Mu opioid receptor and orexin/hypocretin mRNA levels in the lateral hypothalamus and striatum are enhanced by morphine withdrawal

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Abstract

In this study, we investigated the effects of acute morphine administration, chronic intermittent escalating-dose morphine administration and spontaneous withdrawal from chronic morphine on mRNA levels of mu opioid receptor (MOP-r), and the opioid peptides pro-opiomelanocortin (POMC) and preprodynorphin (ppDyn) in several key brain regions of the rat, associated with drug reward and motivated behaviors: lateral hypothalamus (lat.hyp), nucleus accumbens (NAc) core, amygdala, and caudate–putamen (CPu). There was no effect on MOP-r mRNA levels in these brain regions 30 min after either a single injection of morphine (10 mg/kg, i.p.) or chronic intermittent escalating-dose morphine (from 7·5 mg/kg per day on day 1 up to 120 mg/kg per day on day 10). Activation of the stress-responsive hypothalamic–pituitary–adrenal axis by 12 h withdrawal from chronic morphine was confirmed; both POMC mRNA levels in the anterior pituitary and plasma adrenocorticotropic hormone levels were significantly elevated. Under this withdrawal-related stress condition, there was an increase in MOP-r mRNA levels in the lat.hyp, NAc core, and CPu. Recent studies have demonstrated a novel role for the lat.hyp orexin (or hypocretin) activation in both drug-related positive rewarding, and withdrawal effects. Around 50% of lat.hyp orexin neurons express MOP-r. Therefore, we also examined the levels of lat.hyp orexin mRNA, and found them increased in morphine withdrawal, whereas there was no change in levels of the lat.hyp ppDyn mRNA, a gene coexpressed with the lat.hyp orexin. Our results show that there is an increase in *MOP-r* gene expression in a region-specific manner during morphine withdrawal, and support the hypothesis that increased lat.hyp orexin activity plays a role in morphine-withdrawal-related behaviors.

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Introduction

Endogenous opioidergic systems, especially pro-opiomelanocortin (POMC)-derived β-endorphin, exert inhibitory effects on the hypothalamic–pituitary–adrenal (HPA) axis in both humans (Volavka *et al.* 1979, Schluger *et al.* 1998) and rodents (Eisenberg 1980, Zhou *et al.* 2005). β-Endorphin immunoreactive (ir) fibers and corticotropin-releasing hormone (CRH)-ir perikarya are colocalized in the paraventricular nucleus (PVN) of the hypothalamus (e.g. Pilcher & Joseph 1984). To exert a tonic inhibition on CRH neuronal activity, it is suggested that β-endorphin acts primarily at the mu opioid receptor (MOP-r; Nikolarakis *et al.* 1987). In the arcuate nucleus of the mediobasal hypothalamus, MOP-rs are presynaptic autoreceptors in β-endorphin neurosecretory neurons that regulate the release of β-endorphin from these cells (Nikolarakis *et al.* 1987, Kelly *et al.* 1990). It has been reported that a downregulation of MOP-r binding following chronic morphine treatment is associated with decreased potency and/or efficacy of MOP-r agonists in mediobasal hypothalamic neurosecretory neurons (including β-endorphin and dopamine neurons) in the guinea pig (Zhang *et al.* 1996). In conjunction with this finding, MOP-r mRNA levels are found to decrease in the mediobasal hypothalamus of the female guinea pig following chronic morphine treatment (Ronnekleiv *et al.* 1996). Chronic methadone maintenance in the male rat, however, does not alter the MOP-r mRNA levels in the hypothalamus, nucleus accumbens (NAc) core, or caudate–putamen (CPu; Leri *et al.* 2006).

In the male rat, acute (1 or 2 day) administration of morphine stimulates β-endorphin and adrenocorticotropic hormone (ACTH) secretion from the anterior pituitary, and then corticosterone secretion from the adrenal gland, while tolerance (even suppression) of HPA activity develops in the rat following long-term (5 days or longer) morphine treatment (Buckingham & Cooper 1984, Ignar & Kuhn 1990, Martinez *et al.* 1990, Zhou *et al.* 1999). Both spontaneous and opioid antagonist-precipitated morphine-withdrawal results in an elevation of plasma β-endorphin, ACTH, and glucocorticoid levels in both rodents (Lightman & Young 1988, Ignar & Kuhn 1990, Martinez *et al.* 1990) and humans (Kreek & Hartman 1982, Kosten *et al.* 1986, Culpepper-Morgan & Kreek 1997). In humans, an earlier study from our laboratory has found that naloxone-precipitated withdrawal in opiate-dependent individuals results in an increase in HPA hormonal release, which

precedes subjective and objective symptoms of withdrawal (Culpepper-Morgan & Kreek 1997).

The lateral hypothalamus (lat.hyp) is an important brain region for reward and other motivated behaviors (e.g. Harris *et al*. 2005). However, the question of whether morphine withdrawal influences *MOP-r* gene expression in the lat.hyp has not yet been studied. An administration paradigm of chronic intermittent escalating-dose morphine was recently developed in our laboratory in order to mimic the multiple and escalating doses that human heroin abusers seek daily to achieve rewarding effects and to prevent symptoms of withdrawal from a 12 h between-dose interval (Kreek *et al*. 2002). The present studies were, therefore, undertaken to determine the effects of acute single-dose morphine administration, 10-day chronic intermittent escalating-dose morphine administration or its 12 h spontaneous withdrawal, on MOP-r mRNA levels in the lat.hyp. We also examined the effects of this morphine withdrawal on mRNA levels of MOP-r in several other brain regions associated with drug-seeking or drug-withdrawal behaviors, including the NAc core, amygdala, and CPu. In the medial portion of the hypothalamus and anterior pituitary, the regions associated with HPA regulation, we also measured POMC and CRH type 1 receptor (CRH-R1) mRNA levels.

