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Spinal administration of lipoxygenase inhibitors suppresses behavioural and neurochemical manifestations of naloxone-precipitated opioid withdrawal

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1 This study investigated the role of spinal lipoxygenase (LOX) products in the induction and expression of opioid physical dependence using behavioural assessment of withdrawal and immunostaining for CGRP and Fos protein expression in the spinal cord.

2 Administration of escalating doses (5–50 mg kg^{-1}; i.p.) of morphine for 5 days markedly elevated CGRP-like immunoreactivity in the dorsal horn of the rat spinal cord. Naloxone (2 mg kg^{-1}; i.p.) challenge precipitated a robust withdrawal syndrome that depleted CGRP-like immunoreactivity and increased the number of Fos-like immunoreactive neurons in the dorsal horn.

3 Intrathecal administration of NDGA (10, 20 μg), a nonselective LOX inhibitor, AA-861 (1.5, 3 μg), a 5-LOX selective inhibitor, or baicalein (1.4, 2.8 μg), a 12-LOX selective inhibitor, concurrently with systemic morphine for 5 days or as a single injection immediately preceding naloxone challenge, blocked the depletion of CGRP-like immunoreactivity, prevented increase in the number of Fos-like immunoreactive neurons in the dorsal horn, and significantly attenuated the morphine withdrawal syndrome.

4 The results of this study suggest that activity of LOX products, at the spinal level, contributes to the expression of opioid physical dependence, and that this activity may be expressed through increased sensory neuropeptide release.
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Keywords: Opioid physical dependence; opioid withdrawal; lipoxygenase

Abbreviations: CGRP, calcitonin gene-related peptide; COX, cyclooxygenase; LOX, lipoxygenase; MAPK, mitogen-activated protein kinase; NMDA, *N*-methyl-D-aspartate

Introduction

Opioid drugs constitute potent analgesics that are clinically used in the treatment of moderate to severe pain. However, repeated administration of these agents can result in loss of analgesic potency (tolerance), while cessation of treatment precipitates a withdrawal syndrome characterized by a state of neuronal hyperactivity, which manifests as autonomic and somatic hyperactivity (physical dependence).

Considerable evidence has implicated the activity of L-glutamate, expressed *via* spinal NMDA receptors, in the genesis of opioid tolerance and physical dependence (Trujillo & Akil, 1991; Dunbar & Yaksh, 1996; Jhamandas *et al*., 1996; Shimoyama *et al*., 1996). Recent evidence has demonstrated that activity of neuropeptide transmitters (CGRP, substance P) coexisting with L-glutamate in the high-threshold primary afferent terminals also contributes to the development of opioid tolerance dependence. In the rat spinal dorsal horn and in dorsal root ganglion neurons, the expression of both CGRP and substance P is markedly elevated by chronic morphine treatment (Menard *et al*., 1996; Ma *et al*., 2000; Powell *et al*., 2000). Coadministration of morphine with a CGRP receptor antagonist or a neurokinin-1 (NK-1) receptor antagonist blocks this effect (Powell *et al*., 2000), prevents development of tolerance to the analgesic actions of morphine (Powell *et al*., 2000), and inhibits induction of opioid physical dependence (Trang *et al*., 2002). In CGRP-deficient transgenic mice, there is a marked decrease in the intensity of opioid withdrawal (Salmon *et al*., 2001), while in NK-1 receptor-deficient mice both morphine reward and morphine withdrawal are significantly attenuated (Murtra *et al*., 2000). Collectively, these studies suggest that an adaptive increase in the activity of spinal nociceptive transmitters contribute to the development of opioid tolerance dependence.

In the spinal cord, activation of neuropeptide and glutamate receptors liberates prostaglandins (Hua *et al*., 1999; Pitcher & Henry, 1999), which in turn, activates presynaptic receptors on primary afferents to facilitate the release of CGRP, substance P, and L-glutamate (Malmberg & Yaksh, 1992; Vasko *et al*., 1994). There is evidence that mobilization of these sensory transmitters and prostaglandins contributes to the autonomic and behavioural hyperactivity characterizing the antagonist-precipitated opioid withdrawal syndrome (Tiong *et al*., 1992; Welch *et al*., 1992; Jhamandas *et al*., 1996). We recently

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reported that during the naloxone-precipitated morphine withdrawal, there is depletion of CGRP and substance P-like immunoreactivity from the dorsal horn region, reflecting increased neuropeptide release from spinal afferents in which these transmitters are localized (Trang *et al*., 2002). Spinal administration of nonselective COX inhibitors or COX-2 selective inhibitors reduces the depletion of CGRP (Trang *et al*., 2002), attenuates the naloxone-precipitated morphine withdrawal (Dunbar *et al*., 2000; Trang *et al*., 2002), and inhibits the development of tolerance to morphine analgesia (Powell *et al*., 1999). Thus, these studies suggest a contributory role of spinal prostaglandins in the development of opioid tolerance and physical dependence.

