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Brain Regional Fos Expression Elicited by the Activation of μ- but not δ-Opioid Receptors of the Ventral Tegmental Area: Evidence for an Implication of the Ventral Thalamus in Opiate Reward

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Both μ-opioid receptors (MORs) and δ-opioid receptors (DORs) are expressed in the ventral tegmental area (VTA) and are thought to be involved in the addictive properties of opiates. However, their respective contributions to opiate reward remain unclear. We used intracranial self-administration (ICSA) to study the rewarding effects of morphine microinjections into the VTA of male and female MOR−/− and DOR−/− mice. In brains of mice tested for intra-VTA morphine self-administration, we analyzed regional Fos protein expression to investigate the neural circuitry underlying this behavior. Male and female WT and DOR−/− mice exhibited similar self-administration performances, whereas knockout of the *MOR* gene abolished intra-VTA morphine self-administration at all doses tested. Naloxone (4 mg/kg) disrupted this behavior in WT and DOR mutants, without triggering physical signs of withdrawal. Morphine ICSA was associated with an increase in Fos within the nucleus accumbens, striatum, limbic cortices, amygdala, hippocampus, the lateral mammillary nucleus (LM), and the ventral posteromedial thalamus (VPM). This latter structure was found to express high levels of Fos exclusively in self-administering WT and DOR−/− mice. Abolition of morphine reward in MOR−/− mice was associated with a decrease in Fos-positive neurons in the mesocorticolimbic dopamine system, amygdala, hippocampus (CA1), LM, and a complete absence within the VPM. We conclude that (i) VTA MORs, but not DORs, are critical for morphine reward and (ii) the role of VTA-thalamic projections in opiate reward deserves to be further explored.
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INTRODUCTION

A large body of neuropharmacological evidence suggest that opiate reward depends upon μ-opioid receptors (MORs; Bardo, 1998; van Ree *et al*, 1999; De Vries and Shippenberg, 2002). Genetic studies using knockout mice lacking MOR−/− support this view: morphine-conditioned place preference (CPP) and intravenous morphine self-administration are abolished in MOR−/− mice (Matthes *et al*, 1996; Becker *et al*, 2000; Sora *et al*, 2001). In contrast, the contribution of δ-opioid receptors (DORs) to opiate reward remains controversial. DOR agonists induce CPP, and the administration of μ- or δ-agonists into the ventral tegmental area (VTA) is rewarding, potentiates brain stimulation reward, and increases DA release in the nucleus accumbens (Shippenberg *et al*, 1987; Leone *et al*, 1991; Pothos *et al*, 1991; Spanagel *et al*, 1992; Devine *et al*, 1993a, b; Heidbreder *et al*, 1996; Suzuki *et al*, 1996, 1997; Duvauchelle *et al*, 1997; Longoni *et al*, 1998; Yoshida *et al*, 1999). However, δ-agonists have similar but weaker effects than μ-agonists (Devine *et al*, 1993b; Devine and Wise, 1994; Heidbreder *et al*, 1996; Duvauchelle *et al*, 1997). The specific δ-agonist deltorphine II does not induce CPP in MOR−/− mice (Hutcheson *et al*, 2001). To date, opiate self-administration has not been tested in DOR−/− mice.

Overwhelming evidence suggests that DA is involved in opiate reward (Pierce and Kumaresan, 2006). Intracranial self-administration (ICSA) and CPP studies have revealed the potent rewarding effects of intra-VTA morphine injections (Phillips and LePiane, 1980; Bozarth and Wise, 1981; Welzl *et al*, 1989; David and Cazala, 1994a; Devine and Wise, 1994; David *et al*, 2002). This stimulatory effect is

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thought to reflect the disinhibition of DA-A10 neurons, via a μ-dependent hyperpolarization of GABA interneurons (Gysling and Wang, 1983; Kalivas *et al*, 1990; Johnson and North, 1992; Klitenick *et al*, 1992). Mice lacking the dopamine D2 receptor fail to exhibit morphine-induced CPP, and intravenous morphine self-administration is disrupted in these mice (Maldonado *et al*, 1997; Elmer *et al*, 2002). However, morphine may be rewarding in animals pretreated with DA antagonists, following a 6-OHDA lesion of DA terminals or in mice lacking DA itself (Pettit *et al*, 1984; Stinus *et al*, 1985; David *et al*, 2002; Hnasko *et al*, 2005). Importantly, GABAergic projections from the VTA to the prefrontal cortex have been described (Carr and Sesack, 2000a). Studies using immunohistochemical detection of the Fos protein, product of the immediate early gene c-*fos*, show that acute or chronic morphine administration, or exposure to a morphine-associated context, induce Fos in DA-related structures (Nye and Nestler, 1996; Schroeder *et al*, 2000; Schroeder and Kelley, 2002). Deletion of the DA transporter results in increased morphine reward and morphine-induced c-*fos* in the NAc shell (Spielewoy *et al*, 2000).

Here we have investigated the respective contribution of VTA opioid receptor subtypes in opiate reward. Male and female KO mice lacking either the MOR−/− or DOR−/− were tested for intra-VTA morphine self-administration. A regional analysis of Fos protein expression was then conducted to determine which brain regions were associated with morphine self-administration. Reward-related areas of the mesocorticolimbic DA system were analyzed first. Since reward was assessed through spatial discrimination learning, the dorsal hippocampus was also studied. The mammillary bodies of the hypothalamus, which are increasingly implicated in emotional processes, were investigated (Beracochea, 2005). Finally, considering the recent demonstration of rich DA innervation of the ventral thalamus in the primate and human brain (Sanchez-Gonzalez *et al*, 2005; Garcia-Cabezas *et al*, 2007), we studied Fos expression within this thalamic area.

METHODS

Ethical Statement

All surgical and experimental procedures were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Production of MOR and DOR Null Mutants

The generation and characterization of the MOR and DOR null mutant mice have been described in previous papers (Matthes *et al*, 1996; Filliol *et al*, 2000). Mutations were obtained on the 129SVJ background and then backcrossed with C57BL/6 for at least 10 successive generations (MOR or DOR mutant mice). Animals used in the experiments were homozygote and wild-type littermates from heterozygote animal crossing, as recommended by the Banbury Conference in 1997. In both MOR and DOR mutants, the level of expression and distribution of remaining opioid receptors were mostly unchanged, and transcription of opioid peptides was unaltered (Matthes *et al*, 1996; Kitchen *et al*, 1997; Slowe *et al*, 2001; Goody *et al*, 2002). All analyses were performed blind.

Maintenance and Surgery

At 12 weeks of age, mice were housed individually with *ad libitum* access to food and water in a temperature-controlled room (23°C) with a light–dark cycle (12–12 h, light on at 0800 hours) and sawdust bedding changed weekly. The animals were aged 12–16 weeks (males: 27–30 g; females: 23–26 g) at the beginning of the experiments. The subjects were anesthetized with a ketamine/xylazine mixture (Ketamine 1000 Virbac®: 100 mg/kg i.p./Rompun® 2%: 8 mg/kg i.p.), and lidocaine HCl (Xylocaine®, 5%) was applied locally both before opening the scalp and trepanation. The animals were implanted in a counterbalanced left and right order, and unilaterally since it has previously been demonstrated that the magnitude of the motivational effects of unilaterally applied opioids into the VTA is equivalent to that observed when bilateral injections are used (Phillips and LePiane, 1980; Bozarth, 1987). The tip of the guide cannula (outer diameter 0.460 mm or gauge 25; inner diameter 0.255 mm or gauge 30) was positioned 1.5 mm above the VTA. The stereotaxic coordinates used were the following: 0.40 mm anterior to the interaural line, $\pm$0.30 mm lateral to the sagittal line, and 3.30 mm vertically below the surface of the skull. The incisor bar was leveled with the interaural line. Mice were allowed to recover from surgery for at least 1 week.

Material and Experimental Protocol

Intracranial self-injection procedure. Self-administration behavior was studied in a gray Plexiglas Y-maze, the two arms of which were separated by an angle of 90°. The stem and the arms were 31 cm long and 12 cm high. The starting box (14 × 8 cm) was separated from the stem by a sliding door. Sliding doors were also located at the entrance of each arm. A photoelectric cell was situated 6 cm from the end of each arm. On each day of the experimental period, a stainless-steel injection cannula (outer diameter 0.229 mm or gauge 31, inner diameter 0.127 mm or gauge 36) was inserted into the VTA and was held in a fixed position, by means of a small connector. The injection cannula was connected by a flexible polyethylene tubing to the microinjection system, which housed a 5 µl Hamilton syringe. The tip of the injection cannula projected beyond the guide cannula by 1.5 mm. By interrupting the photocell beam in one of the two target arms, mice could trigger an injection of morphine sulfate dissolved in polyionic Ringer solution; the other arm being neutral (no injection). Intracranial injections were carried out using an automatic computer-controlled apparatus, which provided, via a microvernier system, a precise and highly reproducible descent of the microsyringe piston. Each self-injection (50 nl) lasted 4 s. Normal drug flow was verified visually both before and after each ICSA session for each animal. The movement of the animals in the Y-maze was detected by an optical system. This information was transmitted to a microcomputer, which in turn rotated the injector in the same direction as the animal's movement. This process avoided the twisting of the flexible tubing. The number of self-administrations

per daily session was noted and an automatic equipment, triggered by opening the door to the stem, recorded the latency to enter the reinforced arm (response latency) or the neutral arm for each subject.

Behavioral protocol, experiment I: morphine ICSA in MOR and DOR mutant mice. A total of six groups were exposed to intra-VTA morphine self-infusions: MOR−/− (male $n=6$; female $n=6$), DOR−/− (male $n=6$; female $n=6$), and WT (wild type; male $n=6$; female $n=6$). Two groups of animals had only vehicle available (polyionic Ringer-Aguettant®, Lyon, France; VTA $n=6$/gender, constituted of two WT, two MOR−/−, and two DOR−/− pooled). Previous experiments have shown that the dose of 50 ng morphine sulfate (or 65 pmol, as referred to the salt) produced optimal ICSA performance in mice (David *et al*, 2002). The protocol consisted of three phases:

(i) *Acquisition phase*. This phase lasted for six daily sessions. A session comprised the following steps. Each daily session was composed of 10 trials separated by a 1-min intertrial interval. To begin a trial, a mouse was placed in the start box and after 1 min the door to the stem and target arms were opened. In each group, half of the animals were assigned to enter the right arm to trigger the injection of morphine, whereas the remainder were assigned to the left arm. Therefore a maximum of 10 injections could be obtained by each subject per daily session. During the first four trials of the 1st session only, if an animal chose the neutral arm, it was immediately allowed to access to the arm enabling an injection of morphine. From the 5th trial onward, the mouse was not allowed to enter the other arm following its first choice. After a 10-s confinement, the mouse was removed and replaced directly into the start box for the following trial.