The orexins/hypocretins are neuropeptides that are mainly expressed in cells of the lat.hyp (de Lecea *et al*. 1998). It has been established that the hypothalamic orexins (orexins A and B) are involved in the regulation of sleep, arousal, feeding, and stress (Saper *et al*. 2005, Winsky-Sommerer *et al*. 2005). There is a growing body of evidence suggesting a novel role for the orexins in regulation of drug seeking-related behaviors. For example: (1) the studies of acute morphine administration have shown an attenuated increase in extracellular dopamine levels in the NAc by orexin receptor blockade in the ventral tegmental area (Narita *et al*. 2006). (2) Animal behavioral studies suggest that the interaction between orexins and their receptors may underlie motivated behaviors induced by morphine, cocaine, or food (Boutrel *et al*. 2005, Harris *et al*. 2005, Borgland *et al*. 2006), and morphine-withdrawal-related behaviors (Georgescu *et al*. 2003). In the present studies, therefore, we also determined whether acute or chronic morphine administration, spontaneous morphine withdrawal would affect orexin mRNA expression in the lat.hyp.

Materials and Methods

Animal maintenance and treatment

Male Fischer rats (190–220 g, Charles River Labs, Kingston, NY, USA) were housed individually in a stress–minimized facility with free access to water and food. Animals were adapted to a standard 12 h light:12 h darkness cycle (lights off from 0700 to 1900 h) for 7 days.

Experiment I: acute morphine Animals received i.p. injections of saline (1 ml/kg) for 7 days in their home cages. On the experimental day, animals were randomly assigned to two treatment groups, and given either one single injection of morphine (10 mg/kg) or an equal volume of saline (1 ml/kg) in their home cages, beginning 4 h after the start of their daily dark cycle (1100 h). Thirty minutes after the injection, rats were exposed to CO_2 for 15 s and killed by decapitation.

The time of killing is around the peak time point of plasma ACTH and corticosterone levels during the day in the rat. The morphine dose chosen was based on a pilot study, in which a single 10 mg/kg dose, but not 5 mg/kg, was observed to moderately increase ACTH and corticosterone levels in morphine naïve rats (Y Zhou, J Bendos & L Hofmann, unpublished observations).

Experiment II: chronic morphine and its acute withdrawal Animals were randomly assigned to two groups, and given either morphine or an equal volume of saline (1 ml/kg) three times daily with two 6-h intervals and one 12-h interval, beginning 4 h after the start of their daily dark cycle (1100, 1700, and 2300 h) for 10 days. The paradigm of chronic (10 days) escalating-dose morphine included a dose increase every second day: animals received $7 \cdot 5$ ($3 \times 2 \cdot 5$) mg/kg per day on days 1–2, 15 ($3 \times 5 \cdot 0$) mg/kg per day on days 3–4, 30 (3×10) mg/kg per day on days 5–6, 60 (3×20) mg/kg per day on days 7–8, and 120 (3×40) mg/kg per day on days 9–10.

We conducted our studies in two separate experiments, due to timing, space, and manpower requirements involved in such chronic experiments: (1) *Experiment IIa*: chronic (10 days) intermittent escalating-dose morphine (CIEM). (2) *Experiment IIb*: acute spontaneous withdrawal from chronic intermittent escalating-dose morphine (ASWCIEM). We had two separate saline-control groups, one for each experiment, which were by necessity identical. In each experiment, animals were assigned to two groups of six animals. In Experiment IIa, (1) CIEM experimental group: the animals received intermittent escalating-dose morphine for 10 days (administration paradigm as detailed earlier) and then received the last injection of morphine at a final dose (40 mg/kg) at 1100 h on day 11. (2) CIEM control: the animals received identical injections of saline for 10 days and then received the last injection of saline at 1100 h on day 11. In Experiment IIb, (1) ASWCIEM experimental group: the animals received chronic escalating-dose morphine for 10 days as described in Experiment I, and then received a last injection of saline (instead of morphine) at 1100 h on day 11. (2) ASWCIEM control: the animals received chronic saline for 10 days and then received the last injection of saline at 1100 h on day 11, as described in the Experiment IIa. Since the saline-control groups are identical in Experiments IIa and IIb, the data are considered together as one single group ('saline control') for analysis. The animals' body weights were recorded for 10 days during the injections. Animals were killed at 1130 h (30 min after the last injection).

Since 40 mg/kg is a relatively high dose of morphine in opiate-naïve animals, possibly leading to symptoms of morphine overdose, including respiratory depression (Hurwitz & Fischer 1984) and lethality (Borron *et al.* 2002), we could not examine the effects of acute morphine treatment at 40 mg/kg. We followed the Principles of Laboratory Animal Care (NIH Publication No. 86-23, 1996), and the specific protocols were approved by the Rockefeller University Animal Care and Use Committee.

