Induction of c-Fos and zif268 in the nociceptive amygdala parallel abstinence hyperalgesia in rats briefly exposed to morphine

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Received 3 December 2006; received in revised form 25 April 2007; accepted 21 May 2007

Abstract

Opioid-induced analgesia can be followed by spontaneous pain in humans, and hyperalgesia in rodents. In this study, opioid-induced hyperalgesia was measured by the tail-flick test when acute abstinence was precipitated by administering naloxone to drug naive rats that had experienced morphine analgesia for only 30 min. In a further experiment, the drug treatment that previously caused opioid-induced hyperalgesia was found to increase neurons expressing nuclear c-Fos or zif268 proteins in extended amygdalar regions targeted by projections of the ascending spino-parabrachio-amygdaloid nociceptive pathway. Transcription factor induction, however, was not detected in multiple brain regions known to respond in parallel with the same extended amygdalar structures when (1) rats are exposed to interoceptive/physical stressors, or (2) naloxone is used to precipitate abstinence in opioid dependent rats. Surprisingly, in many regions c-Fos induction by morphine was reduced or blocked by naloxone, even though these subjects had also experienced the effects of morphine for 30 min prior to antagonist administration. It is suggested transcription factor induction during opioid hyperalgesia in non-dependent rats could support the induction or consolidation of neural plasticity in nociceptive amygdaloid circuitry previously suggested to function in bi-directional control of pain and expression of pain-related behaviors. © 2007 Published by Elsevier Ltd.

Keywords: Opioid analgesia; Opioid induced hyperalgesia; Central pain; Acute abstinence; Transcriptional activation; Central amygdala

1. Introduction

Opioid-induced hyperalgesia has been reported by a large number of clinical and animal studies (Compton et al., 2004; Martin and Sloan, 1977; McNally, 1999; Ossipov et al., 2004; Simonnet and Rivat, 2003). In humans, it is most commonly identified by masking of the analgesic opioid effect, or a rebound increase in pain sensitivity that develops when the analgesic opioid effect has diminished (see Angst and Clark, 2006). Hyperalgesia is also a well-established sign of early withdrawal or acute abstinence syndromes in opioid-dependent subjects; but chronic or repeated exposure to opioids is not a prerequisite for this, as opioid antagonists also precipitate hyperalgesia in drug naive subjects following a first exposure to opioids (Martin and Sloan, 1977; Simonnet and Rivat, 2003; Compton et al., 2004; Harris, 2004). However, chronic or repeated administrations do facilitate the magnitude and duration of opioid-induced hyperalgesia, and may expand the anatomical sites at which hyperalgesia is induced. For example, it appears that abstinence hyperalgesia induced in rats after a first exposure to morphine is mediated by a supraspinal mechanism, as it is blocked by microinjections of lidocaine into the rostral ventromedial medulla (Kaplan and Fields, 1991). In contrast, spinal mechanisms are revealed by chronic intrathecal administration of morphine, which increases basal sensitivity to heat (hyperalgesia) or touch (allodynia), and inhibits analgesia (anti-analgesia) (Ossipov et al., 2004; Simonnet and Rivat, 2003).

Abstinence hyperalgesia develops rapidly, and can be induced when previously drug-naive subjects have been exposed to morphine for only 30 min (Célèrier et al., 1999; Devillers et al., 1995; Goldfarb et al., 1978; Kaplan and Fields, 1991; Kim et al., 1990; Larcher et al., 1998). This rapid time course...
of induction does not necessarily preclude the initial trigger for hyperalgesia being rebound activation of opioid-sensitive neurons that become dependent during this brief exposure; but it is not consistent with cellular mechanisms of opioid dependence that only develop after chronic or repeated exposures to opioids (Kieffer and Evans, 2002; Williams et al., 2001). Alternate hypotheses propose that hyperalgesic mechanisms are triggered as an adaptive compensatory physiological response to the effects of opioid drugs, or are induced by learning (Colpaert, 1996; McNally, 1999; Siegel et al., 2000; Simonnet and Rivat, 2003; Vanegas and Schaible, 2004).

The present study used induction of the immediate early gene (IEG) proteins c-Fos and zif268 (Herdegen and Leah, 2000; West et al., 2002) to examine the effect of an opioid treatment which induced abstinence hyperalgesia. This was precipitated in opioid naive rats by administering naloxone 30 min after a single morphine injection as described previously (Kaplan and Fields, 1991; Kim et al., 1990). Our study focused on the central nucleus of the amygdala (CeA) as analgesia induced in rats and primates by systemic morphine administration is mostly eliminated by unilateral or bilateral lesions of the CeA (Manning and Mayer, 1995a,b). The CeA processes noxious information received from cortex and the spino-parabrachial-amygdalar pathway, and can function in pain facilitation as well as regulating hypoalgesia in response to fear-inducing stimuli (Neugebauer et al., 2004).

2. Methods

2.1. Animals and drug treatments

Seventy-two experimentally naive, male Wistar rats (300–400 g) were obtained from a commercial supplier (Gore Hill Laboratories, Sydney, Australia) and were housed in plastic boxes (65 cm length × 40 cm width × 32 cm depth) in groups of four with food and water continuously available. The boxes were kept in an air-conditioned colony room maintained at 23 °C on a 12 h light–dark cycle with lights on at 0700 h. Experimental procedures occurred between 0900 and 0930 h each day, and were approved by the Animal Care and Ethics Committees of the University of Sydney and the UNSW.

