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Modulation of anxiety by μ-opioid receptors of the lateral septal region in mice

Julie Le Merrer *, Barbara Cagniard [1], Pierre Cazala

Laboratoire de Neurosciences Cognitives, CNRS UMR 5106, Université Bordeaux I, Avenue des Facultés, 33405 Talence, France

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Abstract

Morphine and opiates are known to exert anxiolytic effects, probably by interacting with the GABAergic system. The lateral septum (LS), mainly constituted of GABA neurons, exhibits high densities of μ-opiate receptors and could thus represent one the brain sites where opiates interact with GABAergic transmission to modulate anxiety. We examined the effects of intra-LS morphine injections on measures of anxiety using the elevated plus-maze and hole-board tests. Fos imaging was used to identify neural circuits involved in anxiety modulation. Unilateral intra-LS morphine (100 or 500 ng/100 nl) decreased open-arm exploration in the plus-maze and reduced head-dipping frequency in the hole-board, an anxiogenic-like effect associated with decreased Fos expression in the ventral LS, the dorsal hippocampus and the anterior hypothalamus. Anatomical specificity was assessed by injecting morphine into the medial septum, which failed to produce anxiogenesis. Pre-injection of the μ-opioid receptor antagonist naloxonazine (100 ng/100 nl) into LS reversed morphine-induced anxiogenesis and the associated pattern of Fos expression, indicating a specific recruitment of μ-opioid receptors by morphine. Surprisingly, bilateral morphine injections (20 to 500 ng/100 nl) were not found anxiogenic, perhaps due to their stimulant effect. Taken together, these results suggest that LS μ-opioid receptors participate to the modulation of anxiety.

Keywords: Septum; Intra-cranial injections; Opiates; Naloxonazine; Elevated plus-maze; Hole-board; Dorsal hippocampus; Hypothalamus

1. Introduction

Several studies have reported an anxiolytic action of morphine and mu opiate receptor agonists when injected peripherally (Asakawa et al., 1998; Koks et al., 1999; Millan and Duka, 1981; Zarrindast et al., 2005), whereas μ-opioid receptor antagonists tend to be anxiogenic (Tsuda et al., 1996; Zhang et al., 1996). It has been suggested that the anxiolytic action of opiates is mediated by its interaction with the GABAergic system in some specific brain areas (Sasaki et al., 2002), the amygdala being one of these (Kang et al., 2000; Sasaki et al., 2002). We hypothesise that the septal region, and more particularly the lateral septum (LS), could represent another brain site for GABA/opioid interaction in the modulation of anxiety. Several authors have stressed the important role played by the LS in the control of anxiety, although the inhibitory (Thomas, 1988; Yadin et al., 1993) or facilitatory (Treit and Menard, 2000) nature of such control remains a matter of debate. The LS is mainly constituted of GABAergic neurons (Jakab and Leranth, 1995; Panula et al., 1984; Risold and Swanson, 1997a) and expresses high densities of GABA receptors (Pirker et al., 2000). Anxiolysis is observed when LS GABAergic transmission is facilitated by means of local injections of either the benzodiazepine anxiolytic midazolam or the $GABA_A$ agonist muscimol (Degroot et al., 2001; Pesold and Treit, 1996), demonstrating the involvement of the LS GABAergic system in the modulation of anxiety. Interestingly, opioid peptides, either released by septal afferents (Barna et al., 1997; Lewis et al., 1985; Onteniente et al., 1989; Szeidemann et al., 1995) or synthesized locally (Risold and Swanson, 1997a) are also widely expressed in the LS, as well as mu, delta or kappa opioid receptors (Mansour et al., 1995). However, little attention has been given to the potential role

* Corresponding author. Current address: Department of Psychology, School of Life Science, University of Sussex, Falmer, Brighton, BN1 9QG, UK. Tel.: +44 1273 606755; fax: +44 1273 876619.
E-mail address: J.Le-Merrer@sussex.ac.uk (J. Le Merrer).
[1] Current address: Department of Neurobiology, Pharmacology, and Physiology, University of Chicago, Chicago, IL 60637, USA.

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played by the septal opioidergic system in the modulation of anxiety.

The purpose of the present study was to examine the behavioural effects resulting from activating the opioidergic system of the LS, which, to the best of our knowledge, have not previously been explored, and to further elucidate the functional role played by μ-opioid receptors of the LS in the modulation of anxiety. Activation of μ-opioid receptors was induced by infusions of morphine, directly into the dorsolateral part of the LS (Risold and Swanson, 1997a), an anatomical subdivision of the septal region that have been targeted in previous pharmacological studies (Cheeta et al., 2000; Ouagazzal et al., 1999; Treit and Menard, 2000). As there is growing evidence for a functional lateralization of crucial structures for the modulation of anxiety (Belcheva et al., 1994; Coleman-Mesches and McGaugh, 1995; Zald, 2003), we tested the hypothesis of such an asymmetry for the LS by comparing the effects of unilateral or bilateral morphine injections. Anxiety and locomotor activity were evaluated in mice using the elevated plus-maze (Lister, 1987; Pellow et al., 1985) followed by the four-hole-board exploration task (Takeda et al., 1998). The anatomical specificity of intra-LS morphine was assessed by measuring anxiety-like behaviours following injections into the medial septum. Finally, in order to better understand how activation of μ-opioid receptors by morphine may modulate local septal circuitry, but also to identify larger neural circuits recruited in the modulation of anxiety by the septal region, we examined the expression of the product of the transcription factor c-*fos*. Induction of the Fos protein has been reported following various environmental stimuli and drug treatments (Herrera and Robertson, 1996; Hughes et al., 1999; Singewald et al., 2003) and is widely used as an indirect correlate of neuronal activation (Dragunow and Faull, 1989; Sagar et al., 1988).

2. Materials and methods

2.1. Animals and surgery

154 male mice of the inbred strain BALB/cByJICO obtained from Iffa-Credo (Lyon, France) were used throughout. At 9 weeks of age, they were housed individually with ad libitum food and water access, in a temperature-controlled room (23 ± 1 °C) maintained on a 12-h light/dark cycle (lights on at 7:00 am). All experiments were carried out during the light period of the cycle. Mice were aged 14–18 weeks and weighed about 28–32 g at the beginning of the experiments. Under deep anesthesia (Avertin, 300 mg/kg i.p.; local Xylocaine®, 5%), animals were placed in a stereotaxic frame (La Précision Cinématographique, Paris, France) and implanted either unilaterally or bilaterally with one or two guide cannulas (8 mm long, O.D. = 0.460 mm, I.D. = 0.255 mm) into the lateral part of the septum. The stereotaxic coordinates for unilateral (LS group) as well as bilateral (biLS group) implantations into the LS were: antero-posterior (AP) referring to bregma +0.80 mm; lateral (L) referring to sagittal line ±0.40 mm; vertical (V) from the surface of the skull −1.60 mm. The unilateral intra-LS implantations were carried out in a counter balanced left and right order. The stereotaxic coordinates for implantations into the medial septum (MS group) were: AP = +0.80 mm, L = 0 mm; V = −2.00 mm. In order to minimize tissue damage, the tip of each guide cannula was positioned 1.5 mm above the targeted structure. The cannula was anchored to the skull with two stainless steel screws and rapid-setting acrylic dental cement. Patency was maintained by inserting a stylet that fit the length of each guide cannula. Mice were allowed 10 days to recover from the operation. All surgical and experimental procedures were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Compounds

Morphine sulfate (donated by Francopia, Sanofi Synthelabo Laboratories, Paris, France) was dissolved in sterile Ringer solution (Meram®, Cooper laboratories, Melun, France) and administered intracerebrally (20–500 ng/100 nl) into either the dorsal part of the LS or the MS. The final pH of the solution was 6.1–6.2. The selective μ-opioid receptor antagonist naloxonazine (bichlorhydrate; 100 ng/100 nl) was purchased from Sigma (Lyon, France), dissolved in Ringer and administered into the LS 25 min prior to morphine infusion.

2.3. Intraseptal injection procedure

Mice were handled and submitted to a daily intracerebral injection simulation during 4 consecutive days prior to testing in order to minimize the effects of stress on subsequent behavioural testing and Fos expression. Intraseptal injections (morphine or vehicle) were carried out in freely moving mice by inserting one (LS and MS groups) or two (biLS group) injection cannula(s) (9.5 mm long, O.D. = 0.230 mm) through guide cannula(s) 20 min before the behavioural session. The injection cannula protruded 1.5 mm beyond the tip of the guide cannula. The injected volume (100 nl) was delivered over a 3-min period, via a 1-μl Hamilton syringe mounted on an infusion pump and connected to injection cannula by means of a polyethylene tubing. The injection cannula was left in place for an additional 2 min after the end of the infusion to ensure proper diffusion. Animals receiving the μ-opioid receptor antagonist naloxonazine (or vehicle) were injected in the same manner 25 min before receiving morphine into the LS.

2.4. Behavioural testing

2.4.1. Elevated plus-maze

The elevated plus-maze test is based on the natural aversion of rodents for open spaces and heights (Montgomery, 1955). It is considered a useful and reliable test to investigate the effects of both anxiolytic and anxiogenic agents in rats (Pellow et al., 1985) as well as in mice (Lister, 1987). In the present study, the apparatus, which was placed in a quiet room and uniformly illuminated at 100 lx, was

constructed using grey PVC. The maze was elevated to a height of 55 cm above the floor and consisted of two opposite open arms (30×7 cm^2) and two opposite enclosed arms (30×7 cm^2) enclosed by 17 cm high opaque walls. Arms were connected by a central platform (7×7 cm^2). The observer was situated in a separate neighbouring room and each behavioural session was videotaped. The test started when the mouse was placed into a mobile cylinder on the central platform of the maze. The cylinder was removed 10 s later, allowing the mouse to freely explore the apparatus for an 8-min period. Behavioural parameters to evaluate anxiety were the number of open and closed arm entries, the time spent in different arms, and, as suggested by Pellow et al. (1985) and Lister (1987), the percentage of open arm entries (activity ratio) and the percentage of time spent in open arms (time ratio). The number of closed arm entries was also used as a measure of locomotor activity in the plus-maze (Rodgers and Johnson, 1995). A mouse was considered to have entered an arm when all four paws had crossed into the arm. The maze was cleaned with distilled water and dried after each mouse was tested.

