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Effects of U-50,488H and U-50,488H withdrawal on c-*fos* expression in the rat paraventricular nucleus. Correlation with c-*fos* in brainstem catecholaminergic neurons

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1 In the present work, we have studied the expression of Fos during acute and chronic administration of the κ-opioid receptor agonist U-50,488H and after U-50,88H withdrawal in the rat hypothalamic paraventricular nucleus (PVN). Fos production was also studied in brainstem regions that innervate the PVN: the A_2 cell group of the nucleus of solitary tract (NTS-A_2) and the A_1 cell group of the ventrolateral medulla (VLM-A_1), combined with immunostaining for tyrosine hydroxylase (TH) for immunohistochemical identification of active neurons after acute U-50,488H administration.

2 For acute experiments, male rats were treated with saline i.p. for 4 days. On day 5, rats were given saline or U-50,488H (15 mg kg^{-1}, i.p.). Other groups of rats were rendered tolerant/dependent on U-50,488H by injecting the drug twice daily (15 mg kg^{-1}, i.p.) for 4 days. Control animals received saline i.p. on the same time schedule. On day 5, rats were treated with vehicle i.p., with U-50,488H (15 mg kg^{-1}) or with the selective κ opioid-receptor antagonist nor-binaltorphimine (Nor-BNI, 5 mg kg^{-1}, i.p.).

3 Using immunohistochemical staining of Fos, present results indicate that acute administration of U-50,488H produced an increase in Fos expression in the PVN and in the noradrenergic A_1 and A_2 cell groups. Moreover, when double-label immunohistochemistry was used to identify Fos and catecholaminergic-positive neurons in the brainstem, it was found that catecholaminergic-positive neurons in the NTS and VLM showed a significant increase in Fos expression in response to acute U-50,488H injection. Chronic application of U-50,488H leads to the development of tolerance towards their effects on Fos expression in the PVN as well as in the NTS and VLM. However, administration of Nor-BNI to U-50,488H-dependent rats did not induce any changes in Fos immunoreactivity in the PVN or in the brainstem.

4 These findings demonstrate that acute activation of κ-opioid receptors results in different altered patterns of immediate-early gene expression in the PVN, which occurs concurrently with an increased activity of their inputs from the brainstem. Interestingly in contrast to morphine withdrawal, present results demonstrate that rats withdrawn from U-50,488H did show no changes in Fos-immunoreactivity in the PVN, NTS or VLM, indicating the absence of dependence on the κ-agonist under the present experimental conditions.

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Abbreviations: HPA, hypothalamus – pituitary – adrenocortical axis; IR, immunoreactivity; NTS-A_2, nucleus tractus solitarius-A_2; TH, tyrosine hydroxylase; VLM-A_1, ventrolateral medulla-A_1

Introduction

Fos protein forms part of the activator protein 1 transcription factor, which regulates the expression of other delayed response genes (Morgan & Curran, 1991). Basal c-*fos* levels in brain are low, and c-*fos* is induced by a wide variety of stimuli including opioids (Hughes & Dragunow, 1995). This implies that c-*fos* could be a marker of neuronal activation leading to longer term adaptive responses mediated by the regulation of gene expression.

Many drugs of abuse induce immediate-early gene (IEG) expression in the central nervous system. Previous studies from our laboratory and others have demonstrated that acute activation of μ- and κ-opioid receptor causes induction of the IEG c-*fos* in localized areas of the brain (Gutstein *et al*., 1998; Laorden *et al*., 2000a). In addition, chronic morphine administration has been shown to alter the IEGs expressed in specific brain areas that are normally responsive to acute morphine treatment (Frankel *et al*., 1999; Laorden *et al*., 2002).

On the other hand, morphine withdrawal also produces a complex endocrine alteration in rats, including the activation of the hypothalamus – pituitary – adrenocortical (HPA) axis.