The effect of inducing acute abstinence by reversing the effect of morphine with naloxone was examined using a behavioural measure in experiment 1, focused on the central nucleus of the amygdala (CeA) as analgesia induced in rats and primates by systemic morphine administration is mostly eliminated by unilateral or bilateral lesions of the CeA (Manning and Mayer, 1995a,b). The CeA processes noxious information received from cortex and the spino-parabrachial-amygdalar pathway, and can function in pain facilitation as well as regulating hypoalgesia in response to fear-inducing stimuli (Neugebauer et al., 2004).

2.2. Immersion tail-flick test for thermal hyperalgesia

The tail-flick apparatus consisted of a water bath set at 51 °C (±0.5 °C) by an open bath thermoregulator (Ratek Instruments, Melbourne, Australia), which was in a laboratory maintained at an ambient temperature of 21–23 °C. During testing, rats were transported to the laboratory and placed in individual plastic buckets (26 cm diameter × 45 cm high) with air holes drilled in the lid and sides. To minimize any stress-induced alterations in tail-flick latencies, on days 1–4 of the experiment, rats were transported to the laboratory and placed in the plastic buckets for 20 min, removed, handled, and returned to the buckets. This handling was repeated a further three times at 5 min intervals to familiarize the rats with procedures to be used on test. On day 5 of the experiment, rats were transported to the laboratory and placed in the plastic buckets for 20 min. Baseline tail-flick latencies were determined by taking the average of the last three of four tail-flick trials spaced 5 min apart. For tail-flick testing, the distal 4 cm portion of the rat’s tail was immersed in the water bath, and latency to completely remove the tail was recorded using stop-watch. A cut-off of 10 s was used to avoid tissue damage. At the conclusion of each trial, the tail was wiped with a flannel cloth to prevent hot water from clinging to the tail. After baseline-testing, rats received subcutaneous morphine (10 mg/kg) or saline (1 ml/kg) and placed back into the buckets. Tail-flick testing commenced 15 min following injection and was repeated 3 times at 5 min intervals. Thirty-minutes after the first injection rats received a subcutaneous injection of naloxone (5, or 0.05 mg/kg) or saline (1 ml/kg) and were placed back into buckets. Tail-flick testing began 5 min following injection and was repeated 6 times at 5 min intervals.

2.3. Tissue processing and immunohistochemistry

A well documented dual immunoperoxidase technique was used to visualize nuclear c-Fos immunoreactivity and cytoplasmic markers (Hamlin et al., 2001, 2004; Buller et al., 2005). Two hours following naloxone or saline injection, rats were deeply anaesthetised with sodium pentobarbital (100 mg/kg i.p.) and transcardially perfused with 50 ml of 0.9% saline containing 1% sodium nitrite and heparin (5000 IU/ml), followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4.Brains were preserved for 1 h in the same fixative and placed in 20% sucrose solution overnight. Tissue blocks cut using a matrix (Stoelting, Wood Dale, II) aligned to the Atlas of Paxinos and Watson (1997) were frozen and 40 μm coronal sections were cut using a freezing microtome (Leica 1320, Wetzlar, Germany) in four serially adjacent sets and stored in 0.1% sodium azide in 0.1 M phosphate buffered saline. Two series of sections were selected from each rat and two-colour immunohistochemistry was used to reveal c-Fos and tyrosine hydroxylase (TH) or c-Fos and calcitonin gene-related peptide (CGRP). Free floating sections were washed repeatedly in 0.1 M PB (pH 7.4), followed by two 30 min washes in 50% ethanol, the second of which contained 3% H2O2, and were then incubated in 5% normal horse serum (NHS) in PB (pH 7.4) for 30 min. Sections were then incubated in rabbit antiserum against c-Fos (1:5000, c-Fos (4), sc-52, Santa Cruz Biotechnologies), which were mixed with either a sheep antiserum against TH (1:3000, AB5142, Chemicon) or a goat antiserum against C GRP (1:3000, Biogeness). These primary antibodies were diluted in 0.1 M PB (pH 7.4) containing 2% NHS and 0.2% Triton X-100 (PB-TX), and incubations were for 48 h at 4 °C, with gentle agitation. After washing off unbound primary antibodies, sections were incubated overnight at room temperature in biotinylated donkey anti-rabbit IgG (1:1,000; Jackson Immunoresearch Laboratories) diluted in 2% NHS PB-TX. After washing, sections were then incubated for 2 h at room temperature in ABC reagent (Vector Elite kit: 6 μl/ml avidin and 6 μl/ml biotin; Vector Laboratories). Black immunoreactive nuclei labelled for c-Fos were revealed by a nickel-intensified diaminobenzidine reaction, with peroxidase being generated by glucose oxidase. To do this sections were washed in PB, followed by 0.1 M acetate buffer (pH 6.0), and then incubated for 15 min in 0.1 M acetate buffer (pH 6.0) containing 2% nickel sulphate, 0.0025% 3,3′ diaminobenzidine, 0.004% ammonium chloride, and 0.02% D-glucose. The peroxidase reaction was started by adding 0.2 μl glucose oxidase and stopped using acetate buffer (pH 6.0). Brain sections were then washed in PB and processed again, in a similar manner using biotinylated donkey anti-sheep IgG (1:1000; Jackson Immunoresearch Laboratories) but without nickel-intensification to localise immunoreactivity for TH and C GRP, revealed as a brown reaction product. Sections were mounted onto chrome alum/gelatin-treated slides, dehydrated, cleared in xylene, and coverslipped with DePeX (Gurr).