Preparation of RNA extracts

Each rat brain was removed from the skull and placed in a chilled rat brain matrix (Electron Microscopy Sciences, Ft Washington, PA, USA). Coronal slices containing the brain regions of interest were removed from the matrix and placed on a chilled Petri dish. Dissection was carried out under a dissecting microscope using razor blades and forceps. The brain regions of interest were identified according to the Rat Brain Stereotaxic Coordinates (Paxinos & Watson 1986), as described in detail in an earlier publication (Maggos *et al.* 1997). Six brain and pituitary regions, including the anterior pituitary, neurointermediate lobe/posterior lobe of the pituitary (NIL/PL), CPu, nucleus accumbens (NAc) core and amygdala, lat.hyp, and medial portion of hypothalamus (MH, including the PVN and arcuate nucleus) were dissected on ice, homogenized in guanidinium thiocyanate buffer and extracted with acidic phenol and chloroform as described earlier (Chomczynski & Sacchi 1987). After the final ethanol precipitation step, each extract was resuspended in DEPC-treated H_2O and stored at $-80\,^\circ C$.

Solution hybridization ribonuclease (RNase) protection–trichloroacetic acid (TCA) precipitation assay

The solution hybridization RNase protection–TCA precipitation protocol has been described in detail in earlier reports (Branch *et al.* 1992, Zhou *et al.* 1996). A 2100 bp fragment from the rat MOP-r cDNA, a 538 bp fragment from the rat POMC cDNA, or a 1700 bp fragment from the rat preprodynorphin (ppDyn) cDNA was cloned into the polylinker region of either pSP64 or pSP65 plasmids (Promega) in both the sense and the antisense orientations. A 531 bp fragment from rat hypocretin (or orexin) cDNA was cloned into the polylinker region of pBC SK+ (Stratagene, La Jolla, CA, USA). A 2·5 kb fragment from the rat CRH-R1 cDNA was cloned into the polylinker region of pcDNA (Promega). The plasmid pS/E (a pSP65 derivative) was used to synthesize riboprobe for the 18S rRNA to determine total RNA. ^{33}P-labeled cRNA antisense probes and unlabeled cRNA sense standards were synthesized using a SP6, T3, or T7 transcription system. A denaturing agarose gel containing 1·0 M formaldehyde showed that a single full-length transcript had been synthesized from each plasmid.

RNA extracts were dried in 1·5 ml Eppendorf tubes and resuspended in 30 μl 2×TESS (10 mM *N*-tris[hydroxy-methyl]methyl-2-aminoethane sulfonic acid (pH 7·4); 10 mM EDTA; 0·3 M NaCl; 0·5% SDS) that contained 150 000–300 000 c.p.m. of a probe. The samples were covered with mineral oil and hybridized overnight at 75 °C. For RNase treatment, 250 μl buffer that contained 0·3 M NaCl; 5 mM EDTA; 10 mM Tris–HCl (pH 7·5), 40 μg/ml RNase A (Worthington, Biochemicals, Freehold, NJ, USA) and 2 μg/ml RNase T1 (Calbiochem, San Diego, CA, USA) were added and each sample was incubated at 30 °C for 1 h. TCA precipitation was effected by the addition of 1 ml solution that contained 5% TCA and 0·75% sodium pyrophosphate. Precipitates were collected onto a filter in sets of 24 by using a cell harvester (Brandel, Gaithersburg, MD, USA) and were measured in a scintillation counter with liquid scintillant (Beckman Instruments, Palo Alto, CA, USA).

The procedure to measure mRNA levels involved comparison of values obtained from experimental samples (brain extracts) to those obtained for a set of calibration standards. The calibration standards had known amounts of an *in vitro* sense transcript, whose concentration was determined by optical absorbance at 260 nm. The set of calibration standards included those with no added sense transcript and those that contained between 1·25 and 80 pg sense transcript (Zhou *et al.* 1996). To determine the total attomoles of each mRNA in each extract, the amounts calculated from the standard curves were multiplied by 5·71 for MOP-r, 2·04 for POMC, 1·3 for orexin, or 1·08 for CRH-R1 to correct for the difference in length between the sense transcript (2100, 538, 531, or 2500 bases for the MOP-r, POMC, orexin, or CRH-R1 respectively) and the full-length mRNA (12, 1·1, 0·7, or 2·7 kb for the MOP-r, POMC, orexin, or CRH-R1). A new standard curve was generated each time the experimental samples were analyzed and all extracts of a particular tissue were assayed for each mRNA as a group in a single assay.

Total cellular RNA concentrations were measured by hybridization of diluted extracts to a ^{33}P-labeled probe complementary to 18S rRNA at 75 °C. The calibration standards for this curve contained 10 μg *Escherichia coli* tRNA plus either 0·0 or from 2·5 to 40 ng total RNA from rat brain, whose concentration was determined by optical absorbance at 260 nm.

Radioimmunoassays

At the time of decapitation, blood from each rat was collected in tubes, placed on ice, and was spun in a refrigerated centrifuge. Plasma was separated and stored at $-40\,^\circ C$ for hormonal measurements by RIA. Corticosterone and testosterone levels were assayed using a rat corticosterone or testosterone ^{125}I kit from ICN Biomedicals (Costa Mesa, CA, USA). ACTH or luteinizing hormone (lat.hyp) immunoreactivity levels were assayed from unextracted plasma by using a kit from Nichols Institute (San Juan Capistrano, CA, USA) or from Amersham Pharmacia Biotech. All values were determined in duplicate in a single assay.