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Such axis alteration is characterized by an increased ACTH and corticosterone secretion possibly because of the overproduction of CRF (Vargas *et al*., 1997; Milanés *et al*., 1998; Fuertes *et al*., 2000). In addition, morphine withdrawal is associated with an increase in hypothalamic noradrenaline (NA) turnover, which has been attributed to an enhanced NA secretion and suggests a stimulatory role of this neurotransmitter in CRF release during opioid dependence (Milanés *et al*., 1998; Fuertes *et al*., 2000; Laorden *et al*., 2000b). In contrast to morphine, U-50,488H withdrawal produces no changes in the HPA axis activity or in hypthalamic NA turnover. It may suggest that μ- and κ-receptors could be regulated through different cellular and molecular mechanisms (Laorden & Milanés, 2000).

The transcription factor Fos has been shown to be altered in several brain areas following morphine withdrawal (Beckmann *et al*., 1995; Frankel *et al*., 1999; Laorden *et al*., 2002). However, to our knowledge, the effects of U-50,488H (a selective κ-opioid receptor agonist) withdrawal on the induction of Fos protein in brain have not been studied. Therefore, the purpose of the present study was to investigate possible changes in the c-*fos* expression after acute and chronic treatment with the selective κ-opioid receptor agonist, U-50,488H, and after antagonist-precipitated withdrawal from U-50,488H in the parvocellular part of the hypothalamic paraventricular nucleus (PVN), which is involved in the neuroendocrine control of the HPA axis. After the same treatments, we also studied Fos production in the ventrolateral medulla (VLM-A_1) and in the nucleus of the solitary tract (NTS-A_2), which project to the PVN (the primary location of tuberoinfundibular CRF cells) to test the possible correlation between Fos expression in these areas *versus* that in the PVN. Additionally, double-label immunostaining was used to investigate Fos expression after acute administration of U-50,488H in brainstem neurons positive for tyrosine hydroxylase (TH), the rate-limiting step in the biosynthesis of catecholamines.

Methods

Male Sprague – Dawley rats (220 – 240 g at the beginning of the experiments) were housed four to five per cage under a 12 h light/dark cycle (L: 8:00 – 20:00h) in a room with controlled temperature ($22 \pm 2^{\circ}$C), humidity ($50 \pm 10\%$), and food and water available *ad libitum*. All surgical and experimental procedures were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Experimental procedure

For acute experiments, groups of rats were treated with saline i.p. (controls) twice daily for 4 days. On day 5, rats ($n = 3 – 5$ per group) received saline i.p. or U-50,488H (15 mg kg^{-1}, i.p.). Another group of rats ($n = 4$) was rendered tolerant/dependent on U-50,488H by injecting the drug twice daily (15 mg kg^{-1}, i.p.) for 4 days. This procedure has been shown to induce tolerance to the different effects of the κ-receptor agonist U-50,488H (Laorden & Milanés, 2000). Control animals ($n = 4$) received saline i.p. on the same time schedule. Groups of rats pretreated with saline or U-50,488H ($n = 3 – 4$ per group) were injected on day 5 with vehicle (Milli-Q water) i.p., with U-50,488H (15 mg kg^{-1}, i.p.) or with the selective κ-opioid receptor antagonist nor-binaltorphimine (Nor-BNI, 5 mg kg^{-1}, i.p.). Rats were observed before and for 90 min after administration of the opioid antagonist or vehicle to determine the possible existence of withdrawal signs (wet dog-shakes, teeth chattering, tremor, salivation, piloerection or ptosis).