A third series of sections was selected from each rat at defined levels along the rostro-caudal axis of the extended amygdala and three-colour
immunofluorescence was used to reveal zif268, CGRP, and anti-neuronal nuclei (NeuN). Free floating sections were washed repeatedly in 0.1 M PBS (pH 7.2), followed by a 2-h incubation in PBS (pH 7.2) containing 10% NHS and 0.5% Triton X-100. Sections were then incubated in rabbit antiserum against zif268 (1:3000; Santa Cruz Biotechnologies), which were mixed with goat anti-serum against CGRP (1:3000; Biogenesis) and a mouse antiserum against NeuN (1:1500; Chemicon). These primary antibodies were diluted in 0.1 M PBS (pH 7.2) containing 0.1% sodium azide, 2% NHS and 0.2% Triton X-100 (PB-T-X), and incubations were for 48 h at room temperature, with gentle agitation. After washing off unbound primary antibodies, sections were incubated for 4 h at room temperature in biotinylated donkey anti-mouse IgG (1:200; Jackson Immunoresearch Laboratories), donkey anti-rabbit Alexa Fluor 488 (1:500; Molecular Probes), and donkey anti-goat Alexa Fluor 594 (1:2000; Molecular Probes) diluted in 2% NHS PB-T-X. After washing off unbound secondary antibodies, sections were further incubated for 1 h in AMCA-Avidin D (1:100; Vector Laboratories). Sections were mounted onto chrome alum/gelatin-treated slides and coverslipped with buffered glycerol (pH 8.6).

2.4. Counting neurons with c-Fos- or zif268-positive nuclei

Sections were viewed under a microscope equipped with a eyepiece graticule by an observer blinded to the experimental condition, and manual counts of neuronal nuclei immunoreactive (IR) for c-Fos were conducted through the rostro-caudal extent of each brain region analysed. All sections counted were 160 μm apart. Chemoarchitecture revealed by TH and CGRP labelling was used to aid in identification of anatomical boundaries. The brain regions ana-lysed were: nucleus accumbens shell (AchSh) and core (AchC) over 5 sections beginning at 2.20 mm; dorsal striatum (dorsomedial and ventromedial) over 4 sections beginning at 1.60 mm; bed nucleus of the stria terminalis lateral-dor- sal (BSTld) and ventral (BSTv) over 4 sections beginning at −0.26 mm; interstitial nucleus of the posterior limb of the anterior commissure lateral (IPACl) and medial (IPACm) over 17 and 12 sections respectively beginning at 1.60 mm; periaqueductal gray, dorsal (dPAG) over 6 sections beginning at −1.80 mm; amygdalar-striatal transition zone (AStr) over 6 sections beginning at −1.40 mm; substantia nigra (SN), ventral tegmental area lateral (VTA) and interfascicular nucleus (IF) over 7 sections beginning at −5.20 mm; periaqueductual gray, dorsal (dPAG) over 7 sections beginning at −5.80 mm, lateral (IPAG) over 6 sections begin- ning at −6.04 mm, and ventrolateral (vIPAG) over 5 sections beginning at −7.64 mm; periallocortical nucleus (PB) over 5 sections beginning at −9.16 mm; rostral ventromedial medulla (RVM) over 10 sections beginning at −10.04 mm; and the nucleus of the solitary tract (NTS) over 18 sections beginning at −12.80 mm. Manual blind counts of neuronal nuclei IR for zif268 were conducted over a single section at defined levels of the extended amygdala, BSTld (−0.26 mm), IPACl (−0.92 mm), ASv (−1.30 mm), CeL, and CeC (−2.12 mm). All co-ordinates given are distance from bregma according to the rat brain atlas of Paxinos and Watson (1997).

2.5. Data analysis

Data from the tail-flick tests were analysed as withdrawal latencies in seconds. The saline pre-treated groups were analysed using a two-way repeated measures ANOVA and trend analyses to identify possible change in tail-flick latency over time. Paired t-tests were used for post hoc pairwise mean comparisons to detect changes in the number of c-Fos or zif268 expressing neurons induced by morphine (S/S vs M/S) or either dose of naloxone (S/S vs S/N0.05 and S/S vs. S/N5). Unless stated otherwise all data are expressed as the mean ± SEM and significance taken as P < 0.05. Statistical analyses were performed using SPSS v11 for Macintosh.