Data analysis

In Experiment I, a Student's *t*-test was carried out for each tissue to evaluate the statistical significance of differences between treatment groups. In Experiments IIa and IIb, one-way ANOVA (saline control (controls combined), CIEM, and ASWCIEM) was used followed by Newman-Keuls *post hoc* tests. To examine the effects of chronic morphine on body weights in Experiment IIa, a Student's *t*-test was carried out. The accepted level of significance was $P < 0.05$.

Results

Effects of acute single-dose morphine (10 mg/kg)

mRNA levels of MOP-r, POMC, orexin, or CRH-R1 in specific brain regions or pituitary As shown in Table 1, acute morphine had no effect on MOP-r mRNA levels in the lat.hyp, amygdala, nucleus accumbens core, or CPu. There were no significant differences in POMC, orexin, or CRH-R1 mRNA levels in the medial hypothalamus, lat.hyp, amygdala, anterior pituitary, or neurointermediate lobe/posterior lobe of the pituitary, following acute morphine.

Plasma levels of ACTH, corticosterone, lat.hyp, or testosterone (Table 1) Although plasma ACTH levels after acute morphine were almost twice as high as in the saline controls, this effect did not reach statistical significance ($t = 3.38$, d.f. = 13, $P = 0.089$). However, there were significantly higher levels of plasma corticosterone after acute morphine ($t = 10.31$, d.f. = 14, $P < 0.01$). Acute morphine had no effects on either plasma lat.hyp or testosterone levels.

Effects of chronic (10 days) intermittent escalating-dose morphine or 12 h spontaneous withdrawal from chronic morphine

MOP-r mRNA levels in four specific brain regions In the lat.hyp, one-way ANOVA showed a significant treatment effect ($F_{(2,21)} = 12.2$, $P < 0.0005$; Fig. 1A). Although the mean level of MOP-r mRNA after chronic morphine was approximately 24% lower than the mean saline control level, a Newman-Keuls *post hoc* test did not show a significant difference. However, spontaneous 12 h withdrawal from chronic intermittent escalating–dose morphine led to a significant increase in MOP-r mRNA levels in the lat.hyp ($P < 0.01$). In addition, the MOP-r mRNA levels in the morphine-withdrawal group were significantly higher than the chronic morphine group ($P < 0.01$).

In the amygdala, neither chronic morphine nor its withdrawal had any effect on MOP-r mRNA levels (Fig. 1A).

In the CPu, one-way ANOVA showed a significant treatment effect ($F_{(2,21)} = 5.71$, $P < 0.05$; Fig. 1B). Chronic morphine had no effects on MOP-r mRNA levels. However, spontaneous morphine withdrawal led to a significant increase in MOP-r mRNA levels in the CPu ($P < 0.05$). In addition, the MOP-r mRNA levels in the morphine-withdrawal group were significantly higher than the chronic morphine group ($P < 0.05$).

In the nucleus accumbens core, one-way ANOVA showed a significant treatment effect ($F_{(2,17)} = 6.03$, $P < 0.05$; Fig. 1B). Chronic morphine had no effects on MOP-r mRNA levels in this region. However, spontaneous withdrawal resulted in a significant increase in MOP-r mRNA levels in the nucleus accumbens core ($P < 0.05$). In addition, the MOP-r mRNA levels in the withdrawal group were significantly higher than the chronic morphine group ($P < 0.05$).

Table 1 Effects of acute single-dose morphine (10 mg/kg, i.p.) on mRNA levels (attomole/µg RNA) of mu opioid receptor (MOP-r), POMC, orexin, or CRH-R1 in the lateral hypothalamus, medial hypothalamus, amygdala, caudate–putamen, nucleus accumbens core, anterior pituitary, or neurointermediate lobe/posterior lobe of the pituitary (NIL/PL), and on plasma levels of ACTH (pg/ml), corticosterone (ng/ml), luteinizing hormone (LH; ng/ml) and testosterone (ng/ml). Data shown in tables are treatment group means ± S.E.M.

		Saline control	Acute morphine
Lateral hypothalamus	MOP-r	0.30 ± 0.03	0.28 ± 0.04
	Orexin	5.1 ± 0.42	4.8 ± 0.34
Medial hypothalamus	POMC	14 ± 1.7	11 ± 1.8
Amygdala	MOP-r	0.17 ± 0.02	0.20 ± 0.01
	POMC	2.0 ± 0.38	1.7 ± 0.28
Caudate–putamen	MOP-r	0.13 ± 0.02	0.15 ± 0.01
Nucleus accumbens core	MOP-r	0.28 ± 0.03	0.31 ± 0.02
Anterior pituitary	POMC	720 ± 42	693 ± 45
	CRH-R1	0.95 ± 0.07	0.93 ± 0.08
NIL/PL	POMC	$21\,631 \pm 1002$	$17\,216 \pm 1535$
Plasma	ACTH	193 ± 35	380 ± 90[†]
	Corticosterone	234 ± 36	398 ± 36*
	LH	0.25 ± 0.04	0.26 ± 0.04
	Testosterone	0.87 ± 0.20	0.54 ± 0.14