Tissue preparation and Fos-immunohistochemistry

Rats were killed with an overdose of pentobarbital (100 mg kg^{-1}, i.p.) 90 min after administration of saline, U-50,488H, Nor-BNI or vehicle. The delay of 90 min after drugs injection was chosen, since it has been demonstrated that the peak effect of stimulated Fos in brain is 90 min (Morgan & Curran, 1991). Following anaesthesia, rats were perfused transcardially with 300 ml of phosphate-buffered saline (PBS; pH 7.4) followed by 500 ml of cold, 4% paraformaldehyde in PBS (pH 7.4). Following perfusion, brains were removed, postfixed in the same fixative, and stored at 4°C overnight. Free floating coronal brain sections (150 μm in thickness) throughout the rostrocaudal extent of the hypothalamus were obtained on a Vibratome. A total of 16 hypothalamic sections were taken from each animal, corresponding to plates 23 – 26 in the atlas of Palkovits & Brownstein (1988), which contain the hypothalamic PVN (plane of sections relative to bregma: -1.8 to -2.12 mm). Since noradrenergic A_1 and A_2 neuronal groups in the VLM and NTS, respectively, extend from the level caudal to the area postrema (AP), rostrally, to upper cervical segment of spinal cord caudally (Sawchenko & Swanson, 1982; Cunningham & Sawchenko, 1988), the brain sections chosen for analysis of Fos and Fos/TH (50 μm in thickness) in the NTS and VLM were taken at various levels, ranging from level of the AP, rostrally, to the pyramidal decussation, caudally (plane of sections relative to bregma: $-14.08 – 14.6$ (Paxinos & Watson, 1998). The data for each of the NTS and VLM were combined for the entire nucleus.

Expression of Fos protein was examined in free floating sections, which were collected serially in adjacent sets, shaken in PBS for at least 30 min to remove the fixative, and processed for immunohistochemistry as described previously (Laorden *et al*., 2000a; 2002). Briefly, the sections were preincubated for 20 min in absolute methanol plus 10% H_2O_2 to block endogenous peroxidase activity. They were rinsed in PBS twice (15 min each) and treated with normal swine serum (NSS) – PBS (PBS containing 1% NSS; Dako, Gostrup, Denmark; and 0.5% Triton X-100) for 30 min. All sections were then reacted with the primary polyclonal Fos antibody (dilution 1:3000 in NSS – PBS; sc253, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 36 h at 4°C. Next, the bound primary antibody was localized by biotinilated anti-rabbit IgG (dilution 1:200 in NSS – PBS, Vector, Burlingame, CA, U.S.A.), and subsequently with the avidin – biotin complex (ABC kits; Vector) at room temperature for 1 h each. Visualization of the antigen – antibody reaction sites used 0.033% 3,3′-diaminobenzidine (DAB; Sigma) and 0.014% H_2O_2 in 0.05 M Tris-HCl buffer (Sigma) for 7 min. The reaction was stopped in PBS. Then the sections were mounted onto glass slides coated with gelatine, air-dried, dehydrated through

graded alcohols, cleared in xylene and cover-slipped with DPX.

For Fos and TH double-label immunohistochemistry, brainstem tissue sections from each rat in each treatment group were processed as follows: Fos was revealed with DAB intensified with nickel in the first position and the enzyme revealed with DAB in the second position. Fos-immunohistochemistry was performed as described previously and Fos antibody – peroxidase complex was visualized with a mixture of $NiSO_4 \cdot 6H_2O$ (33.2 mg ml^{-1}), DAB (0.033%) and 0.014% H_2O_2 in 0.175 M sodium acetate solution (pH 7.5). When staining was appropriate, tissue sections were transferred into distilled water (Milli-Q water) to stop the colour reaction. Following the Fos staining, sections were rinsed in PBS twice (15 min each), treated with NSS – PBS for 30 min, then incubated in the mouse monoclonal anti-TH antibody (dilution 1 : 6000 in NSS – PBS; LNC1, Diasorin, Stillwater, MN, U.S.A.) overnight. The same immunohistochemistry procedures described above were followed, except horse anti-mouse IgG (1:400, 1 h; Vector) that was used as a secondary antibody. The TH antibody – peroxidase complex was stained in 0.033% DAB and 0.014% H_2O_2 in 0.05 M Tris-HCl buffer. The reaction was stopped in PBS. The sections were mounted onto glass slides coated with gelatine, air-dried, dehydrated through graded alcohols, cleared in xylene and cover-slipped with DPX.

