, ON, Canada). Three spinal cord sections were randomly taken from three rats in each of the treatment groups outlined above. Relative OD measurements for CGRP or substance P immunoreactivity in the spinal dorsal horn region of all treatment groups were compared to morphine dependent animals challenged with naloxone to determine the effects of drug treatment on neuropeptide expression following withdrawal. Images of the dorsal horn regions were taken at 10 × magnification using a high-resolution CCD camera. To minimize variations in staining densities that can result from tissues stained at different times and/or in different solutions, spinal tissue from all groups were stained at the same time in the same solutions. To further ensure consistency, measurements of relative OD were performed at the same time with identical illumination background intensity settings.

Drugs

Morphine sulphate (BDH Pharmaceuticals, Toronto, Canada), naloxone HCl (DuPont NEN, Rahway, NJ, U.S.A.), and ketorolac tromethamine (Palo Alto, CA, U.S.A.) were dissolved in physiological saline (0.9%). SR 140333 (1-[2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl) piperidin-3-yl]ethyl-4-phenyl-1-azoniabicyclo [2.2.2] octane, chloride) (Sigma Chemical company, St. Louis, MO, U.S.A.), DuP697 (5-Bromo-2[4-fluorophenyl]-3[4-(methyl-sulphonyl)-phenyl]thiophene) (Sigma Chemical Company, St. Louis, MO, U.S.A.), and nimesulide (N-[4-Nitro-2-phenoxyphenyl]-methanesulfonamide) (Sigma Chemical Company, St. Louis, MO, U.S.A.) were dissolved in 5% cyclodextrin (Sigma Chemical Company, St. Louis, MO, U.S.A.).

Data analysis

Tailflick values were converted to a maximum per cent effect (M.P.E.): M.P.E. = 100 × [post-drug response – baseline response]/[cutoff value – baseline response]. Data was expressed as mean (± s.e.mean) in the figures. Statistical analysis of cumulative behavioral data was analysed for homogeneity of variance using the Cochran's test, if variance was homogenous then a one-way ANOVA followed by appropriate *post hoc* tests (Newman-Keuls and Dunnett) was used. $P<0.05$ was considered significant.

Results

Effects of chronic morphine

The antinociceptive effects of systemic morphine treatment in animals receiving ascending doses of morphine alone or in combination with other agents for 7 days was assessed by using the tail flick test (see above). Saline produced no significant effect on nociceptive response over this treatment period (average M.P.E. = 3.7% ± 0.2%) (Figure not shown). Morphine effect reached a maximal value on day 2 (10 mg kg^{-1}; M.P.E. = 95.4% ± 4.0%) and this response was maintained with ascending doses of morphine for the remaining 5 days (average M.P.E. = 97.6 ± 0.7%). Chronic treatment with SR 140333 (average M.P.E. = 98.2 ± 1.0%), $CGRP_{8-37}$ (average M.P.E. = 98.6 ± 1.1%), ketorolac (average M.P.E. = 98.5 ± 0.6%), DuP 697 (average M.P.E. = 98.7 ± 0.7%), or nimesulide (average M.P.E. = 98.2 ± 0.8%) did not significantly influence the antinociceptive action of morphine over the 7 day treatment period. Thus, the efficacy of systemic morphine was not influenced by chronic spinal injections of the agents tested.

Study 1: Acute drug effect on the expression of opioid physical dependence

As illustrated in Figure 1, naloxone (2 mg kg^{-1}; i.p.) challenge to saline ($n=6$) treated animals produced minimal

withdrawal scores (average cumulative withdrawal score of 11). However, a similar challenge to chronic morphine treated groups resulted in an intense withdrawal that produced a 260% increase in cumulative withdrawal scores as compared to those obtained in saline treated groups. Acute intrathecal pre-treatment with substance P receptor antagonist (SR 140333) produced a dose-related reduction in cumulative withdrawal scores. The lower dose of SR 140333 (1.4 μg, $n=7$) did not significantly lower cumulative withdrawal scores, although the incidence of headshakes and wet dog shakes were decreased (Table 1). However, a higher dose of SR 140333 (2.8 μg, $n=6$) reduced cumulative withdrawal scores by approximately 66% and markedly attenuated the incidences of allodynia, chewing/licking, headshakes, and wet dot shakes. Similarly, the effects of $CGRP_{8-37}$, a CGRP receptor antagonist, were also dose-related. At a higher dose of 8 μg ($n=6$), $CGRP_{8-37}$ lowered cumulative withdrawal scores by approximately 52% and significantly reduced the incidence of chewing/licking, headshakes, and wet dog shakes, whereas no effect on cumulative withdrawal scores was seen at a lower dose of 4 μg ($n=5$). Acute intrathecal administration of COX inhibitors also attenuated the symptoms of withdrawal. Ketorolac, a non-selective COX inhibitor, given at a dose of 45 μg ($n=11$), but not at 30 μg ($n=11$), significantly decreased cumulative withdrawal scores by approximately 34%, especially reducing the incidences of chewing/licking, and headshakes. In contrast, the COX-2 selective inhibitor, DuP 697, reduced withdrawal at both doses, lowering withdrawal scores by approximately 46% at a 10 μg dose ($n=5$) and 75% at a 30 μg dose ($n=6$). Treatment with another COX-2 selective inhibitor, nimesulide (30 μg, $n=7$), also decreased withdrawal scores by approximately

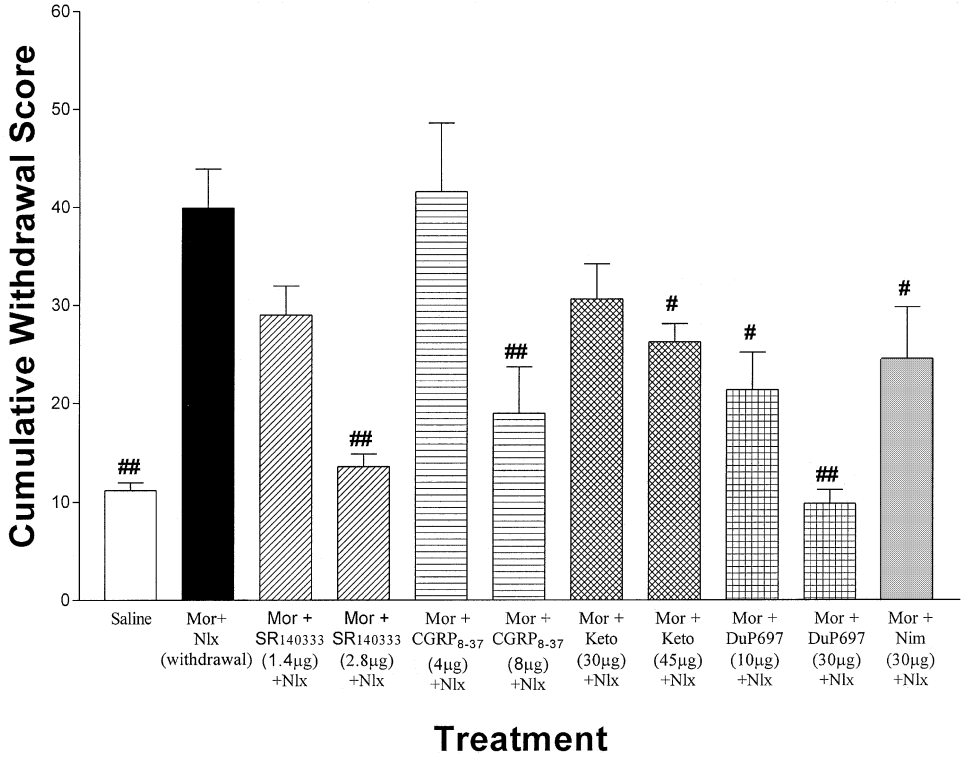

Figure 1 The effects of acute intrathecal drug pre-treatment on naloxone-induced morphine withdrawal. Animals were given systemic morphine for 7 days and given a single intrathecal drug injection 30 min prior to a naloxone challenge. The data are expressed as mean$\pm$s.e.mean. Significant difference from morphine treated animals challenged with naloxone; #($P<0.05$); ##($P<0.01$).