3. Results

3.1. Naloxone induced hyperalgesia in morphine-pre-treated rats

As abstinence-hyperalgesia is induced dose-dependently by systemic naloxone injection after a brief period of morphine analgesia in drug-naive rats (Kim et al., 1990), in this experimen-t we used the immersion tail-flick test to compare the ef-fect of two doses of naloxone (0.05 and 5 mg/kg). The average baseline tail-flick latency was 4.7 ± 0.1 s and did not differ significantly between the 6 experimental groups (one-way ANOVA: F(4,25) = 0.439, P = 0.819). Tail-flick latencies were not different between the three groups of rats given saline as the first drug, but a small linear reduction was measured over the 30 min test period after these groups were subse- quently injected with saline or either dose of naloxone (linear trend: F(1,21) = 9.5, P = 0.006). As expected, tail-flick latencies increased close to the cut-off (10 ± 0.04 s) in the three groups of rats given morphine as the first drug (paired t-test: P < 0.001). This level of analgesia remained unchanged during the 30 min testing period in the group of rats subsequently injected with saline (linear trend: F(1,7) = 0.876, P = 0.38). In contrast, the low dose of naloxone (0.05 mg/kg) reversed morphine antinociception and shortened tail-flick latencies back to baseline after 15 min (Fig. 1), whereas the high dose of naloxone (5 mg/kg) not only reversed morphine antinociception but also induced thermal hyperalgesia measured by a reduction in tail-flick latencies below baseline. This hyperalgesia was apparent 5 min after the antagonist injection, and was maintained throughout the 30 min testing period (Fig. 1).

3.2. Induction of c-Fos in divisions of the central amygdala in parallel with morphine analgesia and abstinence hyperalgesia

The same drug design was used in a second immunohistochemical experiment to determine if induction of c-Fos-positive nuclei in neurons in the central nucleus of the amygdala occurred in parallel with analgesia or hyperalgesia caused by the opioid agonist-antagonist treatments. This was done in a separate parallel experiment to avoid c-Fos being induced by noxious stimulation or motor activity induced during the tail-flick testing. We co-stained sections containing the central nucleus of the amygdala for c-Fos and tyrosine hydroxylase (TH) immunoreactivity (IR) (Fig. 2), which was used as a chemoarchitectural marker (Asan et al., 2005) for identifying the medial (CeM), lateral (CeL) and ventral capsular (CeC) subdivisions of the central nucleus, and the amygdalostrital transition zone (AStr) sometimes referred to as the dorsal amygdala in parallel with morphine analgesia and abstinence hyperalgesia.
capsular region (Alheid et al., 1995; Cassell et al., 1999; McDonald, 2003; Price et al., 1987).

Abstinence induced by the high naloxone dose in morphine pre-treated rats (S/N5 vs. M/N5) increased neurons with c-Fos-positive nuclei in CeC ($P < 0.001$) and AStr ($P < 0.001$) (Figs. 3, 4). When this dose of naloxone was administered to saline pre-treated rats, it induced a smaller increase in c-Fos-positive neurons in CeC (S/S vs. S/N5, $P < 0.05$) but had no effect in AStr (S/S vs. S/N5, $P = 0.104$). No effect was detected in CeC or AStr when saline was administered following morphine pre-treatment, or when the low naloxone dose was administered following morphine or saline (Fig. 3).

Systemic morphine when followed by saline (S/S vs M/S) increased neurons with c-Fos-positive nuclei in CeM ($P < 0.01$) and CeL ($P < 0.001$) (Fig. 3). The increase in CeM was not affected by the low naloxone dose, but was not detected when the high naloxone dose was administered following morphine (S/S vs. M/N5, $P = 0.865$). Neither dose of naloxone induced c-Fos-positive neurons in CeM when administered to saline pre-treated rats (Fig. 3). In CeL, both doses of naloxone increased c-Fos-positive neurons when administered to saline-pre-treated rats (S/S vs. S/N0.05 and S/S vs. S/N5, $P < 0.05$) (Fig. 3). The effect of morphine and naloxone in the CeL were independent and additive, as ANOVA detected significant main effects of agonist ($F_{(1,18)} = 29.613, P < 0.001$) and antagonist ($F_{(2,18)} = 8.628, P < 0.05$) treatments, but no agonist x antagonist treatment interaction ($F_{(2,18)} = 0.042, P = 0.841$).

### 3.3. Induction of c-Fos in the central extended amygdala

Abstinence induced by the high naloxone dose following morphine (S/N5 vs. M/N) increased c-Fos-positive neurons in lateral IPAC ($P < 0.001$) and lateral-dorsal BST ($P < 0.01$)(Fig. 3). This dose of naloxone also caused smaller increases in both regions when administered following saline (S/S vs. S/N5, $P < 0.01$; BST, $P < 0.05$). Systemic morphine administration had no effect when followed by a saline

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Tyrosine-hydroxylase
Calcitonin gene-related peptide

Fig. 2. Chemoarchitecture of the central amygdala. Photomicrographs of coronal sections through the rostro-caudal extent of the central amygdala showing the distribution of tyrosine hydroxylase (TH) and calcitonin gene-related peptide (CGRP) immunoreactivity. Also shown are c-Fos-positive neurons injected, nor did the low naloxone dose have an effect when administered after morphine or saline. No differences in the number of c-Fos-positive neurons measured in the six experimental groups were detected in medial IPAC, ventral BST (Fig. 3), or the lateral septum (data not shown).