The inhibition of COX activity shunts arachidonic acid into the lipoxygenase (LOX) pathway yielding different LOX metabolites, some of which have significance in opioid action (Kirchner *et al*., 1997; Vaughan *et al*., 1997; Gilroy *et al*., 1998). Subanalgesic doses of morphine delivered in combination with a leucotriene receptor antagonist were reported to produce significant antinociceptive effects in the hot-plate test (Gök *et al*., 1999). In the rat midbrain periaqueductal gray, the presynaptic inhibition of GABA release by opioid peptides is mediated by arachidonic acid metabolites generated by the activity of 12-LOX (Vaughan *et al*., 1997). In isolated guinea-pig ileum, LOX inhibitors block the contractions induced by naloxone challenge following acute opiate exposure (Capasso, 1999). However, the role of LOX activity in opioid withdrawal *in vivo* is not known. Considering that treatment with COX inhibitors suppresses opioid withdrawal only partially, the present study examined whether spinally administered LOX inhibitors can influence the behavioural and neurochemical manifestations of precipitated morphine withdrawal. The behavioural assessment included rating of autonomic and somatic signs of withdrawal, while neurochemical assessment included measurement of changes in CGRP and Fos protein expression in the dorsal horn neurons. Previous studies have established that neuronal expression of Fos, a product of the immediate early gene c-Fos, is markedly elevated during opioid withdrawal. Thus, its measurement serves as a useful quantitative index of the neuronal activity elicited during this state (Chieng *et al*., 1995; Rohde *et al*., 1996; 1997; Jhamandas *et al*., 1998).

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. Adult male Sprague–Dawley rats (Charles River, Quebec) weighing 300–350 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 animals were anaesthetized with halothane (4%) and placed in a stereotaxic frame. A small puncture was made in the atlanto-occipital membrane and a polyethylene catheter (PE-10; 7.5 cm long) inserted caudally such that the tip rested on the lumber enlargement of the spinal cord, and the rostral end was exteriorized 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

Morphine was administered intraperitoneally (i.p.) twice daily, separated by an 8 h interval, in escalating doses for a period of 5 days as follows: day 1: 5, 10 mg kg^{-1}; day 2: 15, 20 mg kg^{-1}; day 3: 25, 30 mg kg^{-1}; and day 4: 35, 40 mg kg^{-1}. On day 5, animals received a morning injection of 50 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.

Assessment of naloxone-precipitated morphine withdrawal

At 1 h 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 (Trang *et al*., 2002). Withdrawal signs included autonomic signs (allodynia, piloerection, ptosis, teeth chattering, weight loss, wet-dog shakes) and somatic motor signs (headshakes, jumping, chewing/licking, tremors). Allodynia to hair deflection or light touch, piloerection, and teeth chattering were assigned a standardized score ranging from 0 to 3 (0 = absent, 1 = mild, 2 = moderate, 3 = severe). The number of bouts of headshakes, jumping, and wet-dog shakes were counted over each 10 min interval and then assigned a standardized score of 0–3 (0 = absent; 1 = 1–3 bouts; 2 = 4–6 bouts; and 3 = 7 bouts and greater). Chewing/licking, ptosis, and tremors were also evaluated, with one point being given to the presence of each sign during each 10 min interval. The number of periods showing the sign were then counted (maximum score of 5). Animals were also weighed before and after naloxone challenge and weight loss (an indicator of urination and defecation) was calculated. Assessment of withdrawal signs was made in a blind fashion by two investigators.

Study 1: Effects of spinal LOX inhibitors on the expression of opioid physical dependence

To examine the role of spinal LOX metabolites in the expression of opioid physical dependence, animals were given morphine according to the 5-day dosing paradigm described above. On the last day of treatment, animals were administered a single intrathecal injection of one of the following agents: NDGA (10, 20 μg), AA-861 (1.5, 3 μg), or baicalein (1.4, 2.8 μg), 15 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: Effects of spinal LOX inhibitors on the induction of opioid physical dependence

To examine the role of spinal LOX metabolites in the induction of morphine physical dependence, intrathecal injection of one of the agents, NDGA (10, 20 μg), AA-861 (1.5, 3 μg), or baicalein (1.4, 2.8 μg), was 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 CGRP and Fos-like immunoreactivity associated with physical dependence

CGRP and Fos-like immunostaining Following naloxone-induced withdrawal, spinal cords from animals in studies 1 and 2 were isolated and immunostained for CGRP or Fos protein (Jhamandas *et al*., 1998; 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 and 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-cFos antibody (1 : 3000) 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.). To minimize variation in staining densities, spinal tissues from all groups were immunostained simultaneously.

Quantification of CGRP-like immunoreactivity

The relative optical density (OD) of CGRP-like immunoreactivity in tissue sections was measured using image analysis software (Imaging Research Inc., St Catherine, ON, Canada). Five spinal cord sections were randomly taken from three rats in each of the treatment groups outlined above. OD measurements were taken from two regions of the spinal dorsal horn: the superficial laminae (I–II) and deeper laminae (III–VI), as described by Molander *et al*. (1984). Relative OD measurements for CGRP-like immunoreactivity in the spinal dorsal horn region of all treatment groups were compared to morphine-treated animals challenged with naloxone to determine the effects of drug treatment on CGRP-like expression following withdrawal. OD readings were performed using identical background intensity settings, and compared between treatment groups to measure the relative changes. Images of the dorsal horn regions were taken at $\times 10$ magnification using a high-resolution CCD camera.

Quantification of Fos-like immunoreactive neurons

Photomicrographs of *Fos*-like immunoreactive neurons were taken at $\times 10$ and $\times 20$ objectives under a bright field microscope. Images were randomly coded and transferred to a computer for neuronal counting by an investigator blinded to the treatment. Five spinal cord sections were randomly obtained from three rats in each of the treatment groups outlined above. Counts of Fos-like immunoreactive neurons were taken from two regions of the spinal dorsal horn: the superficial laminae (I–II) and deeper laminae (III–VI) as described by Molander *et al*. (1984). The number of Fos-like immunoreactive neurons in each region was determined by averaging the counts made in the five sections for each treatment group.

