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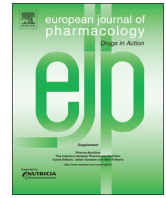
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## Molecular and cellular pharmacology

## Comparison of the transcriptional responses induced by acute morphine, methadone and buprenorphine



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## ABSTRACT

Despite their widespread use in opioid maintenance treatment and pain management, little is known about the intracellular effectors of methadone and buprenorphine and the transcriptional responses they induce. We therefore studied the acute effects of these two opioids in rats, comparing our observations with those for the reference molecule, morphine. We determined the analgesic ED<sub>50</sub> of the three molecules in the tail flick test, to ensure that transcriptional effects were compared between doses of equivalent analgesic effect. We analysed changes in gene expression over time in three cerebral structures involved in several opioid behaviours—the dorsal striatum, thalamus and nucleus accumbens—by real-time quantitative PCR. We analysed the expression of genes encoding proteins of the endogenous opioid system in parallel with that of *Fos*, a marker of neuronal activation. The acute transcriptional effects of methadone resembled those of morphine more closely than did those of buprenorphine, in terms of kinetics and intensities. Our results provide the first evidence that these two drugs widely used in pain management and opioid maintenance treatment can disturb the regulation of endogenous opioid system genes and induce molecular outcomes different from those observed with morphine.

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## 1. Introduction

Mu receptors are essential for all the biological activities elicited by morphine. For instance, analgesia, the main therapeutic activity of these receptors, is not observed in Mu Opioid (mu) receptor mutant mice (Fuchs et al., 1999; Loh et al., 1998; Matthes et al., 1996; Sora et al., 1997). Reward, the other main biological action of morphine, is also abolished in mutant mice (Matthes et al., 1996; Sora et al., 2001). The activation of mu receptor by morphine triggers multiple signal transduction systems. An understanding of the way in which mu receptor agonists, particularly those used in pain management or opioid maintenance treatment, differ in terms of these fundamental aspects of mu receptor activation will contribute to the development of effective treatments.

In this study, we investigated several opioid mu receptor agonists: morphine, methadone and buprenorphine. These drugs are all mu receptor agonists, but they differ in their pharmacological

and pharmacokinetic activity spectra. Morphine and methadone have similar opioid agonist profiles with respect to mu receptor, but methadone also binds NMDA receptors, with a lower affinity, and acts as a non-competitive NMDA receptor antagonist (Ebert et al., 1995; Mannino et al., 2006). Morphine has a shorter biological half-life than methadone (Ing Lorenzini et al., 2012; Lugo et al., 2005). Buprenorphine has a unique and complex pharmacology. It is classified as a partial agonist, and it activates a distinct subset of G proteins, different from those activated by morphine and methadone (Saidak et al., 2006; Wheeler-Aceto and Cowan, 1991). Buprenorphine is also a kappa receptor antagonist and an agonist of the nociception/orphanin FQ (NOP) receptor (Lutfy and Cowan, 2004).

Most preclinical studies have compared the behavioural effects of morphine, methadone and buprenorphine (Erichsen et al., 2005; Glover and Davis, 2008; Kalvass et al., 2007; Meert and Vermeersch, 2005), with only a few having considered the transcriptional responses they induce. Methadone and morphine display structure-specific differences in the induction of *Fos* expression in the nucleus accumbens (Taracha et al., 2006). Similar changes in nerve growth factor mRNA levels in rat striatum are observed following perinatal exposure to methadone and buprenorphine (Wu et al., 2001). In CEMx174 cells, methadone has

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similar effects on mu receptor mRNA levels to morphine but causes a selective upregulation of *CCR5* mRNA (Suzuki et al., 2002). We compared the acute effects of methadone and buprenorphine on transcription of the immediate early gene, *Fos*, with those of the reference opiate, morphine. The endogenous opioid system also plays an important role in many types of behaviour (Kieffer and Gaveriaux-Ruff, 2002; Sora et al., 1997). We also investigated the influence of morphine, methadone and buprenorphine on the transcription of genes encoding three opioid peptides and their receptors.

We first determined ED<sub>50</sub> values for the three opioids in the tail flick analgesic test. We then evaluated the patterns and time course of changes in gene expression in three cerebral structures, the striatum, nucleus accumbens and thalamus, in response to acute morphine, methadone and buprenorphine injections. These brain structures express the mu receptor receptor (Peckys and Landwehrmeyer, 1999) and are involved in the analgesic properties of morphine in the tail flick test (Dong et al., 1999; Saade et al., 1997; Yen et al., 1989). Moreover, two of these brain structures (dorsal striatum and nucleus accumbens) are among those most frequently implicated in the transition from recreational drug use to compulsive consumption of drugs of abuse (Lesscher and Vanderschuren, 2012). Morphine is an addictive drug and the other two molecules are used as maintenance treatments. It is therefore important to understand the early molecular responses to these drugs in these structures, as they are thought to trigger long-lasting changes in neuroplasticity and drug abuse (Robison and Nestler, 2011). We also evaluated the contributions of mu, delta, kappa and NOP opioid receptors to buprenorphine-induced analgesia in our conditions, given the specific pharmacological features of this drug.

## 2. Materials and methods

### 2.1. Animals

Male Sprague-Dawley rats (Janvier, Le Genest-Saint-Isle, France) weighing 220–230 g were housed in groups of four per cage, in a room with a 12-h light/12-h dark cycle and a controlled-temperature environment (20–22 °C). Rats were allowed to get used to the housing facilities over a period of 1 week. Body weight was determined on the day of experimentation. Food and water were provided ad libitum. Animal experiments were carried out in accordance with the European Communities Council Directives of 24 November 1986 (86/609/EEC) and with French law, with standard ethical guidelines and under the control of the faculty ethics committee. Every effort was made to minimise the number of animals used and their discomfort.