Quantification of Fos-IR

Evidence of Fos-like immunoreactivity (Fos-IR) was examined under a light microscope. Density of Fos-like immunopositive nuclei was performed with a computer-assisted image analysis system as described previously (Laorden *et al*., 2000a). This system consists of an Axioskop microscopy (Carl Zeiss, Germany) connected to a videocamera and a Imco 10 computer (Kontron Instrument Ltd., Bildanalyse, Germany) with Microm Image Processings software (Microm, Spain). The three to four sections of each nucleus that showed the highest level of Fos-IR were selected for quantitative image analysis. A square field (93.5 μm side) was superimposed upon the captured image ($\times$ 40 magnification) to be used as reference area. The area of Fos-immunolabelling included in this square was used for estimating the IR, and the percentage of Fos-IR was evaluated. The method used to quantify the IR consists in the division of the immunoreactive area per reference area, as has been previously used in our laboratory (Laorden *et al*., 2000a; 2002). Based on orientation criteria, the medial parvicellular neurosecretory portion of the PVN was defined once the adjacent boundaries of the posterior magnocellular and periventricular parts could be identified (Swanson & Simmons, 1989). For this nucleus, the density of Fos-like immunopositive nucleus at three to four rostrocaudal levels encompassing the parvicellular zone, the primary location of tuberoinfundibular CRF cells, was used for estimating Fos-IR. The percentage of Fos-IR of both right and left sides of three to four correlative sections for each nucleus was averaged per animal. For the NTS-A_2 and VLM-A_1, the right and left sites of five to six sections of each were analysed and averaged per rat. Measures were also averaged in each experimental group for the PVN, VLM and NTS.

Quantification of Fos-positive TH neurons

Nuclei-positive Fos-IR were detected using the same conventional light microscopy described above, and counted at $\times$ 20 magnification. Fos-positive TH cells were identified as cells with brown cytoplasmatic deposits for TH-positive staining and blue-dark nuclear staining for Fos. A square field (260 μm) was superimposed upon captured image to be used as reference area. The number of single- and double-labelled TH neurons observed bilaterally were counted in four to five sections from each animal in the NTS-A_2 and VLM-A_1. Since TH-positive neurons could only be counted as Fos positive whenever the nucleus was visible, the TH-positive cells without a visible nucleus were excluded from the analysis. Data from Fos-TH double-labelled cells from the brainstem were expressed as the percentage of Fos-positive TH neurons.

Drugs

U-50,488H (*trans* ($\pm$)-3,4 dichloro-*N*-methyl-*N*-[2-(1-pyrrolidynyl)ciclohexyl] benzeneacetamide methane sulphonate; a gift from Upjohn, Kalamazoo, MI, U.S.A.) was dissolved in saline; Nor-BNI (Sigma) was dissolved in Milli-Q-sterile water (vehicle). Drugs were administered in volumes of 0.10 ml 100 g^{-1}.

Statistical analysis

All values are expressed as means $\pm$ s.e.m. Data were analysed by analysis of variance followed by the Newman – Keul's *post hoc* test. Two tailed Student's *t*-test was used in the experiments, in which two groups of animals were monitored (control and experimental group). Differences with a *P*-value less than 0.05 were considered significant.

Results

Rats rendered tolerant/dependent on U-50,488H receiving Nor-BNI on day 5 did not show any of the behavioural signs seen during morphine dependence (wet dog-shakes, teeth chattering, tremor, piloerection or ptosis), indicating the absence of abstinence syndrome.

Fos expression in the PVN after acute and chronic U-50,488H administration and after U-50,488H withdrawal

Quantitative analysis of labelling in the PVN was restricted to the parvocellular subdivision. Low but detectable Fos-IR was observed in the PVN from control animals that received saline i.p. Present data show that acute administration of U-50,488H (15 mg kg^{-1}, i.p.) significantly ($P<0.001$, *t*-test) increases Fos-positive cells in the parvocellular subdivision of the PVN (7.56 ± 1.0) compared with control rats receiving saline (1.46 ± 0.15). By contrast, a significant ($P<0.001$) decrease in Fos-IR (1.87 ± 0.41) was found after injection of U-50,488H (day 5) to rats chronically treated with U-50,488H, when compared with the group injected acutely with the κ-agonist. These results indicate that tolerance develops towards the Fos-IR increasing action of U-50,488H.