Drugs

Morphine sulphate (BDH Pharmaceuticals, Canada) and naloxone HC1 (DuPont NEN, U.S.A.) were dissolved in physiological saline (0.9%). Nordihydroguaiaretic acid (NDGA), 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone (AA-861), and baicalein were dissolved in 5% cyclodextrin.

Data analysis

Statistical analysis of cumulative behavioural data, OD measurements, and Fos counts were analysed using a one-way ANOVA followed by appropriate *post hoc* tests (Newman–Keuls and Dunnett). $P<0.05$ was considered significant. Data was expressed as mean ($\pm$ s.e.m.) in the figures.

Results

Study 1: Effects of spinal LOX inhibitors on the expression of opioid physical dependence

Figure 1 shows the effects of *acute* intrathecal administration of LOX inhibitors on the naloxone (2 mg kg^{-1}; i.p.) precipitated morphine withdrawal response. As illustrated in Figure 1, naloxone challenge to rats treated twice daily for 5 days with ascending doses of morphine ($n=6$) resulted in an intense withdrawal that produced a 12-fold increase in cumulative withdrawal scores over those obtained in the saline-treated control ($n=5$). The naloxone-precipitated morphine withdrawal response was characterized by a profound increase in autonomic (allodynia, piloerection, ptosis, teeth chattering, weight loss, wet-dog shakes) and somatic motor (headshakes, jumping, chewing/licking, tremors) signs of withdrawal. Acute intrathecal pretreatment with a nonselective LOX inhibitor, NDGA, significantly reduced cumulative withdrawal scores by 55% (10 μg, $n=6$) and 63% (20 μg, $n=5$). At a dose of 10 μg, NDGA primarily decreased the incidence of headshakes, jumping, ptosis, and tremors, while at the higher dose the drug dramatically reduced allodynia and weight loss (Table 1). Similarly, pretreatment with the 5-LOX selective inhibitor, AA-861, at doses of 1.5 μg ($n=5$) and 3 μg ($n=5$) reduced cumulative withdrawal scores by 50 and 60%, respectively. The incidence of allodynia, headshakes, jumping, ptosis, and tremors was markedly diminished by pretreatment

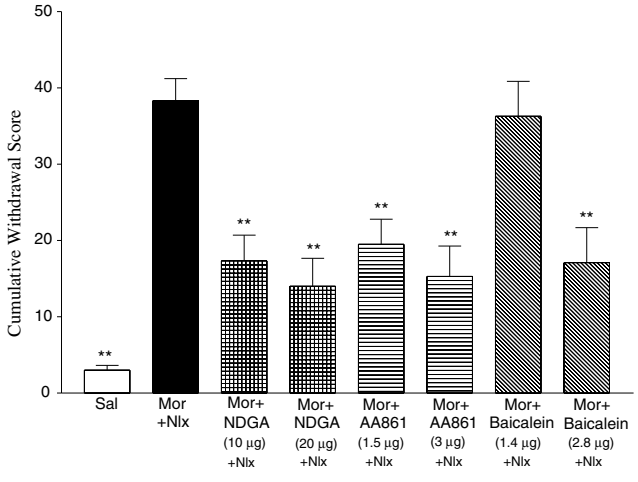

Figure 1 Effect of *acute* intrathecal pretreatment with LOX inhibitors on naloxone-induced morphine withdrawal. Animals were administered systemic morphine for 5 days, and given a single intrathecal drug injection 30 min prior to a naloxone challenge. The data are expressed as mean ± s.e.m. Asterisks represent significant difference from morphine-treated animals challenged with naloxone: $^{**}P<0.01$.