Significant differences are indicated: *$P < 0.01$ ([†]$P = 0.09$).
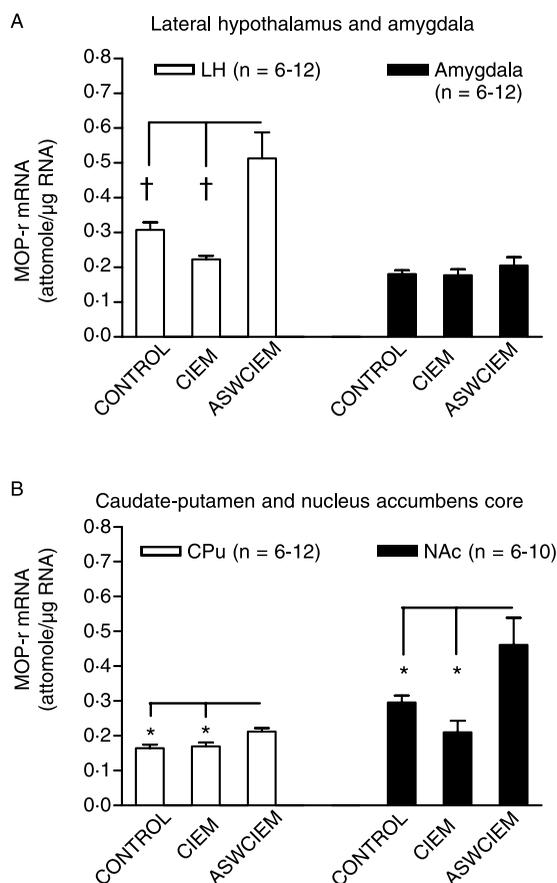
Figure 1 Effects of chronic (10 days) intermittent escalating-dose morphine (CIEM) or its 12 h acute spontaneous withdrawal (ASWCIEM) on mu opioid receptor (MOP-r) mRNA levels (attomole/μg RNA) in the (A) lateral hypothalamus (LH) or amygdala and (B) caudate–putamen (CPu) or nucleus accumbens (NAc) core. Data shown are treatment group means + s.e.m. Significant differences by Newman-Keuls *post hoc* test are indicated: *$P<0.05$ or †$P<0.01$.
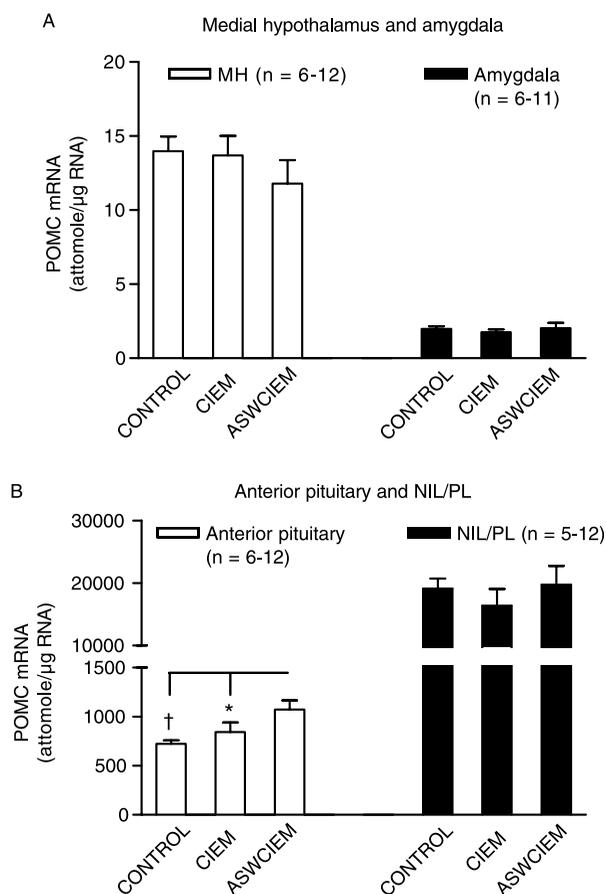

Figure 2 Effects of chronic (10 days) intermittent escalating-dose morphine (CIEM) or its 12 h acute spontaneous withdrawal (ASWCIEM) on POMC mRNA levels (attomole/μg RNA) in the (A) medial hypothalamus (MH) or amygdala and (B) anterior pituitary or neurointermediate lobe/posterior lobe of the pituitary (NIL/PL). Data shown are treatment group means + s.e.m. Significant differences are indicated: *$P<0.05$ or †$P<0.01$.

POMC mRNA levels in the brain and pituitary
Figure 2A shows POMC mRNA levels in the RNA extracts from the medial hypothalamus or amygdala. Neither chronic intermittent escalating-dose morphine nor its spontaneous withdrawal resulted in any change of POMC mRNA levels in these two regions.

In the anterior pituitary, one-way ANOVA showed a significant treatment effect ($F_{(2,23)}=7\cdot96$, $P<0\cdot01$; Fig. 2B). There was no significant effect of chronic morphine on POMC mRNA levels. However, spontaneous withdrawal led to a significant increase in POMC mRNA levels in the anterior pituitary ($P<0\cdot01$). In addition, the POMC mRNA levels in the withdrawal group were significantly higher than the chronic morphine group ($P<0\cdot05$).

No differences were found following chronic morphine or its withdrawal on POMC mRNA levels in the neurointer–mediate lobe/posterior lobe of the pituitary (Fig. 2B).