### 2.2. Drugs and pharmacological treatment

Morphine chlorhydrate and buprenorphine were purchased from Francopia (Paris, France). Methadone and naltrindole (NTI) were purchased from Sigma (Saint Quentin Fallavier, France). Naloxonazine (NLZ), (±)-1-[(3*R*\*,4*R*\*)-1-(cyclooctylmethyl)-3-(hydroxymethyl)-4-piperidinyl]-3-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one (J113397) and nor-binaltorphimine dihydrochloride (*nor*-BNI) were purchased from R&D Systems (Lille, France). Morphine, buprenorphine, methadone, *nor*-BNI and NTI were dissolved in saline (0.9% NaCl). J113397 was dissolved in vehicle (10% DMSO, 10% Tween 80, 80% H<sub>2</sub>O) and NLZ was dissolved in water. Morphine, buprenorphine and methadone were administered subcutaneously (s.c.) and the antagonists were administered intraperitoneally (i.p.), with an injection volume of 0.1 ml/100 g of body weight. The 50% effective dose (ED<sub>50</sub>) was calculated by the Litchfield and Wilcoxon method. As previously described, NTI (3 mg/kg), *nor*-BNI (2 mg/kg) and J113397 (2 mg/kg) were injected 30 min, and NLZ (10 mg/kg) was injected 5 h before morphine or buprenorphine (Liu and Jernigan, 2011; Peana et al., 2011; Xie et al., 2008; Xu et al., 2006; Yamamoto et al., 2006).

### 2.3. Tail-flick test

Antinociceptive responses were determined by measuring the time required to respond to a radiating thermal stimulus. The rat was restrained so that the radiant heat source was focused on the base of the tail. An automatic tail-flick analgesimeter (Applex, France) was used, and the cutoff time was set at 15 s, to minimise tissue damage. For each rat, three determinations were carried out before the experiment, to determine baseline latencies (between 2 and 4 s). An additional determination was performed 20 min after drug injection (to determine test latency). Latency in the tail-flick test is expressed as a percentage of the maximum effect (%MPE), as follows: %MPE = (test latency – baseline latency) / (cutoff time – baseline latency).

### 2.4. Dissection, RNA extraction and reverse transcription for quantitative real-time PCR

Total RNA was isolated from separate groups of rats (*n* = 10–12). Animals were killed 30 min, 1 h and 4 h after the injection of drugs, by a lethal injection of pentobarbital. The rats were decapitated and their brains were rapidly removed, frozen in isopentane at –50 °C, and placed in an acrylic matrix (David Kopf Instruments, Phymep, France) for the reproducible slicing of 1 mm coronal sections. Dorsal striatum (bregma 1.7\_0.7), nucleus accumbens (bregma 1.7\_0.7) and thalamus (bregma –3.3\_–4.3) were then dissected within 2 mm slices, according to the co-ordinates indicated in the Paxinos and Franklin atlas of the brain (Academic

**Table 1**  
Primer sequences used for SYBR Green-based real-time quantitative polymerase chain reaction.

Gene	Forward primer	Reverse primer
<i>Rplpo</i>	GGG GGC CAC CTG GAG AAC AAC	CAG CAG CTG GCA CCT TAT TG
<i>Tbp</i>	TGC ACA GGA GCC AAG AGT GAA	CAC ATC ACA GCT CCC CAC CA
<i>Fos</i>	GGC AAA GTA GAG CAG CTA TCT CCT	TCA GCT CCC TCC TCC GAT TC
<i>Oprd1</i>	CCC CAC CCA AGT GAA GTG AT	CTC CCC CCC CCC GTC TG
<i>Oprk1</i>	ACC CCC ATC CAC ATC TTT ATC	GCT GCT GTT GGT ATA AC
<i>Oprm1</i>	TGT CGG GCT CCA AAG AAA A	GG GGT CCA GCA GAC AAT AAA T
<i>Pdyn</i>	CAG GGC TCC TTC TGA ATC TTG	GGA CCA CGC CAT TCT GTA TC
<i>Penk</i>	CGG CGA CAT CAA CTT CCT	CTG GGG CTT GGA CAC CT
<i>Pomc</i>	GCG ACG GAG GAG AAA AGA	GA GCG ACT GTA GCA GAG TCT

Press, 2nd edition, 2001), and stored at  $-80^{\circ}\text{C}$  until processing. Total RNA was extracted from bilateral structures with the RNeasy Qiagen mini kit plus or the micro kit plus, according to the manufacturer's lipid tissue protocol (Qiagen, Courtaboeuf, France). Total RNA was quantified with a NanoDrop<sup>®</sup> ND-1000 spectrophotometer. The RNA was reverse-transcribed, as previously described (Belkai et al., 2009), in a final volume of 20  $\mu\text{l}$  containing 500 ng of total RNA, and cDNAs were stored at  $-20^{\circ}\text{C}$ .

### 2.5. Real-time quantitative PCR

Opioid peptides and receptors were chosen according to the nomenclature of the International Union of Basic and Clinical Pharmacology (IUPHAR). The nucleotide primer sequences for PCR were selected with Oligo 6.42 software (MedProbe, Oslo, Norway). The sequences of the primers used are described in Table 1. Gene expression was assessed by real-time quantitative PCR on the cDNA. Amplification was detected by SYBR<sup>®</sup> Green fluorescence on an ABI PRISM<sup>®</sup> 7900HT sequence detection system (Applied Biosystems, France). Thermocycling was carried out in a final volume of 20  $\mu\text{l}$  containing 0.5  $\mu\text{M}$  of each of the primers used and 10  $\mu\text{l}$  of Power SYBR<sup>®</sup> Green PCR Master Mix 2X (Applied Biosystems, France). We added 8  $\mu\text{l}$  of diluted cDNA to this mixture. The thermal cycling conditions were as previously described (Benturquia et al., 2008). Quantification was based on a calibration curve generated with cDNA from an untreated rat. In addition to the genes of interest, the *Rplp0* and *Tbp* transcripts were also quantified, and each sample was normalised on the basis of its *Rplp0* and *Tbp* content, as previously described (Vandesompele et al., 2002). For each treated group (morphine, methadone and buprenorphine) the results are presented as a fold-change with respect to the saline group.