Table 1 Effect of acute intrathecal drug treatment on naloxone-induced withdrawal signs

Withdrawal sign	*Mor*	*SR 140333* (1.4 μg)	*SR 140333* (2.8 μg)	*$CGRP_{8-37}$* (4 μg)	*$CGRP_{8-37}$* (8 μg)	*KETO* (30 μg)	*KETO* (45 μg)	*DuP697* (10 μg)	*DuP697* (30 μg)	*Nim* (30 μg)
Allodynia	10$\pm$2.2	7.7$\pm$2.5	0$\pm$0*	18.8$\pm$3.3	11.3$\pm$5.6	9.2$\pm$3.1	4.6$\pm$1.6	5.4$\pm$2.8	1.3$\pm$0.4*	9.6$\pm$3.2
Chewing/licking	6.6$\pm$0.7	5.4$\pm$0.8*	3.6$\pm$0.9*	3.2$\pm$0.8*	2.0$\pm$0.4*	3.1$\pm$0.7*	3.4$\pm$0.7*	2.6$\pm$0.5*	2.3$\pm$0.7*	1.1$\pm$0.4*
Headshakes	4.6$\pm$0.7	0.8$\pm$0.4*	0.8$\pm$0.7*	0$\pm$0*	0$\pm$0*	1.6$\pm$0.7*	2.8$\pm$0.6*	1.0$\pm$0.6*	0.1$\pm$0.1*	0.6$\pm$0.3*
Jumping	1.7$\pm$0.7	0.4$\pm$0.4	0.4$\pm$0.4	0.2$\pm$0.2	0.2$\pm$0.2	0.9$\pm$0.5	0.4$\pm$0.2	0.6$\pm$0.4	0$\pm$0*	0.3$\pm$0.3
Piloerection	4.0$\pm$0.8	4.6$\pm$0.5	3.2$\pm$1.3	5.4$\pm$1.0	3.2$\pm$1.5	3.6$\pm$0.8	2.1$\pm$0.6	2.0$\pm$0.9	3.4$\pm$0.6	5.6$\pm$1.6
Teeth chattering	2.3$\pm$1.4	2.9$\pm$0.5	1.0$\pm$0.6	0.8$\pm$0.5	0.3$\pm$0.2	2.4$\pm$0.9	1.6$\pm$0.7	0.6$\pm$0.6	0.6$\pm$0.4	1.3$\pm$1.0
Wet dog shakes	2.8$\pm$1.0	0.1$\pm$0.1*	0.2$\pm$0.2*	0.8$\pm$0.5	0.2$\pm$0.2*	0.9$\pm$0.3*	1.1$\pm$0.4	1.2$\pm$0.7	0$\pm$0*	0.3$\pm$0.2*
Weight loss (grams)	12.3$\pm$1.6	8.0$\pm$1.6	8.0$\pm$2.0	10.0$\pm$1.4	9.7$\pm$1.5	8.8$\pm$1.3	11.3$\pm$1.5	9.3$\pm$1.8	9.4$\pm$1.5	9.0$\pm$2.0

Animals were treated with morphine (i.p.) for 7 days and given a single intrathecal drug injection 30 min before naloxone challenge (i.p.). Data are expressed as mean$\pm$s.e.m. values. *Represents a significant difference from morphine group ($P<0.05$).

38%. Both COX-2 selective inhibitors markedly attenuated the incidences of chewing/licking, headshakes, and wet dog shakes, with the higher dose of DuP697 having a significant effect on allodynia and jumping behaviors. The incidence of piloerection, teeth chattering, and weight loss was not significantly reduced by acute pre-treatment with COX inhibitors or the neuropeptide receptor antagonists.

Study 2: Chronic drug effect on the induction of opioid physical dependence

Figure 2 shows the effects of chronic spinal administration of a substance P receptor antagonist, CGRP receptor antagonist, and COX inhibitors on the naloxone (2 mg kg^{-1}, i.p.) precipitated morphine withdrawal. As illustrated in Figure 2, naloxone challenge in animals treated with morphine for 7 days produced a 300% increase in average cumulative withdrawal scores as compared to those obtained in the saline treated group. Chronic intrathecal administration of SR 140333 (1.4 μg; $n=5$), which had minimal effect when given acutely, reduced cumulative withdrawal scores by approximately 50%, decreasing allodynia, chewing/licking, headshakes, jumping, and weight loss (Table 2). Similarly, chronic treatment with intrathecal $CGRP_{8-37}$ at doses of 4 μg ($n=6$) and 8 μg ($n=4$) decreased cumulative withdrawal scores by approximately 47 and 61%, respectively. $CGRP_{8\text{-}37}$ markedly attenuated the incidence of headshakes and jumping at both doses, while allodynia and teeth chattering were reduced at higher doses. Treatment with all three COX inhibitors (ketorolac, DuP 697, nimesulide) also effectively reduced the symptoms of withdrawal. At doses of 15 μg ($n=5$) and 30 μg ($n=5$), ketorolac decreased cumulative withdrawal scores by approximately 57 and 65%, respectively. Ketorolac lowered the incidence of headshakes, jumping, and weight loss, but allodynia was only attenuated with the higher dose. Interestingly, chronic treatment with COX-2 selective inhibitors produced the greatest reductions in cumulative withdrawal scores. DuP 697 at doses of 10 μg ($n=5$) and 30 μg ($n=4$), decreased cumulative withdrawal scores by approximately 71 and 77%, respectively. Similarly, nimesulide (30 μg; $n=5$) reduced these scores by approximately 71%. Both agents noticeably attenuated withdrawal-induced allodynia, headshakes, jumping, piloerection, and weight loss.

Study 3: Relative changes in sensory neuropeptide immunoreactivity associated with physical dependence

CGRP-like immunostaining Representative photomicrographs of CGRP immunoreactive fibres in the L4-L5 dorsal horn region of rats given chronic intrathecal drug treatment (Study 2) are shown in Figure 3. The corresponding semi-quantitative data from measurements of mean optical density (OD) for CGRP immunoreactivity are represented in Figure 4. As shown in Figure 3A, in saline treated (control) animals, CGRP immunoreactivity was confined largely in the superficial laminae of the dorsal horn. Chronic morphine treatment produced a dramatic increase in CGRP immunoreactivity that was discernible in both the superficial and deeper laminae of the dorsal horn ($P<0.001$) (Figure 3B).

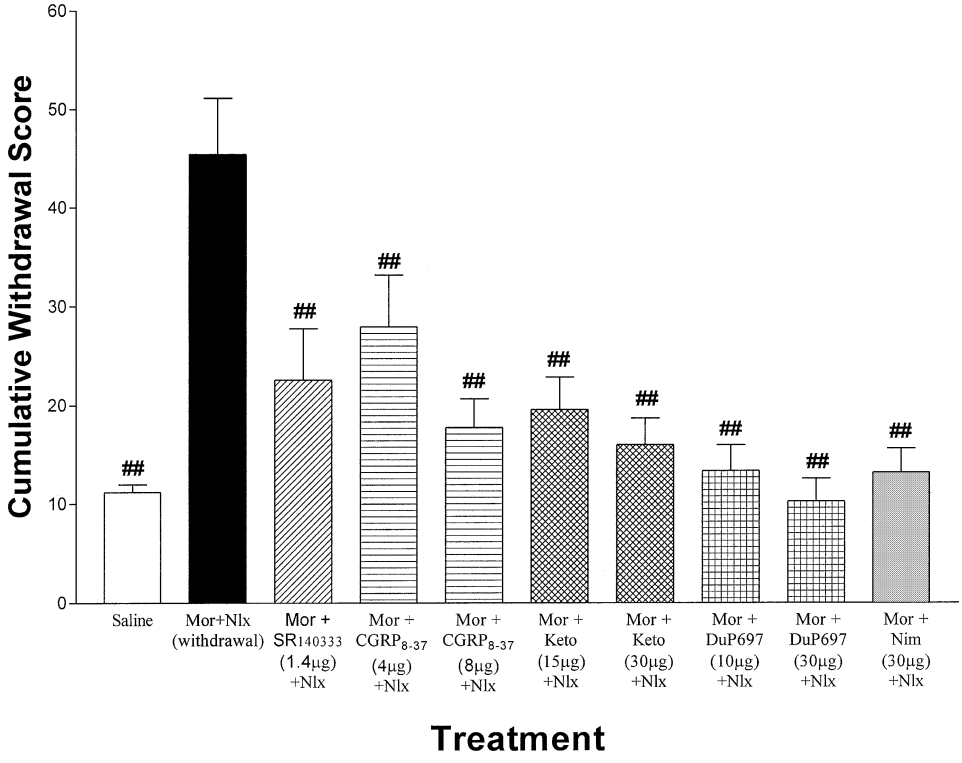

Figure 2 The effects of chronic intrathecal drug pre-treatment on naloxone induced morphine withdrawal. Drugs were administered intrathecally in combination with morphine (i.p.) for 7 days. 3–4 h following the final dose on day 7, withdrawal was induced by naloxone (i.p.). The data are expressed as mean ± s.e.mean. Significant difference from morphine treated animals challenged with naloxone; #($P<0.05$); ##($P<0.01$).