A series of sections were co-stained for CGRP-IR (Fig. 3) to determine the distribution of c-Fos-positive neurons in CeC, CeL and lateral-dorsal BST relative to terminal fields arising from the spino-parabrachial-amygdalar pathway (Xu et al., 2003). The high naloxone dose administered to saline or morphine pre-treated rats induced c-Fos-positive neurons, which were preferentially localised to extended amygdala regions containing dense CGRP-IR terminals (Figs. 2, 4).

3.4. Induction of zif268 in morphine pre-treated rats injected with naloxone

Another series of sections from the same experiment were used to determine if neurons with zif268-positive nuclei in the central amygdala also increased in parallel with morphine analgesia or naloxone-induced hyperalgesia. For this analysis, zif268-positive neurons were identified by immunofluorescence in a single section taken from a mid-rostro-caudal level of the CeA corresponding to −2.12 mm from Bregma, and at levels of the AStr, IPAC and BST corresponding to −1.30 mm, −0.92 mm and 0.26 mm from Bregma respectively.

Abstinence induced by the high dose of naloxone following morphine (S/N5 vs. M/S) increased zif268-positive neurons in CeC (P < 0.001), AStr (P < 0.001) and lateral IPAC (P < 0.001) (Fig. 5). This dose of naloxone also induced smaller increases in zif268-positive neurons in AStr (P < 0.05) and lateral IPAC (P < 0.05) when administered following saline.

Increases in zif268-positive neurons were also detected in CeL and lateral-dorsal BST but these were not specific to abstinence associated with hyperalgesia as ANOVA detected separate main effects of Agonist (CeL: F(1,18) = 10.505, P < 0.01; BST: F(1,18) = 29.364, P < 0.001) and antagonist (CeL: F(2,18) = 32.054, P < 0.001; BST: F(2,18) = 62.322, P < 0.001) treatments, but no agonist × antagonist interaction (CeL: F(2,18) = 0.100, P = 0.758; BST: F(24,5) = 2.634, P = 0.131). In the CeL, zif268-positive neurons were increased by systemic morphine (S/S vs. M/S, P < 0.01) and when the high naloxone dose was administered following saline (S/S vs. S/N5, P < 0.001). In the lateral-dorsal BST, zif268-positive neurons were increased by systemic morphine (S/S vs. M/S, P < 0.01) and when the low naloxone dose (S/S vs. S/N0.05, P < 0.05) or the high naloxone dose followed induced by a morphine-saline treatment. Lines indicate the anatomical boundaries of the divisions of the central amygdala used for analysis. Arrows indicate examples of c-Fos-positive neuronal nuclei. Abbreviations: AStr, amygdalostraiatal transition zone; CeC, capsular division of the central amygdala; CeL, lateral division of the central amygdala; CeM, medial division of the central amygdala. Distance from bregma (Paxinos and Watson, 1997). Scale bar = 200 μm.

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Fig. 3. Abstinence hyperalgesia activates specific regions of the central extended amygdala. Shown are the effects of naloxone (5, 0.05 mg/kg) or saline (1 ml/kg) injection given 30 min following morphine (10 mg/kg) (closed circles) or saline (1 ml/kg) (open circles) injection on the number of c-Fos-positive neurons. Lines show the mean ± SEM number of c-Fos-positive neurons counted across the rostro-caudal extent of the lateral-dorsal and ventral bed nucleus of the stria terminalis (BST; 4 sections), medial and lateral interstitial nucleus of the posterior limb of the anterior commissure (IPAC; 17 and 12 sections respectively), amygdalostratal transition zone (6 sections), and central amygdala (CeA; 5 sections) in each of the treatment groups.

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Fig. 4. Illustration of the distribution of c-Fos-positive neurons relative to CGRP-immunoreactive terminals in the central extended amygdala following acute morphine (10 mg/kg), high dose of naloxone (5 mg/kg), and high dose of naloxone (5 mg/kg) in morphine (10 mg/kg) pre-treated rats. Abbreviations: ac, anterior commissure; AStr, amygdalostrital transition zone; BSTld, lateral dorsal bed nucleus of the stria terminalis; BSTma, anterior medial bed nucleus of the stria terminalis; BSTv, ventral bed nucleus of the stria terminalis; BLA, basolateral amygdala; CeC, capsular division of the central amygdala; CeL, lateral division of the central amygdala; CeM, medial division of the central amygdala; ct, commissural stria terminalis; IPACl, lateral interstitial nucleus of the posterior limb of the anterior commissure; IPACm, medial interstitial nucleus of the posterior limb of the anterior commissure; GP, globus pallidus; ot, optic tract; Str, striatum. Dark shading represents dense CGRP terminal labelling, light shading represents sparse CGRP terminal labelling. Distance from bregma (Paxinos and Watson, 1997).
saline (S/S vs. S/N5, \(P < 0.001\)). Fig. 5B shows that the distribution of zif268-positive neurons induced by a high dose of naloxone (5 mg/kg) following a brief exposure to morphine were also confined to regions of dense CGRP-immunoreactive terminals.

3.5. Induction of c-Fos in the basolateral amygdala, midbrain dopamine areas and striatum requires prolonged activation by morphine

We next examined regions known to be activated in parallel with the extended amygdala following abstinence withdrawal in morphine dependent rats (Georges et al., 2000; Hamlin et al., 2001; Hayward et al., 1990; Milanes et al., 1997), or by interoceptive stressors such as cytokine induced sickness or haemorrhage.