Orexin and ppDyn mRNA levels in the lateral hypothalamus Figure 3A shows orexin and ppDyn mRNA levels in the same RNA extracts from the lat.hyp as those used in assays of MOP-r mRNA (Fig. 1A). One-way ANOVA showed a significant treatment effect on orexin mRNA levels ($F_{(2,21)}=18\cdot16$, $P<0\cdot0001$). While there was no significant effect of chronic morphine on orexin mRNA levels, spontaneous morphine withdrawal led to a significant increase in orexin mRNA levels in the lat.hyp ($P<0\cdot001$). In addition, orexin mRNA levels in the morphine-withdrawal group were significantly higher than the chronic morphine group ($P<0\cdot001$).

Neither chronic intermittent escalating-dose morphine nor its spontaneous withdrawal resulted in any change of the ppDyn mRNA levels in the lat.hyp (Fig. 3A).

Orexin mRNA in the lat.hyp coexpress with ppDyn (Chou *et al.* 2001) and MOP-r (Georgescu *et al.* 2003).
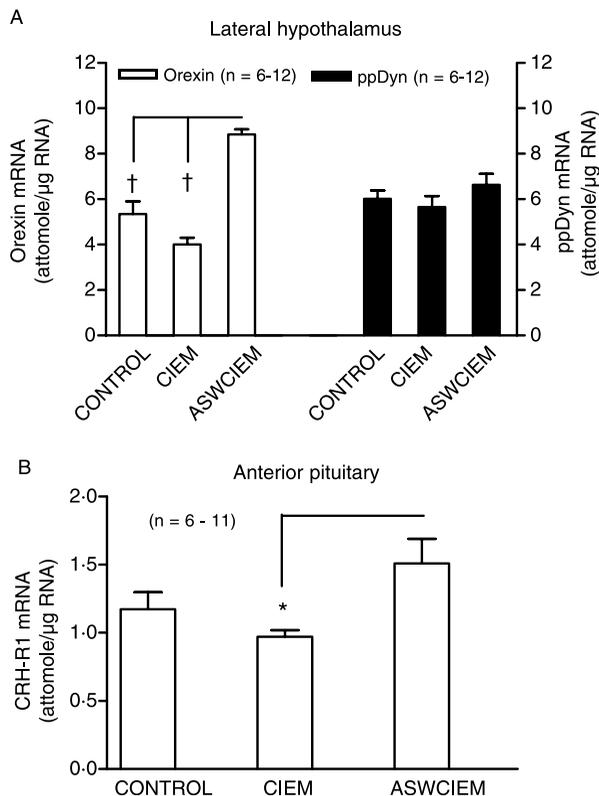
A

B

Figure 3 Effects of chronic (10 days) intermittent escalating-dose morphine (CIEM) or its 12 h acute spontaneous withdrawal (ASWCIEM) on mRNA levels (attomole/μg RNA) of (A) orexin or preprodynorphin (ppDyn) in the lateral hypothalamus (LH) and (B) CRH type 1 receptor (CRH-R1) in the anterior pituitary. Data shown are treatment group means + s.e.m. Significant differences are indicated: *$P < 0.05$ or †$P < 0.001$.

Since all of MOP-r, orexin, and ppDyn mRNA levels were determined in the lat.hyp of each rat, we were able to examine the relationship between the levels of mRNAs of three genes. Therefore, we examined whether orexin mRNA levels were related to either MOP-r or ppDyn mRNA levels. There was no correlation between the orexin, MOP-r, or ppDyn mRNA levels in the lat.hyp (data not shown).

CRH-R1 mRNA levels in the anterior pituitary
One-way ANOVA just failed to show a significant treatment effect on CRH-R1 mRNA levels in the anterior pituitary ($F_{(2,19)} = 3.25$, $P = 0.06$; Fig. 3B). Chronic morphine had no effects on CRH-R1 mRNA levels. Spontaneous morphine withdrawal did not lead to a significant increase in the CRH-R1 mRNA levels. However, there were significantly higher CRH-R1 mRNA levels in the morphine-withdrawal group than in the chronic morphine group in the anterior pituitary ($P < 0.05$).

Effects of chronic (10 days) intermittent escalating-dose morphine or 12 h spontaneous withdrawal from chronic morphine on body weight, and plasma levels of ACTH, corticosterone, lat.hyp, and testosterone

Body weight Rats that received saline injections ($n = 12$) had a body weight gain from day 1 (257 ± 4 g) to day 10 (271 ± 4 g). However, the animals that received morphine injections ($n = 12$) showed no body weight gain from day 1 (257 ± 2 g) to day 10 (255 ± 3 g). By day 10, body weights in the chronic morphine group were significantly lower than those in the saline-control group ($t = 9.97$, d.f. $= 22$, $P < 0.005$).