Table 2 Effect of CHRONIC intrathecal drug treatment on naxolone-induced withdrawal signs

Withdrawal sign	*Mor*	*SR 140333* (1.4 μg)	*$CGRP_{8-37}$* (4 μg)	*$CGRP_{8-37}$* (8 μg)	*KETO* (15 μg)	*KETO* (30 μg)	*DuP697* (10 μg)	*DuP697* (30 μg)	*Nim* (30 μg)
Allodynia	12.9±3.6	6.4±2.4*	9.1±3.4	4.0±3.4*	7.8±2.4	0.8±0.8*	3.5±1.5*	1.0±0.6*	5.2±1.7*
Chewing/licking	4.6±0.7	0.4±0.3*	3.4±0.5	4.5±2.3	2.0±0.7	2.0±0.5	2.2±0.9	1.8±0.6	1.8±0.4
Headshakes	3.8±1.0	0.2±0.2*	0.3±0.3*	0.5±0.5*	1.0±0.5*	0.8±0.7*	0.5±0.3*	0.3±0.3*	0.4±0.2*
Jumping	2.8±0.8	0.2±0.2*	0±0*	0.3±0.3*	0.2±0.2*	0.4±0.4*	0.8±0.6*	0.8±0.6*	0±0*
Piloerection	4.5±0.9	4.3±1.1	6.9±1.8	3.0±0.9	2.8±1.0	4.8±0.7	2.2±0.9	0±0*	0.4±0.2*
Teeth chattering	1.4±0.6	1.0±0.5	1.0±0.6	0±0*	0.7±0.5	0.8±0.4	1.8±0.9	2.0±1.4	1.6±0.8
Wet dog shakes	1.6±0.6	0.4±0.4	0±0*	0.5±0.5	1.2±0.7	0.2±0.2*	0.2±0.2*	0.5±0.3	0±0*
Weight loss (grams)	12.6±2.0	4.2±0.8*	5.1±1.0*	8.5±3.1	5.6±1.4*	5.4±1.0*	6.8±1.6	5.0±2.6*	5.2±1.9*

Animals were given intrathecal drug injections in conjunction with morphine (i.p.) for 7 days and challenged with naloxone (i.p.) on day 7. Data are expressed as mean±s.e.m. values. *Represents a significant difference from morphine group ($P<0.05$).

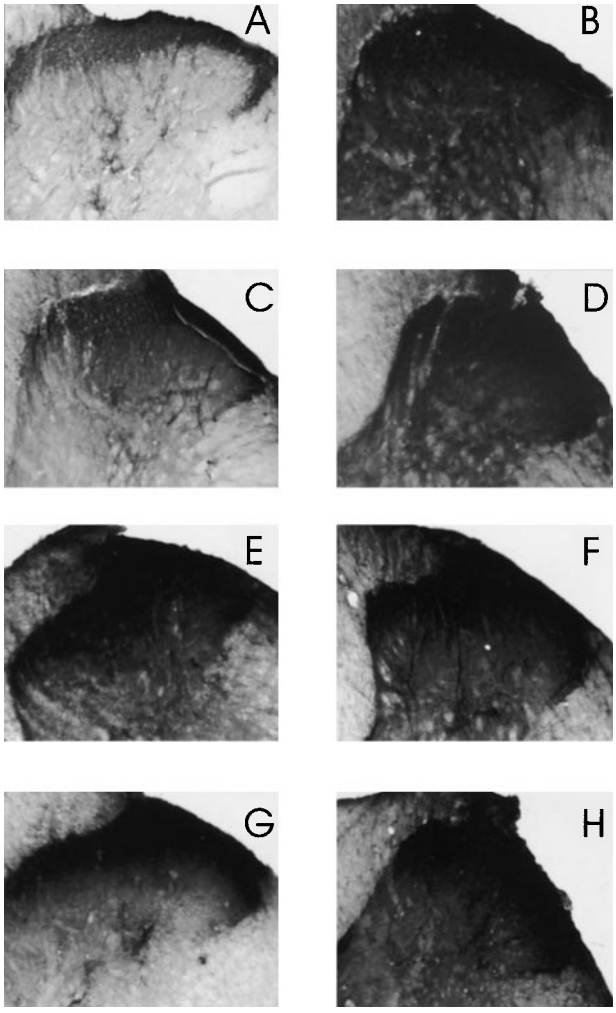

Figure 3 Photomicrographs of CGRP immunoreactive axons in the dorsal horn of L4-L5 spinal cords in naive rats (A), following daily systemic injections of morphine for 7 days (B), and 1 h after naloxone induced withdrawal (C). Withdrawal associated depletion in CGRP immunoreactivity was attenuated by chronic intrathecal treatment with SR140333 (1.4 g) (D), $CGRP_{8-37}$ (8 g) (E), ketorolac (30 g) (F), DuP 697 (30 g) (G), and nimesulide (30 g) (H). Scale bar 100 μm.

The OD value in these animals showed a 2.4 fold increase relative to that in the saline treated control group (Figure 4). Naloxone challenge in morphine treated animals resulted in a depletion of CGRP immunoreactivity, reflected by a 2.1 fold decrease in OD value compared to that in morphine treated animals not receiving naloxone. In marked contrast, animals that had been treated chronically with morphine and intrathecal injections of SR 140333 (1.4 μg), $CGRP_{8-37}$ (8 μg), ketorolac (30 μg), DuP 697 (30 μg), or nimesulide (30 μg), a challenge with naloxone produced a much lower level of peptide depletion (Figure 3). Indeed, as shown in Figure 4, the OD values for CGRP immunoreactivity in these groups was comparable to the values in chronic morphine treated groups that did not receive the naloxone challenge. Thus, in the chronic study, all agents tested prevented the depletion of CGRP immunoreactivity from the dorsal cord and effectively reduced the symptoms of naloxone-precipitated withdrawal. In contrast with the chronic treatment, acute treatment with these agents (Study 1) was less effective in preventing naloxone-induced depletion of CGRP immunoreactivity (photomicrographs not shown). Examination of OD values (Figure 5) showed that treatment with SR 140333 (1.4 μg) had no effect on naloxone-induced CGRP depletion in the dorsal horn. Acute intrathecal injections of COX inhibitors, ketorolac (30 μg), DuP 697 (30 μg), and nimesulide (30 μg) reduced the peptide depletion but their effects did not reach statistical significance. In contrast, animals treated with $CGRP_{8-37}$ (8 μg) showed similar OD values as morphine treated animals, indicating that acute treatment with this receptor antagonist significantly inhibited CGRP depletion in the dorsal horn.