2.4.2. 4-hole-board

The four-hole-board test allows distinct measures of the effects of pharmacological treatments on anxiety and locomotor activity (File and Wardill, 1975; Takeda et al., 1998). Pellow et al. (1985) and Lister (1987) submitted their animals to the hole-board test immediately before the elevated plus-maze test, arguing that exposure to a novel environment increased subsequent exploration in the plus-maze. However, in highly emotive mice of the DBA/2 strain, previous exposure to an open-field decreased exploration in the elevated plus-maze (Rodgers and Cole, 1993). Since BALB/c mice are considered as highly emotive animals (Belzung and Berton, 1997; Griebel et al., 1993), we chose to expose them first to the elevated plus-maze with the aim of prioritizing behavioural measures in this test. The hole-board, located in a quiet and dimly illuminated room (15 lx), consisted of a grey Plexiglas box ($45 \times 45 \times 30$ cm^3), roof-less, which had four holes, 3 cm in diameter, equally spaced in the centre of each floor quadrant. Infrared photocells, directly beneath each hole, provided automatic measures of the number of head-dips and time spent head-dipping. Behavioural sessions were observed from a separate room and videotaped. Mice were placed immediately after the plus-maze test into a mobile cylinder at the centre of the board for 10 s. Following withdrawal of the cylinder, they were allowed to explore the hole-board for 6 min. Three parameters were measured: 1) head-dipping frequency and 2) head-dipping duration (anxiety parameters); and 3) number of crossings between the four quadrants (locomotion parameter).

2.5. Immunohistochemistry and histology

All injection sites were verified histologically on either Cresyl violet- or Fos-stained sections, by using the track of the guide and the injection cannula. Fos immunocytochemistry was performed for all the animals submitted to behavioural testing except for the group of mice injected bilaterally into the LS with the 50 ng dose of morphine. Animals were deeply anaesthetised (Avertin, 400 mg/kg, i.p.) 90 min after the beginning of the elevated plus-maze test 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 (ABC) peroxidase method (Elite Vectastain Kit, Vector Laboratories, Burlingame, USA) as described by Bontempi and Sharp (1997). The peroxidase was detected with diaminobenzidine (Sigma, Lyon, France) as chromogen. The primary antibody was a rabbit polyclonal antibody (Ab-5, 1:20 000, Oncogene Science, Cambridge, USA) raised to a synthetic peptide derived from amino acid sequences 4–17 of the Fos protein. The secondary antibody

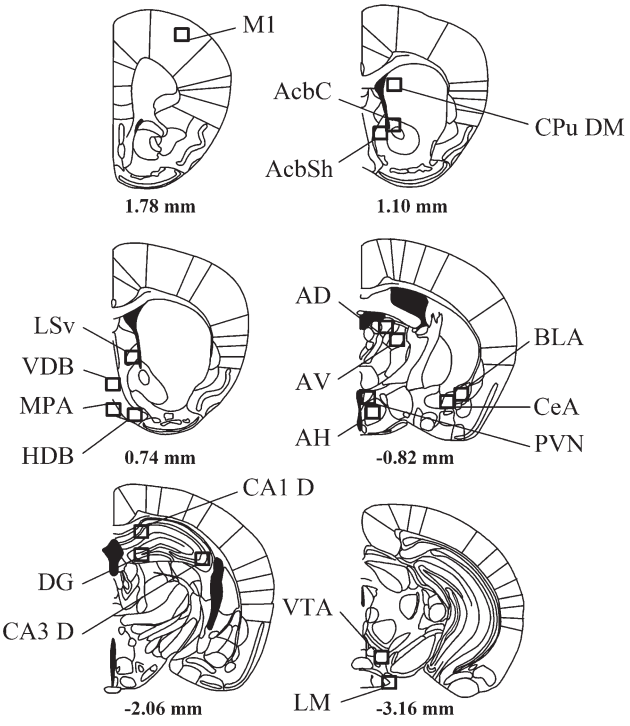

Fig. 1. Schematic diagrams of coronal sections adapted from the mouse brain atlas (Paxinos and Franklin, 2001) indicating the approximate antero-posterior levels (to Bregma) where regions of interest were sampled. AcbC: nucleus accumbens, core; AcbSh: nucleus accumbens, shell; AD: thalamus, antero-dorsal nucleus; AH: hypothalamus, anterior nucleus; AV: thalamus, antero-ventral nucleus; BLA: amygdala, basolateral nucleus; CA1 D: hippocampus, CA1, dorsal part; CA3 D: hippocampus, CA3, dorsal part; CeA: amygdala, central nucleus; CPu MD: caudate putamen, medio-dorsal part; DG: hippocampus, dentate gyrus; HDB: diagonal band of Broca, horizontal limb; LM: mammillary bodies, lateral nucleus; LSv: lateral septum, ventral part; M1: primary motoric cortex; MPA: hypothalamus, medial preoptic area; PVN: hypothalamus, paraventricular nucleus, parvocellular subdivision; VDB: diagonal band of Broca, vertical limb; VTA: ventral tegmental area.

was a biotinylated goat anti-rabbit IgG (1:2000, Jackson Immunoresearch, West Grove, USA).

2.6. 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. Fos-positive nuclei were counted using computer-assisted (Biocom Visiol@b 2000) software, in a 0.14 mm^2 window (×20 magnification). Data were expressed as the number of Fos-positive nuclei/mm^2 for all regions studied. Fos immunostaining was evaluated in 19 cerebral regions, belonging to 7 anatomical groups (septal region, hippocampus, hypothalamus, thalamus, dopaminergic pathways, amygdala and cortices) defined according to the mouse atlas of (Paxinos and Franklin, 2001). The approximate anatomical levels of each region of interest are shown in Fig. 1.

Quantification of Fos expression within the dorsal part of the LS (LSd) or in the MS nucleus was not possible due to the presence of an inflammatory response around the injection site. Counting of Fos-positive nuclei was therefore limited to the LSv, VDB and HDB (see Fig. 1 a for complete list of abbreviations). Fos expression was also examined in the dorsal part of the hippocampus (CA1 D, CA3 D, and DG D), which represent the major afference of the LS (Jakab and Leranth, 1995). In the hypothalamus, Fos staining was quantified within the anterior area (MPA and AH nuclei), which constitutes direct anatomical target of the LSd (Chiba and Murata, 1985; Jakab and Leranth, 1995; Risold and Swanson, 1997b). In addition, Fos expression was evaluated within the PVN nucleus, as the origin of the HPA axis, and the mammillary bodies (LM nucleus), as well as in the anterior thalamus (AD and AV), to which the mammillary bodies massively project via the mammillothalamic tract (Shibata, 1992). The number of Fos-positive nuclei was evaluated in the VTA and its limbic brain targets, the nucleus accumbens (mesolimbic pathway: AcbSh and AcbC) and caudate–putamen (mesostriatal pathway: CPu, mediodorsal part; see Oades and Halliday, 1987). Finally, Fos-positive nuclei were counted within the two main divisions of the amygdala, namely the central and basal nuclei (CeA and BLA) and in the primary motor cortex (M1).

2.7. Statistical analysis

Behavioural data expressed as ratios (activity and time ratios) were first submitted to an angular transformation (ArcSin√) to ensure a normalized distribution (Zar, 1999). Similarly, head-dipping duration was submitted to log(x) transformation (Zar, 1999). Behavioural parameters and Fos immunostaining were compared across groups using an analysis of variance (ANOVA), followed by post hoc comparisons using the Newman–Keuls test (Statistica 5.5 software). For all comparisons, values of $p<0.05$ were considered significant.

3. Results

3.1. Histology

Cannula placements within the septum were verified histologically on Cresyl violet- or Fos-stained sections. Animals with incorrect cannula placement were excluded from the analysis. Representative injection sites are shown in Fig. 2.

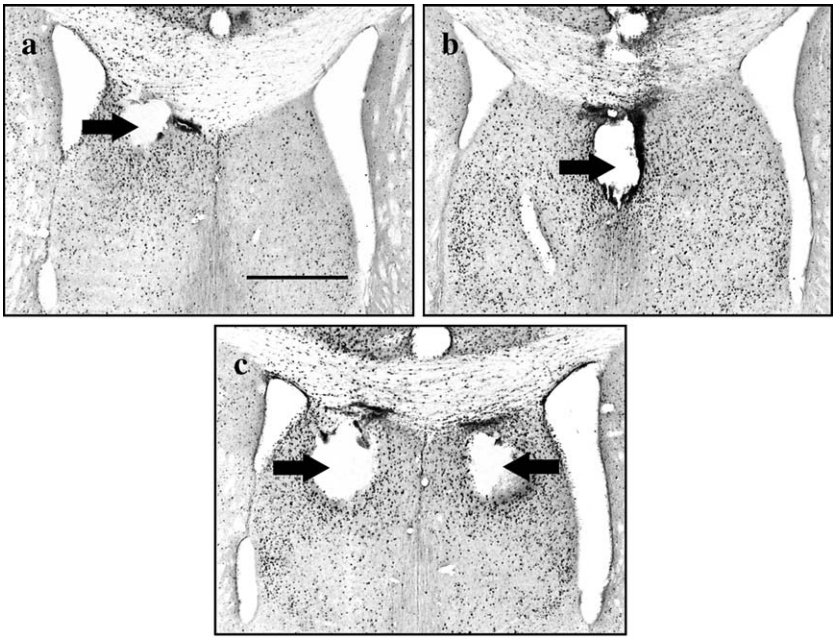

Fig. 2. Photomicrographs of Fos immunostained coronal brain sections showing locations of the cannula track and tip of the injection cannula (black arrows) within (a) the LS (unilateral injection), (b) the MS (unilateral injection), and (c) the LS (bilateral injection). Scale bar: 500 μm.

3.2. Behavioural study

3.2.1. Effects of unilateral morphine injection into the LS

3.2.1.1. Elevated plus-maze task. As illustrated in Fig. 3, unilateral morphine injections into the LS at doses of 100 and 500 ng globally reduced the number of arm entries in the plus-maze. A repeated measure of ANOVA showed a main effect of treatment on the number of arm entries ($F_{2,26}=9.93$, $p<0.001$), but also a main effect of arm (open vs. closed: $F_{1,26}=26.61$, $p<0.0001$), all animals visiting more closed arms than the open arms. Consequent separate analysis revealed that, whereas morphine reduced the number of entries in the open arms (one-way ANOVA: $F_{2,26}=26.24$, $p<0.0001$), it failed to decrease significantly the number of entries in the closed arms ($F_{2,26}=3.01$, $p=0.07$). In accordance with this result, the activity ratio was decreased by intra-LS morphine when compared to vehicle injection ($F_{2,26}=6.17$, $p<0.01$). Morphine treatment did not globally affect the time animals spent in the arms of the plus-maze. A repeated measure of ANOVA revealed no main effect of treatment on this parameter ($F_{2,26}=0.30$, NS), but a significant effect of arm ($F_{1,26}=46.67$, $p<0.0001$), all animals spending more time in the closed arms. When analysed separately, it appeared that intra-LS morphine at 100 and 500 ng decreased time spent in the open arms ($F_{2,26}=7.31$, $p<0.01$) but not in the closed arms ($F_{2,26}=0.35$, NS). However, intra-LS morphine failed to significantly reduce the time ratio ($F_{2,26}=1.64$, NS). Animals receiving either 100 ng or 500 ng morphine within the left hemisphere did not show a difference in behavioural measures in the plus-maze as compared to those implanted in the right hemisphere (data not shown).