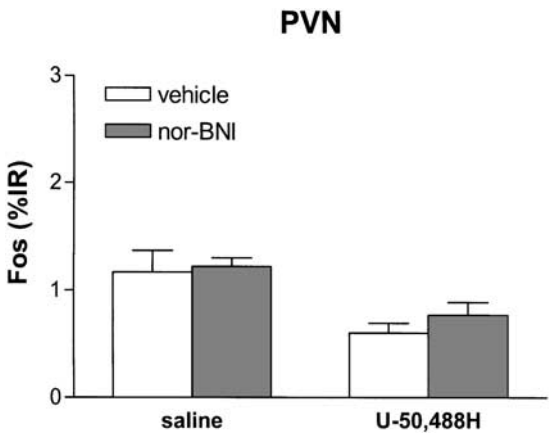

Figure 1 Quantitative analysis of Fos-immunoreactivity in the PVN 90 min after vehicle (i.p.) or Nor-BNI (5 mg kg^{-1}, i.p.) administration to rats pretreated for 4 days with saline or U-50,488H.

Figures 1 and 2 summarize the Fos-IR after administration of Nor-BNI or vehicle to rats chronically treated with U-50,488H or saline. Administration of Nor-BNI did not modify the Fos-IR in the PVN (0.77 ± 0.12) compared with control animals receiving saline plus Nor-BNI (1.22 ± 0.08) and with chronically U-50,488H-treated rats receiving vehicle (0.60 ± 0.09).

Effects of acute U-50,488H administration on Fos expression in the brainstem catecholaminergic A_1 and A_2 cell groups

Quantitative analysis of labelling in the NTS-A_2 and VLM-A_1 was confined to coronal sections that included the rostrocaudal extent from AP to obex, the level at which catecholaminergic neurons are predominantly noradrenergic. In control rats injected with saline, few neurons with Fos-IR were found in the NTS (0.55 ± 0.02). Acute administration of U-50,488H led to significant ($P < 0.001$; *t*-test) increases in Fos-IR (2.24 ± 0.09; Figures 3 and 4). Stimulation of κ-receptors by acute U-50,488H injection also elicited a significant ($P < 0.001$; *t*-test) increase in the Fos-IR (2.47 ± 0.10) in the VLM compared with control rats receiving saline (0.47 ± 0.10; Figures 3 and 4).

To determine the specificity and magnitude of κ-receptors activation of catecholaminergic neurons that innervate the PVN, sections of the NTS-A_2 and VLM-A_1 cell groups were immunohistochemically double labelled for both Fos and the rate-limiting catecholaminergic biosynthetic enzyme, TH. Figure 5 depicts the staining pattern of Fos-positive TH neurons in both regions from rats acutely injected with saline or U-50,488H. Quantitative analysis showed that after acute U-50,488H administration, both regions (NTS and VLM) exhibited significant ($P < 0.001$; *t*-test) increase in the percentage of TH-containing cells expressing Fos (93 ± 1.4 and $96 \pm 0.69\%$, respectively) when compared to the control group injected with saline (55 ± 2.3 and $60 \pm 3.2\%$, respectively).