Substance P-like immunostaining Representative photomicrographs of substance P immunoreactive fibres in the L4-L5 dorsal horn region of rats given chronic spinal drug treatment (study 2) are shown in Figure 6. The corresponding semi-quantitative data from measurements of mean optical density (OD) for substance P immunoreactivity are represented in Figure 4. As in the case of CGRP, substance P immunoreactivity in saline treated (control) animals was observed mainly in the superficial laminae of the dorsal horn (Figure 6A). Chronic morphine treatment produced a 3.3 fold increase in substance P immunoreactivity, the increase being discernable even in deeper layers of the dorsal horn ($P<0.001$) (Figure 4). However, examination of the photomicrographs and OD values showed that substance P immunoreactivity in the dorsal horn (Figure 4) was considerably less intense than CGRP immunoreactivity. Naloxone challenge in morphine treated animals produced a significant decrease in substance P immunoreactivity, the OD values indicating a 2.2 fold-reduction in comparison to

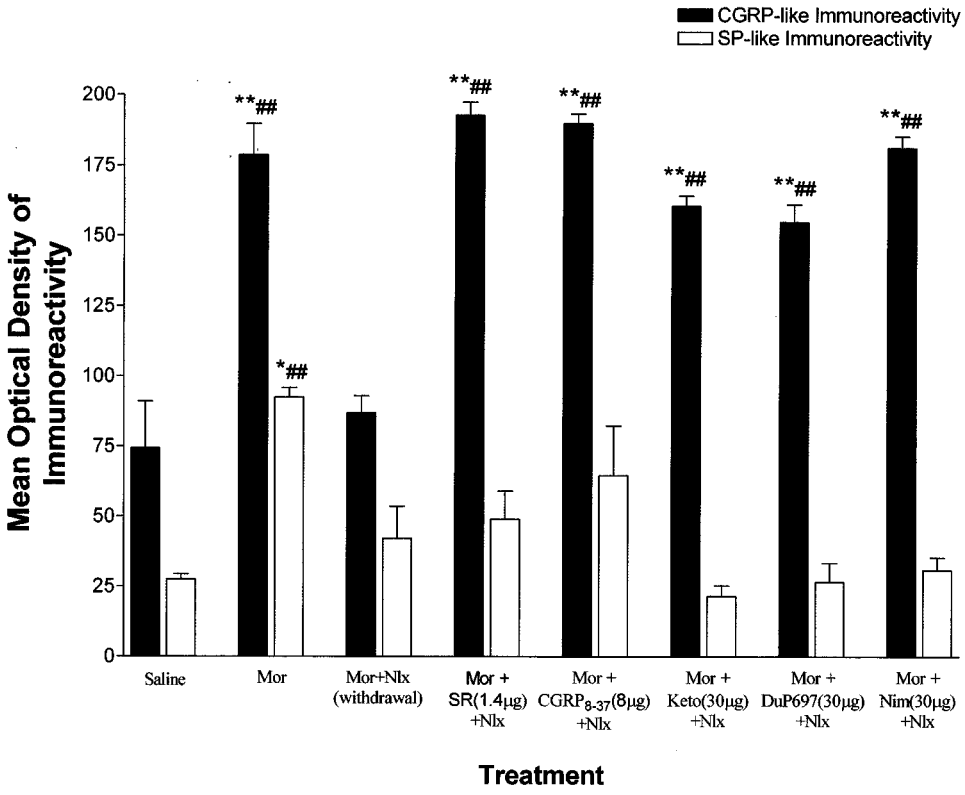

Figure 4 Effect of chronic spinal drug treatment on the mean optical density of CGRP and SP immunoreactive axons in the dorsal horn of L4-L5 spinal cords of rats. Significant difference from morphine treated animals challenged with naloxone; #($P<0.05$); ##($P<0.01$). Significant differences from saline *($P<0.05$); **($P<0.001$).

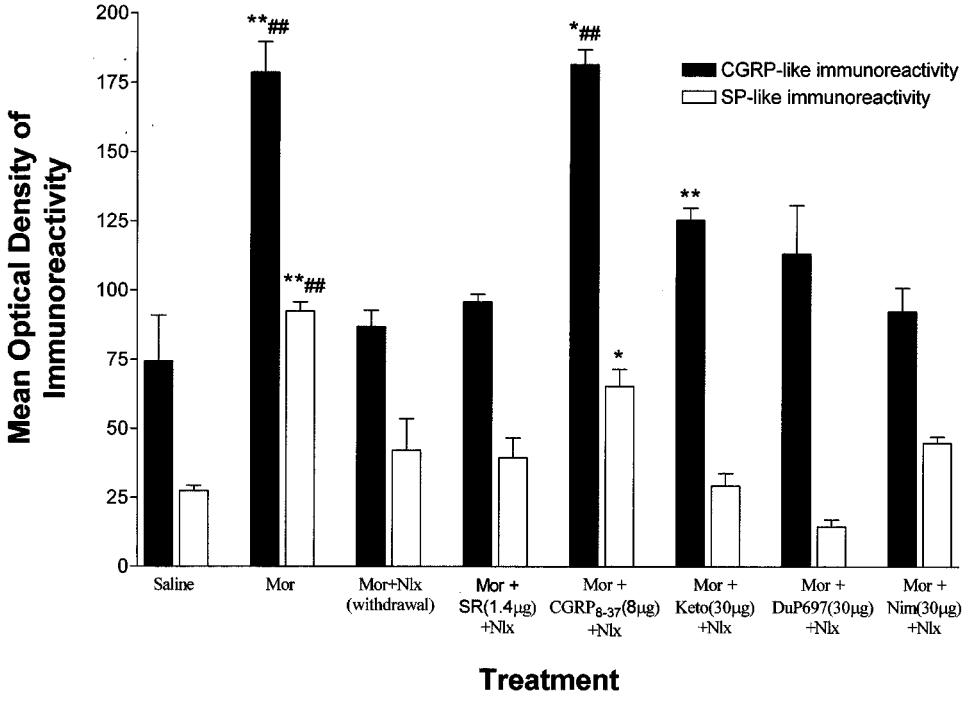

Figure 5 Effect of acute spinal drug treatment on the mean optical density of CGRP and SP immunoreactive axons in the dorsal horn of L4-L5 spinal cords of rats. Significant difference from morphine treated animals challenged with naloxone; #($P<0.05$); ##($P<0.01$). Significant difference from saline *($P<0.05$); **($P<0.001$).

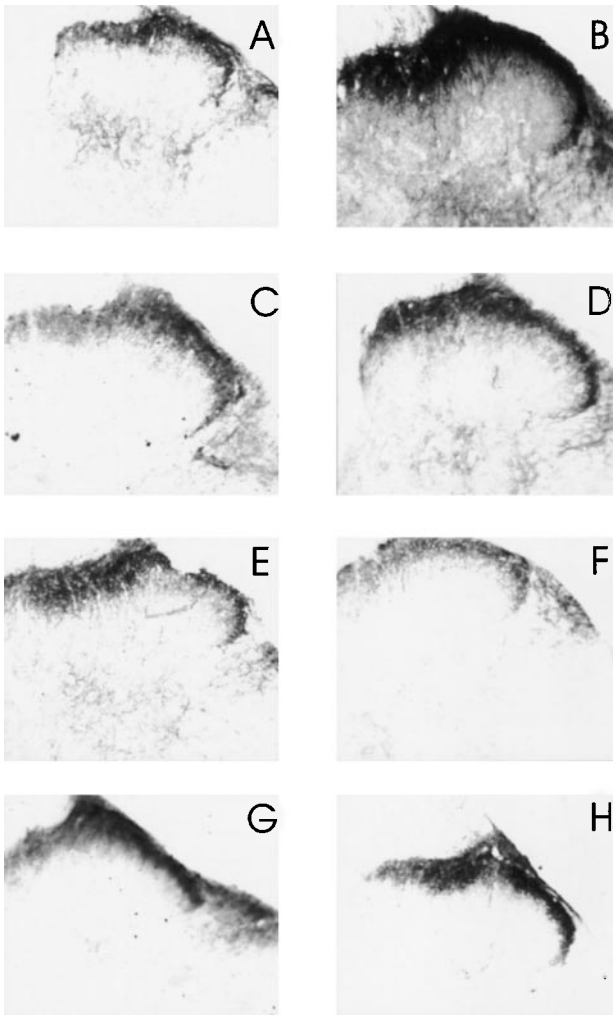

Figure 6 Photomicrographs of SP immunoreactive axons in the dorsal horn of L4-L5 spinal cords in naive rats (A), following daily systemic injections of morphine for 7 days (B), and 1 h after naloxone induced withdrawal (C). Withdrawal associated depletion in SP immunoreactivity was not blocked by chronic intrathecal treatment with SR140333 (1.4 g) (D), $CGRP_{8-37}$ (8 g) (E), ketorolac (30 g) (F), DuP 697 (G), and nimesulfide (30 g) (H). Scale bar 100 μm.

the values seen in morphine treated animals not receiving naloxone ($P<0.05$) (Figure 6C). In animals given chronic spinal injections of SR 140333 (1.4 μg), $CGRP_{8-37}$ (8 μg), ketorolac (30 μg), DuP 697 (30 μg), or nimesulide (30 μg) (Figure 6D–H), there appeared to be less depletion of substance P immunoreactivity following the naloxone-precipitated withdrawal. However, relative OD measurements failed to reveal a significant treatment effect on the naloxone-induced response. Similarly, acute intrathecal injections of the agents tested in study 1 did not block the depletion of substance P immunoreactive neurons in the dorsal horn (Figure 5) (photomicrograph not shown).