Plasma levels of ACTH, corticosterone, lat.hyp, and testosterone One-way ANOVA showed a significant treatment effect on plasma ACTH levels ($F_{(2,30)} = 45.64$, $P < 0.0001$; Fig. 4A). While there was no significant effect of chronic morphine, morphine withdrawal led to a significant increase in plasma ACTH levels, compared both with the
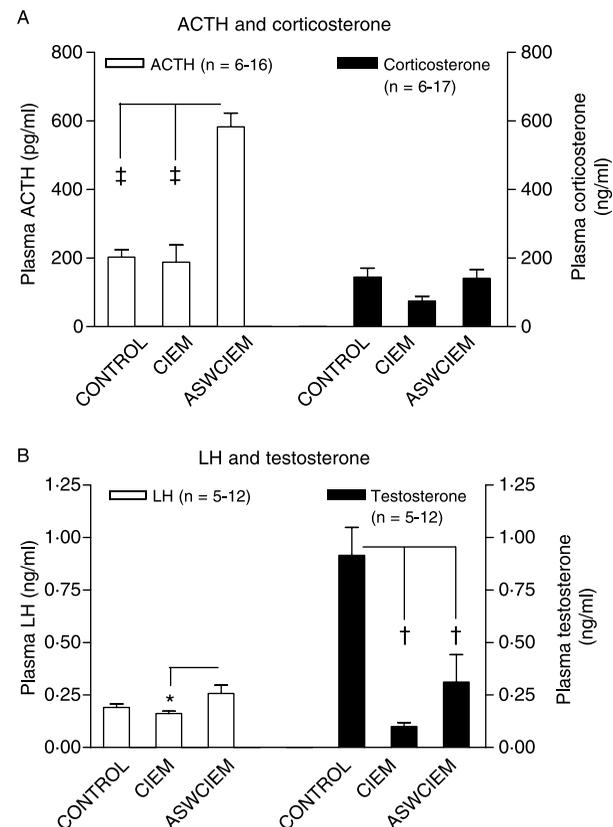
A

B

Figure 4 Effects of chronic (10 days) intermittent escalating-dose morphine (CIEM) or its 12 h acute spontaneous withdrawal (ASWCIEM) on plasma hormonal levels of (A) ACTH (pg/ml) or corticosterone (ng/ml) or (B) luteinizing hormone (LH; ng/ml) or testosterone (ng/ml). Data shown are treatment group means + s.e.m. Significant differences are indicated: *$P < 0.05$, †$P < 0.01$, or ‡$P < 0.001$.

saline–control group ($P<0\cdot001$), and to the chronic morphine group ($P<0\cdot001$). Neither chronic morphine nor its withdrawal resulted in any change in plasma corticosterone levels (Fig. 4A).

One-way ANOVA showed a marginally significant treatment effect on plasma lat.hyp levels ($F_{(2,19)}=3\cdot36$, $P=0\cdot05$; Fig. 4B). While there was no significant effect of either chronic morphine or its withdrawal on plasma lat.hyp levels compared with controls, plasma lat.hyp levels were significantly higher in the withdrawal group than in the chronic morphine group ($P<0\cdot05$).

One-way ANOVA showed a significant treatment effect on plasma testosterone levels ($F_{(2,21)}=11\cdot6$, $P<0\cdot0005$; Fig. 4B). Chronic morphine led to a significant decrease in plasma testosterone levels ($P<0\cdot01$), which was still found following 12 h withdrawal ($P<0\cdot01$).

Discussion

MOP-r mRNA responses in acute morphine withdrawal

In several studies of coupling of MOP-rs to K+ channels, it has been reported that arcuate nucleus β-endorphin neurons in the female guinea pig develop a high degree of tolerance to morphine compared with some other neurons in the same region, and the decrease in the efficacy and/or potency of the response to MOP-r agonists in the mediobasal hypothalamus is due in part to the downregulation of the MOP-r (Kelly et al. 1990, Zhang et al. 1996). Furthermore, a decreased MOP-r mRNA level was found in the mediobasal hypothalamus of the female guinea pig after chronic morphine (Ronnekleiv et al. 1996). In this study, we examined the rat lat.hyp after 10 days of chronic intermittent escalating-dose morphine administration, and found no significant effect on MOP-r mRNA levels. We extended our studies to investigate the effects of 12 h spontaneous morphine withdrawal, and found an increase in MOP-r mRNA levels in the lat.hyp. This increase in MOP-r mRNA levels after morphine withdrawal suggests an enhanced MOP-r biosynthesis, although it cannot be determined from assays of mRNA levels alone which steps (*MOP-r* gene transcription, processing, and/or degradation of mRNA) are affected. To the best of our knowledge, no effect of either chronic morphine or its withdrawal on MOP-r mRNA stability has been reported. Indeed, neither chronic morphine nor opiate antagonists have been reported to change the size of the MOP-r mRNA molecule, which suggests that the MOP-r mRNA stability is unaltered by chronic opiate treatment (Brodsky et al. 1995).

Different studies have examined the effect of chronic opioid agonists or their withdrawal on MOP-r mRNA levels in different brain regions (Brodsky et al. 1995, Buzas et al. 1996, Ronnekleiv et al. 1996, Castelli et al. 1997, Sehba et al. 1997, Duttaroy & Yoburn 2000). The results obtained are conflicting; a decrease (Ronnekleiv et al. 1996, Duttaroy & Yoburn 2000), an increase (Sehba et al. 1997), or no change

(Brodsky et al. 1995, Buzas et al. 1996, Castelli et al. 1997) have been reported, and these apparently contradictory results may depend on differences in the brain regions examined, exposure time, dose, and route of the opioid agonist administered. In many of the previous studies, morphine or opioid agonists were chronically administered by minipumps or pellets (Brodsky et al. 1995, Ronnekleiv et al. 1996, Sehba et al. 1997, Duttaroy & Yoburn 2000). This differs from our morphine experiments using an intermittent pattern of administration, in which the animal could experience both rewarding effects and chronic stress induced by repeated morphine injection and withdrawal.