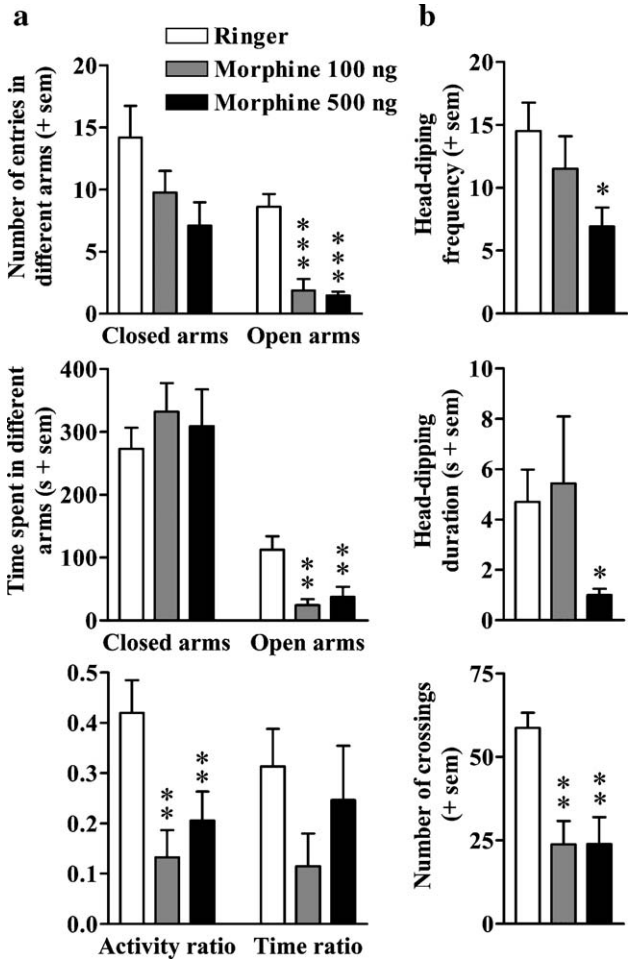

Fig. 3. Effects of unilateral intra-LS injections of morphine (100 or 500 ng) or vehicle (Ringer) on anxiety as measured in (a) the elevated plus maze (8 min total exploration session) and (b) the hole-board (6 min total exploration session) tasks. Decreased number of entries in open arms and of time spent in these arms, as well as decreased activity ratio, indicate an anxiogenic response. Data represent means ± S.E.M. ($n=8$–11 per group). *$p<0.05$, **$p<0.01$ vs. vehicle.

3.2.2. Hole-board task

A one-way ANOVA showed a significant effect of the dose of morphine on head-dipping frequency ($F_{2,26}=3.62$, $p<0.05$) and head-dipping duration ($F_{2,26}=5.00$, $p<0.05$). Post hoc analyses revealed that the highest dose of morphine decreased both measures of exploration in the hole-board when compared to vehicle-treated animals ($p<0.05$). Both doses of morphine decreased locomotor activity as revealed by a decrease in the number of crossings ($F_{2,26}=8.66$, $p<0.01$) (see Fig. 3).

3.3. Effects of unilateral pre-injection of naloxonazine into the LS

3.3.1. Elevated plus-maze task

As shown in Fig. 4, intra-LS vehicle or naloxonazine pre-injection had no significant effect on the number of arm entries. A repeated measure of ANOVA revealed no main effect of pharmacological treatment ($F_{2,24}=0.13$, NS) or arm ($F_{1,24}=1.86$, NS) on this parameter, and the treatment × arm interaction failed to reach significance ($F_{2,24}=2.90$, NS). However, the morphine-induced decrease in the activity ratio following infusion into the LS (100 ng dose) was reversed by intra-LS pre-injection of 100 ng of naloxonazine administered 25 min before morphine. A one-way ANOVA revealed a significant effect of drug pre-treatment on activity ratio ($F_{2,24}=4.21$, $p<0.05$), with post hoc analysis confirming that this ratio was decreased in animals pre-treated with saline, but not with naloxonazine, before intraseptal morphine when compared to double-blank controls ($p<0.05$). Although a repeated measure of ANOVA showed no main effect of treatment ($F_{2,24}=0.43$, NS) or arm ($F_{1,24}=0.24$, NS) on the time spent in the arms of the plus-maze, the treatment × arm interaction was significant ($F_{2,24}=6.14$, $p<0.01$). Indeed, when using separated analysis, pharmacological treatment appeared to have a significant effect on the number of closed arm ($F_{2,24}=4.09$, $p<0.05$) and open arm entries ($F_{2,24}=4.41$, $p<0.05$). Post hoc analyses showed that morphine, when preceded by saline injection, increased the time spent in closed arms ($p<0.05$) whilst reduced the time spent in open arms ($p<0.05$), an effect that was completely reversed by naloxonazine pre-treatment. Moreover, pharmacological treatment had a significant effect on time ratio ($F_{2,24}=5.35$, $p<0.05$), with post hoc analysis revealing that time ratio was

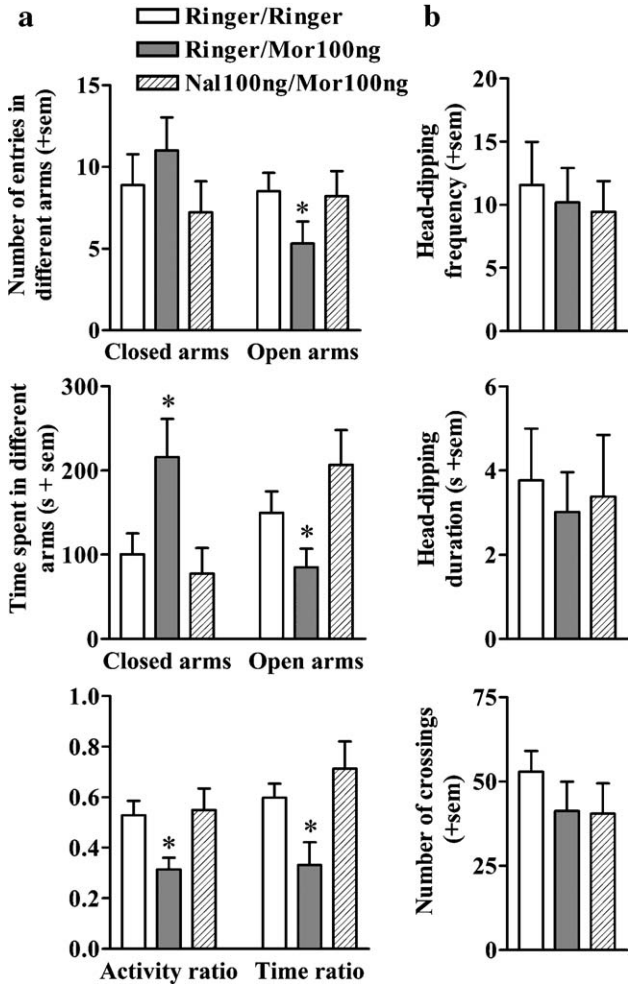

Fig. 4. Effects of pre-treatment with naloxonazine on the morphine-induced increase in anxiety as measured (a) in the elevated plus maze and (b) in the hole-board. All mice were injected unilaterally into the lateral septum and received either vehicle (Ringer) or naloxonazine (100 ng) followed 25 min later by vehicle or morphine (100 ng). Data represent means±S.E.M. (n=8–11 per group). *p<0.05 vs. vehicle.

decreased in animals pre-treated with saline, but not with naloxonazine, before intra-LS morphine when compared to double-blank controls (p<0.05).

3.3.2. Hole-board task

Pre-injection of naloxonazine had no effect on either head-dipping frequency ($F_{2,24}$=0.88, NS), head-dipping duration ($F_{2,24}$=0.23, NS) or transitions between quadrants ($F_{2,24}$=0.77, NS) when compared with Ringer pre-injection (see Fig. 4), consistent with the observation that unilateral morphine injection into the LS at the dose of 100 ng failed to modify head-dipping frequency and duration in the hole-board task.

3.4. Effects of morphine injection into the MS

3.4.1. Elevated plus-maze task

As shown in Fig. 5, morphine injections into the MS modified locomotor activity in the plus-maze. A repeated measure of ANOVA showed a main effect of treatment ($F_{2,25}$=6.11, p<0.01) and arm ($F_{1,25}$=13.75, p<0.01) on the global number of entries in the arms of the plus-maze. Post hoc analyses revealed that only morphine at the dose of 100 ng increased this number (p<0.05), open arms being less visited than closed arms in all groups. Intra-MS morphine had no effect on activity ratio ($F_{2,25}$=0.21, NS). Likewise, it failed to modify the time spent in the arms of the plus-maze (effect of treatment: $F_{2,25}$=0.84, NS), all animals spending more time in the closed arms than in the open arms (effect of arm: $F_{2,25}$=14, 46, p<0.0001). Accordingly, intra-MS morphine had no effect on time ratio ($F_{2,25}$=0.03, NS).

3.4.2. Hole-board task

Morphine injection into the MS at doses of either 100 ng or 500 ng had contrasting effects on parameters of exploration in

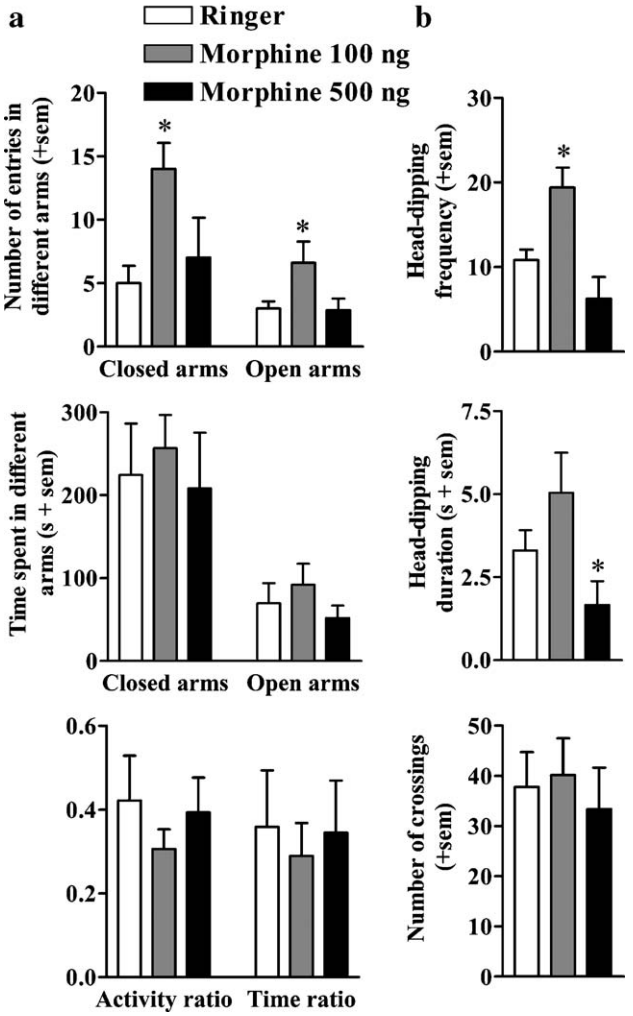

Fig. 5. Effects of morphine injections into the MS on anxiety as measured (a) in the elevated plus maze and (b) hole-board tasks. Morphine was injected at doses of 100 or 500 ng. Ringer was used as vehicle. No signs of increased anxiety were detected. Data are presented as means±S.E.M. (n=8–10 per group). *p<0.05 vs. vehicle.

the hole-board. One-way analysis showed a significant effect of the dose of morphine on head-dipping frequency ($F_{2,25}=10.12$, $p<0.001$) and head-dipping duration ($F_{2,25}=5.07$, $p<0.05$). Post hoc analyses revealed an increase in head-dipping frequency after morphine injection at 100 ng as compared to vehicle injections ($p<0.01$). In contrast, morphine injected at the dose of 500 ng reduced head-dipping duration as compared to vehicle injection (post hoc: $p<0.05$). Intra-MS morphine had no effect on locomotor activity in the hole-board ($F_{2,25}=0.20$, NS) (see Fig. 5).