### 2.6. Data analysis

All series of behavioural data were analysed with Statview 5.0 software (SAS Institute Inc., NC, USA). The data for real-time were analysed in a one-way ANOVA between subjects, followed by a Tukey test for *post hoc* comparisons. In the case of real-time PCR fold-change data, the S.D. was calculated as follows:  $\text{S.D.} = (\text{mean of treated group})^2 / (\text{mean of control group})^4 \times (\text{S.E.M. of mean of control group})^2 + (\text{S.E.M. of mean of treated group})^2 / (\text{mean of control group})^2$ . The S.E.M. was calculated as:  $\text{S.D.} / \sqrt{[(\text{number of control animals} + \text{number of treated animals}) - 1]}$ . Values of  $P < 0.05$  were considered significant.

## 3. Results

### 3.1. Determination of the effective dose 50 ( $ED_{50}$ ) for morphine, methadone and buprenorphine in the tail-flick test

The tail-flick test was performed, to determine the dose-dependence of the antinociceptive effects and the  $ED_{50}$  values of the three subcutaneously administered opioids and their 95% confidence limits. Three doses of morphine (1 mg/kg, 2.5 mg/kg and 6.25 mg/kg), methadone (0.5 mg/kg, 1 mg/kg and 2 mg/kg) and buprenorphine (0.0025 mg/kg, 0.05 mg/kg and 0.1 mg/kg) were used. As shown in Table 2, the  $ED_{50}$  of morphine was 3.25 mg/kg, with a confidence interval of 4.26–2.48 mg/kg. The  $ED_{50}$  of methadone was 1.2 mg/kg, with confidence interval of 1.70–0.84 mg/kg. The  $ED_{50}$  of buprenorphine was 0.065 mg/kg, with a confidence interval of 0.088–0.041 mg/kg. The dose of morphine (10 mg/kg, s.c.) used in the transcriptional studies was based on previous studies in the same rat strain (Garcia et al., 1995; Gutstein et al., 1998). The corresponding doses of

**Table 2**  
Morphine, buprenorphine and methadone  $ED_{50}$  values in the tail-flick test.

Molecule	$ED_{50}$ (95% CI)
Morphine	3.25 mg/kg (2.48–4.26)
Methadone	1.2 mg/kg (0.84–1.70)
Buprenorphine	0.065 mg/kg (0.041–0.088)

Drugs were injected s.c 30 min before testing in the tail-flick apparatus.  $n = 8$ –10 per group.

buprenorphine (0.2 mg/kg, s.c.) and methadone (3.7 mg/kg, s.c.) giving an equivalent level of analgesia were calculated.

### 3.2. Involvement of the four opioid receptors in morphine- and buprenorphine-induced analgesia

Buprenorphine has unusual pharmacological characteristics, interacting with several opioid receptors, depending on the experimental design. We investigated the role of the four opioid receptors in buprenorphine-induced analgesia in our conditions, by carrying out a detailed study with specific antagonists of the  $\mu$ ,  $\delta$ ,  $\kappa$  and NOP receptors. Four independent experiments were carried out. Rats were first injected with one of the four opioid antagonists, as described in the materials and methods section, and then with either morphine (10 mg/kg, s.c.) or buprenorphine (0.2 mg/kg, s.c.), and thermal nociception was then investigated in the tail-flick test. One-way ANOVA showed a significant difference between treatments (Fig. 1). In *post hoc* comparisons, both buprenorphine and morphine were found to have a significant antinociceptive effect in the four independent experiments. *Post hoc* testing also indicated that, in our conditions, NLZ was the only antagonist blocking the antinociceptive response of morphine ( $p < 0.001$ ) or buprenorphine ( $p < 0.001$ ).

### 3.3. Acute morphine-, methadone- and buprenorphine-induced changes to mRNA levels in the nucleus accumbens

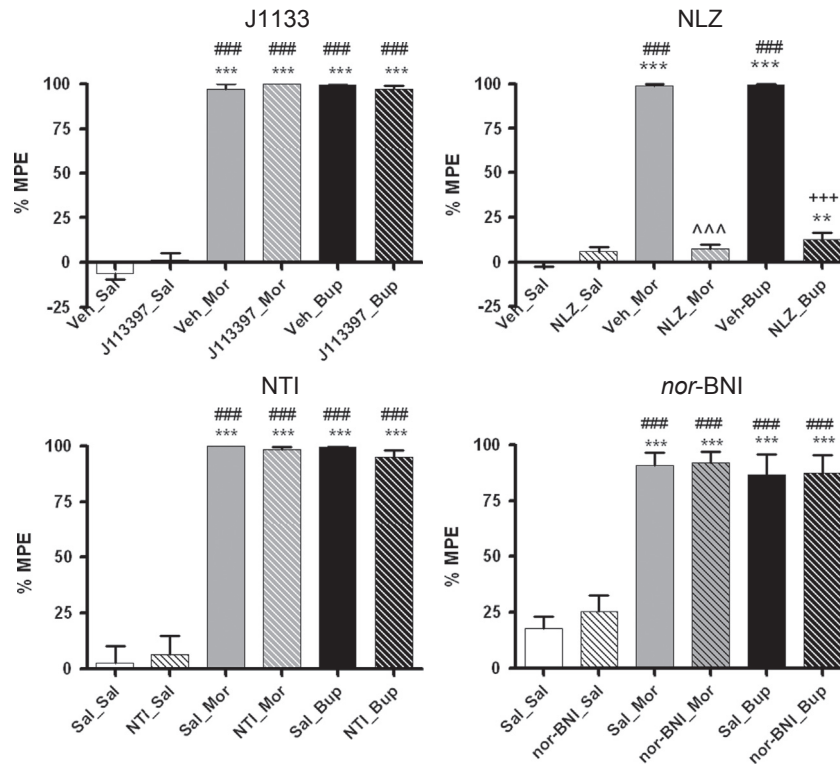
The kinetics of the effects of morphine, methadone and buprenorphine on the expression of the *Fos*, *Penk*, *Pdyn*, *Oprm1*, *Oprd1* and *Oprk1* genes at 30 min, 1 h and 4 h post-injection were analysed in nucleus accumbens (Fig. 2). The administration of morphine, methadone or buprenorphine had no effect on opioid receptor mRNA levels in this structure, at any of the time points tested. *Pomc* mRNA was undetectable in this structure in our conditions.