Fig. 5. (A) Induction of zif268 by abstinence hyperalgesia. Effects of naloxone (5, 0.05 mg/kg) or saline (1 ml/kg) injection given 30 min following morphine (10 mg/kg) (closed circles) or saline (1 ml/kg) (open circles) injection on the number of zif268-positive neurons. Lines show the mean ± SEM number of zif268-positive neurons counted for each treatment group in the: lateral-dorsal bed nucleus of the stria terminalis (BST) at −0.26 mm from bregma, lateral interstitial nucleus of the posterior limb of the anterior commissure (IPAC) at −0.92 mm from bregma, amygdalostratal transition zone at −1.3 mm from bregma, and central amygdala (CeA) at −2.12 mm from bregma. (B) Photomicrographs of the central amygdala showing the relative distribution of zif268-positive neuronal nuclei (green) and CGRP-immunoreactive terminals (red) following acute morphine (10 mg/kg), high dose of naloxone (5 mg/kg), and high dose of naloxone (5 mg/kg) in morphine (10 mg/kg) pre-treated rats. Abbreviations: CeA, central amygdala; BLA, basolateral amygdala; CeC, capsular division of the central amygdala; CeL, lateral division of the central amygdala; CeM, medial division of the central amygdala. Scale bar = 200 µm.

Systemic morphine administration increased c-Fos-positive neurons in the basolateral amygdala (S/S vs. M/S, \(P < 0.001\)) (Fig. 6). Abstinence did not cause further increases. Instead, significantly fewer c-Fos-positive neurons were induced when morphine pre-treated rats were injected with the low dose of naloxone (M/S vs. M/N0.05, \(P < 0.05\), and no induction was detected when the high dose of naloxone was injected (M/S vs. M/N5, \(P < 0.001\)) (Fig. 6). Neither dose of naloxone had an effect on c-Fos-positive neurons in the basolateral amygdala when injected into control saline pre-treated rats.

Sections containing the midbrain A9 and A10 dopamine cell groups were co-stained for c-Fos and TH-IR. Morphine followed by a saline injection (S/S vs. M/S) increased c-Fos-positive neurons in the substantia nigra (P < 0.05) and interfacicular nucleus (P < 0.05) (Fig. 7). No significant change was detected in the ventral tegmental area (P = 0.192) but it
can be seen that the variance in c-Fos-positive neurons counted in the M/S group in this region was large (Fig. 7). In the substantia nigra, only non-dopamine (TH-negative) neurons were c-Fos-positive. In contrast, the interfascicular nucleus only contains dopamine neurons, and so all of the c-Fos-positive neurons were also TH-positive (Fig. 7). The increase in c-Fos-positive neurons that was induced by systemic morphine in the substantia nigra and interfascicular nucleus when followed by saline, was not detected when the high naloxone dose was injected 30 min after the agonist (M/S vs. M/N5: SN, \( P < 0.01 \); IF, \( P < 0.01 \) (Fig. 7).

As morphine can induce both c-Fos mRNA and protein in the accumbens and striatum of rats (Barrot et al., 1999; Bonnetti and Sharp, 1997; Curran et al., 1996; D’Este et al., 2002; Garcia et al., 1995; Liu et al., 1994; Nye and Nestler, 1996; Tolliver et al., 2000), we also examined these targets of the striatonigral and mesolimbic dopamine projections. Morphine followed by a saline injection, increased c-Fos-positive neurons in the dorsomedial and ventromedial striatum, (S/S vs. M/S: dorsomedial, \( P < 0.001 \); ventromedial, \( P < 0.01 \) but not in the shell and core of the nucleus accumbens (Fig. 7). These increases in c-Fos-positive neurons could no longer be detected in the striatum when the high naloxone dose was administered 30 min after morphine (M/S vs. M/N5: dorsomedial, \( P < 0.01 \); ventromedial, \( P < 0.05 \) (Fig. 7).

Fig. 6. (A) Photomicrographs of the basolateral amygdala showing the distribution of c-Fos-positive neurons (5 sections) after a saline challenge, low dose of naloxone (0.05 mg/kg), and high dose of naloxone, in morphine pre-treated rats. Abbreviations: CeA, central amygdala; BLA, basolateral amygdala; LA, lateral amygdala. Scale bar = 200 μm. (B) Effects of naloxone (5, 0.05 mg/kg) or saline (1 ml/kg) injection given 30 min following morphine (10 mg/kg) (closed circles) or saline (1 ml/kg) (open circles) injection on the number of c-Fos-positive neurons. Lines show the mean ± SEM number of c-Fos-positive neurons counted across the rostro-caudal extent basolateral amygdala.