Discussion

In a previous study using a combination of immunocytochemical and behavioural techniques, it was found that sensory neuropeptide and prostaglandin activity contribute to the development of opioid tolerance (Menard *et al*., 1995; 1996; Powell *et al*., 1999; 2000). The results of the present study provide evidence for the involvement of spinal neuropeptides (CGRP, substance P) and prostaglandins in the development of opioid physical dependence. Chronic treatment with systemic morphine markedly elevated CGRP and substance P immunoreactivity in the dorsal horn region of the spinal cord and a naloxone challenge depleted this immunoreactivity and precipitated a well-defined withdrawal syndrome. Spinal administration of CGRP and NK-1 receptor antagonists or COX inhibitors (non-selective and COX-2 selective), blocked both the neuropeptide depletion and the behavioural manifestations of precipitated morphine withdrawal. Although these agents were delivered acutely prior to the naloxone challenge and chronically with morphine for 7 days, the latter treatment was clearly more effective in influencing the manifestations of withdrawal. The results of the present study suggest that increased activity of spinal neuropepetides and prostaglandins governs the induction of opioid physical dependence. These results are interpreted in terms of a model that involves a positive feedback between the sensory neuropeptide transmitters (CGRP, substance P) and prostaglandins in the dorsal horn region of the spinal cord (Figure 7). Chronic morphine conceivably increases neuropeptide levels, which results in an over-stimulation of CGRP and NK-1 receptors. This activity leads to the generation of prostaglandins, which in turn activate presynaptic prostaglandin receptors and provoke neuropeptide release from the terminals of primary afferent fibres. Thus, augmentation of the positive feedback between neuropeptides and prostaglandins in the dorsal horn, under the influence of chronic opioid exposure, may be an important factor in the genesis of tolerance and physical dependence.

Role of CGRP in opioid physical dependence

Repeated spinal morphine administration was previously shown to increase neuropeptide immunoreactivity in the spinal dorsal horn region of rats (Menard *et al*., 1995; Powell *et al*., 2000) and in cultured dorsal root ganglion neurons (Ma *et al*., 2000). Chronic systemic morphine administration in this study reproduced this effect, however, the opioid effect in this case appeared stronger than seen preivously with spinal morphine (Powell *et al*., 1999; 2000). Unlike control animals, in which the CGRP immunoreactivity was confined to superficial layers of the dorsal cord, morphine treated animals showed a wider distribution of CGRP that was discernable in the deeper laminae of the dorsal horn. This may reflect *de novo* expression of CGRP in the fibres of neurons that do not normally contain this neuropeptide. Naloxone challenge, at a dose that precipitated an intense withdrawal syndrome, produced a significant decrease in spinal CGRP immunoreactivity from superficial as well as deeper laminae, a response that likely reflects increased mobilization of peptides from terminals of primary afferent fibres. The ability of intrathecal $CGRP_{8-37}$, a CGRP-receptor antagonist, to attenuate the symptoms of withdrawal and to prevent depletion of CGRP immunoreactivity suggests that activation of CGRP receptors contributes to certain signs of withdrawal (see below). Other studies performed in

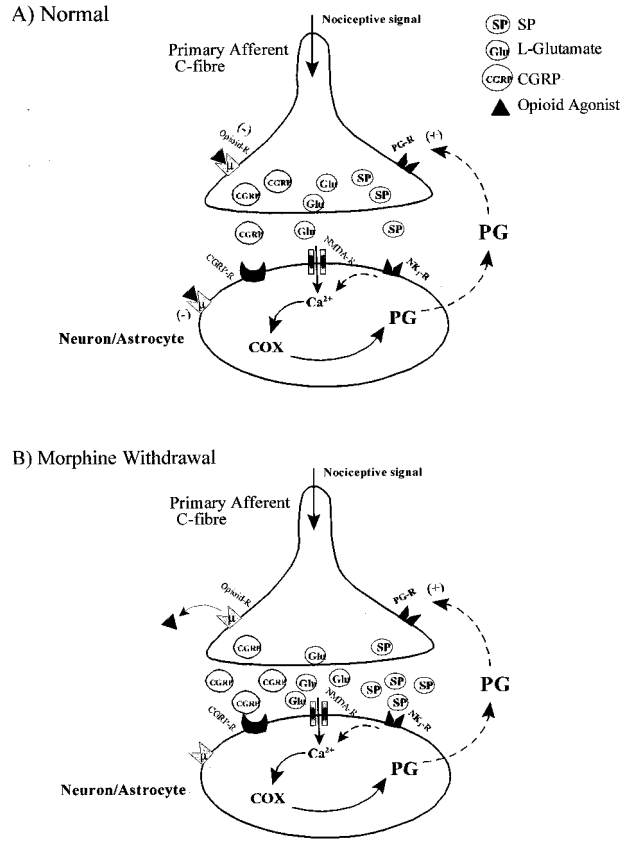

Figure 7 Model illustrating the reciprocal relationship between sensory transmitters and prostaglandins in the dorsal horn. (A) Activity of primary afferents in the dorsal horn releases glutamate, substance P, and CGRP, which activate specific receptors located on neurons and astrocytes and mobilizes prostaglandins (PG), which act on presynaptic receptors to release sensory transmitters. (B) In response to chronic morphine, there is an increase in neuropeptide and L-glutamate levels. Naloxone challenge displaces the opioid ligand from opioid receptors and results in a greater mobilization of neuropeptides and PG. Activation of tachykinin and excitatory amino acid receptors leads to the formation of PG, which act as retrograde second messengers to further augment the release of sensory transmitters. This increased neuronal activity contributes to the opioid withdrawal syndrome.

rats have also shown that CGRP levels are markedly elevated in the medulla oblongata (Tiong *et al*., 1992) and in the corpus striatum (Welch *et al*., 1992) during precipitated opioid withdrawal. More recently, Salmon *et al*. (2001) reported that CGRP deficient mice display a marked reduction in withdrawal associated jumping. These and the present findings provide evidence for the involvement of spinal CGRP neurons in opioid physical dependence. How CGRP activity influences the induction and expression of opioid physical dependence is not clear. However, studies have shown that CGRP receptor activation increases cyclic AMP levels *via* a G-protein coupled mechanism (van Rossum *et al*., 1997). Increased levels of cyclic AMP have been implicated in the genesis of opioid physical dependence (Lane-Ladd *et al*., 1997; Nestler, 1996), thus CGRP-receptor mediated increases in the levels of this cyclic nucleotide could play a major role in the expression of opioid withdrawal. CGRP has also been shown to stimulate the release of L-glutamate (Kangrga *et al*., 1990) and previous studies have provided evidence for increased spinal L-glutamate and L-aspartate release during naloxone-precipitated morphine withdrawal (Jhamandas *et al*., 1996; Dunbar & Yaksh, 1996). CGRP induced increases in cyclic AMP levels could therefore contribute to the excitatory amino acid release response elicited during morphine withdrawal. In the spinal cord, CGRP is co-localized with substance P (Gibbins *et al*., 1987; Gibbins & Morris, 1987), and it has been reported that CGRP potentiates substance P release (Oku *et al*., 1987), augments substance P-induced nociception and prolongs the actions of substance P by inhibiting its degradation (Le Greves *et al*., 1985). In view of these observations, CGRP may contribute to the opioid withdrawal response by increasing substance P levels and indirectly stimulating substance P receptors in the dorsal horn of the spinal cord. Indeed, previous findings and the result of this study strongly point to the involvement of substance P in the genesis of opioid physical dependence (Kreeger & Larson, 1993; 1996).

Role of substance P in opioid physical dependence

Like CGRP, substance P-containing primary afferent fibres terminate in laminae I and II of the lumbar dorsal horn (Hokfelt *et al*., 1975). Chronic morphine significantly increased substance P-like immunoreactivity in these and deeper laminae of the dorsal horn, which were clearly depleted following naloxone challenge. The ability of SR 140333, an NK-1 receptor antagonist, to inhibit the opioid withdrawal response suggests that the release of this peptide from primary afferents contribute to the opioid withdrawal response. This finding is in accord with results of a recent study demonstrating significant reductions in morphine reward and morphine withdrawal in NK-1 receptor deficient mice (Murtra *et al*., 2000). It is also consistent with results of previous studies implicating supraspinal (Elliott & Iversen, 1986; Jung *et al*., 1994; Johnston & Chahl, 1991; Yip & Chahl, 1999) and spinal substance P in opioid withdrawal (Bergström *et al*., 1984; Ueda *et al*., 1987; Nylander *et al*., 1991). Other studies that have mimicked the depletion of substance P using capsaicin have also demonstrated a reduction in the symptoms of opioid withdrawal (Sharpe & Jaffe, 1986; 1989; Donnerer, 1989). Thus, the evidence presented in this and in previous studies supports the role of substance P activity in the genesis of opioid physical dependence.