#### 3.3.1. Fos mRNA levels

One-way ANOVA revealed significant differences in *Fos* transcription levels at 30 min and 4 h. In morphine- and methadone-treated animals, *Fos* gene expression levels 4 h after injection were higher than those in control animals (by factors of 1.82 and 1.7, respectively). *Post hoc* comparisons showed that buprenorphine treatment was associated with significantly lower (0.76-fold) levels of *Fos* mRNA level than for control rats at 30 min. The amounts of this mRNA were significantly larger in the morphine and methadone groups than in the buprenorphine group (by factors of 2.02 and 1.88, respectively).

#### 3.3.2. Penk mRNA levels

One-way ANOVA revealed a significant treatment effect for *Penk* at 30 min. *Post hoc* comparisons showed *Penk* mRNA levels to be higher in morphine-treated animals than in controls (1.33 times higher). Methadone induced a 1.47-fold increase in the amount of



**Fig. 1.** Effects of NTI, Nor-BNI, NLZ and J113397 on the analgesia induced by acute morphine (10 mg/kg) and buprenorphine (0.2 mg/kg) in the tail flick test. Rats were pre-treated with the antagonists, as described in the materials and methods, and then with morphine or buprenorphine. They were tested 20 min after the opiate injection. Results are expressed as %MPE. Mean  $\pm$  S.E.M. ( $n=10-12$  per group). Significantly different from: control \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; antagonist ### $P < 0.001$ ; buprenorphine +++ $P < 0.001$ ; ^^^ $P < 0.001$ .

this mRNA, and buprenorphine induced a 1.37-fold increase in the amount of this mRNA with respect to control animals.

### 3.3.3. *Pdyn* mRNA levels

One-way ANOVA for *Pdyn* showed a significant treatment effect at 30 min. *Post hoc* comparisons showed *Pdyn* gene expression levels to be significantly lower in methadone-treated animals than in the control (0.77-fold), morphine (0.8-fold) and buprenorphine (0.8-fold) groups at this time point.

### 3.4. Acute morphine-, methadone- and buprenorphine-induced changes in mRNA levels in the striatum

The effects of morphine, methadone and buprenorphine on the expression of the *Fos*, *Penk*, *Pdyn*, *Pomc*, *Oprm1*, *Oprd1* and *Oprk1* genes in the striatum were analysed at 30 min, 1 h and 4 h post-injection (Fig. 3). Morphine, methadone and buprenorphine had no effect on the level of *Oprm1* transcription in this structure, at any of the time points considered.

#### 3.4.1. *Fos* mRNA levels

A one-way ANOVA of *Fos* expression revealed significant differences between treatments in terms of mRNA levels at 4 h. *Post hoc* comparisons showed *Fos* gene expression levels to be significantly higher in the morphine-treated (2.13-fold) and methadone-treated (1.93-fold) animals than in the control group. Furthermore, the levels of *Fos* mRNA were 1.47 times higher in morphine-treated animals than in buprenorphine-treated animals.

#### 3.4.2. *Penk* mRNA levels

For *Penk*, one-way ANOVA revealed a significant difference in mRNA levels at 1 h and 4 h  $F(3,44)=3.032$ ,  $P=0.04$ . *Post hoc*

comparisons showed the levels of this mRNA to be significantly higher after morphine treatment (1.47-fold) than in the control group at 1 h. *Penk* mRNA levels were significantly lower (0.55-fold) in morphine-treated animals than in controls at 4 h. By contrast, the levels of this mRNA were 1.24 times higher in methadone-treated animals than in the control group.