(ii) *Opiate antagonist (naloxone) challenge*. This second phase lasted 5 days. Starting on the 6th session, all subjects who exhibited morphine self-administration were injected subcutaneously (s.c.) with the competitive opiate receptor antagonist naloxone (4 mg/kg; s.c.), 10 min before the self-administration session. Injections were administered in a volume of 0.1 ml/10 g body weight. In mice that did not acquire morphine ICSA (MOR mutants), the dose of morphine was raised to 100 ng for five more consecutive sessions.

(iii) *Reacquisition test: replacement of naloxone by NaCl.* Following the last session of naloxone-induced extinction, the opiate antagonist was then replaced by pretrial injections of vehicle alone for three consecutive daily sessions.

Behavioral protocol, experiment II: Fos protein expression elicited by morphine ICSA. Male MOR−/−, DOR−/−, and WT mice were submitted to the acquisition of morphine self-administration for four consecutive sessions, according to a behavioral protocol that was identical to the one used in experiment I. Following completion of the last session, mice were perfused and their brain removed for Fos immunohistochemistry. Groups were constituted as follows: morphine groups: $n=6$ per genotype; and control (vehicle) groups: $n=6$ per genotype. Vehicle-injected mice, and morphine-injected MOR null mutants, remained active enough to trigger the injections 'en-passant', that is without being forced. Therefore, Fos expression cannot be considered as a mere function of morphine dose, as some MOR−/− subjects received as much morphine as WT or DOR−/− mice.

Experiment III: intra-VTA self-administration of a GABA-A receptor antagonist (bicuculline). The aim of this experiment was to test whether MOR−/− mice would learn a spatial discrimination task to obtain a reward that do not depend on MORs. To stimulate DA neurons without acting on μ-receptors located on GABA interneurons, we used the GABA-A receptor antagonist bicuculline. When injected into the VTA in rodents, bicuculline blocks GABA-A receptor-mediated hyperpolarization of DA neurons, resulting in μ-independent stimulation of DA cells (Ikemoto *et al*, 1997a). Mice (WT, $n=6$ and MOR−/−, $n=6$) were allowed to self-injected (−)-bicuculline methiodide at the dose of 0.5 ng/50 nl for seven sessions. Two WT subjects exhibited either ipsi- or contralateral circling behavior following completion of session 3 and 4, during which they rapidly triggered 10 consecutive injections. All remaining WT and MOR−/− mice self-administered bicuculline without showing circling.

Fos immunohistochemistry. Animals were deeply anesthetized (Avertin®, 600 mg/kg) 90 min after the end of the behavioral session and perfused transcardially with 100 ml of 0.9% NaCl followed by 100 ml of cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were dissected, post-fixed for 12 h in the same fixative, and cryoprotected in 30% sucrose/PB and left overnight at 4°C. They were then frozen and 50 μm frontal sections were cut on a freezing microtome. Immunohistochemistry was performed on free-floating sections using a standard avidin–biotin–peroxidase method. The tissue was rinsed in 0.1 M PB four times each for 5 min. Sections were then incubated overnight at room temperature in normal goat serum containing the primary antibody: a rabbit polyclonal c-fos antibody (Ab-5, Oncogene Science, 1:20000) raised to a synthetic peptide derived from amino-acid sequences 4–17 of the human Fos protein. Sections were rinsed again with 0.1 M PB five times and incubated for 2 h in 2.5 ml of goat serum containing the secondary antibody, a biotinylated goat anti-rabbit IgG (Jackson Immunoresearch; 1 : 2000). Sections were rinsed with 0.1 M PB five times. Following incubation with the avidin–biotin–peroxidase complex reagent (Vecstatain kit, Vector Laboratories Inc., Burlingame, CA) for 2 h, sections were rinsed again with 0.1 M PB five times. The peroxidase was detected with diaminobenzidine (Sigma, St Louis, MO) as chromogen. Reaction was induced with H_2O_2 0.3%, and terminated by rinsing four more times with 0.1 M PB. Sections were then mounted on gelatin-coated slides, dried and dehydrated before coverslipping. Histological verification of cannula placements was realized on these sections for all subjects used in the present experiments.

Cell counting and image analysis. Quantitative analysis of Fos-positive nuclei was performed using a color video camera (Sony® DXC-950P) interfaced with an Olympus®

BX-50 microscope (Tokyo, Japan). Fos-positive nuclei were counted using a computer-assisted software (Biocom Visiolab® 2000). Sections were observed at $\times 20$ magnification. Data were expressed as the mean number of Fos-positive nuclei/mm^2 in three contiguous coronal sections for all regions studied, except for the lateral mamillary nuclei where only two coronal sections could be counted. Fos-expressing neurons were counted within 12 brain regions selected for their involvement in reward or emotional processes, spatial memory, and motor function (Figure 3). These brain regions correspond to the main cortical and subcortical target areas of the DA system, the dorsal hippocampus (CA1 and CA3), the mammillary bodies of the hypothalamus, and the sensory (ventral) thalamus at the level of ventral posterolateral/posteromedial thalamic nuclei. Concerning the mammillary bodies, we focused on the lateral mammillary nucleus, which provided more slices for quantification than the medial mammillary nucleus.

Histology. At the end of experiment I, animals were killed with an overdose of Avertin®. The head was removed, with the guide cannula attached, and placed into 10% formalin for a 72-h period. The guide cannula was then withdrawn, the brain dissected, and placed in a solution of formol containing 30% sucrose for an additional week. Brains were then frozen and cut in a microtome to provide 60-μm frontal sections, which were stained using 0.1% thionine to identify the injection site.

Statistical analysis.

Behavior: Each phase of the behavioral testing was analyzed separately. During acquisition, the number of self-administrations and the time to trigger the self-injections were analyzed using three-way analysis of variance (ANOVA) with genotype and gender as between-subjects factors and session as a within-subjects repeated factor. During naloxone-induced extinction, drug treatment and gender were used as factors. Significant main effects were further analyzed (*post hoc*) using Fischer PLSD tests.

Fos immunostaining: Following counting of Fos-positive cells by the computer-assisted software, the data were entered into StatView 5.0 (Abacus Concepts) for statistical analysis. Groups represent only male mice, since no difference between genders was observed during behavioral tasks. The number of Fos-positive cells was analyzed using three-way ANOVA with genotype, drug, and side of injection as factors for each of the brain area sampled. Since the side of injection (left or right hemisphere) had no significant effects, F-values were not reported in the results and data presented correspond to the mean of both sides. *Post hoc* analyses of significant main effects were further examined using Fischer PLSD tests. A significance level of $p<0.05$ was used for all statistical analyses.

RESULTS

Experiment I: Intra-VTA Morphine Self-Administration

Acquisition of morphine ICSA, dose of 50 ng (sessions A1–A6).

Arm discrimination: No discrimination was observed between the two arms of the Y-maze when only vehicle (Ringer) was available for intra-VTA self-injections in any of the animals tested, whatever the genotype. In contrast, male and female WT and DOR null mutants rapidly discriminated between the drug-reinforced arm and the neutral arm of the Y-maze to self-administer morphine into the VTA, and exhibited similar discrimination performances. MOR−/− mice did not show any preference for the morphine-associated arm of the maze. These observations are supported by a global ANOVA revealing a significant main effect of genotype: $F_{2,31}=77.34$, $p<0.001$; session: $F_{5,155}=43.55$, $p<0.001$; and genotype × session interaction: $F_{10,155}=10.97$, $p<0.001$; but no main effect of gender: $F_{1,31}=0.20$, NS (Figure 1).

Self-injection latencies: In subjects having only vehicle available, this parameter progressively increased over the acquisition sessions, a phenomenon related typically to a lack of motivation (David *et al*, 2002). Compared to vehicle, a significant decrease in self-injection latency was observed in male and female WT and DOR−/− mice over successive acquisition sessions. However, this was not the case for the MOR−/− mice, whatever the gender (Figure 1). These results yielded a significant effect of the genotype on this parameter: $F_{2,31}=69.93$, $p<0.001$; no main effect of gender: $F_{1,31}=1.94$, NS; but an effect of the repeated factor session: $F_{5,155}=3.99$, $p<0.01$; a session × genotype interaction: $F_{5,155}=9.03$, $p<0.001$; and a session × gender interaction: $F_{5,155}=2.46$, $p<0.05$.

Morphine ICSA, dose of 100 ng: None of the MOR−/− mice tested for self-administration exhibited a behavioral response to intra-VTA injections of the 50-ng morphine dose. Although this dose is about 10 times higher than the threshold dose required to elicit a morphine-seeking behavior (David and Cazala, 1994a), we increased the dose up to 100 ng for five more consecutive sessions (sessions A7–A11, not shown). There was no significant effect of this dose on self-administration behavior ($F_{4,20}=1.74$, NS).

Effects of naloxone 4 mg/kg, i.p. (sessions E1–E5).

Arm discrimination: Systemic pre-injection of the competitive opiate antagonist naloxone in groups exhibiting intra-VTA morphine self-administration (WT and DOR−/−) disrupted choice accuracy similarly in both groups, at a rate similar to those undergoing extinction, that is replacement of morphine by vehicle (Cazala *et al*, 1987; David *et al*, 2002). Male mice of both genotypes tested (WT and DOR−/−) were more sensitive to the disrupting effects of naloxone than female mice of the same genotype. These observations are supported by a significant main effect of naloxone treatment: $F_{4,60}=55.20$, $p<0.0001$; no main effect of genotype: $F_{1,15}=0.39$, NS; and no main effect of gender but a naloxone × gender interaction: $F_{5,155}=3.86$, $p<0.01$ (Figure 1).

Self-injection latencies: Concomitantly to the decrease in discrimination performance, the time to trigger the injection increased progressively over the five sessions following naloxone treatment. This effect was similar in WT and DOR−/− mice. As for discrimination performance, male mice were more sensitive than female mice to the disruptive effects of naloxone on self-injection latency.