3.5. Effects of bilateral injections of morphine into the LS

3.5.1. Elevated plus-maze task

As shown in Fig. 6, bilateral injections of morphine (20 to 500 ng) into the LS tended to increase activity in the plus-maze. A repeated measure of ANOVA revealed a significant main effect of treatment ($F_{4,48}=2.57$, $p=0.05$) and of arm ($F_{1,48}=17.06$, $p<0.0001$) on the number of arm entries in this apparatus. However, when analysed separately, the number of closed arm entries (as a measure of locomotor activity) failed to be affected by morphine treatment ($F_{4,48}=1.49$, NS). Bilateral morphine into the LS failed to modify the activity ratio ($F_{4,48}=1.39$, NS). Neither the pharmacological treatment ($F_{4,48}=0.19$, NS), nor the type of arm ($F_{1,48}=3.37$, NS) had effect on the time spent in the arms of the maze. The time ratio was not affected by morphine bilateral injections.

3.5.2. Hole-board task

In the hole-board test, morphine injected bilaterally into the LS tended to decrease both head-dipping frequency and

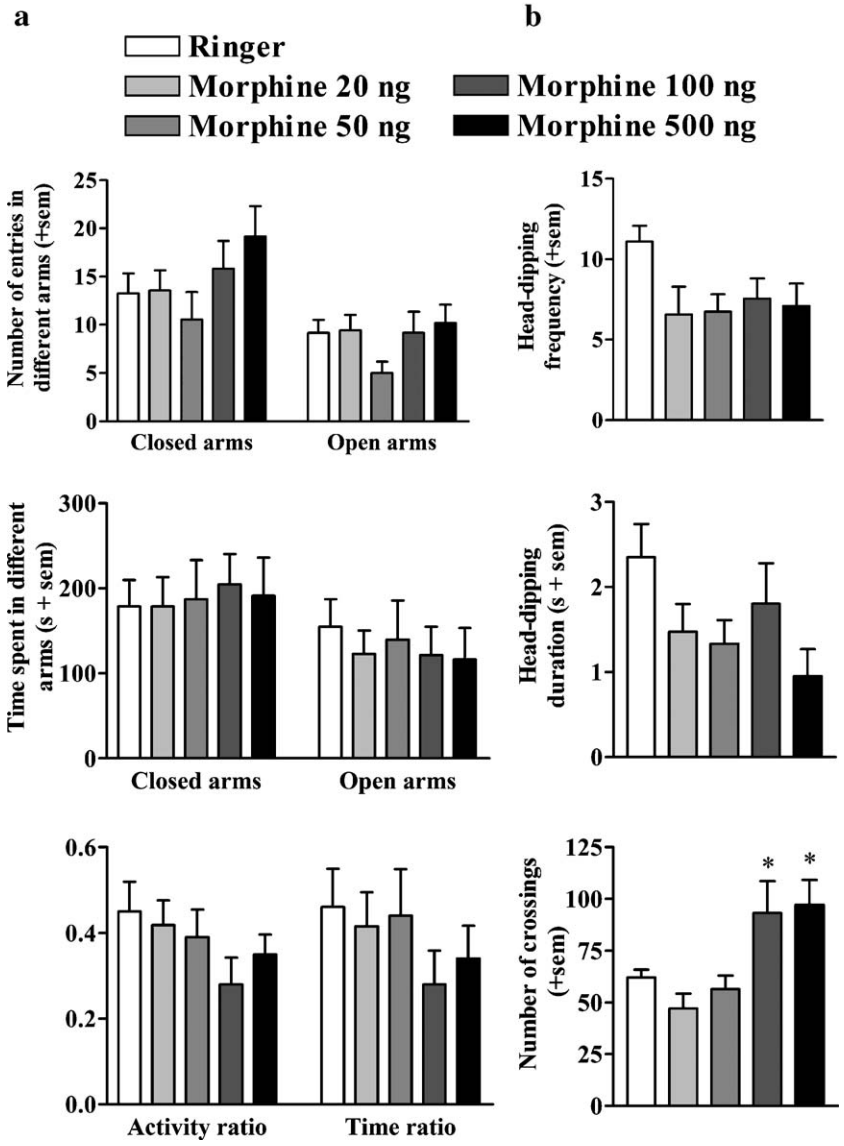

Fig. 6. Effects of bilateral injections of morphine into the LS on anxiety as measured (a) in the elevated plus maze and (b) the hole-board tasks. Morphine was injected at doses from 20 ng to 500 ng. Ringer was used as vehicle. At high doses, morphine increased locomotor activity as measured by crossings in the hole-board. Data are presented as means±S.E.M. ($n=9$–11 per group). *$p<0.05$ vs. vehicle.

head-dipping duration, although these effects failed to reach significance ($F_{4,48}=2.16$, $p=0.09$; $F_{4,48}=2.17$, $p=0.09$, respectively). On the other hand, bilateral morphine increased locomotor activity. A one-way ANOVA shows a significant effect of the dose of morphine on the number of crossings recorded in the hole-board ($F_{4,48}=4.81$, $p<0.01$). Post hoc analyses confirmed that both highest doses of morphine induced an increase in the number of crossings (100 ng vs. control: $p<0.05$, 500 ng vs. control: $p<0.01$) (see Fig. 6).

Table 1
Effects of unilateral morphine injection (100 and 500 ng) or pre-injection of the μ-opioid receptor antagonist naloxonazine (100 ng) into the dorsal part of the lateral septum on Fos expression

Structures	Vehicle	Morphine 100 ng	Morphine 500 ng	Vehicle/Vehicle	Vehicle/Morphine	Naloxonazine/Morphine
Septum						
LSv ipsi	2767.7±151.7	2287.6±137.6*	2411.0±192.3*	2920.2±182.9	2277.8±120.3††	2861.5±157.7°°
LSv contra	2654.9±148.6	2368.4±151.6*	2195.2±123.5*	2754.4±95.4	2378.7±145.4††	2566.3±128.4°°
HDB ipsi	1028.3±184.9	1018.8±115.3	471.7±77.6***	839.8±118.6	804.4±159.5	698.9±112.0
HDB contra	990.2±172.6	1022.0±96.6	447.4±69.0***	794.4±87.4	873.2±159.9	714.6±121.8
VDB	932.7±121.2	682.4±74.0	410.4±82.7**	782.0±102.5	576.7±120.3	581.0±121.3
Hippocampus						
CA1 D ipsi	3667.1±177.2	3382.8±139.7	2916.8±111.9***	3137.6±195.1	3030.8±281.6	3393.6±188.1
CA1 D contra	3783.7±194.1	3453.5±185.5	2987.4±113.4***	3163.9±200.6	3094.5±300.1	3538.9±179.5
CA3 D ipsi	1336.4±93.1	1120.3±128.7*	1007.8±42.8***	1134.5±59.1	1148.2±129.7	1306.6±163.6
CA3 D contra	1431.4±107.6	1119.2±148.2*	1016.9±60.7***	1106.9±83.8	1207.2±139.5	1347.2±137.1
DG D ipsi	752.2±97.5	574.4±68.8*	350.1±53.2***	392.5±48.9	491.5±57.2	504.5±29.9
DG D contra	732.6±107.2	577.2±65.9*	396.7±43.2***	449.8±59.6	526.4±72.8	572.9±31.0
Hypothalamus						
MPA ipsi	1551.6±130.3	1670.0±77.0	1084.1±59.6***	1360.5±138.1	1383.7±107.4	1422.5±145.7
MPA contra	1512.1±133.0	1594.2±101.9	1025.2±90.1***	1362.5±84.1	1481.7±138.9	1521.3±123.0
AH ipsi	1354.9±101.0	1331.1±84.4	952.0±66.2**	1343.0±36.5	955.3±135.2††	1174.5±126.6°
AH contra	1275.2±106.0	1259.5±84.1	1006.3±78.7**	1344±51.2	906.5±133.8††	1257.9±142.6°
PVN ipsi	3056.1±234.7	2475.1±385.4	2430.9±306.9	3061.4±159.8	2527.8±329.3	2221.8±341.72†
PVN contra	2973.4±278.0	2696.7±345.4	2449.0±289.5	3099.0±227.8	2467.0±385.6	2320.8±316.2†
LM ipsi	625.6±145.9	1436.9±366.0**	1359.3±226.0**	319.2±76.4	1283.1±272.8†††	697.4±162.7°
LM contra	580.0±124.3	1393.3±280.7**	1321.3±248.6**	363.2±63.2	1095.9±199.9†††	682.7±187.3°
Anterodorsal thalamus						
AD ipsi	279.9±57.5	508.7±84.4**	651.5±65.0***	238.0±42.4	453.6±166.4†	223.6±60.4°
AD contra	276.0±45.2	541.1±50.6**	602.7±92.7***	161.8±20.7	381.6±94.4†	154.9±46.6°
AV ipsi	2081.2±176.1	1931.6±166.8	2040.3±95.1	996.4±126.5	1481.8±257.3†	1207.5±175.4
AV contra	1962.0±198.2	2001.5±176.6	1990.3±103.6	1059.5±123.8	1610.6±276.7†	1110.6±168.4
Dopaminergic pathways						
AcbC ipsi	1722.0±181.6	1281.0±188.4	1022.5±123.8*	1416.0±105.0	1045.5±134.1	1076.3±65.3
AcbC contra	1561.1±177.5	1973.4±200.8	1453.7±136.6*	1409.2±99.4	1627.6±190.6	1389.4±95.0
AcbSh ipsi	1798.1±140.8	2356.8±100.9***	2701.7±139.3***	1609.0±107.7	1891.7±176.5†	1855.4±158.3
AcbSh contra	1856.2±150.7	2481.3±140.2***	2836.9±145.6***	1556.0±81.5	2104.1±204.4†	1783.3±171.2
CPu ipsi	1266.5±142.6	1214.1±169.7	1503.4±112.7	924.5±96.1	1055.7±187.8	766.4±116.8
CPu contra	1335.8±179.0	1435.0±195.8	1473.4±112.5	1172.5±69.3	1027.6±159.4	726.0±71.8
VTA ipsi	923.8±105.5	1369.3±112.2***	1344.4±94.6***	650.8±31.3	1155.5±108.8†††	911.2±100.64†
VTA contra	876.7±96.9	1253.6±90.7***	1315.6±127.4***	620.0±54.8	1103.1±96.8†††	843.6±96.4†
Amygdala						
BLA ipsi	1228.2±194.2	1095.2±86.4	1542.8.5±177.0	1655.5±292.2	1439.7±242.0	1387.8±206.1
BLA contra	1053.5±71.9	1218.9±81.0	1076.8±62.2	1248.2±212.9	1310.2±211.6	1020.0±93.8
CeA ipsi	887.8±247.8	830.7±136.2	1526.1±267.7	1820.7±368.7	1220.5±342.9†	941.4±259.6††
CeA contra	706.6±102.8	1030.4±137.4	890.0±65.2	1728.4±449.1	971.4±244.4†	733.0±67.0††
Motor cortex						
M1 ipsi	1895.7.7±252.7	2003.3±245.9	1858.8±241.8	1590.9±366.5	1908.5±417.9	1777.6±321.2
M1 contra	1526.7±287.0	1720.3±242.8	1348.0±141.0	1210.8±352.5	1821.7±427.8	1363.1±236.4