#### 3.4.3. *Pdyn* mRNA levels

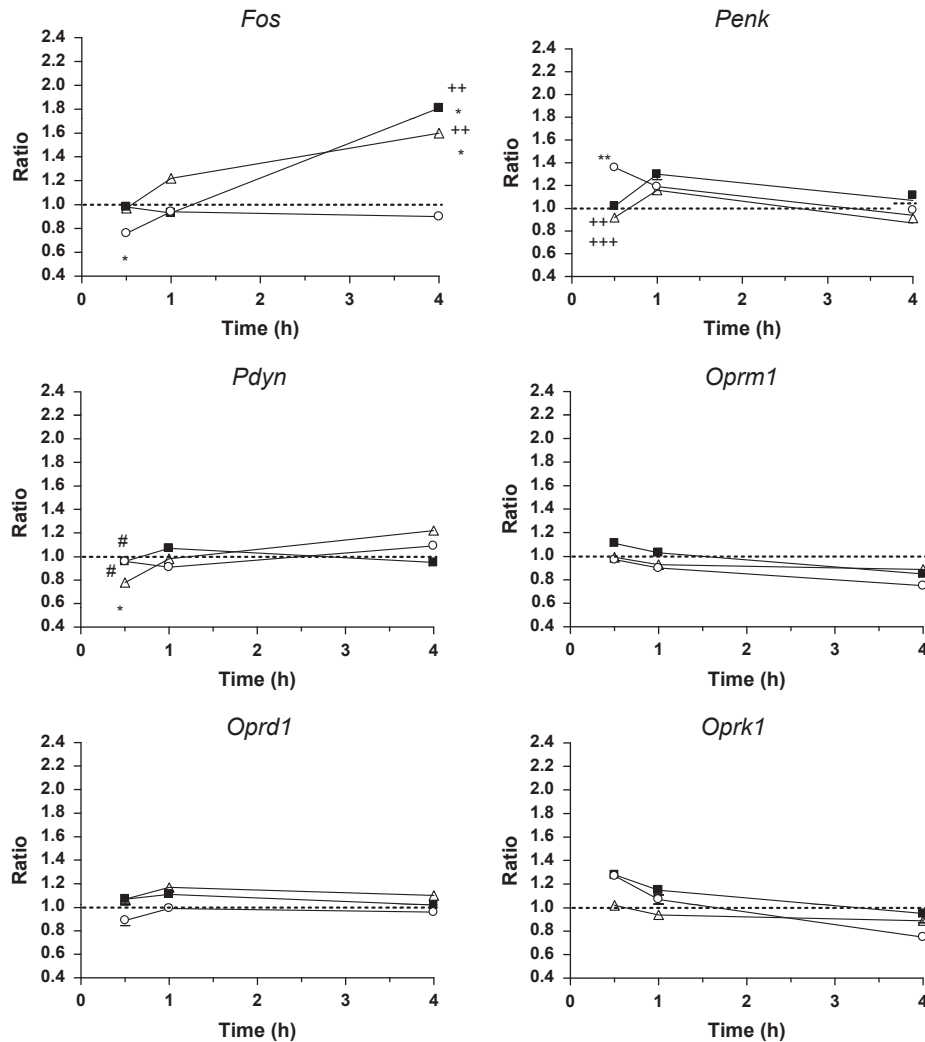
For *Pdyn*, one-way ANOVA showed a significant difference in mRNA levels between the treatments, at 4 h. *Post hoc* comparisons revealed that the levels of this mRNA were significantly lower in morphine-treated than in control animals (0.53-fold) at this time point. At 4 h, morphine-treated animals had 0.5 times more *Pdyn* mRNA than buprenorphine-treated rats (0.5-fold).

#### 3.4.4. *Pomc* mRNA levels

For *Pomc*, one-way ANOVA showed a significant difference in mRNA levels between treatments at 30 min and 4 h. *Post hoc* comparisons showed that *Pomc* mRNA levels at 4 h were lower in the morphine group than in the controls (0.6-fold). Methadone treatment resulted in lower levels of this mRNA than were found in the control (0.6-fold) and morphine (0.6-fold) groups, at 30 min. In addition, *post hoc* comparisons at 4 h also showed *Pomc* mRNA levels to be lower in methadone-treated animals than in control rats (0.48). Buprenorphine-treated rats had *Pomc* expression levels lower than those of control rats by a factor of 0.53.

#### 3.4.5. *Oprd1* mRNA levels

For *Oprd1*, one-way ANOVA revealed a significant difference in mRNA levels between treatments only at 30 min. *Post hoc* comparisons revealed that *Oprd1* gene expression was stronger in the



**Fig. 2.** Time-course of changes in mRNA levels in the nucleus accumbens, as assessed by quantitative real-time PCR after the acute injection of morphine, methadone or buprenorphine. Rats ( $n=10-12$  per group) received morphine (10 mg/kg; ■), methadone (3.7 mg/kg; Δ) and buprenorphine (0.2 mg/kg; ○) and were killed 30 min, 1 h or 4 h later. Results are expressed as fold-change  $\pm$  S.E.M. with respect to the control group. Significantly different from: saline \* $P < 0.05$ , \*\* $P < 0.01$ ; methadone # $P < 0.05$ ; buprenorphine ++ $P < 0.01$ , +++ $P < 0.001$ .

morphine group than in the control (1.28-fold), methadone (1.27-fold) and buprenorphine (1.44-fold) groups.

#### 3.4.6. *Oprk1* mRNA levels

For *Oprk1*, one-way ANOVA showed a significant difference in mRNA levels between treatments only at 1 h. *Post hoc* comparisons showed *Oprk1* mRNA levels to be higher (1.56-fold) in morphine-treated animals than in control animals at this time point.

#### 3.5. Acute morphine-, methadone- and buprenorphine-induced changes in mRNA levels in the thalamus

We assessed the effects of morphine, methadone and buprenorphine on the expression of *Fos*, *Penk*, *Oprm1*, *Oprd1* and *Oprk1* in the thalamus at 30 min, 1 h and 4 h post-injection (Fig. 4). The three opioid treatments had no effect on the expression of the other two opioid receptors in the thalamus, at any of the time points tested. We detected no *Pdyn* or *Pomc* mRNA in this structure in our conditions.

##### 3.5.1. *Fos* mRNA levels

One-way ANOVA revealed significant differences in the amounts of *Fos* mRNA between treatments at 1 h and 4 h.