Substance P activity induces spinal prostaglandin release

At the spinal level, substance P acts as a potent inducer of prostaglandin release (Marriott *et al*., 1991a, b; Hua *et al*., 1999). In the rat, substance P induced firing of dorsal horn neurons (Pitcher & Henry, 1999a, b) and the nociception produced by intrathecal injections of substance P is effectively blocked by spinal injections of COX inhibitors (Malmberg & Yaksh, 1992). As well, intrathecal administration of prostaglandin E_2 elicits behaviors not unlike those produced by substance P or those elicited by opioid withdrawal (Malmberg *et al*., 1994). Matsumura *et al*. (1992) have demonstrated that prostaglandin receptors are located on primary afferents, while Vasko *et al*. (1994) have shown that activation of these receptors by prostaglandin E_2 releases both CGRP and substance P from spinal cord slices.

Interestingly, this neuropeptide release response in the spinal cord is attenuated by intrathecal administration of COX inhibitors (Southall *et al*., 1998). Thus, these findings provide strong evidence for an intermediary role of prostaglandins in substance P-mediated responses. During naloxone-precipitated withdrawal, it is likely that increased substance P activity promotes the release of prostaglandins, which in turn activate primary afferents and further release neuropeptides. This increased neuronal activity contributes to the autonomic and somatic responses that constitute the opioid withdrawal syndrome (Rohde *et al*., 1996; 1997a, b).

Inhibitors of prostaglandin synthesis reduce opioid withdrawal

The findings with ketorolac, a non-selective COX inhibitor, are consistent with results of a previous study by Dunbar *et al*. (2000) showing that acute intrathecal administration of ibuprofen inhibits morphine withdrawal-associated hyperalgesia. However, ketorolac is a more potent enzyme inhibitor (Cryer & Feldman, 1998) and in this study suppressed several withdrawal signs that included allodynia, chewing/licking, headshakes, jumping and weight loss. In addition, ketorolac prevented the depletion of spinal CGRP immunoreactivity following naloxone challenge. Ketorolac inhibits the formation of prostaglandins and thereby, block the prostaglandin-regulated release of spinal neuropeptides from primary afferents. The effects of ketorolac were reproduced with COX-2 selective inhibitors, DuP 697 and nimesulide, which also exerted inhibitory effects on peptide depletion and withdrawal symptoms. The stronger effects of DuP 697 may be attributed to the fact that it is a potent and irreversible COX-2 inhibitor (Kargman *et al*., 1996; Seibert *et al*., 1996). The results of these experiments suggests that the activity of COX-2 likely mediates opioid withdrawal and the observed effects of ketorolac may in fact be related to a weaker inhibition of the activity of this enzyme isoform. Recent studies have revealed the presence of COX-2 in the spinal cord (Beiche *et al*., 1996; Grubb *et al*., 1997) and administration of COX-2 inhibitors was found to reduce pain behaviours associated with inflammation (Hay & de Belleroche, 1997). Thus, it is likely that an adaptive increase in the expression or activity of COX-2 at the spinal level contributes to the state of opioid physical dependence, however, this possibility needs to be evaluated in future experiments.

Common underlying mechanism of neuropeptides and prostaglandins in opioid physical dependence

Intrathecal treatment with CGRP and substance P receptor antagonists, and inhibitors of COX exerted similar effects on the opioid withdrawal response. Behaviourally, acute treatment consistently reduced incidences of chewing/licking, headshakes, and wet dog shakes, whereas chronic treatment affected a spectrum of signs that encompassed sensory, autonomic, and motor components of withdrawal including allodynia, headshakes, jumping, and weight loss. While all the agents tested influenced CGRP immunoreactivity, they failed to affect the decrease in substance P immunoreactivity. The differential response of these two neuropeptides to drug treatment has also been reported in morphine tolerant rats spinally treated with $CGRP_{8-37}$ (Powell *et al*., 2000) and in neuropathic animals given MK-801 (an NMDA-receptor antagonist) (Garrison *et al*., 1993). The discrepancy between CGRP and substance P immunostaining may be due to differences in their distribution at the spinal level: CGRP is most abundant in primary afferents (Gibson *et al*., 1984) although there is limited representation in interneurons within the dorsal horn (Tie-Jun *et al*., 2001; Conrath *et al*., 1989). Relative to CGRP, substance P is present in lower amounts and is distributed in primary afferents (Hokfelt *et al*., 1975), descending fibres (Hokfelt *et al*., 2000), and interneurons (Tessler *et al*., 1980; 1981). Interneurons account for nearly half of the substance P containing terminals in laminae I and II of the dorsal horn (Howe *et al*., 1987). Increased mobilization of substance P from these sites during withdrawal may have contributed to the depletion in substance P immunostaining. Thus, spinal blockade of CGRP and NK-1 receptors, and inhibition of COX activity may have attenuated neuropeptide release from primary afferents while release from descending and intrinsic neurons was unaffected. Since a substantial proportion of substance P is present in interneuron terminals, increased mobilization of this neuropeptide from these sites is reflected by a significant reduction in immunostaining. Despite the discrepancy between behavioural and immunohistochemical data regarding substance P, the results of experiments with the NK-1 receptor antagonist favour the participation of this neuropeptide in the genesis of opioid withdrawal.

In conclusion, chronic morphine exposure increased CGRP and substance P immunoreactivity in spinal dorsal horn fibres, while naloxone challenge markedly depleted these levels and precipitated a robust withdrawal syndrome. The results indicate that an increase in the activity of these sensory neuropeptides contributes to the opioid withdrawal syndrome and that prostaglandins generated *via* COX activity may mediate this activity at the spinal level.

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References

BEICHE, F., SCHEUERER, S., BRUNE, K., GEISSLINGER, G. & GOPPELT-STRUEBE, M. (1996). Up-regulation of cyclooxygenase-2 mRNA in the rat spinal cord following peripheral inflammation. *FEBS Lett.,* **390,**165 – 169.

BERGSTRÖM, L., SAKURADA, T. & TERENIUS, L. (1984). Substance P levels in various regions of the rat central nervous system after acute and chronic morphine treatment. *Life Sci.,* **35,** 2375 – 2382.

BUCCAFUSCO, J.J. & SHUSTER, L.C. (1997). Effect of intrathecal pretreatment with the neurokinin receptor antagonist CP-99994 on the expression of naloxone-precipitated morphine withdrawal symptoms. *Brain Res. Bull.,* **43,** 321 – 326.

CONRATH, M., HELENE, T., POHL, M. & CARAYON, A. (1989). Immunocytochemical evidence for calcitonin gene-related peptide-like neurons in the dorsal horn and lateral spinal nucleus of the rat cervical spinal cord. *J. Chem. Neuroanat.,* **2,** 334 – 337.

CRYER, B. & FELDMAN, M. (1998). Cyclooxygenase-1 and cyclooxygenase-2 selectivity of widely used nonsteroidal anti-inflammatory drugs. *Am. J. Med.,* **104,** 413 – 421.

D'AMOUR, F.E. & SMITH, D.D. (1941). A method for determining loss of pain sensation. *J. Pharmacol. Exp. Ther.,* **72,** 74 – 79.

DAMBISYA, Y.M. & LEE, T.L. (1996). Role of nitric oxide in the induction and expression of morphine tolerance and dependence in mice. *Br. J. Pharmacol.,* **117,** 914 – 918.

DONNERER, J. (1989). Primary sensory neurones and naloxone-precipitated morphine withdrawal. *Br. J. Pharmacol.,* **96,** 767 – 772.

DUNBAR, S. & YAKSH, T.L. (1996). Effect of spinal infusion of L-NAME, a nitric oxide synthase inhibitor, on spinal tolerance and dependence induced by chronic intrathecal morphine in the rat. *Neurosci. Lett.,* **207,** 33 – 36.