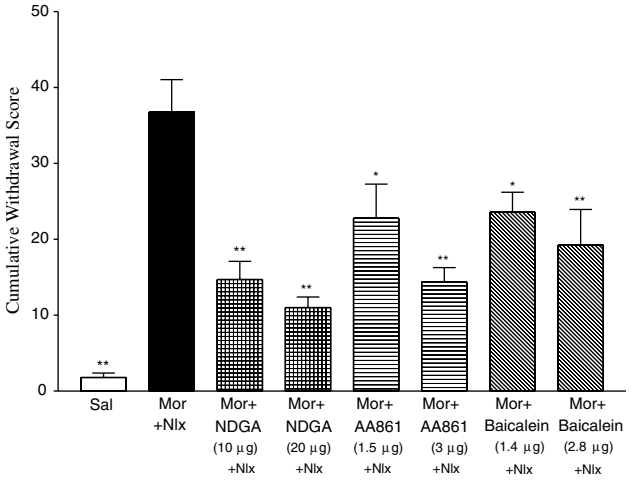

Figure 2 Effect of *chronic* intrathecal treatment with LOX inhibitors on naloxone-induced morphine withdrawal. LOX inhibitors were administered intrathecally in combination with systemic morphine for 5 days. At 3 h following the final dose on day 5, withdrawal was induced by naloxone (i.p.). The data are expressed as mean ± s.e.m. Asterisks represent 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	*Saline* (n = 5)	*Morphine* (n = 6)	*NDGA* (10 μg) (n = 6)	*NDGA* (20 μg) (n = 5)	*AA861* (1.5 μg) (n = 5)	*AA861* (3.0 μg) (n = 5)	*Baicalein* (1.4 μg) (n = 5)	*Baicalein* (2.8 μg) (n = 5)
Allodynia	0 ± 0**	10.7 ± 1.9	5.0 ± 2.2	2.3 ± 1.4*	3.5 ± 2.2*	2.7 ± 1.7*	6.9 ± 1.9	3.0 ± 1.6*
Chewing/Licking	2.6 ± 0.7	5.8 ± 0.7	3.7 ± 1.1	2.8 ± 0.6	3.2 ± 0.8	3.0 ± 0.6	5.4 ± 0.7	2.4 ± 1.2
Headshakes	0 ± 0**	3.6 ± 1.4	0.2 ± 0.2**	1.3 ± 1.3	0.4 ± 03*	1.2 ± 0.6	0.8 ± 0.4	0.2 ± 0.2*
Jumping	0 ± 0**	2.2 ± 0.6	0 ± 0**	0 ± 0**	0 ± 0**	0.4 ± 0.4*	2.0 ± 1.3	0 ± 0**
Piloerection	0.2 ± 0.2*	5.7 ± 2.1	4.8 ± 1.4	4.5 ± 1.0	5.2 ± 1.2	0.6 ± 0.4*	5.9 ± 1.2	6.6 ± 0.8
Ptosis	0 ± 0*	3.7 ± 0.8	0.5 ± 0.5*	2.5 ± 1.4	0 ± 0*	1.0 ± 1.0	1.6 ± 0.9	1.8 ± 0.8
Teeth chattering	0 ± 0	1.2 ± 0.6	1.0 ± 0.4	0.5 ± 0.5	2.2 ± 1.1	1.8 ± 1.3	5.2 ± 1.9*	1.4 ± 0.9
Tremors	0 ± 0**	1.7 ± 0.5	0 ± 0**	1.0 ± 0.0	0 ± 0**	1.4 ± 0.6	1.4 ± 0.5**	0 ± 0**
Wet-dog shakes	0 ± 0	1.0 ± 0.8	0.2 ± 0.2	0.3 ± 0.3	0.8 ± 0.6	1.0 ± 0.8	0.4 ± 0.2	0.4 ± 0.2
Weight loss (g)	3.4 ± 0.4**	12.8 ± 1.4	10.0 ± 1.4	6.5 ± 1.0**	8.4 ± 1.4	7.6 ± 1.1*	8.8 ± 1.4	3.2 ± 1.1**

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 ± s.e.m. values. Asterisks represent a significant difference from morphine group: $^{*}P<0.05$; $^{**}P<0.01$.

with AA-861 at a dose of 1.5 μg, while allodynia, jumping, piloerection, and weight loss was affected by a higher dose of 3 μg. Intrathecal pretreatment with a 12-LOX selective inhibitor, baicalein, also produced a reduction in cumulative withdrawal scores. Although the lower dose of baicalein (1.4 μg, $n=5$) did not have a significant affect on the withdrawal response, the higher dose (2.8 μg, $n=5$) decreased cumulative withdrawal score by 55% and markedly attenuated the incidence of allodynia, jumping, headshakes, tremors, and weight loss. The incidence of chewing/licking, teeth chattering, and wet-dog shakes was not significantly affected by acute pretreatment with any of the LOX inhibitors tested in this study.

Study 2: Effects of spinal LOX inhibitors on the induction of opioid physical dependence

Figure 2 shows the effects of *chronic* intrathecal administration of LOX inhibitors on naloxone (2 mg kg^{-1}; i.p.) precipitated morphine withdrawal. As shown in Figure 2, naloxone challenge in animals treated with morphine ($n=5$) for 5 days produced a significant 13-fold increase in cumulative withdrawal scores over that in the saline-treated control group ($n=5$). Chronic intrathecal administration of NDGA reduced the cumulative withdrawal score by 60% when given at a dose of 10 μg ($n=5$); however, at 20 μg ($n=5$), NDGA produced an even greater reduction (70%) in withdrawal. NDGA treatment effectively attenuated signs such as allodynia, headshakes, ptosis, and weight loss (Table 2), with the higher dose of NDGA also reducing chewing/licking behaviours. Similarly, chronic intrathecal injections of AA-861 decreased cumulative withdrawal scores by 50 and 60% at 1.5 μg ($n=5$) and 3.0 μg ($n=5$), respectively. In particular, AA-861 attenuated the incidence of chewing/licking, jumping, and ptosis, while higher doses also reduced allodynia and weight loss. Interestingly, chronic intrathecal administration of the lower dose of baicalein (1.4 μg; $n=5$), an agent that had minimal effect when given acutely, reduced cumulative withdrawal scores by 35%. An even greater reduction (54%) in cumulative withdrawal scores was observed when baicalein was administered at a dose of 2.8 μg ($n=6$). Baicalein attenuated the expression of several withdrawal signs that included chewing/licking,

Table 2 Effect of chronic intrathecal drug treatment on naloxone-induced withdrawal signs

Withdrawal sign	*Saline* (n=5)	*Morphine* (n=5)	*NDGA* (10 μg) (n=5)	*NDGA* (20 μg) (n=5)	*AA861* (1.5 μg) (n=5)	*AA861* (3.0 μg) (n=5)	*Baicalein* (1.4 μg) (n=5)	*Baicalein* (2.8 μg) (n=5)
Allodynia	0±0**	10.1±2.4	2.4±1.1**	3.0±1.1*	4.4±1.7	3.8±1.7*	7.6±1.0	3.2±1.5*
Chewing/licking	1.8±0.6*	5.2±1.5	2.2±0.7	1.0±0.8**	1.6±0.8*	0.4±0.4**	1.8±0.9*	1.7±0.7*
Headshakes	0±0**	4.1±1.3	1.6±0.5	1.0±0.5	3.4±1.5	1.0±0.8	0.8±0.4*	0.8±0.7*
Jumping	0±0**	2.0±0.5	0±0**	0±0**	0±0**	0.6±0.6**	0±0**	0±0**
Piloerection	0±0*	4.2±1.1	2.4±0.5	1.6±0.6	7.2±1.0	3.8±1.4	7.0±1.1	3.8±0.7
Ptosis	0±0**	4.0±0.3	0.6±0.6**	1.6±0.7*	1.0±0.6**	0±0**	2.0±0.6*	0.8±0.5**
Teeth chattering	0±0	1.0±0.6	2.0±1.4	0.2±0.2	0.4±0.4	1.4±0.7	2.4±1.2	0.8±0.7
Tremors	0±0	1.4±0.5	1.2±0.7	0.2±0.2	0.2±0.2	0.2±0.2	1.0±0.6	0.8±0.5
Wetdog shakes	0±0	1.0±0.8	1.2±0.7	1.2±1.0	0.4±0.4	0.8±0.8	0.4±0.4	0.3±0.3
Weight loss (g)	2.8±1.0**	12.6±0.5	6.0±1.4**	5.0±1.3**	9.2±1.6	5.0±1.7**	9.2±1.5	3.5±1.3**