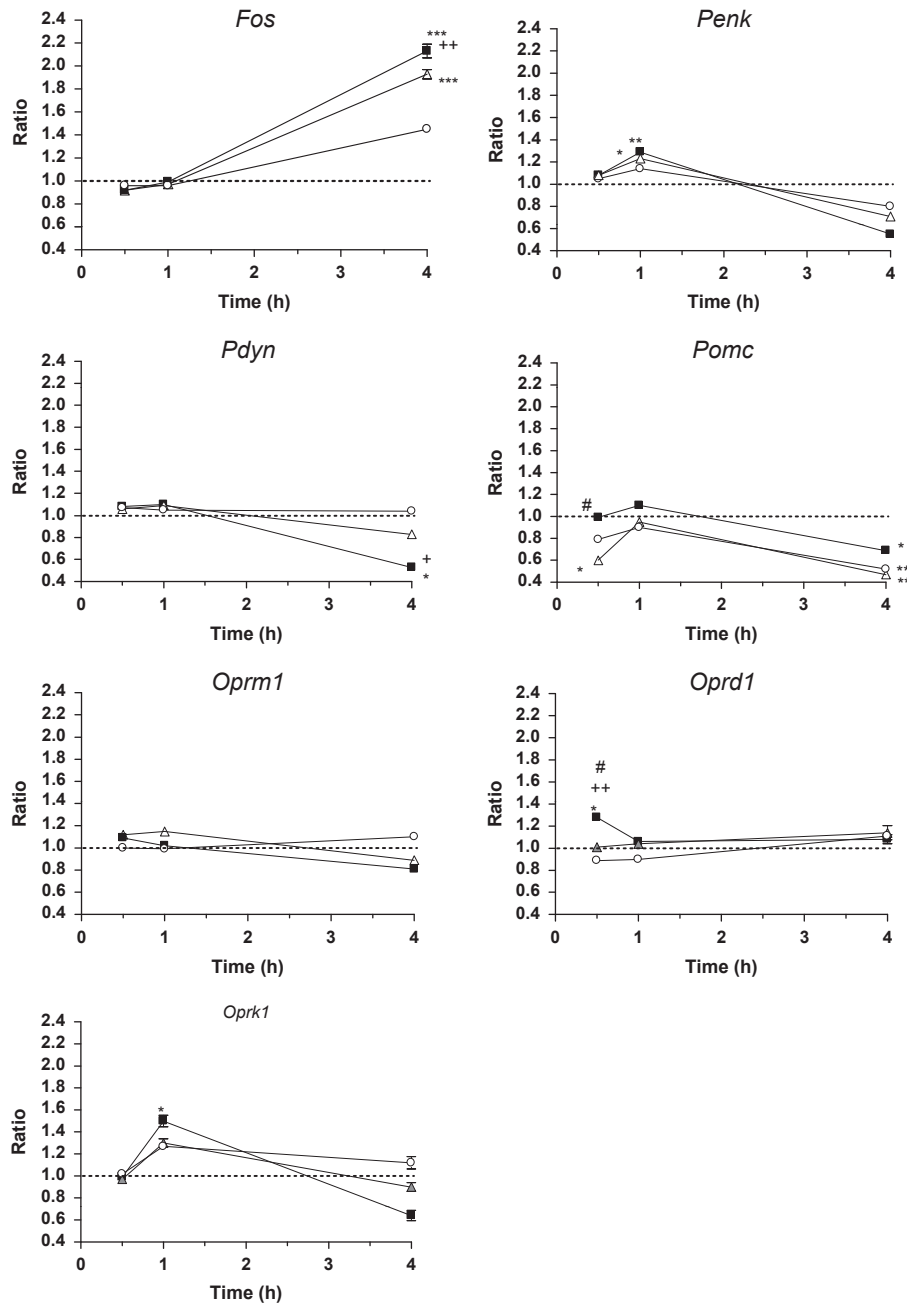
Morphine treatment resulted in significantly higher levels of *Fos* mRNA (1.91-fold) at 4 h than were detected in the control group. *Post hoc* comparison revealed a significant up-regulation of *Fos* expression 4 h after methadone treatment at 4 h, resulting in mRNA levels 1.83 times higher than those in saline-treated rats. *Post hoc* comparisons showed *Fos* mRNA levels to be 1.45 times higher at 1 h and 1.89 times higher at 4 h after buprenorphine treatment than in control rats. Morphine and buprenorphine treatments induced significantly different (1.42-fold) changes in *Fos* expression at 1 h in this structure.

##### 3.5.2. *Penk* mRNA levels

One-way ANOVA showed a significant treatment effect for *Penk* at 30 min only. *Post hoc* comparisons showed *Penk* mRNA levels to be significantly lower (0.52-fold) in buprenorphine-treated animals than in methadone-treated animals.

##### 3.5.3. *Oprk1* mRNA levels

For *Oprk1*, one-way ANOVA showed a significant difference in mRNA levels between treatments only at 30 min. *Post hoc* comparisons showed that *Oprk1* mRNA levels were higher in methadone-treated animals than in the control (2.11-fold), morphine (1.78-fold) and buprenorphine (1.95-fold) groups at this time point.