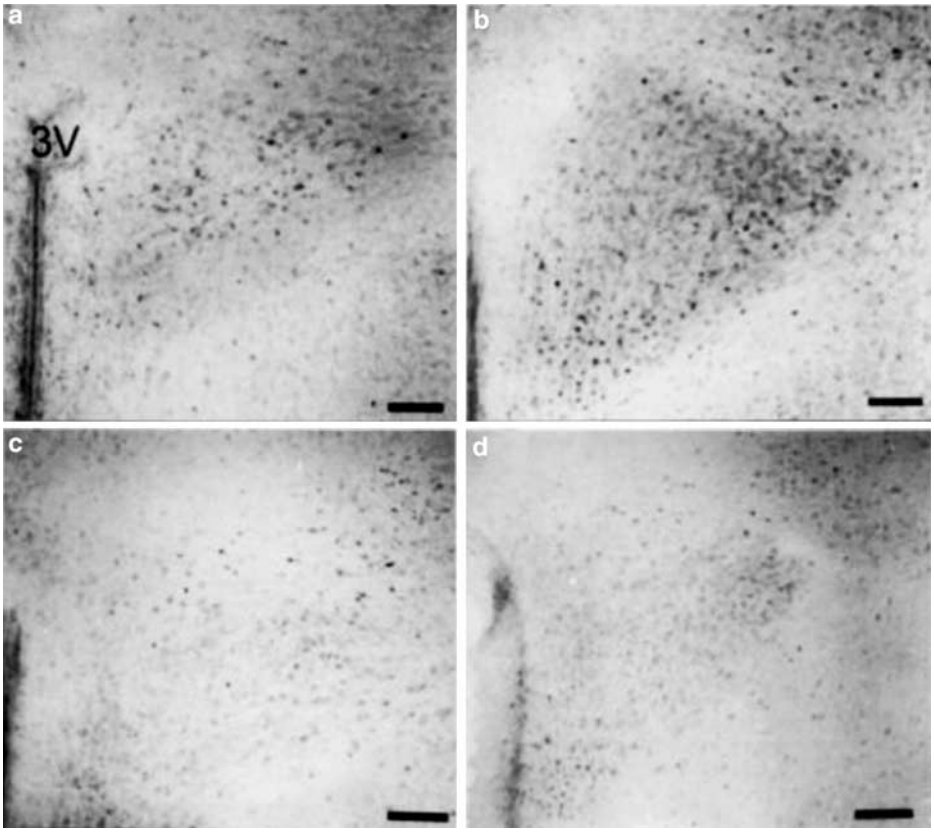

Figure 2 Representative photomicrographs of Fos-IR in the PVN 90 min after vehicle (i.p.) or Nor-BNI (5 mg kg^{-1}, i.p.) administration to rats pretreated for 4 days with saline or U-50,488H (15 mg kg^{-1}, i.p.). (a) saline + vehicle, (b) saline + nor-BNI, (c) U-50,488H + vehicle and (d) U-50,488H + Nor-BNI. (*Scale bar*) 111 μm; 3V: third ventricle.