DUNBAR, S.A., KARAMOV, I.G. & BUERKLE, H. (2000). The effect of spinal ibuprofen on opioid withdrawal in the rat. *Anesth. Analg.,* **91,** 417 – 422.

ELLIOTT, P.J. & IVERSEN, S.D. (1986). Behavioural effects of tachykinins and related peptides. *Brain Res.,* **381,** 68 – 76.

GARRISON, C., PATRICK, D.M. & CARLTON, S.M. (1993). Quantitative analysis of substance P and calcitonin gene-related peptide immunohistochemical staining in the dorsal horn of neuropethic MK-801-treated rats. *Brain Res.,* **607,** 205 – 214.

GIBBINS, I.L., FURNESS, J.B. & COSTA, M. (1987). Pathway-specific patterns of the co-existence of substance P, calcitonin gene-related peptide, cholecystokinin and dynorphin in neurons of the dorsal root ganglia of the guinea-pig. *Cell Tissue Res.,* **248,** 417 – 437.

GIBBINS, I.L. & MORRIS, J.L. (1987). Co-existence of neuropeptides in sympathetic, cranial autonomic and sensory neurons innervating the iris of the guinea-pig. *J. Auton. Nerv. Syst.,* **21,** 67 – 82.

GIBSON, S.J., POLAK, J.M., BLOOM, S.R., SABATE, I.M., MULDERRY, P.M., GHATEI, M.A., McGREGOR, G.P., MORRISON, J.F., KELLY, J.S. & EVANS, R.M. (1984). Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and of eight other species. *J. Neurosci.,* **4,** 3101 – 3111.

GRAY, A.M. (1996). Effect of alprazolam on opiate withdrawal: a combined behavioural and microdialysis study. *Eur. J. Pharmacol.,* **313,** 73 – 77.

GRUBB, B.D., WILLINGALE, H.L., GARDINER, N.J. & GIBLETT, S. (1997). Functional consequences of the identification and localisation of cyclooxygenase isoforms in dorsal horn of rat spinal cord. *Adv. Exp. Med. Biol.,* **433,** 209 – 212.

HAY, C. & DE BELLEROCHE, J. (1997). Carrageenan-induced hyperalgesia is associated with increased cyclo-oxygenase-2 expression in spinal cord. *Neuroreport,* **8,** 1249 – 1251.

HOKFELT, T., ARVIDSSON, U., CULLHEIM, S., MILLHORN, D., NICHOLAS, A.P., PIERIBONE, V., SEROOGY, K. & ULFHAKE, B. (2000). Multiple messengers in descending serotonin neurons: localization and functional implications. *J. Chem. Neuroanat.,* **18,** 75 – 86.

HOKFELT, T., KELLERTH, J.O., NILSSON, G. & PERNOW, B. (1975). Substance P: localization in the central nervous system and in some primary neurons. *Science,* **190,** 889 – 890.

HOWE, J.R., YAKSH, T.L. & GO, V.L.W. (1987). The effect of unilateral dorsal root ganglionectomies or ventral rhizotomies on α2-adrenoceptor binding to, and the substance P, enkephalin, and neurotensin content of, the cat lumbar spinal cord. *Neuroscience,* **21,** 385 – 394.

HUA, X.Y., CHEN, P., MARSALA, M. & YAKSH, T.L. (1999). Intrathecal substance P-induced thermal hyperalgesia and spinal release of prostaglandin E2 and amino acids. *Neuroscience,* **89,** 525 – 534.

JHAMANDAS, K.H., MARSALA, M., IBUKI, T. & YAKSH, T.L. (1996). Spinal amino acid release and precipitated withdrawal in rats chronically infused with spinal morphine. *J. Neurosci.,* **16,** 2758 – 2766.

JHAMANDAS, K.H., POWELL, K.J., QUIRION, R. & DE MILNE, B. (2000). Opioid tolerance and physical dependence: Role of spinal neuropeptides, excitatiory amino acids and their messengers. *Pain Res. Manage.,* **5,** 25 – 32.

JOHNSTON, P.A. & CHAHL, L.A. (1991). Tachykinin antagonists inhibit the morphine withdrawal response in guinea-pigs. *Naunyn-Schmiedebergs Arch. Pharmacol.,* **343,** 283 – 288.

JUNG, M., CALASSI, R., MARUANI, J., BARNOUIN, M.C., SOUILHAC, J. PONCELET, M., GUEUDET, C., EMONDS-ALT, X., SOUBRIE, P. & BRELIERE, J.C. (1994). Neuropharmacological characterization of SR 140333, a non peptide antagonist of NK1 receptors. *Neuropharmacology,* **33,** 167 – 179.

KANGRGA, I., LAREW, J.S. & RANDIC, M. (1990). The effects of substance P and calcitonin gene-related peptide on the efflux of endogenous glutamate and aspartate from the rat spinal dorsal horn in vitro. *Neurosci. Lett.,* **108,** 155 – 160.

KARGMAN, S., WONG, E., GREIG, G.M., FALGUEYRET, J.P., CROMLISH, W., ETHIER, D., YERGEY, J.A., RIENDEAU, D., EVANS, J.F., KENNEDY, B., TAGARI, P., FRANCIS, D.A. & O'NEILL, G.P. (1996). Mechanism of selective inhibition of human prostaglandin G/H synthase-1 and -2 in intact cells. *Biochem. Pharmacol.,* **52,** 1113 – 1125.

KREEGER, J.S. & LARSON, A.A. (1993). Substance P-(1-7), a substance P metabolite, inhibits withdrawal jumping in morphine-dependent mice. *Eur. J. Pharmacol.,* **238,** 111 – 115.

KREEGER, J.S. & LARSON, A.A. (1996). The substance P amino-terminal metabolite substance P(1-7), administered peripherally, prevents the development of acute morphine tolerance and attenuates the expression of withdrawal in mice. *J. Pharmacol. Exp. Ther.,* **279,** 662 – 667.

LANE-LADD, S.B., PINEDA, J., BOUNDY, V.A., PFEUFFER, T., KRUPINSKI, J., AGHAJANIAN, G.K. & NESTLER, E.J. (1997). CREB (cAMP response element-binding protein) in the locus coeruleus: biochemical, physiological, and behavioral evidence for a role in opiate dependence. *J. Neurosci.,* **17,** 7890 – 7901.

LE GREVES, P., NYBERG, F., TERENIUS, L. & HOKFELT, T. (1985). Calcitonin gene-related peptide is a potent inhibitor of substance P degradation. *Eur. J. Pharmacol.,* **115,** 309 – 311.

MA, W., ZHENG, W.H., KAR, S. & QUIRION, R. (2000). Morphine treatment induced calcitonin gene-related peptide and substance P increases in cultured dorsal root ganglion neurons. *Neuroscience,* **99,** 529 – 539.

MALDONADO, R., GIRDLESTONE, D. & ROQUES, B.P. (1993). RP 67580, a selective antagonist of neurokinin-1 receptors, modifies some of the naloxone-precipitated morphine withdrawal signs in rats. *Neurosci. Lett.,* **156,** 135 – 140.

MALMBERG, A.B., RAFFERTY, M.F. & YAKSH, T.L. (1994). Antinociceptive effect of spinally delivered prostaglandin E receptor antagonists in the formalin test on the rat. *Neurosci. Lett.,* **173,** 193 – 196.

MALMBERG, A.B. & YAKSH, T.L. (1992). Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat. *J. Pharmacol. Exp. Ther.,* **263,** 136 – 146.

MAO, J. (1999). NMDA and opioid receptors: their interactions in antinociception, tolerance and neuroplasticity. *Brain Res. Brain Res. Rev.,* **30,** 289 – 304.

MAO, J., PRICE, D.D. & MAYER, D.J. (1994). Thermal hyperalgesia in association with the development of morphine tolerance in rats: roles of excitatory amino acid receptors and protein kinase C. *J. Neurosci.,* **14,** 2301 – 2312.

MARRIOTT, D., WILKIN, G.P., COOTE, P.R. & WOOD, J.N. (1991a). Eicosanoid synthesis by spinal cord astrocytes is evoked by substance P; possible implications for nociception and pain. *Adv. Prostaglandin Thromboxane Leukot. Res.,* **21B,** 739 – 741.