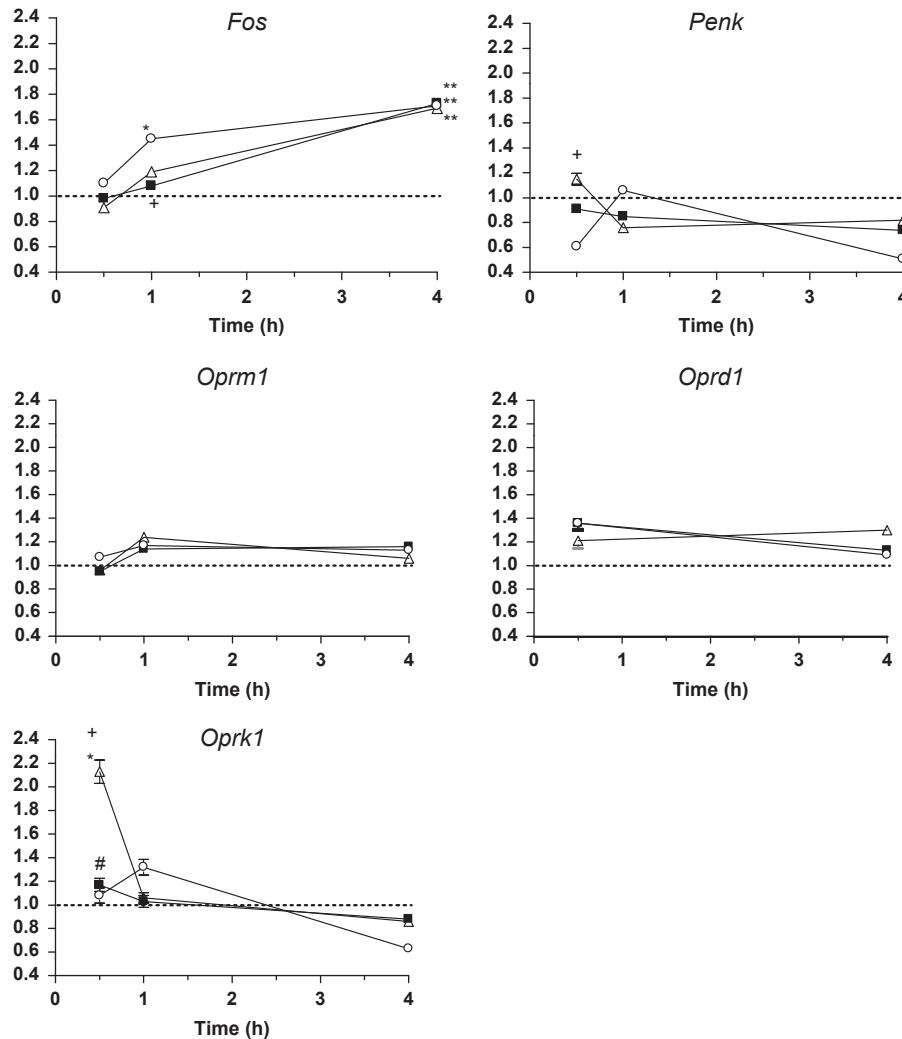


**Fig. 3.** Time-course of changes in mRNA levels in the dorsal striatum, as assessed by quantitative real-time PCR after acute injections of morphine, methadone or buprenorphine. Rats ( $n=10$ – $12$  per group) received morphine (10 mg/kg; ■), methadone (3.7 mg/kg; Δ) and buprenorphine (0.2 mg/kg; ○) and were killed 30 min, 1 h or 4 h later. Results are expressed as fold-change  $\pm$  S.E.M. with respect to the control group. Significantly different from: saline \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; methadone # $P < 0.05$ ; buprenorphine + $P < 0.05$ , ++ $P < 0.01$ .

#### 4. Discussion

Despite their widespread use as analgesics and maintenance treatments for patients addicted to opioids, little is known about the intracellular mechanisms of action or effects on transcription of methadone and buprenorphine. We investigated these aspects, by comparing the acute effects of methadone and buprenorphine with those of the best studied opiate, morphine. We performed a time-course study of the transcriptional responses to these drugs in three cerebral structures involved in opioid-induced behaviour: locomotion (striatum), nociception (striatum and thalamus) and dependence (nucleus accumbens and striatum) (Chudler and Dong, 1995; Dankova et al., 1975; Galtress and Kirkpatrick, 2010; Tang et al., 2009).

The published  $ED_{50}$  values for methadone and buprenorphine were obtained with diverse routes of administration in various rodent models. For comparison of the effects of the three opioids in our study, we first had to determine the doses giving equivalent levels of analgesia. The widely used tail-flick test was carried out and the  $ED_{50}$  values obtained are consistent with the findings of previous studies in rodents (Paronis and Holtzman, 1992; Yu et al., 2006). We chose to use a dose of 10 mg/kg morphine, because this dose has been extensively used in preclinical studies and is known to alter gene transcription when administered acutely or chronically (Fan et al., 2002, 2003; Garcia et al., 1995; Hamlin et al., 2007; Marie-Claire et al., 2003; Salas et al., 2011; Spijker et al., 2004). The corresponding buprenorphine (0.2 mg/kg) and methadone (3.7 mg/kg) doses are within the range commonly used in various



**Fig. 4.** Time-course of changes in mRNA levels in the thalamus, as assessed by quantitative real-time PCR, after acute injections of morphine, methadone and buprenorphine. Rats ( $n=10-12$  per group) received morphine (10 mg/kg; ■), methadone (3.7 mg/kg; Δ) and buprenorphine (0.2 mg/kg; ○) and were killed 30 min, 1 h and 4 h later. Results are expressed as fold-change  $\pm$  S.E.M. with respect to the control group. Significantly different from: saline \* $P < 0.05$ , \*\* $P < 0.01$ ; buprenorphine + $P < 0.05$ .

analgesic tests (Capuano et al., 2009; Erichsen et al., 2005; Hayes and Flecknell, 1999; Lemberg et al., 2006).