Results are expressed as mean number of Fos-positive nuclei per mm^2±S.E.M. counted in each hemisphere (ipsilateral or contralateral). Effects of the dose of morphine or pharmacological treatment: */†$p<0.05$, **/††$p<0.01$, ***/†††$p<0.001$ as compared to control animals (vehicle* or double-blank†), °$p<0.05$, °°$p<0.01$ as compared to vehicle/morphine-injected animals; symbols are quoted for each hemisphere for clarity. See text for the effects of the side of injection. See Fig. 1 for complete list of abbreviations.

4. Fos imaging study

4.1. Effects of unilateral injection of morphine into the LS on Fos expression

The following data were analysed for each studied structure (except VDB) using a two-way ANOVA with dose of morphine (vehicle, 100 ng or 500 ng) and side of the injection (contra- vs. ipsilateral) as main factors, followed by post hoc analysis. However, F values were only reported below when a significant effect of one of these factors was detected. VDB was considered as an odd structure, and Fos immunoreactivity was quantified in a median window. Data from this region were analysed using a one-way ANOVA with dose of morphine as main factor.

4.1.1. Septum and efferents

As shown in Table 1, unilateral injection of morphine into the dorsal part of the LS was associated with a significant modification of Fos expression in the LSv ($F_{2,52}=4.45$, $p<0.05$, see also Fig. 7). Post hoc analyses detected that Fos expression was lower in animals injected with morphine than in vehicle-treated animals ($p<0.05$). At the highest dose only, intra-LS morphine was associated with a decreased number of Fos-positive nuclei in the HDB ($F_{2,52}=13.31$, $p<0.0001$; post hoc: $p<0.001$) and in the VDB ($F_{2,26}=7.81$, $p<0.01$; post hoc: $p<0.01$), as well as in the main anatomical target of the LSd region, the anterior hypothalamic region, namely the AH ($F_{2,52}=9.79$, $p<0.001$; post hoc: $p<0.01$) and MPA ($F_{2,52}=18.61$, $p<0.0001$; post hoc: $p<0.001$).

In the hippocampus, Fos expression following unilateral administration of morphine into the LS at both doses of 100 ng and 500 ng was significantly lower in the CA3 D ($F_{2,52}=8.52$, $p<0.001$; post hoc: $p<0.01$) and DG D ($F_{2,52}=12.79$, $p<0.0001$; post hoc: 100 ng vs. vehicle $p<0.05$, 500 ng vs. vehicle $p<0.001$) than after vehicle injection. In the CA1 D, only the highest dose of morphine was associated with a significant reduction of Fos expression ($F_{2,52}=13.78$, $p<0.0001$; post hoc: $p<0.001$).

4.1.2. Mammillothalamic tract

Morphine injection into the LSd (doses of 100 and 500 ng) resulted in higher Fos staining than vehicle injection into the lateral nucleus of the mammillary bodies (LM: $F_{2,51}=7.62$, $p<0.01$; post hoc: $p<0.01$) and the dorsal part of the anterior thalamus (AD: $F_{2,52}=14.14$, $p<0.0001$; post hoc: 100 ng vs. vehicle $p<0.01$, 500 ng vs. vehicle $p<0.001$).

4.1.3. Dopaminergic structures and projection areas

Animals receiving a unilateral injection of morphine into the LSd displayed higher Fos expression throughout the mesolimbic dopaminergic pathway. Morphine infusion at either 100 ng or 500 ng resulted in higher Fos expression than vehicle in the VTA ($F_{2,52}=10.50$, $p=0.0001$; post hoc: $p<0.001$) and its anatomical target, the shell of the nucleus accumbens (AcbSh: $F_{2,52}=24.55$, $p<0.0001$; post hoc: $p<0.001$). In contrast, Fos expression in the core of the nucleus accumbens (AcbC) was decreased after morphine infusion at the highest dose ($F_{2,52}=4.06$, $p<0.05$; post hoc: $p<0.05$). Moreover, the number of Fos-positive nuclei in this region was found to differ depending on the hemisphere as regard to guide-cannula implantation (ipsilateral vs. contralateral: $F_{1,52}=5.16$, $p<0.05$), with a significant dose × hemisphere interaction ($F_{2,52}=3.34$, $p<0.05$). Indeed, morphine-treated animals displayed significantly lower Fos expression in the ipsilateral than in the contralateral AcbC ($F_{1,36}=10.49$, $p<0,01$), whereas control animals did not ($F_{1,18}=0.4$, NS).

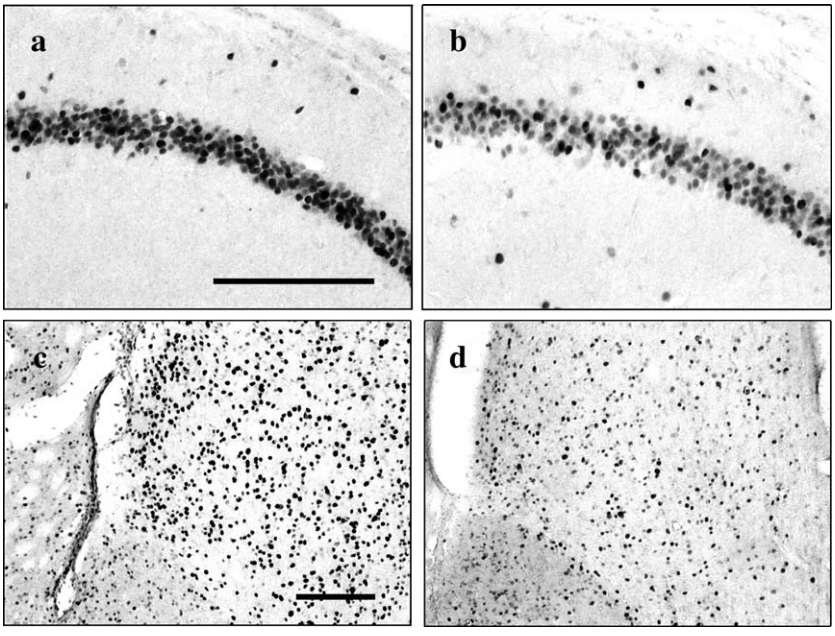

Fig. 7. Photomicrographs of Fos immunoreactivity in coronal sections taken through the dorsal hippocampus (CA1 field, a, b) and the ventral part of the lateral septum (c, d) following either vehicle (Ringer, a, c) or morphine (500 ng, b, d) infusions into the dorsal part of the lateral septum. Note that morphine injections resulted in lower level of Fos expression than vehicle. Scale bar: 100 μm.

4.1.4. Motor cortex

Levels of Fos expression in the primary motor cortex, although this was not dependant on the dose of morphine, were found to be higher in the hemisphere in which the guide-cannula was implanted than in the contralateral hemisphere ($F_{1,52}=4.84$, $p<0.05$).

4.2. Effects of unilateral pre-injections of naloxonazine into the LS on morphine-induced Fos expression

The following data were systematically analysed for each studied structure (except VDB) using a two-way ANOVA with pharmacological treatment (double vehicle, vehicle/morphine or naloxonazine/morphine) and side of the injection (contra- vs. ipsilateral) as main factors, followed by post hoc analysis. However, *F* values were only reported below when a significant effect of one of these factors was detected.

4.2.1. Septum and efferents

As shown in Table 1, Fos expression was lower in the LSv ($F_{2,48}=7.31$, $p<0.01$; post hoc: $p<0.01$) and AH nucleus ($F_{2,48}=6.44$, $p<0.01$; post hoc: $p<0.05$) after morphine intra-LS injection preceded by vehicle pre-treatment than after double vehicle infusion, whereas pre-treatment with naloxonazine restored Fos expression to a level similar to that observed after control injections (post hoc: NS). Morphine injection failed to significantly modify Fos expression in the hippocampus.

4.2.2. Mammillothalamic tract

Naloxonazine pre-treatment antagonized the morphine-induced increased of Fos expression observed in the LM ($F_{2,48}=10.33$, $p<0.001$; post hoc: vehicle/morphine vs. double vehicle, $p<0.05$), the AD ($F_{2,48}=3.93$, $p<0.05$; post hoc: $p<0.05$) and the AV ($F_{2,48}=3.41$, $p<0.05$; post hoc $p<0.05$).

4.2.3. Dopaminergic structures and projection areas

Naloxonazine pre-treatment failed to reverse completely the increase of Fos staining induced by intra-LS morphine injection in the VTA. A two-way ANOVA showed a significant effect of pharmacological treatment ($F_{2,48}=14.82$, $p<0.0001$). Post hoc analyses revealed that Fos expression in the VTA was higher in mice treated with vehicle followed by morphine ($p<0.01$) or naloxonazine followed by morphine ($p<0.05$) than in the double-blank animals; however, the number of Fos-positive nuclei in the VTA was higher in vehicle/morphine animals than in naloxonazine/morphine animals ($p<0.001$). In contrast, naloxonazine reversed the morphine-induced increase in Fos expression in the AcbSh ($F_{2,48}=3.20$, $p<0.05$; post hoc: vehicle/morphine vs. double vehicle, $p<0.05$). Fos immunoreactivity in the AcbC appeared to be different depending on the hemisphere ($F_{1,48}=8.03$, $p<0.01$); the dose × hemisphere interaction failed to reach significance ($F_{2,48}=2.63$, NS). However, while control animals displayed no difference between hemispheres ($F_{1,14}=0.002$, NS), morphine-treated animals showed lower Fos expression in the ipsilateral than in the contralateral AcbC ($F_{1,36}=11.55$, $p<0.01$).