MARRIOTT, D.R., WILKIN, G.P. & WOOD, J.N. (1991b). Substance P-induced release of prostaglandins from astrocytes: regional specialisation and correlation with phosphoinositol metabolism. *J. Neurochem.,* **56,** 259 – 265.

MATSUMURA, K., WATANABE, Y., IMAI-MATSUMURA, K., CONNOLLY, M., KOYAMA, Y., ONOE, H. & WATANABE, Y. (1992). Mapping of prostaglandin E2 binding sites in rat brain using quantitative autoradiography. *Brain Res.,* **581,** 292–298.

MENARD, D.P., VAN ROSSUM, D., KAR, S., JOLICOEUR, F.B., JHAMANDAS, K. & QUIRION, R. (1995). Tolerance to the antinociceptive properties of morphine in the rat spinal cord: alteration of calcitonin gene-related peptide-like immunostaining and receptor binding sites. *J. Pharmacol. Exp. Ther.,* **273,** 887–894.

MENARD, D.P., VAN ROSSUM, D., KAR, S., ST PIERRE, S., SUTAK, M., JHAMANDAS, K. & QUIRION, R. (1996). A calcitonin gene-related peptide receptor antagonist prevents the development of tolerance to spinal morphine analgesia. *J. Neurosci.,* **16,** 2342–2351.

MERIGHI, A., POLAK, J.M. & THEODOSIS, D.T. (1991). Ultrastructural visualization of glutamate and aspartate immunoreactivities in the rat dorsal horn, with special reference to the co-localization of glutamate, substance P and calcitonin-gene related peptide. *Neuroscience,* **40,** 67–80.

MURTRA, P., SHEASBY, A.M., HUNT, S.P. & DE FELIPE, C. (2000). Rewarding effects of opiates are absent in mice lacking the receptor for substance P. *Nature,* **405,** 180–183.

NESTLER, E.J. (1996). Under siege: the brain on opiates. *Neuron,* **16,** 897–900.

NYLANDER, I., SAKURADA, T., LE GREVES, P. & TERENIUS, L. (1991). Levels of dynorphin peptides, substance P and CGRP in the spinal cord after subchronic administration of morphine in the rat. *Neuropharmacology,* **30,** 1219–1223.

OKU, R., SATOH, M., FUJII, N., OTAKA, A., YAJIMA, H. & TAKAGI, H. (1987). Calcitonin gene-related peptide promotes mechanical nociception by potentiating release of substance P from the spinal dorsal horn in rats. *Brain Res.,* **403,** 350–354.

PITCHER, G.M. & HENRY, J.L. (1999a). Mediation and modulation by eicosanoids of responses of spinal dorsal horn neurons to glutamate and substance P receptor agonists: results with indomethacin in the rat in vivo. *Neuroscience,* **93,** 1109–1121.

PITCHER, G.M. & HENRY, J.L. (1999b). NSAID-induced cyclooxygenase inhibition differentially depresses long-lasting versus brief synaptically-elicited responses of rat spinal dorsal horn neurons in vivo. *Pain,* **82,** 173–186.

POWELL, K.J., HOSOKAWA, A., BELL, A., SUTAK, M., MILNE, B., QUIRION, R. & JHAMANDAS, K. (1999). Comparative effects of cyclo-oxygenase and nitric oxide synthase inhibition on the development and reversal of spinal opioid tolerance. *Br. J. Pharmacol.,* **127,** 631–644.

POWELL, K.J., MA, W., SUTAK, M., DOODS, H., QUIRION, R. & JHAMANDAS, K. (2000). Blockade and reversal of spinal morphine tolerance by peptide and non-peptide calcitonin gene-related peptide receptor antagonists. *Br. J. Pharmacol.,* **131,** 875–884.

ROHDE, D.S., DETWEILER, D.J. & BASBAUM, A.I. (1996). Spinal cord mechanisms of opioid tolerance and dependence: Fos-like immunoreactivity increases in subpopulations of spinal cord neurons during withdrawal [corrected]. *Neuroscience,* **72,** 233–242.

ROHDE, D.S., DETWEILER, D.J. & BASBAUM, A.I. (1997a). Formalin-evoked Fos expression in spinal cord is enhanced in morphine-tolerant rats. *Brain Res.,* **766,** 93–100.

ROHDE, D.S., MCKAY, W.R., ABBADIE, C. & BASBAUM, A.I. (1997b). Contribution of sacral spinal cord neurons to the autonomic and somatic consequences of withdrawal from morphine in the rat. *Brain Res.,* **745,** 83–95.

SALMON, A.M., DAMAJ, M.I., MARUBIO, L.M., EPPING-JORDAN, M.P., MERLO-PICH, E. & CHANGEUX, J.P. (2001). Altered neuroadaptation in opiate dependence and neurogenic inflammatory nociception in alpha CGRP-deficient mice. *Nat. Neurosci.,* **4,** 357–358.

SEIBERT, K., MASFERRER, J.L., NEEDLEMAN, P. & SALVEMINI, D. (1996). Pharmacological manipulation of cyclo-oxygenase-2 in the inflamed hydronephrotic kidney. *Br. J. Pharmacol.,* **117,** 1016–1020.

SHARPE, L.G. & JAFFE, J.H. (1986). Neonatal capsaicin modifies morphine withdrawal signs in the rat. *Neurosci. Lett.,* **71,** 213–218.

SHARPE, L.G. & JAFFE, J.H. (1989). Captopril and capsaicin modify opioid withdrawal in the morphine-dependent rat. *Pharmacol. Biochem. Behav.,* **33,** 899–902.

SHIMOYAMA, N., SHIMOYAMA, M., INTURRISI, C.E. & ELLIOTT, K.J. (1996). Ketamine attenuates and reverses morphine tolerance in rodents. *Anesthesiology,* **85,** 1357–1366.

SOUTHALL, M.D., MICHAEL, R.L. & VASKO, M.R. (1998). Intrathecal NSAIDS attenuate inflammation-induced neuropeptide release from rat spinal cord slices. *Pain,* **78,** 39–48.

TESSLER, A., GLAZER, E., ARTYMYSHYN, R., MURRAY, M. & GOLDBERG, M.E. (1980). Recovery of substance P in the cat spinal cord after unilateral lumbosacral deafferentation. *Brain Res.,* **191,** 459–470.

TESSLER, A., HIMES, B.T., ARTYMYSHYN, R., MURRAY, M. & GOLDBERGER, M.E. (1981). Spinal neurons mediate return of substance P following deafferentation of cat spinal cord. *Brain Res.,* **230,** 263–281.

TIE-JUN, S., XU, Z. & HOKFELT, T. (2001). The expression of calcitonin gene-related peptide in dorsal horn neurons of the mouse lumbar spinal cord. *Neuroreport,* **12,** 739–743.

TIONG, G.K., PIERCE, T.L. & OLLEY, J.E. (1992). Sub-chronic exposure to opiates in the rat: effects on brain levels of substance P and calcitonin gene-related peptide during dependence and withdrawal. *J. Neurosci. Res.,* **32,** 569–575.

TRUJILLO, K.A. & AKIL, H. (1991). Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science,* **251,** 85–87.

UEDA, H., TAMURA, S., SATOH, M. & TAKAGI, H. (1987). Excess release of substance P from the spinal cord of mice during morphine withdrawal and involvement of the enhancement of presynaptic Ca2+ entry. *Brain Res.,* **425,** 101–105.

VAN ROSSUM, D., HANISCH, U.K. & QUIRION, R. (1997). Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. *Neurosci. Biobehav. Rev.,* **21,** 649–678.

VASKO, M.R., CAMPBELL, W.B. & WAITE, K.J. (1994). Prostaglandin E2 enhances bradykinin-stimulated release of neuropeptides from rat sensory neurons in culture. *J. Neurosci.,* **14,** 4987–4997.

WELCH, S.P., BASS, P.P., OLSON, K.G. & PUGH, G. (1992). Morphine-induced modulation of calcitonin gene-related peptide levels. *Pharmacol. Biochem. Behav.,* **43,** 1107–1116.

YAKSH, T.L. & RUDY, T.A. (1976). Analgesia mediated by a direct spinal action of narcotics. *Science,* **192,** 1357–1358.

YIP, J. & CHAHL, L.A. (1999). Distribution of Fos-like immunoreactivity in guinea-pig brain following administration of the neurokinin-1 receptor agonist, [SAR9,MET(O2)11] substrate P. *Neuroscience,* **94,** 663–673.

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