Animals were given intrathecal drug injections in conjunction with morphine (i.p.) for 5 days and challenged with naloxone (i.p.) on day 5. Data are expressed as mean±s.e.m. values. Asterisks represent a significant difference from morphine group: $*P<0.05$; $**P<0.01$.

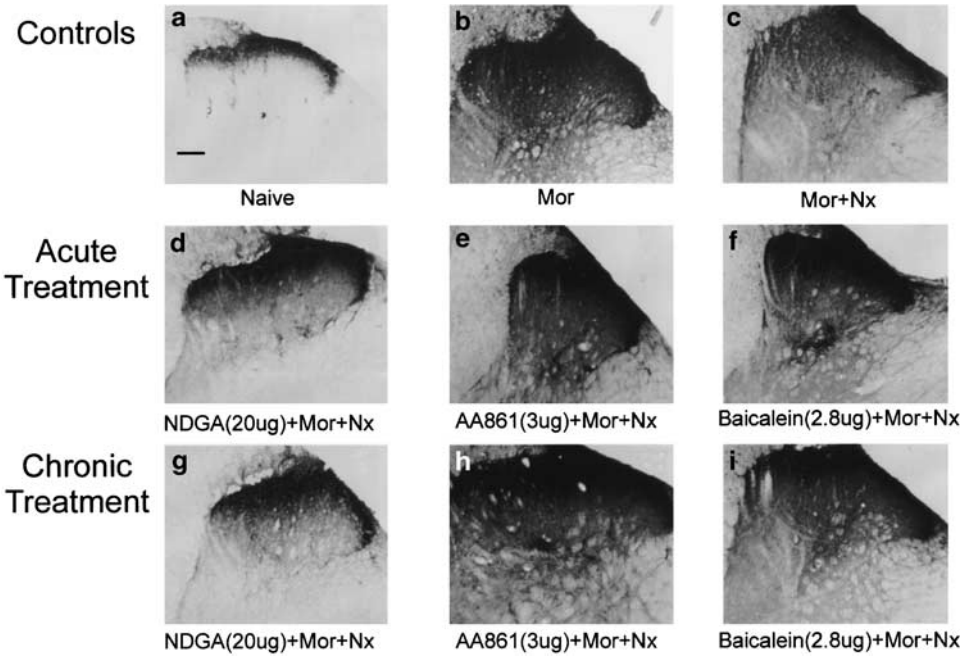

Figure 3 Photomicrographs of CGRP-like immunoreactive axons in the dorsal horn of lumbar spinal cords in naive rats (a), following daily systemic injections of morphine for 5 days (b), and 1 h after naloxone-induced withdrawal (c). Withdrawal-associated depletion in CGRP-like immunoreactivity in the superficial laminae of the dorsal horn was attenuated by acute intrathecal treatment with NDGA (20 g) (d), AA-861 (3 g) (e), or baicalein (2.8 g) (f). This effect was also seen following chronic intrathecal administration with NDGA (20 g) (g), AA-861 (3 g) (h), or baicalein (2.8 g) (i). Scale bar 100 μm.

headshakes, jumping, and ptosis, while higher doses also reduced signs of allodynia and weight loss.

Collectively, chronic treatment with the LOX inhibitors tested in this study failed to significantly affect the incidence of piloerection, teeth chattering, tremors, or wet-dog shakes. Although chronic administration of LOX inhibitors suppressed a broad spectrum of autonomic and somatic motor withdrawal signs, the effects on cumulative withdrawal scores were comparable to those seen following acute treatment with the LOX inhibitors.

Study 3: Relative changes in CGRP and Fos-like immunoreactivity during opioid withdrawal

CGRP-like immunostaining Representative photomicrographs of CGRP-like immunoreactive fibres in the L4–L5 dorsal horn region of rats given intrathecal drug treatment are shown in Figure 3. The corresponding semiquantitative data from measurements of mean OD for CGRP-like immunoreactivity are represented in Figure 4. As shown in Figure 3a, in saline-treated (control) animals, CGRP-like immunoreactivity was largely visualized in the superficial laminae of the dorsal horn. Chronic morphine treatment produced a dramatic increase in CGRP-like immunoreactivity throughout the dorsal horn (Figure 3b). The OD values showed a 50% increase in the superficial laminae and a 300% increase in the deeper laminae of the dorsal horn over the values obtained in the saline-treated control group (Figure 4). Naloxone challenge in the chronic morphine-treated group resulted in a 30% reduction (both layers) in OD values in comparison to values in morphine-treated animals not receiving naloxone. This reduction was evident throughout the entire dorsal horn, and