We specifically selected several brain regions considered to play an important role in the reinforcing or motivational effects of drugs of abuse, such as the nucleus accumbens core, amygdala, lat.hyp, and CPu (e.g. Kreek & Koob 1998). Consistent with several earlier reports (Brodsky et al. 1995, Buzas et al. 1996, Castelli et al. 1997), we found that chronic morphine did not modify the steady-state MOP-r mRNA levels in the brain regions listed earlier. In contrast, spontaneous morphine withdrawal led to an increase in MOP-r mRNA levels in the nucleus accumbens core, lat.hyp, and CPu, but not in the amygdala. Our data clearly showed that morphine withdrawal increases MOP-r mRNA levels in a region-specific manner, and therefore suggest that the observed increase of MOP-r agonist responses may be due to an increased *MOP-r* gene expression in these brain regions (Alcaraz et al. 1993, Johnson & Napier 2000). A body of evidence suggests that opioid agonists exert inhibitory effects on endogenous opioid peptide gene expression through an opioid receptor-mediated mechanism (e.g. Morris et al. 1988). Here, our finding of increased *MOP-r* gene expression by morphine withdrawal suggests that opioid agonists also have an inhibitory effect on *MOP-r* gene expression.

Of note, there is no consensus that chronic opioid agonist administration disrupts MOP-r binding, signal transduction, or gene expression in different rat brain regions. For instance, an earlier study has found an increased MOP-r-binding density after chronic heroin self-administration in the hypothalamus, but not amygdala (Sim-Selley et al. 2000). However, a decrease in MOP-r-stimulated G-protein levels (as reflected by GTPγS binding) is only found in the amygdala, but not in the hypothalamus, after chronic morphine or heroin self-administration (Sim et al. 1996, Sim-Selley et al. 2000, Kruzich et al. 2003). In this study, spontaneous withdrawal from chronic morphine altered MOP-r mRNA levels in the hypothalamus, but not in the amygdala.

Stress responses in acute morphine withdrawal

In this study, we also found that: (1) acute morphine exposure to opiate–naïve rats stimulated the HPA activity, as reflected by an elevation of plasma ACTH and corticosterone levels 30 min after a single dose of morphine at 10 mg/kg dose. (2) After 10 days of chronic intermittent escalating-dose morphine administration, however, the levels of both plasma ACTH and

corticosterone did not show any responses to morphine, indicating adaptation or tolerance of HPA activity to chronic escalating-dose morphine. (3) Following spontaneous withdrawal for 12 h from this chronic morphine administration, there was a significant elevation of plasma ACTH levels. These results confirm earlier findings of HPA axis activation by morphine withdrawal after tolerance has developed following chronic morphine exposure (Buckingham & Cooper 1984, Ignar & Kuhn 1990, Martinez *et al.* 1990, Milanes *et al.* 2002). An elevation of corticosterone levels by morphine withdrawal has been reported (Lightman & Young 1988, Martinez *et al.* 1990), but this is observed after immediate (15 min to 4 h) morphine withdrawal precipitated by naloxone. In our study, which examined spontaneous withdrawal at the 12 h time point, there was no elevation in plasma corticosterone levels, suggesting that corticosterone levels had already returned to the basal levels at this time point.

It has recently been reported that morphine-induced place preference is not seen in orexin knockout mice, suggesting that the excitation of brain orexin neurons and/or the tonic activation of orexin receptors are specifically required for the rewarding effect of opiates (Narita *et al.* 2006). In addition, naloxone-precipitated morphine withdrawal induces orexin gene expression in orexin-LacZ reporter mice, and orexin knockout mice have attenuated opiate physical dependence as reflected by reduced withdrawal signs (Georgescu *et al.* 2003). In the lat.hyp, a subpopulation of orexin neurons (around 50%) expresses MOP-r (Georgescu *et al.* 2003), and nearly all orexin neurons (94%) express ppDyn mRNA (Chou *et al.* 2001).

In this study, quantitative and sensitive solution hybridization assays were used to measure mRNA levels of orexin, MOP-r, and ppDyn in each lateral hypothalamic RNA extract. We found a significant increase in orexin and MOP-r mRNA levels after acute spontaneous withdrawal, with no change in ppDyn mRNA levels. Within the same animals, however, there was no correlation between these two parallel increases. Therefore, this suggests that the regulation by acute morphine withdrawal of lat.hyp orexin gene expression is not directly related to either the *MOP-r* or *ppDyn* gene regulation in the same region.

MOP-r is also expressed in the lat.hyp neurons, which do not express orexin, but which also respond to morphine withdrawal (Georgescu *et al.* 2003). Since our assays could not differentiate these cells from the orexin neuron subpopulation, the MOP-r mRNA increases observed in this study may be due to both the orexin and the non-orexin cells. This may also account for the lack of correlation between the orexin and the MOP-r mRNA increases in morphine withdrawal. Although melanin-concentrating hormone (MCH) neurons are highly expressed in the lat.hyp and several studies have suggested them to be involved in reward and motivated behaviors (e.g. Duncan *et al.* 2005, Georgescu *et al.* 2005), it has been found that the MCH neurons do not express MOP-r, and do not respond to morphine withdrawal (Georgescu *et al.* 2003).

In summary, the present study showed that morphine withdrawal increases MOP-r mRNA levels in a brain region-specific manner. In animal behavioral studies, a growing body of evidence suggests a novel role for the orexins in the neurobiological processes of drug reward and other motivated behaviors (Boutrel *et al.* 2005, Harris *et al.* 2005). Our finding of an increase in orexin gene expression in the lat.hyp in the setting of morphine withdrawal supports this possible role.

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