We investigated the modulation of gene transcription, by carrying out a time-course study of the amount of *Fos* mRNA as a non-specific marker of neuronal activation. Early changes to *Fos* gene expression have been studied in detail in the dorsal striatum, after acute morphine administration (Chang et al., 1988; Curran et al., 1996; Gutstein et al., 1998; Liu et al., 1994). However, some studies have reported an absence of change in *Fos* mRNA levels, even with high doses of morphine (Erdtmann-Vourliotis et al., 1999; Taracha et al., 2006). Only a few *Fos*-positive neurons are observed in the dorsal striatum after morphine injection and they can be observed only by in situ hybridisation (Harlan and Garcia, 1998), potentially accounting for our results. Moreover, morphine is the drug of abuse inducing the smallest change in *Fos* expression in the dorsal striatum (Erdtmann-Vourliotis et al., 1999). We dissected dorsal striatum samples within a 1 mm slice, and any increase in *Fos* mRNA levels in these particular neurons might be masked in the whole sample at early time points (30 min and 1 h). The lack of *Fos* regulation might also be due to the non-specific circadian regulation of its expression or injection stress at early time points. Control and treated rats were handled in parallel, so all the groups should undergo parallel circadian changes. Stress-induced interference at early time points was ruled out by

performing an additional experiment in which animals were subjected to daily handling and subcutaneous injections of saline for four days before the injection of opiates, in the same chamber, by the same experimenter, to minimise the stress associated with the acute drug injections. In these conditions too, *Fos* expression, 30 min after injection was not modulated by any of the three drugs studied in the three brain structures tested (data not shown). Nevertheless, the results obtained here are consistent with published results obtained with the same technique. In a time course study, Piechota and coworkers reported that opioids (morphine and heroin) significantly increased *Fos* mRNA level in mouse striatum, but only four hours after intraperitoneal injection (Piechota et al., 2010).

We also studied the regulation of six opioid system genes (encoding peptides and receptors) in three brain structures (striatum, nucleus accumbens and thalamus). We found that acute injections of the three opioids modulated the expression of the genes encoding endogenous opioid peptides and receptors in the striatum and nucleus accumbens. Thus, despite being administered at equivalent analgesic doses, morphine, methadone and buprenorphine induced transcriptional responses with different time courses in the three brain structures studied. The expression of the genes studied was modulated preferentially in the nucleus accumbens at the earliest time point and in the dorsal striatum at



the latest time point after injection studied. Our results suggest that the transcriptional effects of methadone resemble those of morphine more closely than do those of buprenorphine in these structures.

These results may be accounted for by the pharmacology of the two molecules, which act on several receptors. The behavioural effects of buprenorphine and methadone are mediated principally by the mu-opioid receptor, but these two drugs have been shown to interact with other receptors, such as the NOP and kappa opioid receptors for buprenorphine and the NMDA receptor for methadone. However, it has been shown that NMDA receptors play no role in the analgesic properties of methadone injected systemically at a dose in the analgesic range, instead these receptors are involved in the hyperalgesic effects of this drug at doses lower than that used here by a factor of 370–3700 (Chizh et al., 2000; Holtman and Wala, 2007). We studied the role of the four opioid receptors in buprenorphine-induced analgesic effects in our conditions. As expected, the mu receptor-specific antagonist abolished the analgesic effects of morphine and buprenorphine. The NOP receptor has been shown to contribute to buprenorphine-induced analgesia in the tail-flick test (Lutfy et al., 2003). However, in our conditions, NOP receptors played no role in buprenorphine-induced analgesia. The observed differences between these results can be attributed to the different species used (mice vs. rats) and differences in the doses of buprenorphine and J113397. Moreover, it has been shown that the intraperitoneal injection of J113397 cannot antagonise the analgesic effects of buprenorphine in the rat formalin test (Yamamoto et al., 2006). Studies in mu receptor-knockout mice have implicated kappa receptors in morphine-induced nociception, but extremely high doses of morphine are required to achieve analgesia in the tail-flick test after systemic injection (Loh et al., 1998). In our conditions, kappa receptors did not contribute to morphine-induced analgesia. The mild effect of morphine on spinal kappa receptors is probably concealed by the major mu opioid receptor central effect. Buprenorphine dissociates more slowly from the mu receptor than morphine and methadone (Heel et al., 1979). Pharmacokinetic–pharmacodynamic (PK/PD) modelling has suggested that biophase distribution, rather than receptor association/dissociation, is the rate-limiting step in the kinetics of the analgesic effects of buprenorphine (Yassen et al., 2005). However, the observed differences in the molecular effects of the drugs may reflect differences in the kinetics of dissociation from the mu opioid receptor. In the analysed time frame, the transcriptional effects of buprenorphine did not appear to occur later than those of morphine and methadone, but further studies involving a longer time course, a wider dose range and the use of several infusion rates would be required for modelling of the PK/PD of the three opioids.

Buprenorphine and morphine have been shown to regulate G-protein coupling to mu receptor differently (Saidak et al., 2006), suggesting a possible difference in the cellular responses triggered by the binding of the two molecules to the mu receptor. This is consistent with our results showing differences in the kinetics and specificity of transcriptional responses to acute morphine and buprenorphine.

In conclusion, our results show that despite the mediation of their analgesic effects via the same mu receptor, morphine, methadone and buprenorphine, when injected in amounts giving equal levels of analgesia, regulate the expression of diverse opioid genes in different manners. The observed changes in gene expression are not only specific to the interaction of each drug with its receptor, but also to complex intracellular outcomes, and they are thought to highlight or to trigger long-lasting changes in neuroplasticity (Robison and Nestler, 2011). Our results indicate that single-drug exposure to each of these drugs separately results in different changes to the molecular machinery of the brain. The

endogenous opioid system is involved in many key behavioural functions, including pain, respiration, learning, stress, addiction, eating, locomotion, sexual activity and immunological responses (Bodnar, 2011). It is therefore important to understand how these long-term major analgesic and/or maintenance treatments might disturb the homeostasis of this system. Such studies might contribute to elucidation of the molecular mechanisms underlying aspects of specific methadone and buprenorphine treatment strategies. The transcriptional effects of chronic treatment with these three opioids are currently being studied to improve our understanding of the neurobiological pathways regulated by chronic buprenorphine and methadone treatments and the differences between the effects of these drugs and those of morphine.

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