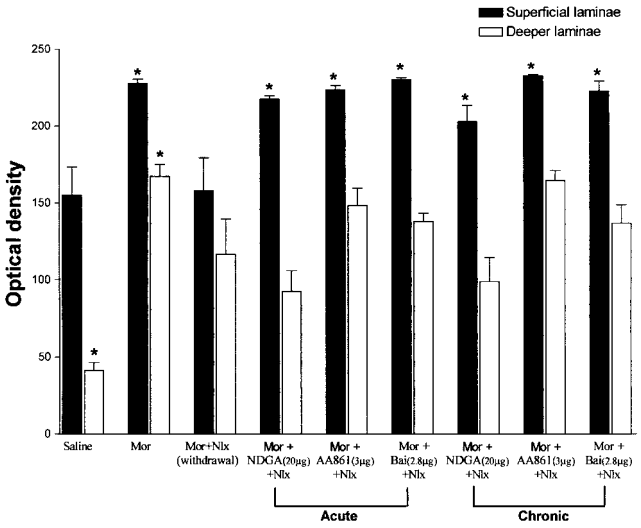

Figure 4 Effect of intrathecal treatment with LOX inhibitors on the mean optical density of CGRP-like immunoreactive axons in the lumbar region of the rat spinal dorsal horn. Asterisks represent significant difference from morphine-treated animals challenged with naloxone: $^{**}P<0.01$.

reflects increased mobilization of CGRP from terminals of primary afferent fibres. In marked contrast, animals that had been treated acutely with an intrathecal injection of NDGA (20 μg), AA-861 (3 μg), or baicalein (2.8 μg), a challenge with naloxone did not exhibit a significant decrease in peptide immunoreactivity in the superficial laminae of the dorsal horn (Figure 3). The OD values for CGRP-like immunoreactivity in the superficial laminae for these groups were comparable to the values obtained in the control group treated with morphine alone (Figure 4), suggesting that spinal treatment with LOX inhibitors very likely blocked peptide release associated with the withdrawal response. Similarly, chronic treatment with the LOX inhibitors tested in this study effectively prevented naloxone-induced depletion of CGRP-like immunoreactivity mainly in the superficial laminae of the dorsal horn (Figures 3e, f). Although visual examination suggested that there was less depletion of CGRP-like immunoreactivity in the deeper laminae, measurements of OD failed to reveal a significant treatment effect in this region of the dorsal horn.

Fos-like immunostaining

Representative photomicrographs of Fos-like immunoreactive neurons in the superficial and deeper laminae of the L4–L5 dorsal horn region of rats given intrathecal drug treatment are shown in Figure 5. The corresponding counts of Fos-like immunostained neurons are represented in Figure 6. As illustrated in Figure 5, low level of Fos expression in both the superficial and deeper laminae of the spinal dorsal horn was detected in saline and saline–naloxone-treated control groups. This pattern of Fos expression likely resulted from basal expression, or from induction through manipulation related to the experimental paradigm. Repeated systemic morphine administration did not significantly affect spinal Fos-like immunoreactivity, an effect that has been attributed to a desensitization of Fos protein expression (Nye & Nestler, 1996). In marked contrast, naloxone-precipitated withdrawal in morphine-treated animals was associated with a profound increase in Fos expression, as compared to morphine-treated controls that had not been given naloxone (Figure 6). The striking increase in the number of Fos-like immunoreactive neurons in the morphine–naloxone withdrawal group was evident in both the superficial (240%) and deeper laminae

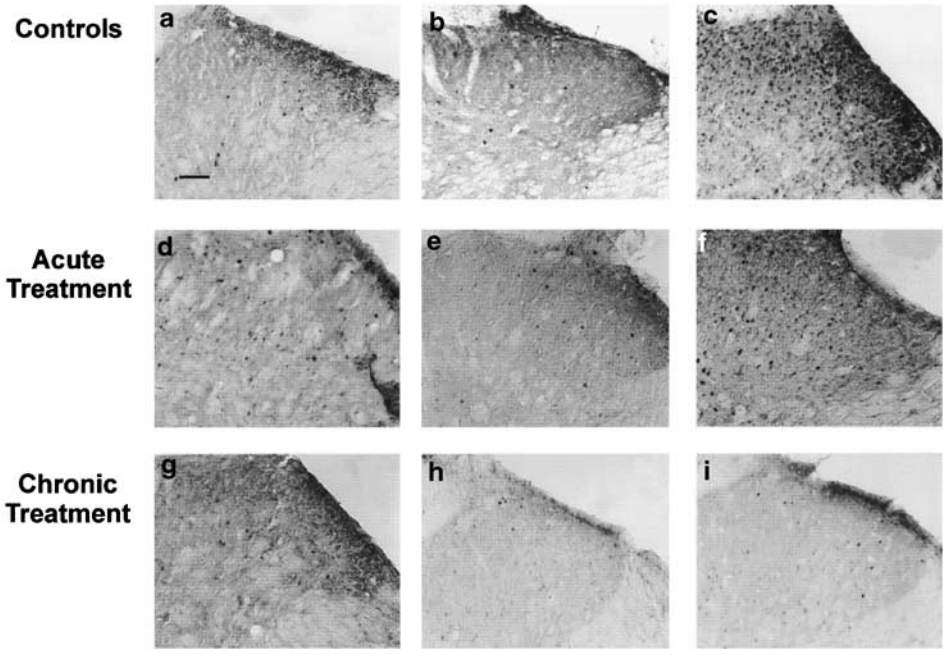

Figure 5 Photomicrographs of Fos-like immunoreactive neurons in the dorsal horn of lumbar spinal cords in naive rats (a), following daily systemic injections of morphine for 5 days (b), and 1 h after naloxone-induced withdrawal (c). Induction of Fos during naloxone challenge was markedly suppressed by either acute or chronic intrathecal treatment with NDGA (20 g) (d; g), AA-861 (3 g) (e; h), or baicalein (2.8 g) (f; i). Scale bar 100 μm.