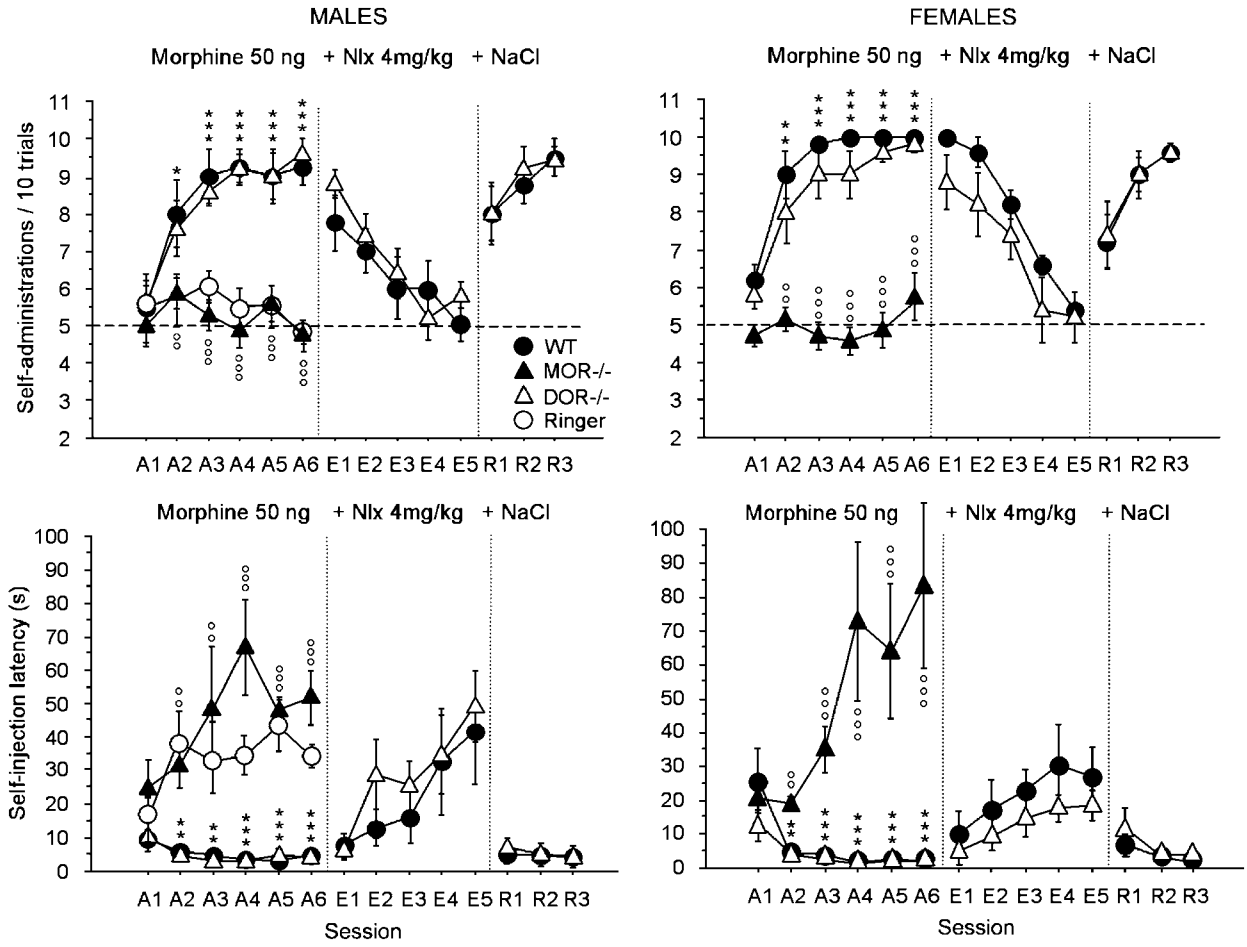

Figure 1 Intra-VTA morphine self-administration in male (left) and female (right) MOR−/−, DOR−/− and WT mice. Top: mean number (±SEM) of intra-VTA morphine self-administrations (dose of 50 ng). $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$: comparison with vehicle (Ringer). $^{\circ}p<0.05$; $^{\circ\circ}p<0.01$; $^{\circ\circ\circ}p<0.001$: comparison with WT. Bottom: mean value of the latency (seconds±SEM) to trigger intra-VTA morphine self-injections (dose of 50 ng). $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$: comparison with vehicle (Ringer). $^{\circ\circ}p<0.01$; $^{\circ\circ\circ}p<0.001$: comparison with WT. A, acquisition; E, extinction; R, reacquisition.

Therefore, the global ANOVA yielded no significant main effect of genotype: $F_{1,15}=1.00$, NS, but a main effect of gender: $F_{1,15}=11.56$, $p<0.01$; naloxone treatment: $F_{4,60}=14.39$, $p<0.0001$; and a naloxone × gender interaction: $F_{4,60}=13.82$, $p<0.01$.

Reacquisition test, replacement of naloxone by NACL (sessions R1–R3). The replacement of naloxone by its vehicle (NaCl) led to immediate recovery of intra-VTA morphine self-administration in DOR−/− and WT mice regardless of gender, as measured both by the preference for the morphine-associated arm and the decrease in the time to trigger morphine self-injections (Figure 1). Discrimination performance as well as latency significantly improved over the three sessions similarly in all groups (main effect of reacquisition sessions on the number of self-administrations: $F_{2,26}=29.11$, $p<0.0001$; with no main effect of genotype: $F_{1,13}=0.05$, NS; or gender: $F_{1,13}=0.19$, NS). Self-administration was observed from the first testing session (Figure 1; comparison of mean number of self-administrations during E5 and R1 in WT $t(8)=4.04$, $p<0.01$; and DOR−/− mice: $t(9)=6.73$, $p<0.001$). Latency was also significantly reduced from the first reacquisition session both in WT mice: $t(8)=2.41$, $p<0.05$; and DOR−/− mice: $t(9)=4.80$, $p<0.001$.

Histological control. Injection sites were precisely located by following the track of each injection cannula. Injection sites were located mainly in the caudal part of the target structure (VTA), between 0.72 and 0.16 mm from the interaural line (Paxinos and Franklin, 2004). A representative example is shown in Figure 2.

Experiment II: Fos Protein Expression Elicited by Intra-VTA Morphine Self-Administration

Behavioral results. Mice subsequently used for Fos immunostaining exhibited self-administration performances similar to those of experiment I. Once again, only MOR−/− failed to acquire intra-VTA morphine self-administration as measured by preference for the drug-reinforced arm of the Y-maze as well as decreasing self-injection latencies. Since data obtained were similar to the acquisition phase of behavioral experiment I, only statistical analysis is provided. The global ANOVA yielded a significant main effect of genotype: $F_{2,29}=100.9$,

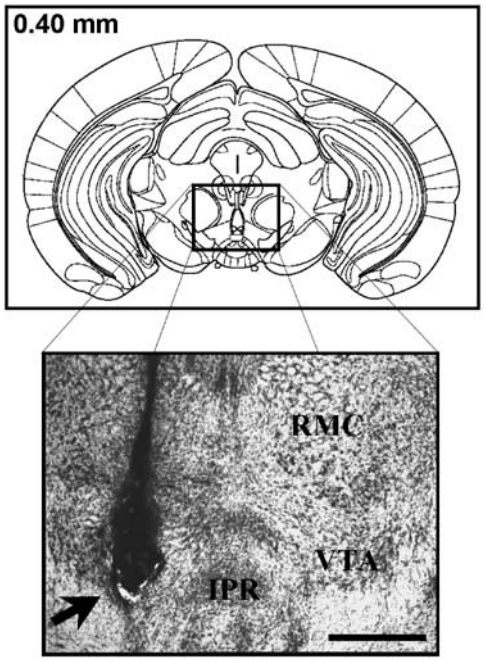

Figure 2 Photomicrograph of a thionine-stained frontal brain section (60 μm) through the cannula track and the injection site within the VTA of a representative mouse (IPR interpeduncular nucleus, rostral subnucleus; RMC, red nucleus magnocellular) (Paxinos and Franklin, 2004). Distance from the interaural line. Scale bar 0.5 mm.

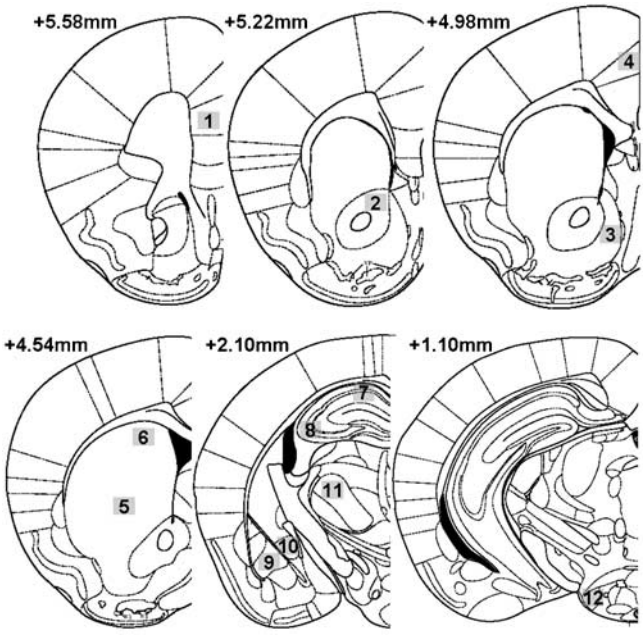

Figure 3 Brain areas sampled for Fos protein expression induced by intra-VTA morphine self-administration in MOR and DOR null mutants and their distance from interaural line (Paxinos and Franklin, 2004). Quantification was performed in the prelimbic area of the prefrontal cortex (PrL) (1), nucleus accumbens core (AcbC) (2) and shell (dorsal part) (AcbSh) (3), anterior cingulate cortex (CG1) (4), ventromedial (vCPu) (5) and dorsomedial (dCPU) (6) part of the caudate-putamen nucleus, dorsal CA1 (7) and CA3 (8) of the hippocampus, basolateral (BLA) (9) and central (CeA) (10) amygdala, ventroposteromedial thalamic nucleus (VPM) (11) and lateral mammillary nucleus (LM) (12).

$p<0.0001$; a main effect of session: $F_{3,87}=33.82$, $p<0.001$; as well as a significant genotype × session interaction. Comparison of the performances displayed by male and female mice during acquisition of intra-VTA morphine self-administration shows that there was no effect of gender: $F_{1,29}=3.55$, NS.

Fos immunohistochemistry (Figure 3).