Table 1 compares c-Fos-positive neurons in the M/S and M/N groups counted in the parabrachial nucleus, periaqueductal...
Fig. 7. (A) Effects of naloxone (5 mg/kg) or saline (1 ml/kg) injection given 30 min following morphine (10 mg/kg) or saline (1 ml/kg) injection on the number of c-Fos-positive neurons. Bars show the mean ± SEM number of c-Fos-positive neurons counted in the dorsomedial and ventromedial striatum (4 sections); accumbens shell and core (5 sections); substantia nigra, ventral tegmental area, and interfascicular nucleus (7 sections), and paraventricular hypothalamus (2 sections) in each of the treatment groups. (B) Photomicrographs showing the distribution of c-Fos-positive neurons and tyrosine-hydroxylase-immunoreactivity in the dorsal striatum, midbrain dopamine regions, and paraventricular hypothalamus after acute morphine (10 mg/kg) and following a naloxone injection (5 mg/kg) 30 min after morphine. Abbreviations: IF, interfascicular nucleus; SN, substantia nigra; VTA, ventral tegmental area; PVN, paraventricular hypothalamus; 3V, third ventricle. Scale bar = 200 μm.
gray, rostral ventromedial medulla, area postrema, the A2/C2 and non-catecholamine cell groups in the nucleus of the solitary tract, and the A1/C1 and non-catecholamine cell groups in the ventrolateral medulla. Administering naloxone after morphine did not increase c-Fos-positive neurons in any of these brain regions. Significant reductions in the number of c-Fos-positive neurons were detected in the ventrolateral periaqueductal gray (P < 0.05), nucleus of the solitary tract (P < 0.05), and the ventrolateral medulla (P < 0.01). A reduction in c-Fos-positive neurons caused by naloxone in the rostral ventromedial medulla following morphine administration was not statistically significant (P = 0.074).

4. Discussion

This study has examined the effect of opioid hyperalgesia induced by acute abstinence in non-dependent rats. A preliminary behavioural experiment confirmed previous reports that naloxone can induce hyperalgesia when administered after a brief 30-min period of morphine analgesia (Céleri et al., 1999; Devillers et al., 1995; Goldfarb et al., 1978; Kaplan and Fields, 1991; Kim et al., 1990; Larcher et al., 1998). In a parallel anatomical experiment, the treatment that induced hyperalgesia increased neurons with nuclei expressing the IEG proteins c-Fos and zif268 in CeC, AStr, lateral IPAC and dorsal-lateral BST (Figs. 3, 5). The CeC and AStr in the CeA, are the major targets of the nociceptive area in the pontine parabrachial nucleus, which also supplies lighter projections to lateral IPAC and dorsal-lateral BST in the extended amygdala (Shammah-Lagnado et al., 1999; Bourgeais et al., 2001; Gauriau and Bernard, 2002). We found evidence of tonic opioid inhibition affecting these regions, as naloxone evoked smaller increases in IEG expressing neurons when administered to saline pre-treated rats, consistent with previous reports (Gestreau et al., 2000; Hamlin et al., 2001; Veinanete et al., 2003). The CeL, which receives only light projections from the nociceptive parabrachial area (Bourgeais et al., 2001, Gauriau and Bernard, 2002), was disinhibited by naloxone but did not respond in parallel with abstinence hyperalgesia. However, in contrast to the regions that showed transcription factor induction in parallel with abstinence hyperalgesia, significant increases in IEG expressing neurons were induced by morphine pre-treatment in CeL and CeM, both of which are targets of autonomic and gustatory areas of the parabrachial nucleus (Gauriau and Bernard, 2003).

The high naloxone dose which precipitated abstinence hyperalgesia in non-dependent rats did not increase c-Fos in the structures outside of the extended amygdala that we examined. This included the paraventricular hypothalamus, ventral BST, parabrachial nucleus, and catecholamine cell groups in the RVM and NTS—all of which show increased Fos mRNA or protein expression in parallel with the CeA and BST after induction of abstinence in dependent rats (Georges et al., 2000; Hamlin et al., 2001; Hayward et al., 1990; Milanes et al., 1997), or (2) stimulation of hypothalamic-pituitary-adrenal (HPA) axis by physical stressors (Day et al., 2005; Dayas et al., 2001; Herman et al., 2003). Abstinence precipitated by opioid antagonists in dependent rats induces a syndrome defined by well-characterised physical and behavioural symptoms (Maldonado et al., 1992), in addition to these widespread localised increases in c-Fos expression in a large number of brain regions. However, in contrast to acute abstinence in non-dependent subjects, withdrawal symptoms are at best only weakly expressed when abstinence is precipitated in drug-naive subjects after a brief opioid exposure (Harris and Gewirtz, 2005).

Further examination of the brain regions where IEG expressing neurons were increased by the combination of morphine and naloxone that precipitated hyperalgesia did not detect an effect of morphine pre-treatment followed by saline. However, administering a high dose of naloxone after saline pre-treatment did cause small increases of c-Fos neurons in the CeC, lateral IPAC and dorsal-lateral BST; and zif268 neurons in the AStr and lateral IPAC. Similar effects have been reported previously, and it has been suggested increased IEG expression is caused by a block of ongoing tonic inhibition maintained in these brain regions by endogenous opioid peptides (Buller et al., 2005; Carr et al., 1999; Gestreau et al., 2000; Hamlin et al., 2001, 2004). There is evidence this could be maintained by kappa opioid receptors (OPRs), as intracerebroventricular administration of the kappa-OPR selective antagonist nor-binaltorphimine (nor-BNI) and the non-selective opioid antagonist naltraxone cause similar increases in c-Fos-positive neurons in the CeA and lateral-dorsal BST, whereas the mu-opioid selective antagonist CTAP has no effect (Carr et al., 1999). Our data provides indirect support for this view, as only the high naloxone dose used to precipitate abstinence hyperalgesia is likely to effectively block kappa-OPRs (e.g. Schmauss and Yaksh, 1984), as the antagonist has approximately a 10-fold lower affinity for this receptor subtype relative to the mu-OPR (Magnan et al., 1982). Irrespective of whether kappa receptor subtypes are involved, the data presented here suggests that morphine facilitates the disinhibitory mechanism that leads to IEG induction in nociceptive amygdalar regions when endogenous opioid systems are blocked. This effect of morphine can persist for prolonged...
periods as c-Fos continues to be induced in regions of the CeA and BST even when naloxone is administered 24 h after a single morphine injection (Jin et al., 2004, 2005). It has been suggested these and other changes in the central amygdalar circuitry may be related to altered expression of conditioned aversive behaviours induced by naloxone treatments (see review by Harris and Gewirtz, 2005).