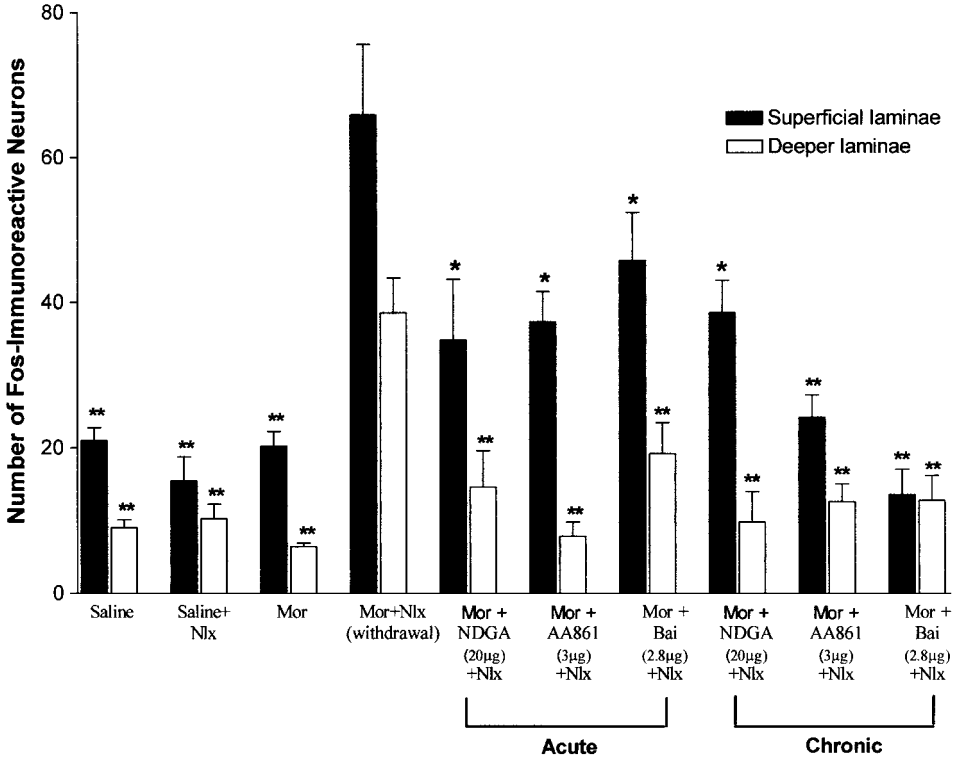

Figure 6 Effect of intrathecal administration of LOX inhibitors on the number of Fos-like immunoreactive neurons in the superficial and deeper laminae of the dorsal horn in lumbar spinal cords of rats. Asterisks represent significant difference from morphine-treated animals challenged with naloxone: $*P<0.05$; $**P<0.01$.

(310%) of the dorsal horn (Figure 6). Consistent with previous studies, expression of Fos-like immunoreactivity was noticeably higher in the superficial laminae as compared to the deeper laminae of the dorsal horn (Abbadie *et al*., 1994; Gestreau *et al*., 2000; Le Guen *et al*., 2001). Acute intrathecal injection of NDGA (20 μg), AA-861 (3 μg), or baicalein (2.8 μg) partially prevented the induction of Fos expression associated with precipitated morphine withdrawal in both regions of the dorsal horn. Interestingly, intrathecal coadministration of these agents with systemic morphine produced an even more pronounced suppression in Fos expression (Figure 6). In fact, the number of Fos-like immunoreactive neurons in animals chronically treated with LOX inhibitors was comparable to that seen in the control groups.

Discussion

Using different inhibitors of the LOX pathway, the present study provides pharmacological evidence that activity of products yielded by this pathway at the spinal level contributes to both the induction and expression of opioid physical dependence. Chronic intrathecal treatment with nonselective, 5- or 12-LOX selective inhibitors in conjunction with morphine was expected to affect the biochemical changes induced by repeated opioid exposure, while acute prenaloxone administration was expected to influence events that occur during the withdrawal. Both treatments prevented withdrawal-associated depletion of CGRP-like immunoreactivity, suppressed the induction of Fos protein in dorsal horn neurons, and attenuated the autonomic and somatic signs of naloxone-precipitated morphine withdrawal.

In accordance with our previous observations (Menard *et al*., 1995; Powell *et al*., 2000; Trang *et al*., 2002), a 5-day treatment with systemic morphine significantly elevated CGRP-like immunoreactivity in both the superficial (I, II) and deeper laminae (III, VI) of the dorsal horn. The mechanism underlying this response is not known; however, a recent study by Ma *et al*. (2001) showed that in cultured adult dorsal root ganglion neurons, morphine increases CGRP-like immunoreactivity by the mitogen-activated protein kinase (MAPK) pathway involving phosphorylation of CREB, a transcription factor regulating CGRP gene expression (Ma *et al*., 2001). Thus, the morphine-induced increase in peptide immunoreactivity observed *in vivo* could result from CGRP gene expression.