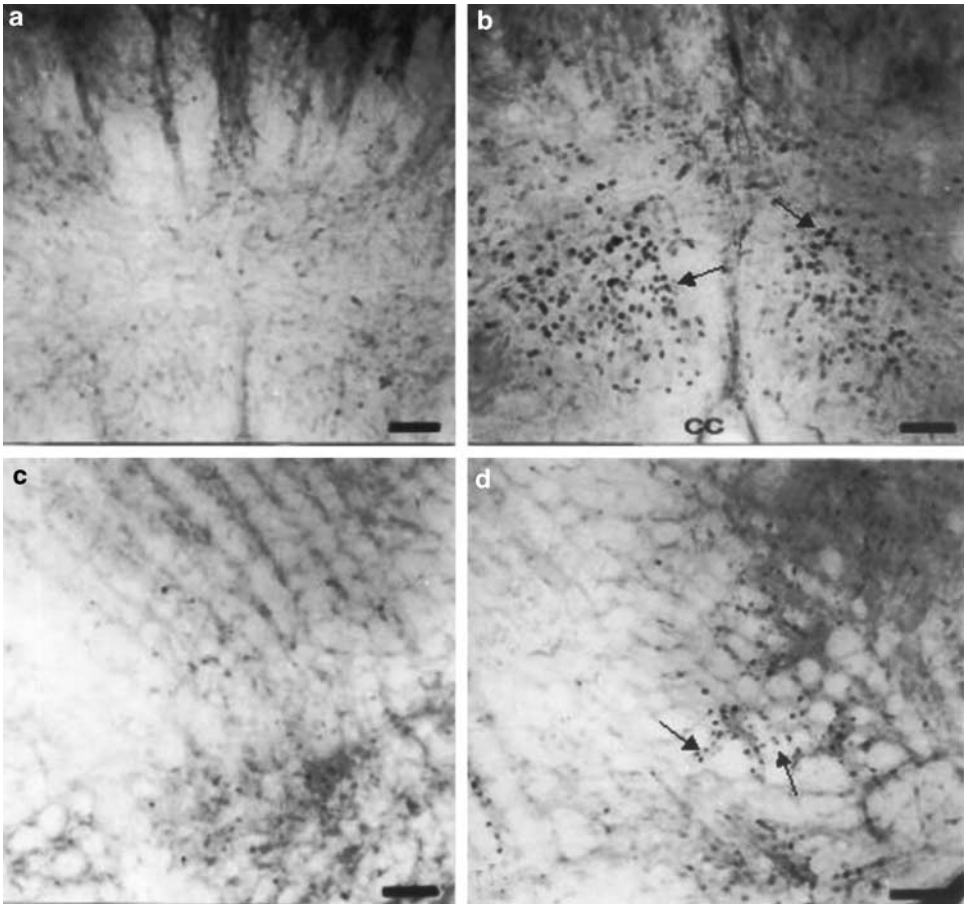

Figure 3 Representative photomicrographs of Fos-IR in the NTS-A_2 and in the VLM-A_1 90 min after acute saline (i.p.) or U-50,488H (15 mg kg^{-1}, i.p.) administration to rats pretreated with saline for 4 days. NTS: (a) saline + saline, (b) saline + U-50,488H. VLM: (c) saline + saline, (d) saline + U-50,488H. (*Scale bar*) 111 μm; CC: central canal.

Fos expression in the brainstem after chronic U-50,488H and after U-50,488H withdrawal

The Fos-IR in the NTS from chronic U-50,488H-treated rats receiving U-50,488H on day 5 was significantly ($P<0.001$) lower (0.45 ± 0.05) than that found in control rats acutely injected with U-50,488H, indicating the development of tolerance towards the Fos-IR increasing action of the κ-agonist. Similarly, the Fos-IR in the VLM from U-50,488H tolerant rats injected with the κ-opioid receptor agonist was lower ($P<0.001$; 0.30 ± 0.01) than that observed in the control rats injected acutely with U-50,488H. The injection of Nor-BNI to rats chronically treated with U-50,488H did not significantly modify the Fos-IR in the NTS (0.53 ± 0.03) or in the VLM (0.44 ± 0.04) when compared with the tolerant rats injected with vehicle or the control group injected with Nor-BNI (0.47 ± 0.005 and 0.41 ± 0.04, respectively) (Figures 6 and 7).

Discussion

In the present study, the IEG product, Fos protein, was used as a marker for neuronal activation to identify whether neuronal populations in the hypothalamus and brainstem are activated by U-50,488H and U-50,488H withdrawal. According to previous results from our laboratory and others (Bot & Chahl, 1996; Laorden *et al*., 2000a), the present immunohistochemical study shows that the acute administration of U-50,488H induces the neuronal expression of Fos protein within neurons of the parvocellular division of the PVN, which regulates the HPA axis. Concomitantly, an increase in Fos production was seen in the catecholaminergic A_1 and A_2 cell groups. As Fos expression is an indicator of neuronal activity, the present results indicate that PVN is activated after κ-opioid receptor activation concomitantly with an activation of the brainstem noradrenergic regions that innervate this nucleus.