4.2.4. PVN and amygdala

Naloxonazine pre-treatment was associated with a decrease in Fos expression in the paraventricular nucleus of the hypothalamus (PVN: $F_{2,48}=3.25$, $p<0.05$; post hoc: $p<0.05$) as compared to control animals (double vehicle infusion). Animals injected with morphine or pretreated with naloxonazine exhibited lower Fos expression in the central nucleus of the amygdala as compared to double-blank animals ($F_{2,48}=4.71$, $p<0.05$; post hoc: $p<0.05$).

4.3. Effects of morphine injection into the MS on Fos expression

Only the results for the structures in which a significant effect of morphine injection was detected are reported below.

4.3.1. Septum and efferent

As shown in Table 2, intra-MS injection of morphine at the dose of 100 ng induced higher Fos expression in the HDB relative to vehicle-injected animals ($F_{2,25}=7.96$, $p<0.001$; post hoc: $p<0.05$). In contrast, the number of Fos-positive nuclei

Table 2
Effects of morphine injection (100 and 500 ng) into the medial septum on Fos expression measured in various brain regions

Structures	Ringer	Morphine 100 ng	Morphine 500 ng
Septum			
LSv	2570.4±107.3	2483.2±126.2	2238.2±166.3
HDB	646.8±123.1	1087.2±119.0*	464.8±77.4
VDB	658.1±102.2	583.8±56.1	277.8±46.8**
Hippocampus			
CA1 D	3079.3±134.3	3623.0±164.6*	2443.5±198.6*
CA3 D	1133.5±60.7	1366.5±112.6	1021.8±115.1
DG D	464.7±35.5	626.5±88.7	364.3±43.5
Hypothalamus			
MPA	1318.3±113.7	1724.7±142.2*	1135.8±101.7
AH	1197.9±55.3	1448.1±118.0	1022.8±58.5
PVN	2059.0±320.6	2545.0±270.3	2468.4±263.9
LM	266.8±66.8	967.7±196.5*	1207.6±235.5**
Thalamus			
AD	278.1±52.5	556.6±136.6	413.8±45.3
AV	1294.7±161.8	2023.8±220.3*	1674.7±224.1
Dopaminergic pathways			
AcbC	1461.3±183.3	1574.7±120.6	1834.5±93.5
AcbSh	1623.8±133.1	2339.6±164.0**	2625.3±224.4**
CPu	976.2±160.2	1362.2±172.3	1346.0±89.8
VTA	728.3±87.6	1313.8±112.4**	1138.1±125.1*
Amygdala			
BLA	1187.8±105.4	1267.9±99.0	1443.6±152.6
CeA	1264.8±259.3	772.0±144.1	1579.2±313.2
Motor cortex			
M1	1389.1±313.3	2224.0±143.3	1487.3±323.4

Results are expressed as mean number of Fos-positive nuclei per mm^2±S.E.M. counted in both hemispheres. Effects of the dose of morphine: *$p<0.05$, **$p<0.01$ vs. vehicle-injected animals. See Fig. 1 for complete list of abbreviations.

within the VDB was lower after intra-MS injection of 500 ng of morphine as compared to controls ($F_{2,25}=6.50$, $p<0.001$; post hoc: $p<0.01$). In the hippocampus, morphine at the dose of 100 ng resulted in higher Fos expression, whereas 500 ng morphine resulted in lower Fos expression, compared to vehicle in the CA1 D ($F_{2,25}=12.33$, $p<0.001$; post hoc: $p<0.05$). Finally, animals receiving 100 ng of morphine into the MS exhibited higher Fos expression within the MPA of the hypothalamus than controls ($F_{2,25}=5.88$, $p<0.01$; post hoc: $p<0.05$).

4.3.2. Mammillothalamic tract

As compared to vehicle-injected animals, animals treated with morphine exhibited an increased number of Fos-positive nuclei in the LM nucleus of the mammillary bodies ($F_{2,24}=7.21$, $p<0.01$; post hoc: 100 ng vs. vehicle $p<0.05$; 500 ng vs. vehicle $p<0.01$). At the dose of 100 ng, morphine injected into the MS induced higher Fos expression in the AV nucleus of the thalamus ($F_{2,25}=3.46$, $p<0.05$; post hoc: $p<0.05$).

4.3.3. Dopaminergic structures and projection areas

Intra-MS injections of morphine at both doses induced higher Fos expression than vehicle in the AcbSh ($F_{2,25}=9.01$, $p<0.01$; post hoc: $p<0.01$) and VTA ($F_{2,25}=8.28$, $p<0.01$; post hoc: 100 ng vs. vehicle $p<0.01$, 500 ng vs. vehicle $p<0.05$).

4.4. Effects of bilateral injections of morphine into the LS on Fos expression

Only the results for the structures in which a significant effect of morphine injection was detected were reported below.

4.4.1. Septum and efferents

As shown in Table 3, bilateral morphine injections into the dorsal part of the LS at the dose of 100 ng resulted in lower Fos expression in the LSv as compared to vehicle injections ($F_{3,30}=3.12$, $p<0.05$; post hoc: $p<0.05$). The highest dose of morphine (500 ng) was required to reduce Fos expression within the VDB ($F_{3,30}=5.15$, $p<0.01$; post hoc: $p<0.01$) and AH ($F_{3,30}=4.11$, $p<0.05$; post hoc: $p<0.05$).

4.4.2. Mammillothalamic tract

The two highest doses of morphine injected bilaterally into the LSd increased Fos expression into the LM when compared to vehicle ($F_{3,30}=4.93$, $p<0.01$; post hoc: $p<0.05$).

4.4.3. Dopaminergic pathways and projection area

Bilateral injections of 500 ng of morphine into the LS induced higher Fos expression than Ringer injections in the VTA ($F_{3,30}=5.97$, $p<0.01$; post hoc $p<0.01$) whereas the two highest doses of morphine were associated with significant increase in Fos expression within the AcbSh ($F_{3,30}=14.33$, $p<0.0001$; post hoc: 100 ng vs. vehicle $p<0.01$, 500 ng vs. vehicle $p<0.001$). Unlike unilateral injections, bilateral morphine injections at doses of 100 and 500 ng increased Fos staining in the CPu ($F_{3,30}=7.37$, $p<0.001$; post hoc: $p<0.01$) when compared to vehicle injection.

Table 3
Effects of bilateral morphine injections (20, 100 and 500 ng) into the dorsal part of the lateral septum on Fos expression measured in various brain regions

Structures	Ringer	Morphine 20 ng	Morphine 100 ng	Morphine 500 ng
Septum				
LSv	2960.2±177.2	2614.9±131.9	2428.9±64.4*	2493.4±125.7
HDB	838.0±124.6	855.0±84.8	1112.6±93.4	682.1±84.9
VDB	804.7±93.6	672.4±75.8	685.3±87.0	356.7±66.5**
Hippocampus				
CA1 D	3770.2±125.5	3332.4±266.3	3725.3±103.4	3173.6±115.1
CA3 D	1274.4±55.0	1110.0±85.1	1364.3±152.7	1128.0±56.7
DG D	595.4±55.5	565.4±64.9	650.3±72.7	475.0±40.6
Hypothalamus				
MPA	1499.1±127.9	1530.7±118.5	1619.7±108.3	1312.3±67.7
AH	1333.9±72.7	1187.9±76.8	1326.2±126.9	933.7±68.2*
PVN	2635.8±270.2	2891.7±200.7	2182.7±379.4	2227.1±269.6
LM	622.6±179.5	868.0±137.6	1634.6±161.4*	1514.3±357.6*
Thalamus				
AD	450.8±74.9	243.5±58.2	557.7±88.0	634.5±96.4
AV	1769.9±119.1	1445.1±98.2	2295.6±200.6	2101.7±214.4
Dopaminergic pathways				
AcbC	1098.7±126.3	1252.4±94.9	1404.8±100.0	929.4±73.2
AcbSh	1725.6±148.4	1722.0±36.0	2298.0±12.9**	2670.9±121.2***
CPu	940.4±73.5	998.4±102.1	1481.1±125.1**	1436.3±108.5**
VTA	827.3±112.4	1102.6±86.7	1216.1±131.7	1534.8±140.1**
Amygdala				
BLA	1697.3±207.6	1850.3±191.1	1568.8±149.6	2162.0±162.5
CeA	1717.3±364.8	1874.7±218.8	1385.7±161.4	2277.7±177.8
Motor cortex				
M1	2462.9±325.0	3245.0±364.3	2919.3±192.1	3730.0±219.4*

Results are expressed as mean numbers of Fos-positive nuclei per mm^2±S.E.M. counted in both hemispheres. Effects of the dose of morphine: *$p<0.05$, **$p<0.01$, ***$p<0.001$ as compared to vehicle-injected animals. See Fig. 1 for complete list of abbreviations.

4.4.4. Motor cortex

Bilateral injections of morphine into the LSd (500 ng) resulted in increased Fos staining in the M1 area as compared to Ringer injections ($F_{3,30}=3.27$, $p<0.05$; post hoc: $p<0.05$).

5. Discussion

The aim of the present study was to examine the role played by μ-opioid receptors in the lateral septal region in the modulation of anxiety. Activation of the LS opioid receptors was obtained by means of intracerebral morphine injections, which represents a stressful procedure for the animals. Despite this, general activity and anxiety ratios measured in the elevated plus-maze for animals receiving a single unilateral injection of Ringer were very similar to those published in the literature for the same strain of mice (Griebel et al., 2000). Animals receiving two successive injections of vehicle (control for naloxonazine

pre-treatment) displayed even higher anxiety ratios, indicating low levels of anxiety. This may be attributable to the double injection simulations and corresponding handling that these animals had to undergo daily for 4 days before the testing session, resulting in better habituation to the stress of injection. In these conditions, unilateral injections of morphine into the LSd resulted in increased anxiety-like behaviour as measured in the plus-maze. More precisely, unilateral injection of morphine into the dorsal part of the LS reduced locomotor activity in the elevated plus-maze, but whereas entries in open arms were massively decreased, entries in the closed arms were not significantly modified. Such a specific reduction of open arm entries, as illustrated by decreased activity ratio, is considered as a sign of increased anxiety (Lister, 1987; Pellow et al., 1985). Exploration (head-dipping) in the hole-board, another sign of increased anxiety, was also reduced (File and Wardill, 1975; Takeda et al., 1998). However, morphine concomitantly reduced locomotor activity during this task, suggesting that a sedative effect of intra-LS morphine cannot be completely ruled out. Indeed, systemic morphine at low doses reduces locomotor activity in mice (Patti et al., 2005). It should be pointed out, however, that in highly emotive strains of mice like BALB/c mice, reduced locomotion is also observed as a consequence of high levels of anxiety. Interestingly, in animals better habituated to the injection procedure, unilateral morphine (preceded by vehicle injection) was not only less anxiogenic in the plus-maze, but also did not reduce locomotor activity in the hole-board. This result supports the idea that the reduction of locomotor activity observed in the hole-board after a single unilateral injection of morphine resulted mainly from increased anxiety-like behaviour.