Mesolimbic and mesostriatal pathways: Fos protein expression was regulated differentially within the mesocorticolimbic projection areas of the VTA, as a function of both drug treatment and genotype (Figures 4 and 5). Specifically, immunostaining increased strongly within the dorsal shell (AcbSh), core (AcbC) of the nucleus accumbens (Figure 5), and caudate-putamen (ventromedial and dorsomedial) of WT and DOR−/−, but not in MOR−/− mice. Accordingly, global ANOVA yielded a significant main effect of drug and genotype for the AcbSh respectively ($F_{1,54}=27.86$, $p<0.0001$; $F_{2,54}=25.35$, $p<0.0001$; drug × genotype interaction: $F_{2,54}=8.17$, $p<0.001$); as well as for the AcbC (main effect of drug: $F_{1,52}=60.05$, $p<0.0001$; main effect of genotype: $F_{2,52}=25.57$, $p<0.001$; and drug × genotype interaction: $F_{1,52}=12.01$, $p<0.001$); the ventromedial part of the caudate (vCPu) (main effect of drug: $F_{1,54}=88.36$, $p<0.0001$; main effect of genotype: $F_{2,54}=17.12$, $p<0.0001$; and drug × genotype interaction: $F_{1,54}=13.08$, $p<0.001$); and the dorsomedial caudate (dCPu) ($F_{1,51}=89.90$, $p<0.0001$; main effect of genotype: $F_{2,51}=13.18$, $p<0.0001$; and drug × genotype interaction: $F_{1,51}=12.52$, $p<0.001$). *Post hoc* analysis revealed that, despite similar self-administration performances, Fos expression within the AcbSh was lower in the DOR mutants than in WT.

Cortical areas: The prelimbic area of the prefrontal cortex (PrL) exhibited a significant increase in Fos-positive neurons as a function of morphine treatment in both WT and DOR−/− mice (Figure 4). A drug-dependent increase was still observed in MOR mutants. These observations are supported by global ANOVA yielding a significant main effect of drug in PrL: $F_{1,50}=31.94$, $p<0.001$; and a main effect of genotype: $F_{1,50}=11.18$, $p<0.001$. In the anterior cingulate cortex (CG1), we observed a similar pattern, although less contrasted than within prefrontal cortex (main effect of drug: $F_{1,50}=13.83$, $p<0.001$; and main effect of genotype: $F_{1,50}=15.19$, $p<0.001$). There was no difference in the number of Fos-positive nuclei between groups at the level of the visual cortex (data not shown).

Amygdaloid complex: The number of Fos-positive neurons within the basolateral amygdala BLA was significantly increased by intra-VTA morphine self-injections in WT mice, whereas this number remained unchanged or decreased respectively in MOR or DOR null mutants, resulting in a weak main effect of drug: $F_{1,50}=4.09$, $p<0.05$; but a strong main effect of genotype: $F_{2,50}=12.45$, $p<0.0001$; and drug × genotype interaction: $F_{2,50}=8.00$, $p<0.001$ (Figures 4 and 5). This pattern was not observed in the CeA, which displayed four times more Fos-positive cells in self-administering animals (both the WT and DOR−/− mice) than in controls and MOR mutants (main effect of drug: $F_{1,50}=94.31$, $p<0.0001$; main effect of

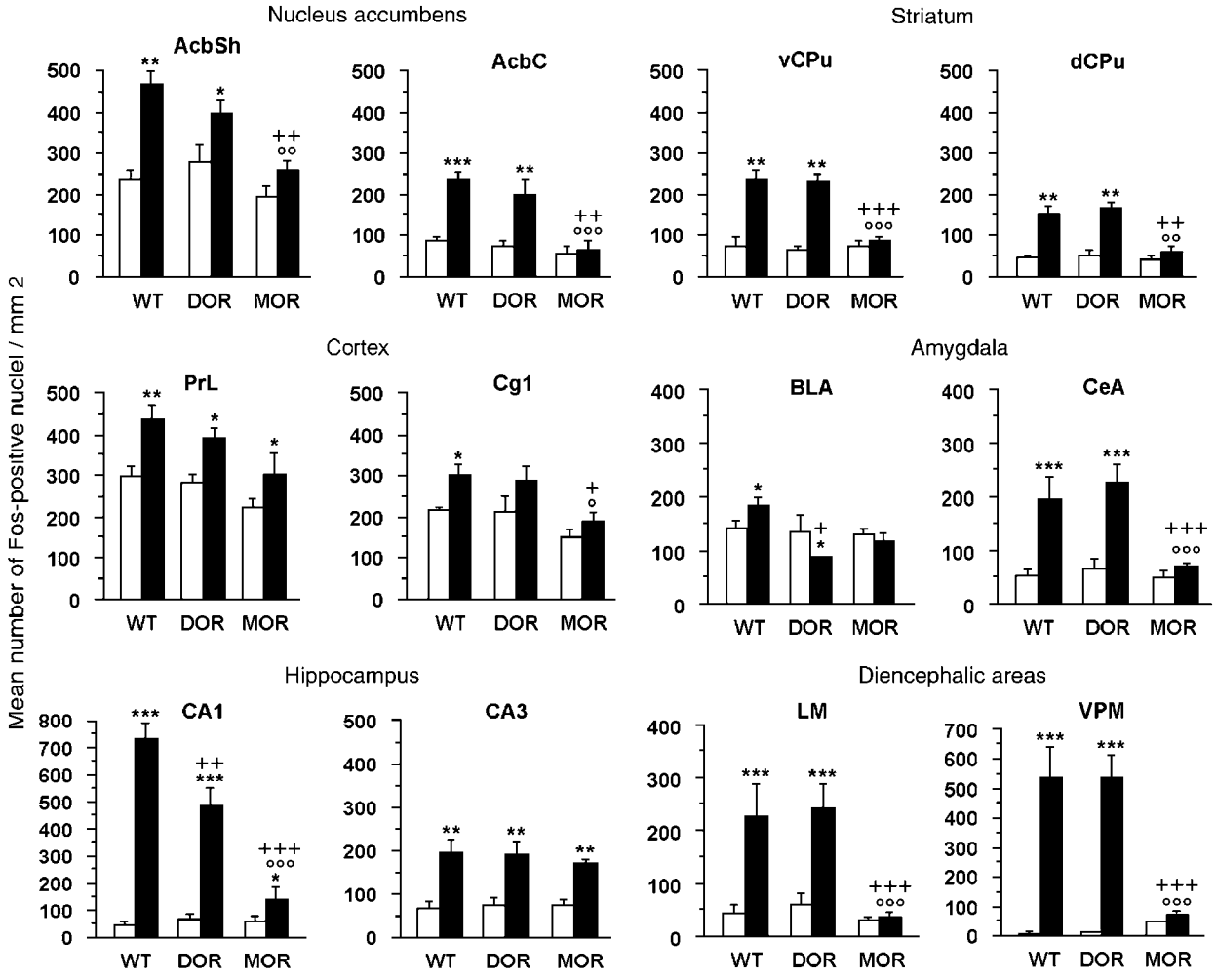

Figure 4 Mean number of Fos-positive nuclei/mm^2 in all brain areas sampled from mice following the last acquisition session of intra-VTA morphine self-administration. An ANOVA was performed for each structure, followed by *post hoc* tests. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$: comparison with vehicle (Ringer). $^{+}p<0.05$; $^{++}p<0.01$; $^{+++}p<0.001$: comparison with WT mice. $^{\circ}p<0.05$; $^{\circ\circ}p<0.01$; $^{\circ\circ\circ}p<0.001$: comparison with DOR mutants. Open bars, vehicle (Ringer); black bars, morphine.

genotype: $F_{2,50}=20.67$, $p<0.0001$; and drug × genotype interaction: $F_{2,50}=11.62$, $p<0.001$).

Dorsal hippocampus: Fos protein was expressed strongly within the CA1 subregion of mice self-administering morphine into the VTA, this effect being similar in WT and DOR−/− (Figures 4 and 6). A significant difference was also observed between vehicle and morphine-exposed MOR null mutants, although to a much lesser extent than in the two previous groups. These observations are supported by a global ANOVA revealing a main effect of drug: $F_{1,54}=30.24$, $p<0.0001$; a main effect of genotype: $F_{2,54}=57.53$, $p<0.0001$; as well as a drug × genotype interaction: $F_{2,54}=57.15$, $p<0.0001$ (Figure 6). There was a significant difference between the two morphine-sensitive genotypes, DOR mutants exhibiting less Fos-positive cells than WT (WT/DOR: $p=0.0085$). The pattern of Fos immunostaining within the CA3 was very different from the CA1. Morphine treatment was effective in inducing a Fos expression in all groups regardless of genotype, although to a much lesser extent than in the CA1 (main effect of drug: $F_{1,54}=59.16$, $p<0.0001$; main effect of genotype: $F_{2,54}=0.53$, NS; and drug × genotype interaction: $F_{2,54}=1.21$, NS). No difference in Fos labeling was observed between groups within the DG (not shown).

Diencephalic brain structures: thalamus and hypothalamus: Several striking results were revealed after counting of Fos-positive cells within diencephalic brain regions (Figures 4 and 6). Morphine-responding WT and DOR−/− mice exhibited an increased number of Fos-positive neurons within the lateral mammillary nucleus (main effect of drug: $F_{1,54}=16.93$, $p<0.001$; main effect of genotype: $F_{2,54}=46.65$, $p<0.001$; and genotype × drug interaction: $F_{2,54}=10.60$, $p<0.001$). A dense population of Fos-positive neurons was observed at the level of the ventral posteromedial thalamic (VPM) nucleus, exclusively in self-administering WT and DOR−/− mice, as compared to both controls (vehicle) of all genotypes and morphine-treated MOR mutants (main effect of drug: $F_{1,54}=34.25$, $p<0.0001$; main effect of genotype: $F_{2,54}=25.68$, $p<0.0001$; and genotype × drug interaction: $F_{2,54}=26.73$, $p<0.0001$; Figure 6). Activation of diencephalic structures clearly depends on MOR activation, since there was no difference in the

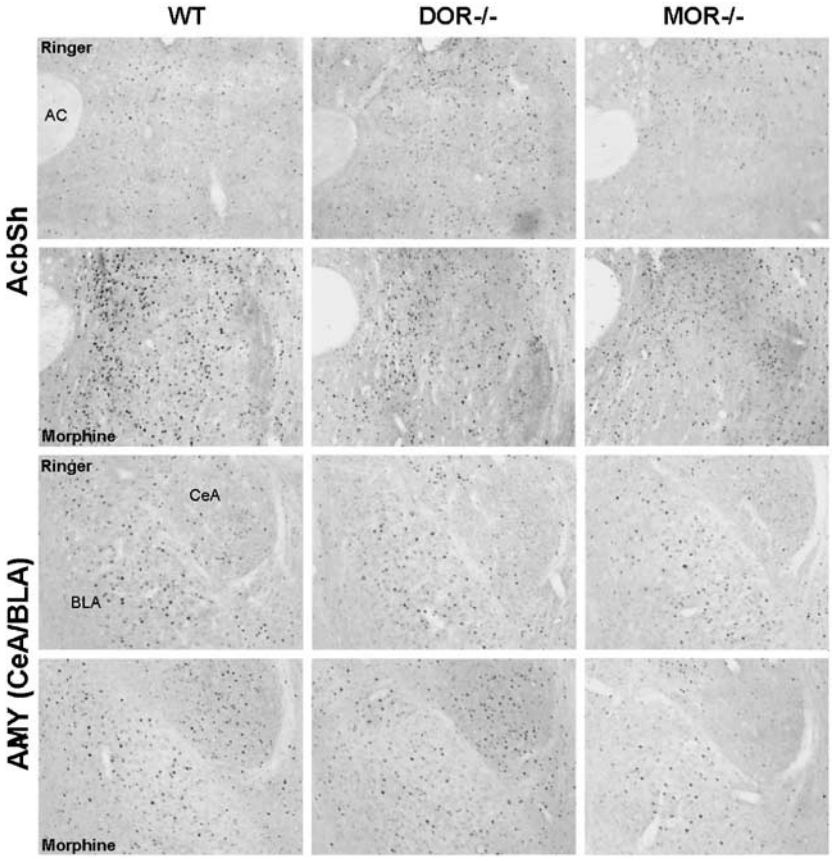

Figure 5 Photomicrographs of morphine ICSA-induced Fos protein expression within the dorsal shell of the nucleus accumbens (AcbSh), basolateral (BLA), and central (CeA) amygdala (AMY). Sections show × 10 magnification. Counting of Fos-positive nuclei was performed at the × 20 magnification. AC, anterior commissure.

number of Fos-positive nuclei counted in DOR null mutants and WT mice.