We found that the CeL was activated independently by morphine and naloxone. It has been demonstrated by in situ hybridisation studies that all CeL neurons are GABA cells that express GAD mRNA and either co-localised proenkephalin or corticotropin releasing-hormone mRNA (Day et al., 1999). As lesion studies show enkephalin terminals in the CeA mostly originate from intrinsic neurons (Cassell et al., 1999), these neurons may be the major source of endogenously released opioids in the CeL. If so, tonic inhibition could be maintained by mu-OPRs as the low naloxone dose increased IEG expressing neurons in the CeL when administered following saline. It is not known if autoreceptors are located on enkephalin neurons, but presynaptic mu-OPRs have been localised to terminals projecting from glutamate neurons in the parabrachial nucleus (Chamberlin et al., 1999), and these correspond to the CGRP-IR terminals that form dense perisomatic baskets around enkephalin neurons in the CeL (Schwaber et al., 1988; Xu et al., 2003).

We were surprised to find in many structures morphine-induced increases in c-Fos neurons were substantially reduced or even absent when the agonist effect was terminated after 30 min with naloxone. As all three groups of rats that received morphine as the first drug experienced analgesia in the following 30 min, we expected the c-fos mRNA levels induced by this stimulus to be close to maximal (Herdegen and Leah, 1998), and would therefore continue to induce c-Fos protein expression 2 h later. However, when naloxone was administered after morphine, no induction of c-Fos was detected in the basolateral amygdala, dorsomedial and ventromedial striatum, paraventricular hypothalumus, substantia nigra, and dopamine neurons in the interfascicular region of the ventral tegmental area. This suggested that c-Fos protein is only induced in these areas after prolonged stimulation by morphine, and may possibly occur in response to secondary processes triggered by the morphine-induced drug state. For example, induction of c-Fos in the medial striatum is dopamine dependent, and extracellular dopamine overflow, typically peaks more than 60 min after systemic morphine administration (Cadoni and Di Chiara, 1999; Forster et al., 2002). However, naloxone treatment could also accelerate the breakdown of c-Fos as Redburn and Leah (1997) report that c-Fos induced in the thalamus by a noxious stimulus is reduced or erased when a second noxious stimulus is administered, which the authors suggest could be due to ubiquination or acceleration of 26S proteosome activity. Given the extensive evidence that IEG induction is required for expression of some of the long-term neuronal and behavioural changes induced by intermittent or continuous morphine treatment (De Vries and Shippenberg, 2002; Vanderschuren and Kalivas, 2000), it would seem important that the temporal basis of this induction be investigated further.

Neugebauer et al., 2004 has recently reviewed a substantial body of experimental evidence, which suggests the CeA can bi-directionally facilitate and inhibit pain. We found morphine induced c-Fos and zif268 neurons in the CeM, and the BLA in the cortical region of the amygdala, both of which are required for normal expression of conditioned fear responding and hyperalgesia. Morphine also increased IEG expression in the CeL, which has been postulated to function in modulating output from the CeM-related circuitry (Cassell et al., 1999; Day et al., 1999, 2005; Petrovich and Swanson, 1997). In contrast, the structures in which IEG expression increased in parallel with abstinence hyperalgesia have extensive interconnections, and form a distinct nociceptive extended amygdalar subcircuit defined by input received from the spino-parabrachial-amygdalar pathway, and cortical areas that do not project heavily to the CeM (McDonald, 1998). Activation of this circuitry could have multiple effects on descending pain regulation. Antinociception could be produced via inhibition of the CeM and other nuclei that participate in descending pain inhibition (Day et al., 1999, 2005; Petrovich and Swanson, 1997). Alternatively, activation of descending pain-facilitation pathways that pass through the amygdala complex (Jasmin et al., 2003; Neugebauer et al., 2004), or feedback projections from the lateral-capsular CeA to the parabrachial nucleus and NTS (Petrovich and Swanson, 1997; Sun and Cassell, 1993; Sun et al., 1994) could function in modulating the transmission of ascending nocuous information. Finally, the CeC, AStr, lateral IPAC and lateral-dorsal BST project directly to targets in the nigrostriatal and basal forebrain systems (Bourgeais et al., 2001; Cassell et al., 1999; Dong et al., 2001a,b; Dong and Swanson, 2004), which could modulate pain processing by thalamocortical systems.