Care was taken in the present study to use a very small injection volume (i.e. 0.1 μl) to limit diffusion. Nevertheless, anatomical specificity was further assessed by injecting morphine in the adjacent septal subdivision, MS, also known for its involvement in anxiety modulation (Degroot and Treit, 2004; McNaughton and Gray, 2000). Animals receiving morphine into the MS did not display anxiety-like behaviours, suggesting that the anxiogenic-like effects of morphine injected into the LSd were specific to that septal subdivision. Moreover, histology revealed that for two animals injected with morphine at 100 ng and one animal injected with morphine at 500 ng, which failed to show morphine-induced increased anxiety-like behaviour, the injection site was located in the lateral ventricle. It seems thus unlikely that behavioural effects of intra-LS morphine were a consequence of diffusion towards the lateral ventricles. Likewise, possible lateralization of the LS in the modulation of anxiety seems very unlikely since unilateral morphine injections were found in our study to be equally anxiogenic, whichever the targeted (left or right) hemisphere. Finally, the anxiogenic-like effects of unilateral intra-LS morphine as well as the concomitant reductions of Fos expression in the ventral LS and anterior hypothalamus, and increases in the mammillary bodies, anterodorsal thalamus and shell of the nucleus accumbens, were completely reversed by pre-treatment with naloxonazine, a potent and selective μ-opioid receptor antagonist (Cruciani et al., 1987; Paul and Pasternak, 1988) suggesting that they were predominantly mediated by μ-opioid receptors.

Surprisingly, bilateral infusions of morphine into the lateral septum failed to reproduce the anxiogenic-like effects of unilateral injections in the plus-maze and hole-board tasks. Although morphine tended to reduce head-dipping frequency in the hole-board, this effect failed to reach significance. But in parallel, bilateral morphine at high doses increased the number of crossings between quadrants in the same apparatus, indicating a stimulant effect of bilateral morphine on locomotor activity. Such a stimulant effect is known to obscure measures of anxiety-like behaviours in the elevated plus-maze (Dawson et al., 1995; Dawson and Tricklebank, 1995). Consistent with behavioural data, immunohistochemical data showed that morphine injected bilaterally induced a strong stimulation of Fos expression not only in the mesolimbic pathway, as seen after unilateral injections, but also in the dorsal caudate putamen and the primary motor cortex. This result suggests that bilateral injections recruited the dopaminergic nigrostriatal pathway, what would account for locomotor stimulation. In such conditions, behavioural measures of anxiety were likely to be overshadowed by locomotor effects, making the results from bilateral injections difficult to interpret.

In the case of unilateral injections, several mechanisms may have mediated morphine-induced anxiogenic-like effects in the LS. At a local level, morphine, by acting mainly on inhibitory μ-opioid receptors putatively located on LS GABAergic projection neurons was expected to exert anxiolytic effects when injected into the LSd. Indeed, data from the literature report anxiolytic effects following lesion of the LS (Menard and Treit, 1996) as well as pharmacological inactivation using local infusion of either indirect (midazolam) or direct (muscimol) GABA receptor agonists (Degroot et al., 2001; Pesold and Treit, 1996), all manipulations resulting in reducing GABAergic output from the LS. A way to reconcile the unexpected anxiogenic effects of intra-LS morphine with the previous results is to assume that mu opioid receptors are located on interposed inhibitory neurons, probably local GABAergic interneurons present in the LS (Jakab and Leranth, 1995; Szeidemann et al., 1995). A similar mechanism has been described in the MS (Alreja et al., 2000). The inhibition of local interneurons would then lead to an activation of the inhibitory GABAergic projection neurons of the LS. Interestingly, results from Fos immunochemistry provide arguments to support this hypothesis. Morphine, especially at the higher dose, produced a decrease in Fos staining in all main projection areas of the LS, namely the anterior hypothalamic area, the ipsilateral core of the nucleus accumbens and the diagonal band of Broca (Jakab and Leranth, 1995; Risold and Swanson, 1997b). Although Fos immunochemistry is a limited method to delineate multi-synaptic pathways, this result indicates that neuronal activity in direct anatomical targets of the LS was depressed, and suggests that LS inhibitory projection neurons were thus activated.

Due to the presence of a cytological inflammatory response around the injection site, we were not able to examine Fos expression in the dorsal part of the LS. We made the assumption that morphine infusion produced an anxiogenic state most likely

by increasing the activity of LS GABAergic projection neurons. However, we found that Fos expression was reduced in the ventral part of the LS, which would rather be indicative of a decreased neuronal activity in this region. Interestingly, Staiger and Nurnberger (1991) proposed that GABAergic projection neurons from the dorsal LS make *en passant* contacts with neurons in the ventral LS. Considering the ventral LS as one of the anatomical targets of the LSd, decreased Fos expression in this region constitutes a further argument supporting the hypothesis of a morphine-induced desinhibition of dorsal inhibitory projection neurons. Moreover, the ventral subdivision of the LS has been shown to be specifically activated by exposure to stressful conditions including exploration of the elevated plus-maze (Chen and Herbert, 1995; Duncan et al., 1996; Martinez et al., 1998; Mongeau et al., 2003; Stamp and Herbert, 1999). Functionally, the recruitment of the ventral subdivision of the LS has been proposed to actively attenuate fear responses rather than directly mediate the expression of anxiety per se (Campeau et al., 1997; but see also Sheehan et al., 2004). In our study, morphine-induced stimulation of GABAergic projection neurons in the LSd would contribute to anxiogenesis by inhibiting the ventral subdivision and thus preventing it from ensuring its suppressive role on fear expression.

Besides the ventral LS, the anterior hypothalamus and the hippocampus also displayed reduced Fos expression following intra-LS morphine injections. The anterior hypothalamic region has been shown to be critically involved in the somatic, endocrine and autonomic correlates of anxiety expression (Canteras, 2002; Risold and Swanson, 1996). This region has also been reported to modulate its major afferent, the LS, through enkephalinergic ascendant projections (Onteniente et al., 1989; Szeidemann et al., 1995), and therefore appears capable of modifying downstream information originating from the hippocampus (Risold and Swanson, 1996; Szeidemann et al., 1995). According to this scenario, the LS would mainly exert its modulatory role on anxiety by gating descending hippocampal outputs projecting to the hypothalamus via the LS (Risold and Swanson, 1996), resulting in a disorganization of the behavioural and neuroendocrine correlates of anxiety expression. In addition, the decrease in hippocampal function, previously described as anxiogenic (File et al., 1998) and mediated in our study by morphine injection into the LS, could generate difficulties for interpreting the emotional value of contextual stimuli (Desmedt et al., 1999; Sparks and LeDoux, 2000). Surprisingly, intra-LS morphine injections failed to significantly modify Fos expression in the amygdala, a brain region known to play a critical role in anxiety and fear modulation in both rodents (LeDoux, 1998) and humans (Zald, 2003). Knowing that Fos immunochemistry reveals only some of the neurons that are activated during a particular manipulation, this lack of effect could reflect technical limitations. However, our result is consistent with Treit and Menard's findings (2000) showing no behavioural effect of either central amygdaloid lesion or local infusions of midazolam in the plus-maze test, and with the proposition that amygdala and septum independently control different aspects of anxiety and fear responses (Desmedt et al., 1999; Sparks and LeDoux, 2000).

In our study, infusions of morphine into the LS were also associated with a significant increase in Fos expression within the mammillary bodies and the anterior thalamus. Interestingly, it has been shown that lesions of the mammillary bodies, which disrupt the mammillothalamic tract, produce anxiolytic effects in the plus-maze test (Beracochea and Krazem, 1991), a result that agrees well with ours. However, it must be pointed out that morphine injections into the MS, which were not found to be anxiogenic, were also associated with increased Fos expression in the mammillothalamic tract. Similarly, intra-LS and intra-MS morphine injections shared the ability of eliciting Fos expression in the mesolimbic pathway (VTA and AcbSh), which could account for their rewarding properties in a comparable range of doses (Cazala et al., 1998). As the mammillothalamic tract is known to receive inputs from the VTA (Oades and Halliday, 1987), its activation after intra-LS morphine injections could thus result from the stimulation of dopaminergic pathways. It is therefore difficult in our study to discriminate between possible involvement of the mammillothalamic tract in rewarding processes or anxiogenesis.

Taken together, the present study provides evidence for an implication of local opioidergic circuitry in the modulatory role of the lateral septal area on anxiety. Unexpectedly, however, the activation of the LS opioidergic system resulted in anxiogenic rather than anxiolytic effects. Activation of μ-opioid receptors in the LS would increase anxiety by inhibiting neuronal activity in the ventral lateral septum, and thus disorganizing the transmission of informations from the hippocampus to the hypothalamus. The role played by these two last structures in the modulation of anxiety exerted by septal opioidergic system will need further investigation.

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References

Alreja M, Shanabrough M, Liu W, Leranth C. Opioids suppress IPSCs in neurons of the rat medial septum/diagonal band of Broca: involvement of mu-opioid receptors and septohippocampal GABAergic neurons. J Neurosci 2000;20:1179–89.

Asakawa A, Inui A, Momose K, Ueno N, Fujino MA, Kasuga M. Endomorphins have orexigenic and anxiolytic activities in mice. Neuroreport 1998; 9:2265–7.

Barna I, Koenig JI, Makara GB. Effects of anterolateral and posterolateral cuts around the medial hypothalamus on the immunoreactive ACTH and beta-endorphin levels in selected brain regions of the rat. Brain Res Bull 1997;42:353–7.

Belcheva I, Belcheva S, Petkov VV, Petkov VD. Hippocampal asymmetry in the behavioral responses to the 5-HT_{1A} receptor agonist 8-OH-DPAT. Brain Res 1994;640:223–8.

Belzung C, Berton F. Further pharmacological validation of the BALB/c neophobia in the free exploratory paradigm as an animal model of trait anxiety. Behav Pharmacol 1997;8:541–8.

Beracochea DJ, Krazem A. Effects of mammillary body and mediodorsal thalamic lesions on elevated plus maze exploration. Neuroreport 1991;2:793–6.

Bontempi B, Sharp FR. 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 1997;17:8596–612.

Campeau S, Falls WA, Cullinan WE, Helmreich DL, Davis M, Watson SJ. Elicitation and reduction of fear: behavioural and neuroendocrine indices and brain induction of the immediate–early gene c-*fos*. Neuroscience 1997;78:1087–104.

Canteras NS. The medial hypothalamic defensive system: hodological organization and functional implications. Pharmacol Biochem Behav 2002;71:481–91.

Cazala P, Norena A, Le Merrer J, Galey D. Differential involvement of the lateral and medial divisions of the septal area on spatial learning processes as revealed by intracranial self-administration of morphine in mice. Behav Brain Res 1998;97:179–88.