Experiment III: Intra-VTA Bicuculline Self-Administration in MOR−/− and WT Mice

Arm discrimination. In contrast to their inability to discriminate the morphine-reinforced arm, MOR null mutants exhibited a rapid discrimination between the drug-reinforced arm and the neutral arm of the Y-maze when bicuculline was infused into the VTA (Figure 7, top). The rate of acquisition was similar in MOR−/− and WT mice. These observations are attested to a global ANOVA revealing no effect of genotype ($F_{1,8} = 0.07$, NS); but a strong main effect of treatment ($F_{5,40} = 39.83$, $p < 0.0001$). Comparison of arm discrimination for morphine and bicuculline in MOR−/− mice confirmed the effect of bicuculline ($F_{1,8} = 20.90$, $p < 0.001$). Choice of the bicuculline-reinforced arm became significant from session 2 for both genotypes (comparison of the mean number of self-administrations during sessions A1/A2; WT mice: $t(5) = 5.09$, $p < 0.01$; MOR−/− mice: $t(5) = 3.65$, $p < 0.05$). The level of performance for bicuculline self-administration in MOR−/− mice was similar to that observed for morphine self-administration in WT and DOR−/−.

Self-injection latencies. Latencies decreased significantly over the course of the six acquisition sessions, both in WT and MOR mutants (no effect of genotype: $F_{1,8} = 0.44$, NS; and main effect of treatment: $F_{5,40} = 16.92$, $p < 0.0001$; Figure 7, bottom). MOR−/− mice triggered bicuculline injections more rapidly than morphine injections ($F_{1,8} = 27.25$, $p < 0.001$), which confirms the positive response of MOR mutants to intra-VTA bicuculline administration. The decrease in latency became significant starting from sessions 2 and 4, respectively, in MOR−/− and WT mice (comparison of the mean self-injection latency during sessions A1/A2 in MOR−/− mice: $t(5) = 4.74$, $p < 0.01$ and sessions A1/A4 in WT: $t(5) = 3.55$, $p < 0.05$).

DISCUSSION

Intra-VTA microinjections of morphine did not serve as a reinforcer in MOR−/− mice, whereas DOR−/− mutants exhibited a normal self-administration response. Therefore, morphine reward elicited from the VTA depends selectively on MORs. Since the behaviorally relevant diffusional spread occurring in the conditions of this study can be estimated as 600 μm laterally and 800 μm dorsally to the injection site, VTA opioid receptors are likely to be the main target of the drug (David and Cazala, 1994a, b). These results are consistent with the previous pharmacological and genetic studies supporting an involvement of MORs in opiate reward (Matthes *et al*, 1996; Bardo, 1998; Shippenberg and

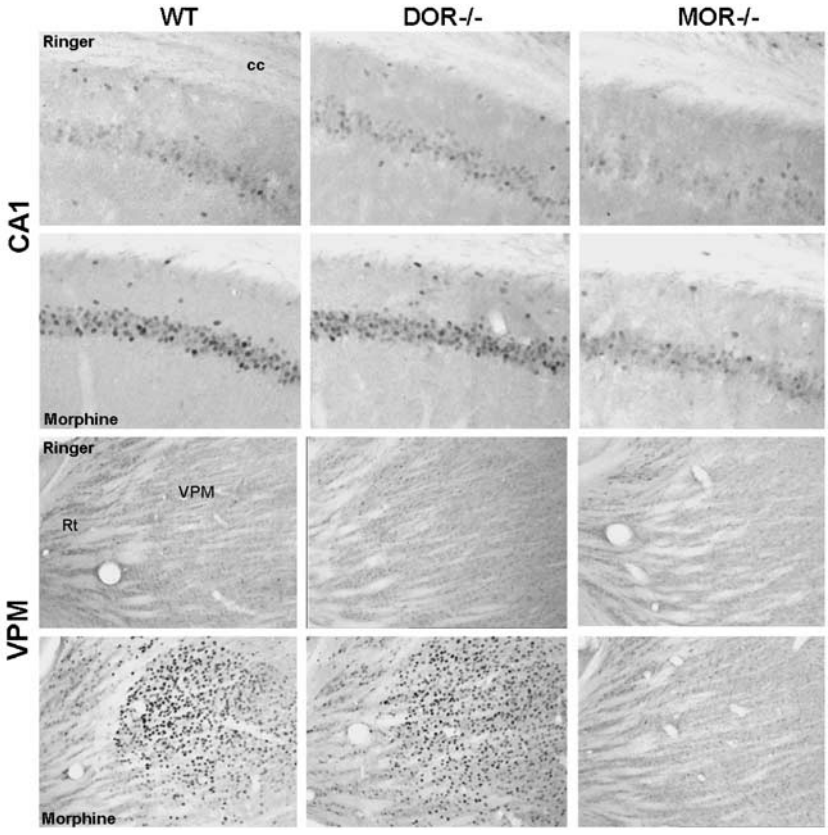

Figure 6 Photomicrographs of morphine ICSA-induced Fos protein expression within the dorsal CA1 of the hippocampus (CA1) and ventral posteromedial thalamic nucleus (VPM) of WT, DOR, and MOR null mutants. Sections show × 20 (CA1) or × 10 magnification (VPM). cc, corpus callosum; Rt, reticular thalamic nucleus.

Elmer, 1998; van Ree *et al*, 1999; Chefer *et al*, 2003; Hall *et al*, 2003). Although preferentially a μ-agonist, morphine also binds DORs with sub-μM affinity. When injected into the VTA, the μ-agonist (DAMGO) and δ-agonist (DPDPE) both increase mesolimbic DA release and support self-administration (Devine *et al*, 1993a, b; Devine and Wise, 1994). However, the effective dose of DPDPE required for maintaining self-administration was 100-fold higher than the effective dose of DAMGO (Devine and Wise, 1994). Non-peptidic δ-agonists (SNC80 and BW373U86) also induce CPP in rats, an effect blocked by pretreatment with the DA-D1 antagonist SCH 23390 (Longoni *et al*, 1998). At the doses effective in eliciting CPP, both compounds failed to affect extracellular DA release in the NAc. Whereas the δ-agonist deltorphine-II induce both reward and physical dependence in mice, these effects are absent in MOR mutants (Hutcheson *et al*, 2001). These data and the present report provide converging evidence that the rewarding effects of δ-agonists may depend on MORs.

The competitive opiate antagonist naloxone disrupted morphine self-administration in WT and DOR−/− mice. Naloxone-induced extinction was not associated with any behavioral signs of withdrawal, suggesting that VTA MORs are not involved in the somatic expression of withdrawal. In rats lever-pressing for intra-VTA morphine infusions, no naloxone-precipitated withdrawal signs were observed, whereas a full syndrome was displayed in rats self-injecting into the periventricular central gray area (Bozarth and Wise, 1984).

Although a growing body of evidence suggests that females are more prone to drug self-administration than males (Alexander *et al*, 1978; Lynch and Carroll, 1999; Carroll *et al*, 2001; Cicero *et al*, 2003; Hu *et al*, 2004; Roth *et al*, 2004), we found no differences in the acquisition of male and female mice. However, it is noteworthy that choice accuracy for the morphine-reinforced arm of the maze reached a maximum in female WT mice, with over 95% of drug-reinforced responses for 4 out of 6 self-administration sessions.

The Fos protein expression study provides new information on the neuroanatomical basis of VTA opiate reward. It is important to recognize first that functional interpretation of these data has methodological limits. The protocol used in the present study does not allow to dissociate direct pharmacological effects from conditioned effects of the drug, passive from self-administration or different processes engaged by the self-administration response. The reinforcing effects of drugs and drug-associated stimuli share many anatomical and cellular substrates, and context of drug administration has a profound impact on the effects of the drug (Badiani *et al*, 1999; Schroeder *et al*, 2000; Crombag *et al*, 2002; Schroeder and Kelley, 2002). Therefore, we cannot exclude morphine effects on various physiological processes. However, the use of well-controlled local infusions certainly contributes to the reduction of non-reward-related effects. Since all subjects completed the task whether or not a preference for the morphine-associated arm was displayed, motor activity cannot