As observed previously (Trang *et al*., 2002), a naloxone challenge that elicited severe signs of opioid withdrawal significantly depleted CGRP-like immunoreactivity in the dorsal spinal cord. This response likely reflects neuropeptide release due to the activation of primary afferent neurons terminating in the superficial laminae of the dorsal horn (Rohde *et al*., 1997). The ability of intrathecal administration of $CGRP_{8-37}$, a CGRP receptor antagonist (Dennis *et al*., 1990), to reduce the peptide depletion and attenuate the precipitated morphine withdrawal syndrome (Trang *et al*., 2002), suggests that activation of CGRP receptors in the dorsal horn underlies this response. These receptors, expressed throughout the spinal dorsal horn, are localized on both presynaptic (Ye *et al*., 1999) and postsynaptic (Menard *et al*., 1995) sites. The cellular targets of CGRP, downstream from its

receptor site, have not been fully elucidated. However, CGRP receptors are G-protein coupled, and their activation increases the intracellular levels of cyclic AMP (van Rossum *et al*., 1997), a response that has been widely implicated in the genesis of opioid physical dependence (Nestler, 1996; Lane-Ladd *et al*., 1997). CGRP receptor-mediated increase in cAMP in spinal dorsal horn neurons increases neuronal activity and underlies the development of the opioid withdrawal response. In cultured astrocytes and microglial cells, CGRP stimulates the production of cyclic AMP, leading to the induction of an immediate early gene *c-fos* and expression of its protein product Fos (Hass *et al*., 1991; Priller *et al*., 1995; 1998a, b; Reddington *et al*., 1995). Thus, the brisk naloxone-induced Fos response in the spinal cord may partly result from a CGRP receptor-dependent increase in cyclic AMP in post-synaptic neurons and/or glial cells.

Le Guen *et al*. (2001) reported that the regional induction of Fos in morphine-dependent rats is correlated with the severity of autonomic signs of withdrawal. Indeed, the suppression of Fos in the superficial and deeper laminae of the dorsal horn observed in the present study was associated with attenuation of autonomic and somatic signs of precipitated morphine withdrawal. Previous studies using other models have reported a reduction in *fos* proto-oncogene expression following treatment with LOX inhibitors. Using 5-LOX selective and nonselective LOX inhibitors, Peppelenbosch *et al*. (1992) observed the blockade of epidermal growth factor-mediated *fos* induction, while Beno *et al*. (1995) reported that these agents suppressed platelet-derived growth factor-induced *fos* expression. It was suggested that LOX inhibitors disrupt nuclear signalling required for proto-oncogene transcription at a step distal or parallel to MAPK activation (Beno *et al*., 1995). However, as LOX inhibitors also reduced the depletion of CGRP from superficial laminae, these agents could have influenced the Fos response by reducing the presynaptic release of CGRP. The depletion of CGRP from deeper laminae was also reduced, but this effect was not statistically significant. The basis for this differential response in the two dorsal horn areas is not clear, but may be related to differences in the nature of afferent input to these areas. Carlton *et al*. (1990) identified two types of CGRP containing afferent terminals in the spinal dorsal horn: small-diameter simple-type CGRP terminals localized in the superficial layers, and large glomerular type CGRP terminals found mainly in the deeper laminae. Whereas the superficial laminae are richly innervated by high-threshold primary afferent fibres expressing abundant levels of CGRP (Gibson *et al*., 1984), deeper laminae receive fewer CGRP afferents and have a limited representation of the peptide in interneurons (Conrath *et al*., 1989; Tie-Jun *et al*., 2001). Thus, the stimulatory action of LOX products may be stronger on CGRP afferents in the superficial laminae and treatment with intrathecal LOX inhibitors primarily exerts action on these afferents. Alternately, the differential action may be related to methodological factors. The representation of CGRP is substantially lower in the deeper laminae, and thus small differences in peptide depletion between treatment groups may not be readily discernible by the immunostaining techniques. Despite this potential limitation, the immunohistochemical approach used in this study provides a useful method for assessing and visualizing relative changes in neuropeptide expression in specific regions whose activity produces the withdrawal response.

The combined biochemical and behavioural approach used in the present study provides the first evidence that activity of metabolites yielded by both the 5- and 12-LOX pathways contributes to the opioid withdrawal syndrome. Since LOX inhibitors were delivered intrathecally, it would appear that LOX-derived metabolites mediate withdrawal at the spinal level. The presence of 12-LOX mRNA has been demonstrated in the spinal cord (Kawajiri *et al*., 2000), but the exact site at which activity of the enzyme generates LOX metabolites is not known. Evidence for a spinal action of LOX metabolites has been demonstrated in a study by Ritchie *et al*. (2000) showing that intrathecal administration of LOX inhibitors effectively suppresses the hyperalgesia produced by spinal substance P or NMDA. Recent studies in our laboratory have also found that direct spinal injections of LOX metabolites (LTB_4 or 15-HPETE) augment the formalin-evoked nociceptive response, and reduce the latency of response in the tailflick thermal nociceptive test (unpublished data). These findings suggest that certain LOX-derived products activate nociceptive afferents that contribute to the hyperalgesic response at the level of the spinal cord. In other models, these products have been shown to act as retrograde messengers to modulate presynaptic transmitter release (Piomelli *et al*., 1987a, b; Lynch *et al*., 1989; Harish & Poo, 1992). However, how LOX metabolites contribute to the opioid withdrawal response remains to be determined.

In conclusion, the results of this study demonstrate that activity of LOX metabolites, at the spinal level, contributes to the induction and expression of opioid physical dependence. This activity likely promotes the release of spinal sensory neurotransmitters, activates dorsal horn neurons, and produces symptoms of opioid withdrawal. Potential treatments aimed at blocking arachidonic acid-generated LOX metabolites could prove useful in the management of the opioid withdrawal syndrome.

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