Cheeta S, Kenny PJ, File SE. The role of 5-HT_{1A} receptors in mediating the anxiogenic effects of nicotine following lateral septal administration. Eur J Neurosci 2000;12:3797–802.

Chen X, Herbert J. Regional changes in c-*fos* expression in the basal forebrain and brainstem during adaptation to repeated stress: correlations with cardiovascular, hypothermic and endocrine responses. Neuroscience 1995;64:675–85.

Chiba T, Murata Y. Afferent and efferent connections of the medial preoptic area in the rat: a WGA–HRP study. Brain Res Bull 1985;14:261–72.

Coleman-Mesches K, McGaugh JL. Differential involvement of the right and left amygdalae in expression of memory for aversively motivated training. Brain Res 1995;670:75–81.

Cruciani RA, Lutz RA, Munson PJ, Rodbard D. Naloxonazine effects on the interaction of enkephalin analogs with mu-1, mu and delta opioid binding sites in rat brain membranes. J Pharmacol Exp Ther 1987;242:15–20.

Dawson GR, Tricklebank MD. Use of the elevated plus maze in the search for novel anxiolytic agents. Trends Pharmacol Sci 1995;16:33–6.

Dawson GR, Crawford SP, Collinson N, Iversen SD, Tricklebank MD. Evidence that the anxiolytic-like effects of chlordiazepoxide on the elevated plus maze are confounded by increases in locomotor activity. Psychopharmacology (Berl) 1995;118:316–23.

Degroot A, Treit D. Anxiety is functionally segregated within the septo-hippocampal system. Brain Res 2004;1001:60–71.

Degroot A, Kashluba S, Treit D. Septal GABAergic and hippocampal cholinergic systems modulate anxiety in the plus-maze and shock-probe tests. Pharmacol Biochem Behav 2001;69:391–9.

Desmedt A, Garcia R, Jaffard R. Vasopressin in the lateral septum promotes elemental conditioning to the detriment of contextual fear conditioning in mice. Eur J Neurosci 1999;11:3913–21.

Dragunow M, Faull R. The use of c-*fos* as a metabolic marker in neuronal pathway tracing. J Neurosci Methods 1989;29:261–5.

Duncan GE, Knapp DJ, Breese GR. Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety. Brain Res 1996;713: 79–91.

File SE, Wardill AG. Validity of head-dipping as a measure of exploration in a modified hole-board. Psychopharmacologia 1975;44:53–9.

File SE, Gonzalez LE, Andrews N. Endogenous acetylcholine in the dorsal hippocampus reduces anxiety through actions on nicotinic and muscarinic1 receptors. Behav Neurosci 1998;112:352–9.

Griebel G, Belzung C, Misslin R, Vogel E. The free-exploratory paradigm: an effective method for measuring neophobic behaviour in mice and testing potential neophobia-reducing drugs. Behav Pharmacol 1993;4:637–44.

Griebel G, Belzung C, Perrault G, Sanger DJ. Differences in anxiety-related behaviours and in sensitivity to diazepam in inbred and outbred strains of mice. Psychopharmacology (Berl) 2000;148:164–70.

Herrera DG, Robertson HA. Activation of c-*fos* in the brain. Prog Neurobiol 1996;50:83–107.

Hughes PE, Alexi T, Walton M, Williams CE, Dragunow M, Clark RG, et al. Activity and injury-dependent expression of inducible transcription factors, growth factors and apoptosis-related genes within the central nervous system. Prog Neurobiol 1999;57:421–50.

Jakab R, Leranth C. Septum. In: Paxinos G, editor. The rat nervous system. New York: Academic Press; 1995. p. 405–42.

Kang W, Wilson SP, Wilson MA. Overexpression of proenkephalin in the amygdala potentiates the anxiolytic effects of benzodiazepines. Neuropsychopharmacology 2000;22:77–88.

Koks S, Soosaar A, Voikar V, Bourin M, Vasar E. BOC-CCK-4, CCK(B) receptor agonist, antagonizes anxiolytic-like action of morphine in elevated plus-maze. Neuropeptides 1999;33:63–9.

LeDoux J. Fear and the brain: where have we been, and where are we going? Biol Psychiatry 1998;44:1229–38.

Lewis ME, Khachaturian H, Watson SJ. Combined autoradiographic–immunocytochemical analysis of opioid receptors and opioid peptide neuronal systems in brain. Peptides 1985;6(Suppl 1):37–47.

Lister RG. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 1987;92:180–5.

Mansour A, Fox CA, Akil H, Watson SJ. Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends Neurosci 1995;18:22–9.

Martinez M, Phillips PJ, Herbert J. Adaptation in patterns of c-*fos* expression in the brain associated with exposure to either single or repeated social stress in male rats. Eur J Neurosci 1998;10:20–33.

McNaughton N, Gray JA. Anxiolytic action on the behavioural inhibition system implies multiple types of arousal contribute to anxiety. J Affect Disord 2000;61:161–76.

Menard J, Treit D. Lateral and medial septal lesions reduce anxiety in the plus-maze and probe-burying tests. Physiol Behav 1996;60:845–53.

Millan MJ, Duka T. Anxiolytic properties of opiates and endogenous opioid peptides and their relationship to the actions of benzodiazepines. Mod Probl Pharmacopsychiatry 1981;17:123–41.

Mongeau R, Miller GA, Chiang E, Anderson DJ. Neural correlates of competing fear behaviors evoked by an innately aversive stimulus. J Neurosci 2003;23:3855–68.

Montgomery KC. The relation between fear induced by novel stimulation and exploratory behavior. J Comp Physiol Psychol 1955;48:254–60.

Oades RD, Halliday GM. Ventral tegmental (A10) system: neurobiology: 1. Anatomy and connectivity. Brain Res 1987;434:117–65.

Onteniente B, Menetrey D, Arai R, Calas A. Origin of the met-enkephalinergic innervation of the lateral septum in the rat. Cell Tissue Res 1989;256:585–92.

Ouagazzal AM, Kenny PJ, File SE. Stimulation of nicotinic receptors in the lateral septal nucleus increases anxiety. Eur J Neurosci 1999;11:3957–62.

Panula P, Revuelta AV, Cheney DL, Wu J-Y, Costa E. An immunohistochemical study on the location of GABAergic neurons in rat septum. J Comp Neurol 1984;222:69–80.

Patti CL, Frussa-Filho R, Silva RH, Carvalho RC, Kameda SR, Takatsu-Coleman AL, et al. Behavioral characterization of morphine effects on motor activity in mice. Pharmacol Biochem Behav 2005;81:923–7.

Paul D, Pasternak GW. Differential blockade by naloxonazine of two mu opiate actions: analgesia and inhibition of gastrointestinal transit. Eur J Pharmacol 1988;149:403–4.

Paxinos G, Franklin K. The mouse brain in stereotaxic coordinates. Second edition. Academic Press; 2001.

Pellow S, Chopin P, File SE, Briley M. Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 1985;14:149–67.

Pesold C, Treit D. The neuroanatomical specificity of the anxiolytic effects of intra-septal infusions of midazolam. Brain Res 1996;710:161–8.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G. GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 2000;101:815–50.

Risold PY, Swanson LW. Structural evidence for functional domains in the rat hippocampus. Science 1996;272:1484–6.

Risold PY, Swanson LW. Chemoarchitecture of the rat lateral septal nucleus. Brain Res Brain Res Rev 1997a;24:91–113.

Risold PY, Swanson LW. Connections of the rat lateral septal complex. Brain Res Brain Res Rev 1997b;24:115–95.

Rodgers RJ, Cole JC. Influence of social isolation, gender, strain, and prior novelty on plus-maze behaviour in mice. Physiol Behav 1993;54:729–36.

Rodgers RJ, Johnson NJ. Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. Pharmacol Biochem Behav 1995;52:297–303.

Sagar SM, Sharp FR, Curran T. Expression of c-*fos* protein in brain: metabolic mapping at the cellular level. Science 1988;240:1328–31.

Sasaki K, Fan LW, Tien LT, Ma T, Loh HH, Ho IK. The interaction of morphine and gamma-aminobutyric acid (GABA)ergic systems in anxiolytic behavior: using mu-opioid receptor knockout mice. Brain Res Bull 2002;57:689–94.

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

Shibata H. Topographic organization of subcortical projections to the anterior thalamic nuclei in the rat. J Comp Neurol 1992;323:117–27.

Singewald N, Salchner P, Sharp T. Induction of c-Fos expression in specific areas of the fear circuitry in rat forebrain by anxiogenic drugs. Biol Psychiatry 2003;53:275–83.

Sparks P, LeDoux J. The septal complex as seen through the context of fear. In: Numan R, editor. The behavioural neuroscience of the septal region. New York: Springer-Verlag; 2000. p. 234–69.

Staiger JF, Nurnberger F. The efferent connections of the lateral septal nucleus in the guinea pig: intrinsic connectivity of the septum and projections to other telencephalic areas. Cell Tissue Res 1991;264:415–26.

Stamp JA, Herbert J. Multiple immediate–early gene expression during physiological and endocrine adaptation to repeated stress. Neuroscience 1999;94:1313–22.

Szeidemann Z, Shanabrough M, Leranth C. Hypothalamic Leu-enkephalin-immunoreactive fibers terminate on calbindin-containing somatospiny cells in the lateral septal area of the rat. J Comp Neurol 1995;358:573–83.

Takeda H, Tsuji M, Matsumiya T. Changes in head-dipping behavior in the hole-board test reflect the anxiogenic and/or anxiolytic state in mice. Eur J Pharmacol 1998;350:21–9.

Thomas E. Forebrain mechanisms in the relief of fear: the role of the lateral septum. Psychobiology 1988;16:36–44.

Treit D, Menard J. The septum and anxiety. In: Numan R, editor. The behavioural neuroscience of the septal region. New York: Springer-Verlag; 2000. p. 210–33.

Tsuda M, Suzuki T, Misawa M, Nagase H. Involvement of the opioid system in the anxiolytic effect of diazepam in mice. Eur J Pharmacol 1996;307:7–14.

Yadin E, Thomas E, Grishkat HL, Strickland CE. The role of the lateral septum in anxiolysis. Physiol Behav 1993;53:1077–83.

Zald DH. The human amygdala and the emotional evaluation of sensory stimuli. Brain Res Brain Res Rev 2003;41:88–123.

Zar J. Biostatistical analysis. Fourth edition. Prentice Hall; 1999.

Zarrindast MR, Rostami P, Zarei M, Roohbakhsh A. Intracerebroventricular effects of histaminergic agents on morphine-induced anxiolysis in the elevated plus-maze in rats. Basic Clin Pharmacol Toxicol 2005;97:276–81.

Zhang HT, Xu ZM, Luo ZP, Qin BY. Anxiogenic effect of naltrexone in social interaction test in rats. Zhongguo Yao Li Xue Bao 1996;17:314–7.