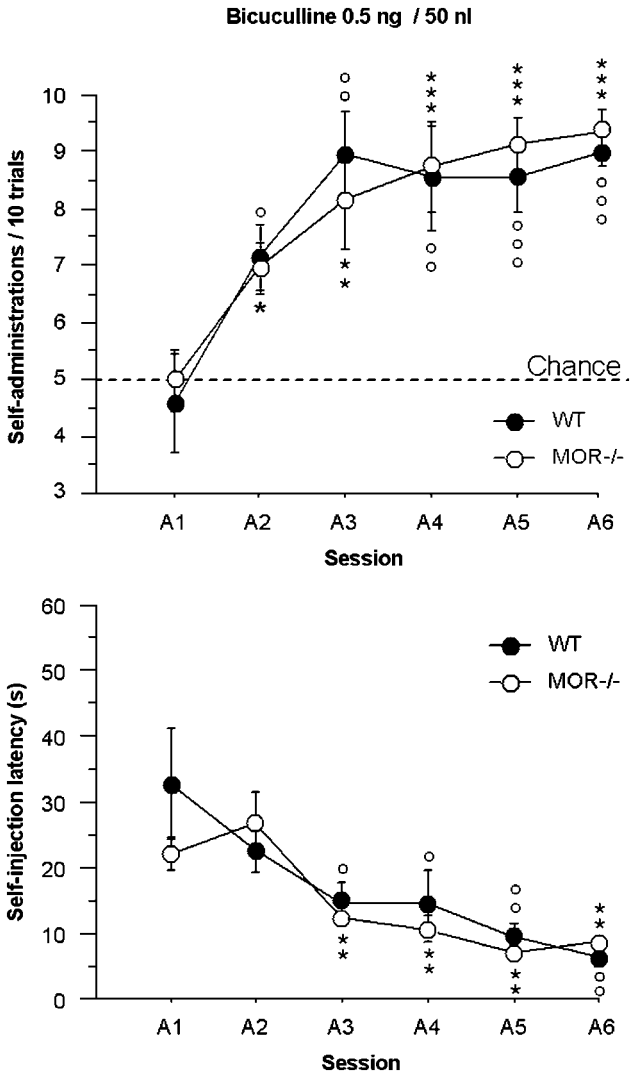

Figure 7 Intra-VTA bicuculline self-administration in MOR−/− and WT mice. Mean number (±SEM) of self-administrations (top) or mean value of the latency (seconds ± SEM) to trigger bicuculline self-injections (bottom). *$p<0.05$; **$p<0.01$; ***$p<0.001$: comparison with morphine (Figure 1). °$p<0.05$; °°$p<0.01$; °°°$p<0.001$: comparison with vehicle (Ringer; Figure 1).

account for the pattern of Fos protein expression observed. Indeed, Fos expression associated with conditioned behavioral activation following morphine treatment is not dependent on motor activity (Schroeder *et al*, 2000). Recent data suggest that Fos induction is a learning-related phenomenon underlying the establishment of specific neural activity rather than locomotor activity (Svarnik *et al*, 2005).

In agreement with the view that intra-VTA opiate administration disinhibits DA-A10 neurons, self-administration was associated with Fos expression within DA brain regions (Acb, CPu, PrL, Cg1). It should be noted however that (i) absence of Fos does not mean absence of neuronal activation and (ii) GABAergic projections from the VTA to the cortex, which parallel the DA system, have been described (Carr and Sesack, 2000a, b; Crombag *et al*, 2002). Yet several lines of evidence point to a role of DA. Activation of DA-D1 receptors by specific agonists or dopaminergic drugs induce striatal Fos expression (Graybiel *et al*, 1990; Robertson *et al*, 1992; Keefe and Gerfen, 1995; Herdegen and Leah, 1998). Systemic or intra-VTA morphine injections induce Fos within the limbic striatum and NAc and this expression is blocked by μ-, D1-, and NMDA antagonists (Bontempi and Sharp, 1997). We further observed that striatal Fos-expressing neurons were restricted to the medial striatum. Prefrontal and anterior cingulate cortices expressed high level of Fos in morphine-sensitive mice. This might be expected given the role of prefrontal cortex in reward expectancy (Watanabe, 1996). Morphine-conditioned locomotor activity is associated with c-fos induction within these two cortical areas (Schroeder *et al*, 2000). The anterior cingulate cortex has been involved also in cue-induced alcohol or cocaine-seeking in rats (Neisewander *et al*, 2000; Ciccocioppo *et al*, 2001).

Analysis of the diencephalic brain regions provided further interesting findings. Fos protein was expressed significantly more in the lateral mammillary nucleus of subjects who were self-administering. This could reflect an activation of the mammillothalamic pathway, which is involved in processing navigation, head-direction signal, spatial memory, and emotional reactivity, possibly through modulation of the anterior thalamic–hippocampal system (Beracochea and Jaffard, 1995; Blair *et al*, 1998; Sziklas and Petrides, 1998; Aggleton and Brown, 1999; Vann and Aggleton, 2004). Since preliminary data show that ibotenic acid lesions of the mammillary bodies do not disrupt intra-VTA morphine self-administration (unpublished observation), this activation could thus be more related to aspects of spatial or cognitive processing rather than to obtaining reinforcement *per se* (Beracochea, 2005).

Another striking observation is the strong and specific Fos expression within the posterior part of the ventral thalamus, particularly at the level of its medial division (ie the ventral posteromedial thalamic nucleus, VPM). VPM neurons expressed Fos exclusively in mice self-administering morphine, whereas Fos is absent within the VPM of active-control subjects, or in morphine-injected mice that did not acquire self-administration (MOR null mutants). Therefore, the VPM Fos immunoreactivity was predictive of self-administration. Despite an early description of DA projections from the VTA to both the ventral and mediodorsal thalamus (Simon *et al*, 1976), these DA pathways have received very little attention. This is likely because DA innervation of the rodent thalamus was considered to be scant (Groenewegen, 1988; Papadopoulos and Parnavelas, 1990). More recently, however, significant DA projections from A10 neurons to the ventral thalamus were demonstrated also in primates and humans (Sanchez-Gonzalez *et al*, 2005; Garcia-Cabezas *et al*, 2007). A μ-dependent inhibition of GABAergic inputs provided by the VTA/SN complex to the ventromedial thalamus could provide an anatomical basis for our observations. Functionally, electrophysiological data suggest that the ventral sensory thalamus may be involved in processing reward-relevant information. In rats discriminating between reward-predicting and non-predicting cues, single neurons of the VPM mediate the acquired affective significance of sensory stimuli (termed 'retrospective coding') and predict the value of upcoming reward ('prospective coding') (Komura *et al*, 2001). It is noteworthy that in humans,

diencephalic amnesia (ie Korsakoff syndrome) result in a difficulty in assessing the affective component of memories and contexts (Oscar-Berman *et al*, 1990; Snitz *et al*, 2002).

The dorsal hippocampus (CA1) also displayed Fos-positive cells in self-administering mice. Given that reward was assessed with a spatial discrimination task, involvement of the CA1 is not surprising. Absence of reward in MOR−/− mice could thus be related to a learning or memory deficit. MOR null mutants exhibit impaired learning in spatial tasks and drug-induced CPP (Kieffer and Gaveriaux-Ruff, 2002). However, learning-associated synaptic plasticity in the CA1 region is normal and MDMA induces CPP in MOR−/− mice (Jamot *et al*, 2003; Robledo *et al*, 2004). To test whether a learning deficit could account for the absence of spatial discrimination, MOR mutants were allowed to self-inject the GABA-A receptor antagonist, bicuculline into the VTA. Bicuculline serves as a powerful reinforcer when infused into the VTA of rats and mice (David *et al*, 1997; Ikemoto *et al*, 1997b). In contrast, the GABA-B receptor agonist baclofen blocks morphine CPP and associated Fos induction in the cortical and limbic brain regions (Kaplan *et al*, 2003). The rapid acquisition of bicuculline self-administration in MOR−/− mice demonstrate that these mutants have no learning deficit in our task, providing that they are efficiently reinforced with a non μ-dependent pharmacological agent. Despite early reports that the hippocampus does not receive a significant DA innervation (Loy *et al*, 1980), projections from the VTA have been described in the stratum moleculare of the CA1 (Gasbarri *et al*, 1994, 1997; Goldsmith and Joyce, 1994). Novel stimuli increase c-fos in the hippocampus (Jenkins *et al*, 2004). The VTA–CA1 pathway was recently proposed to be part of a loop controlling the entry of novel stimuli into long-term memory (Lisman and Grace, 2005). Alternatively, the involvement of the CA1 could occur via dopaminergic modulation of the septo–hippocampal pathway (Sheehan *et al*, 2004).

As concerns other brain regions expressing Fos, the amygdala has long been implicated in aversively motivated associative learning, and there is now a growing body of evidence suggesting that it plays a role in positive reinforcement (Baxter and Murray, 2002; Everitt *et al*, 2003; See *et al*, 2003). In WT mice, the number of Fos-positive neurons within the BLA increased with self-administration. Activation of BLA neurons occurs also during cocaine self-administration (Fuchs and See, 2002; Carelli *et al*, 2003). Tetrodotoxin-induced BLA inactivation suppresses the ability of heroin-paired stimuli to reinstate heroin-seeking behavior (Fuchs and See, 2002; Glass *et al*, 2005). Interestingly, an increase in AMPA GluR1 receptors on BLA dendrites of rats self-administering morphine was reported (Glass *et al*, 2005). DA afferents could enhance the sensory signal driving BLA output neurons, while dampening cortical inhibition (Rosenkranz and Grace, 1999, 2001, 2002). In self-administering DOR−/− mice, however, BLA Fos induction was decreased as compared to vehicle-injected mice, suggesting an indirect effect of DORs in modulating BLA activity. Naloxone-precipitated withdrawal induces an opposite pattern of c-fos mRNA expression within two subpopulations of BLA neurons (Frenois *et al*, 2002, 2005). Involvement of CeA in self-administering subjects could reveal a feedback control of VTA activation by CeA GABAergic output neurons (See *et al*, 2003).

In conclusion, MORs but not DORs, are critical for VTA opiate reward. Morphine self-administration was associated with Fos protein expression within the mesocorticolimbic system, amygdala, dorsal hippocampus (CA1), lateral mammillary nucleus, and the VPM. This latter structure exhibited a strong and selective immunoreactivity in response to intra-VTA morphine self-administration, thus suggesting a role for thalamic DA in opiate reward which may have been overlooked despite the early anatomical description of this innervation. Further experiments are required to investigate the effect of inactivation of the VPM or adjacent thalamic nuclei on morphine-induced behaviors.

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DISCLOSURE/CONFLICT OF INTEREST

We declare that, except for income received from our primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

REFERENCES

Aggleton JP, Brown MW (1999). Episodic memory, amnesia, and the hippocampal-anterior thalamic axis. *Behav Brain Sci* **22**: 425–444; discussion 444–489.

Alexander BK, Coambs RB, Hadaway PF (1978). The effect of housing and gender on morphine self-administration in rats. *Psychopharmacology (Berl)* **58**: 175–179.

Badiani A, Oates MM, Day HE, Watson SJ, Akil H, Robinson TE (1999). Environmental modulation of amphetamine-induced c-fos expression in D1 versus D2 striatal neurons. *Behav Brain Res* **103**: 203–209.

Bardo MT (1998). Neuropharmacological mechanisms of drug reward: beyond dopamine in the nucleus accumbens. *Crit Rev Neurobiol* **12**: 37–67.

Baxter MG, Murray EA (2002). The amygdala and reward. *Nat Rev Neurosci* **3**: 563–573.

Becker A, Grecksch G, Brodemann R, Kraus J, Peters B, Schroeder H *et al* (2000). Morphine self-administration in mu-opioid receptor-deficient mice. *Naunyn Schmiedebergs Arch Pharmacol* **361**: 584–589.

Beracochea D (2005). Interaction between emotion and memory: importance of mammillary bodies damage in a mouse model of the alcoholic Korsakoff syndrome. *Neural Plast* **12**: 275–287.

Beracochea DJ, Jaffard R (1995). The effects of mammillary body lesions on delayed matching and delayed non-matching to place tasks in the mice. *Behav Brain Res* **68**: 45–52.

Blair HT, Cho J, Sharp PE (1998). Role of the lateral mammillary nucleus in the rat head direction circuit: a combined single unit recording and lesion study. *Neuron* **21**: 1387–1397.

Bontempi B, Sharp FR (1997). Systemic morphine-induced Fos protein in the rat striatum and nucleus accumbens is regulated by mu opioid receptors in the substantia nigra and ventral tegmental area. *J Neurosci* **17**: 8596–8612.

Bozarth MA (1987). Neuroanatomical boundaries of the reward-relevant opiate-receptor field in the ventral tegmental area as mapped by the conditioned place preference method in rats. *Brain Res* **414**: 77–84.

Bozarth MA, Wise RA (1981). Intracranial self-administration of morphine into the ventral tegmental area in rats. *Life Sci* **28**: 551–555.

Bozarth MA, Wise RA (1984). Anatomically distinct opiate receptor fields mediate reward and physical dependence. *Science* **224**: 516–517.

Carelli RM, Williams JG, Hollander JA (2003). Basolateral amygdala neurons encode cocaine self-administration and cocaine-associated cues. *J Neurosci* **23**: 8204–8211.

Carr DB, Sesack SR (2000a). GABA-containing neurons in the rat ventral tegmental area project to the prefrontal cortex. *Synapse* **38**: 114–123.

Carr DB, Sesack SR (2000b). Projections from the rat prefrontal cortex to the ventral tegmental area: target specificity in the synaptic associations with mesoaccumbens and mesocortical neurons. *J Neurosci* **20**: 3864–3873.

Carroll ME, Campbell UC, Heideman P (2001). Ketoconazole suppresses food restriction-induced increases in heroin self-administration in rats: sex differences. *Exp Clin Psychopharmacol* **9**: 307–316.

Cazala P, Darracq C, Saint-Marc M (1987). Self-administration of morphine into the lateral hypothalamus in the mouse. *Brain Res* **416**: 283–288.

Chefer VI, Kieffer BL, Shippenberg TS (2003). Basal and morphine-evoked dopaminergic neurotransmission in the nucleus accumbens of MOR- and DOR-knockout mice. *Eur J Neurosci* **18**: 1915–1922.

Ciccocioppo R, Sanna PP, Weiss F (2001). Cocaine-predictive stimulus induces drug-seeking behavior and neural activation in limbic brain regions after multiple months of abstinence: reversal by D(1) antagonists. *Proc Natl Acad Sci USA* **98**: 1976–1981.

Cicero TJ, Aylward SC, Meyer ER (2003). Gender differences in the intravenous self-administration of mu opiate agonists. *Pharmacol Biochem Behav* **74**: 541–549.

Crombag HS, Jedynak JP, Redmond K, Robinson TE, Hope BT (2002). Locomotor sensitization to cocaine is associated with increased Fos expression in the accumbens, but not in the caudate. *Behav Brain Res* **136**: 455–462.

David V, Cazala P (1994a). A comparative study of self-administration of morphine into the amygdala and the ventral tegmental area in mice. *Behav Brain Res* **65**: 205–211.

David V, Cazala P (1994b). Differentiation of intracranial morphine self-administration behavior among five brain regions in mice. *Pharmacol Biochem Behav* **48**: 625–633.

David V, Durkin TP, Cazala P (1997). Self-administration of the GABAA antagonist bicuculline into the ventral tegmental area in mice: dependence on D2 dopaminergic mechanisms. *Psychopharmacology (Berl)* **130**: 85–90.

David V, Durkin TP, Cazala P (2002). Differential effects of the dopamine D2/D3 receptor antagonist sulpiride on self-administration of morphine into the ventral tegmental area or the nucleus accumbens. *Psychopharmacology (Berl)* **160**: 307–317.

De Vries TJ, Shippenberg TS (2002). Neural systems underlying opiate addiction. *J Neurosci* **22**: 3321–3325.

Devine DP, Leone P, Pocock D, Wise RA (1993b). Differential involvement of ventral tegmental mu, delta and kappa opioid receptors in modulation of basal mesolimbic dopamine release: *in vivo* microdialysis studies. *J Pharmacol Exp Ther* **266**: 1236–1246.

Devine DP, Leone P, Wise RA (1993a). Mesolimbic dopamine neurotransmission is increased by administration of mu-opioid receptor antagonists. *Eur J Pharmacol* **243**: 55–64.

Devine DP, Wise RA (1994). Self-administration of morphine, DAMGO, and DPDPE into the ventral tegmental area of rats. *J Neurosci* **14**: 1978–1984.

Duvauchelle CL, Fleming SM, Kornetsky C (1997). DAMGO and DPDPE facilitation of brain stimulation reward thresholds is blocked by the dopamine antagonist cis-flupenthixol. *Neuropharmacology* **36**: 1109–1114.

Elmer GI, Pieper JO, Rubinstein M, Low MJ, Grandy DK, Wise RA (2002). Failure of intravenous morphine to serve as an effective instrumental reinforcer in dopamine D2 receptor knock-out mice. *J Neurosci* **22**: RC224.

Everitt BJ, Cardinal RN, Parkinson JA, Robbins TW (2003). Appetitive behavior: impact of amygdala-dependent mechanisms of emotional learning. *Ann NY Acad Sci* **985**: 233–250.

Filliol D, Ghozland S, Chluba J, Martin M, Matthes HW, Simonin F *et al* (2000). Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. *Nat Genet* **25**: 195–200.

Frenois F, Cador M, Caille S, Stinus L, Le Moine C (2002). Neural correlates of the motivational and somatic components of naloxone-precipitated morphine withdrawal. *Eur J Neurosci* **16**: 1377–1389.

Frenois F, Stinus L, Di Blasi F, Cador M, Le Moine C (2005). A specific limbic circuit underlies opiate withdrawal memories. *J Neurosci* **25**: 1366–1374.

Fuchs RA, See RE (2002). Basolateral amygdala inactivation abolishes conditioned stimulus- and heroin-induced reinstatement of extinguished heroin-seeking behavior in rats. *Psychopharmacology (Berl)* **160**: 425–433.

Garcia-Cabezas MA, Rico B, Sanchez-Gonzalez MA, Cavada C (2007). Distribution of the dopamine innervation in the macaque and human thalamus. *Neuroimage* **34**: 965–984.

Gasbarri A, Packard MG, Campana E, Pacitti C (1994). Anterograde and retrograde tracing of projections from the ventral tegmental area to the hippocampal formation in the rat. *Brain Res Bull* **33**: 445–452.

Gasbarri A, Sulli A, Packard MG (1997). The dopaminergic mesencephalic projections to the hippocampal formation in the rat. *Prog Neuropsychopharmacol Biol Psychiatry* **21**: 1–22.

Glass MJ, Kruzich PJ, Colago EE, Kreek MJ, Pickel VM (2005). Increased AMPA GluR1 receptor subunit labeling on the plasma membrane of dendrites in the basolateral amygdala of rats self-administering morphine. *Synapse* **58**: 1–12.

Goldsmith SK, Joyce JN (1994). Dopamine D2 receptor expression in hippocampus and parahippocampal cortex of rat, cat, and human in relation to tyrosine hydroxylase-immunoreactive fibers. *Hippocampus* **4**: 354–373.

Goody RJ, Oakley SM, Filliol D, Kieffer BL, Kitchen I (2002). Quantitative autoradiographic mapping of opioid receptors in the brain of delta-opioid receptor gene knockout mice. *Brain Res* **945**: 9–19.

Graybiel AM, Moratalla R, Robertson HA (1990). Amphetamine and cocaine induce drug-specific activation of the c-*fos* gene in striosome-matrix compartments and limbic subdivisions of the striatum. *Proc Natl Acad Sci USA* **87**: 6912–6916.

Groenewegen HJ (1988). Organization of the afferent connections of the mediodorsal thalamic nucleus in the rat, related to the mediodorsal-prefrontal topography. *Neuroscience* **24**: 379–431.

Gysling K, Wang RY (1983). Morphine-induced activation of A10 dopamine neurons in the rat. *Brain Res* **277**: 119–127.

Hall FS, Li XF, Goeb M, Roff S, Hoggatt H, Sora I *et al* (2003). Congenic C57BL/6 mu opiate receptor (MOR) knockout mice: baseline and opiate effects. *Genes Brain Behav* **2**: 114–121.

Heidbreder C, Shoaib M, Shippenberg TS (1996). Differential role of delta-opioid receptors in the development and expression of

behavioral sensitization to cocaine. *Eur J Pharmacol* **298**: 207–216.

Herdegen T, Leah JD (1998). Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain Res Brain Res Rev* **28**: 370–490.

Hnasko TS, Sotak BN, Palmiter RD (2005). Morphine reward in dopamine-deficient mice. *Nature* **438**: 854–857.

Hu M, Crombag HS, Robinson TE, Becker JB (2004). Biological basis of sex differences in the propensity to self-administer cocaine. *Neuropsychopharmacology* **29**: 81–85.

Hutcheson DM, Matthes HW, Valjent E, Sanchez-Blazquez P, Rodriguez-Diaz M, Garzon J *et al* (2001). Lack of dependence and rewarding effects of deltorphin II in mu-opioid receptor-deficient mice. *Eur J Neurosci* **13**: 153–161.

Ikemoto S, Kohl RR, McBride WJ (1997a). GABA(A) receptor blockade in the anterior ventral tegmental area increases extracellular levels of dopamine in the nucleus accumbens of rats. *J Neurochem* **69**: 137–143.

Ikemoto S, Murphy JM, McBride WJ (1997b). Self-infusion of GABA(A) antagonists directly into the ventral tegmental area and adjacent regions. *Behav Neurosci* **111**: 369–380.

Jamot L, Matthes HW, Simonin F, Kieffer BL, Roder JC (2003). Differential involvement of the mu and kappa opioid receptors in spatial learning. *Genes Brain Behav* **2**: 80–92.

Jenkins TA, Amin E, Pearce JM, Brown MW, Aggleton JP (2004). Novel spatial arrangements of familiar visual stimuli promote activity in the rat hippocampal formation but not the parahippocampal cortices: a c-fos expression study. *Neuroscience* **124**: 43–52.

Johnson SW, North RA (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J Neurosci* **12**: 483–488.

Kalivas PW, Duffy P, Eberhardt H (1990). Modulation of A10 dopamine neurons by gamma-aminobutyric acid agonists. *J Pharmacol Exp Ther* **253**: 858–866.

Kaplan GB, Leite-Morris KA, Joshi M, Shoeb MH, Carey RJ (2003). Baclofen inhibits opiate-induced conditioned place preference and associated induction of Fos in cortical and limbic regions. *Brain Res* **987**: 122–125.

Keefe KA, Gerfen CR (1995). D1-D2 dopamine receptor synergy in striatum: effects of intrastriatal infusions of dopamine agonists and antagonists on immediate early gene expression. *Neuroscience* **66**: 903–913.

Kieffer BL, Gaveriaux-Ruff C (2002). Exploring the opioid system by gene knockout. *Prog Neurobiol* **66**: 285–306.

Kitchen I, Slowe SJ, Matthes HW, Kieffer B (1997). Quantitative autoradiographic mapping of mu-, delta- and kappa-opioid receptors in knockout mice lacking the mu-opioid receptor gene. *Brain Res* **778**: 73–88.

Klitenick MA, DeWitte P, Kalivas PW (1992). Regulation of somatodendritic dopamine release in the ventral tegmental area by opioids and GABA: an *in vivo* microdialysis study. *J Neurosci* **12**: 2623–2632.

Komura Y, Tamura R, Uwano T, Nishijo H, Kaga K, Ono T (2001). Retrospective and prospective coding for predicted reward in the sensory thalamus. *Nature* **412**: 546–549.

Leone P, Pocock D, Wise RA (1991). Morphine–dopamine interaction: ventral tegmental morphine increases nucleus accumbens dopamine release. *Pharmacol Biochem Behav* **39**: 469–472.

Lisman JE, Grace AA (2005). The hippocampal-VTA loop: controlling the entry of information into long-term memory. *Neuron* **46**: 703–713.

Longoni R, Cadoni C, Mulas A, Di Chiara G, Spina L (1998). Dopamine-dependent behavioural stimulation by non-peptide delta opioids BW373U86 and SNC 80: 2. Place-preference and brain microdialysis studies in rats. *Behav Pharmacol* **9**: 9–14.

Loy R, Koziell DA, Lindsey JD, Moore RY (1980). Noradrenergic innervation of the adult rat hippocampal formation. *J Comp Neurol* **189**: 699–710.

Lynch WJ, Carroll ME (1999). Sex differences in the acquisition of intravenously self-administered cocaine and heroin in rats. *Psychopharmacology (Berl)* **144**: 77–82.

Maldonado R, Saiardi A, Valverde O, Samad TA, Roques BP, Borrelli E (1997). Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. *Nature* **388**: 586–589.

Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I *et al* (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* **383**: 819–823.

Neisewander JL, Baker DA, Fuchs RA, Tran-Nguyen LT, Palmer A, Marshall JF (2000). Fos protein expression and cocaine-seeking behavior in rats after exposure to a cocaine self-administration environment. *J Neurosci* **20**: 798–805.

Nye HE, Nestler EJ (1996). Induction of chronic Fos-related antigens in rat brain by chronic morphine administration. *Mol Pharmacol* **49**: 636–645.

Oscar-Berman M, Hancock M, Mildworf B, Hutner N, Weber DA (1990). Emotional perception and memory in alcoholism and aging. *Alcohol Clin Exp Res* **14**: 383–393.

Papadopoulos GC, Parnavelas JG (1990). Distribution and synaptic organization of dopaminergic axons in the lateral geniculate nucleus of the rat. *J Comp Neurol* **294**: 356–361.

Paxinos G, Franklin KBJ (2004). *The Mouse Brain in Stereotaxic Coordinates*. Elsevier Edition: San Diego, CA.

Pettit HO, Ettenberg A, Bloom FE, Koob GF (1984). Destruction of dopamine in the nucleus accumbens selectively attenuates cocaine but not heroin self-administration in rats. *Psychopharmacology (Berl)* **84**: 167–173.

Phillips AG, LePiane FG (1980). Reinforcing effects of morphine microinjection into the ventral tegmental area. *Pharmacol Biochem Behav* **12**: 965–968.

Pierce RC, Kumaresan V (2006). The mesolimbic dopamine system: the final common pathway for the reinforcing effect of drugs of abuse? *Neurosci Biobehav Rev* **30**: 215–238.

Pothos E, Rada P, Mark GP, Hoebel BG (1991). Dopamine microdialysis in the nucleus accumbens during acute and chronic morphine, naloxone-precipitated withdrawal and clonidine treatment. *Brain Res* **566**: 348–350.

Robertson GS, Vincent SR, Fibiger HC (1992). D1 and D2 dopamine receptors differentially regulate c-fos expression in striatonigral and striatopallidal neurons. *Neuroscience* **49**: 285–296.

Robledo P, Mendizabal V, Ortuno J, de la Torre R, Kieffer BL, Maldonado R (2004). The rewarding properties of MDMA are preserved in mice lacking mu-opioid receptors. *Eur J Neurosci* **20**: 853–858.

Rosenkranz JA, Grace AA (1999). Modulation of basolateral amygdala neuronal firing and afferent drive by dopamine receptor activation *in vivo*. *J Neurosci* **19**: 11027–11039.

Rosenkranz JA, Grace AA (2001). Dopamine attenuates prefrontal cortical suppression of sensory inputs to the basolateral amygdala of rats. *J Neurosci* **21**: 4090–4103.

Rosenkranz JA, Grace AA (2002). Dopamine-mediated modulation of odour-evoked amygdala potentials during pavlovian conditioning. *Nature* **417**: 282–287.

Roth ME, Cosgrove KP, Carroll ME (2004). Sex differences in the vulnerability to drug abuse: a review of preclinical studies. *Neurosci Biobehav Rev* **28**: 533–546.

Sanchez-Gonzalez MA, Garcia-Cabezas MA, Rico B, Cavada C (2005). The primate thalamus is a key target for brain dopamine. *J Neurosci* **25**: 6076–6083.

Schroeder BE, Holahan MR, Landry CF, Kelley AE (2000). Morphine-associated environmental cues elicit conditioned gene expression. *Synapse* **37**: 146–158.

Schroeder BE, Kelley AE (2002). Conditioned Fos expression following morphine-paired contextual cue exposure is environment specific. *Behav Neurosci* **116**: 727–732.

See RE, Fuchs RA, Ledford CC, McLaughlin J (2003). Drug addiction, relapse, and the amygdala. *Ann NY Acad Sci* **985**: 294–307.

Sheehan TP, Chambers RA, Russell DS (2004). Regulation of affect by the lateral septum: implications for neuropsychiatry. *Brain Res Brain Res Rev* **46**: 71–117.

Shippenberg TS, Bals-Kubik R, Herz A (1987). Motivational properties of opioids: evidence that an activation of delta-receptors mediates reinforcement processes. *Brain Res* **436**: 234–239.

Shippenberg TS, Elmer GI (1998). The neurobiology of opiate reinforcement. *Crit Rev Neurobiol* **12**: 267–303.

Simon H, Le Moal M, Galey D, Cardo B (1976). Silver impregnation of dopaminergic systems after radiofrequency and 6-OHDA lesions of the rat ventral. *Brain Res* **115**: 215–231.

Slowe SJ, Clarke S, Lena I, Goody RJ, Lattanzi R, Negri L *et al* (2001). Autoradiographic mapping of the opioid receptor-like 1 (ORL1) receptor in the brains of mu-, delta- or kappa-opioid receptor knockout mice. *Neuroscience* **106**: 469–480.

Snitz BE, Hellinger A, Daum I (2002). Impaired processing of affective prosody in Korsakoff's syndrome. *Cortex* **38**: 797–803.

Sora I, Elmer G, Funada M, Pieper J, Li XF, Hall FS *et al* (2001). Mu opiate receptor gene dose effects on different morphine actions: evidence for differential *in vivo* mu receptor reserve. *Neuropsychopharmacology* **25**: 41–54.

Spanagel R, Herz A, Shippenberg TS (1992). Opposing tonically active endogenous opioid systems modulate the mesolimbic dopaminergic pathway. *Proc Natl Acad Sci USA* **89**: 2046–2050.

Spielewoy C, Gonon F, Roubert C, Fauchey V, Jaber M, Caron MG *et al* (2000). Increased rewarding properties of morphine in dopamine-transporter knockout mice. *Eur J Neurosci* **12**: 1827–1837.

Stinus L, Winnock M, Kelley AE (1985). Chronic neuroleptic treatment and mesolimbic dopamine denervation induce behavioural supersensitivity to opiates. *Psychopharmacology (Berl)* **85**: 323–328.

Suzuki T, Tsuji M, Mori T, Ikeda H, Misawa M, Nagase H (1997). Involvement of dopamine-dependent and -independent mechanisms in the rewarding effects mediated by delta opioid receptor subtypes in mice. *Brain Res* **744**: 327–334.

Suzuki T, Tsuji M, Mori T, Misawa M, Nagase H (1996). The effects of dopamine D1 and D2 receptor antagonists on the rewarding effects of delta 1 and delta 2 opioid receptor agonists in mice. *Psychopharmacology (Berl)* **124**: 211–218.

Svarnik OE, Alexandrov YI, Gavrilov VV, Grinchenko YV, Anokhin KV (2005). Fos expression and task-related neuronal activity in rat cerebral cortex after instrumental learning. *Neuroscience* **136**: 33–42.

Sziklas V, Petrides M (1998). Memory and the region of the mammillary bodies. *Prog Neurobiol* **54**: 55–70.

van Ree JM, Gerrits MA, Vanderschuren LJ (1999). Opioids, reward and addiction: an encounter of biology, psychology, and medicine. *Pharmacol Rev* **51**: 341–396.

Vann SD, Aggleton JP (2004). The mammillary bodies: two memory systems in one? *Nat Rev Neurosci* **5**: 35–44.

Watanabe M (1996). Reward expectancy in primate prefrontal neurons. *Nature* **382**: 629–632.

Welzl H, Kuhn G, Huston JP (1989). Self-administration of small amounts of morphine through glass micropipettes into the ventral tegmental area of the rat. *Neuropharmacology* **28**: 1017–1023.

Yoshida Y, Koide S, Hirose N, Takada K, Tomiyama K, Koshikawa N *et al* (1999). Fentanyl increases dopamine release in rat nucleus accumbens: involvement of mesolimbic mu- and delta-2-opioid receptors. *Neuroscience* **92**: 1357–1365.