Regulatory control of the HPA axis originates principally from the hypothalamic PVN. The parvocellular neurosecretory neurons of this nucleus serve as the origin of a cascade of events by delivering the two main corticotropin releasing factors, CRF and arginine vasopressin, resulting in the ACTH-mediated release of corticosterone (Antoni, 1986). A primary component of the rat response to μ- and κ-opioid receptor agonists is known to involve activation of the HPA axis (Ignar & Kuhn, 1990; Martínez *et al*., 1990; Milanés *et al*., 1998; Laorden *et al*., 2000b; for review see Pechnick, 1993). Although the Fos-positive nuclei in the PVN were largely confined to the parvocellular region, the precise identification of the cell groups that respond to opioids awaits further study using double-labelling

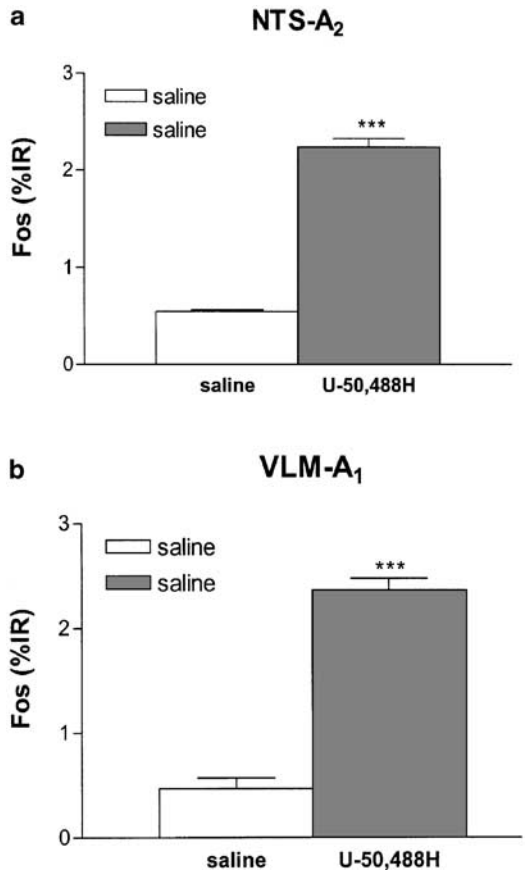

Figure 4 Quantitative analysis of Fos-IR in the NTS-A_2 and in the VLM-A_1, 90 min after acute saline (i.p.) or U-50,488H (15 mg kg^{-1}, i.p.) administration to rats pretreated with saline for 4 days. ***$P<0.001$ *versus* saline + saline.

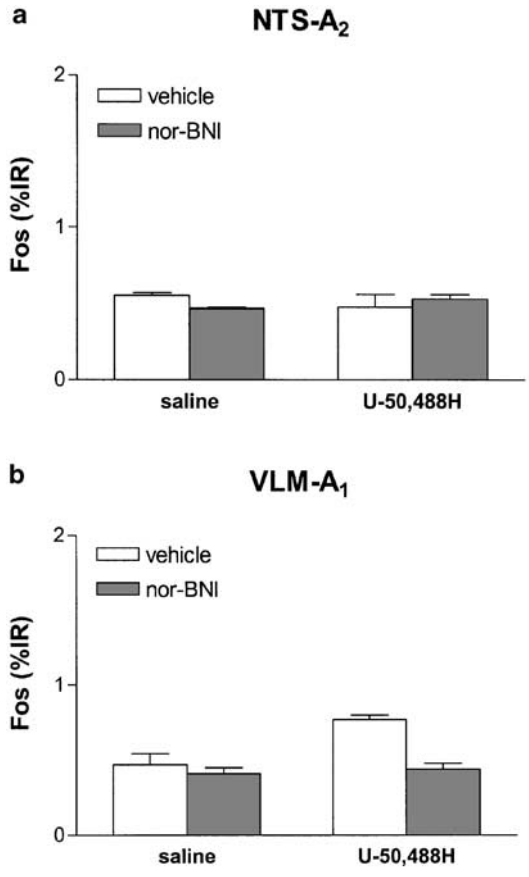

Figure 6 Quantitative analysis of Fos-IR in the NTS-A_2 (a) and VLM-A_1 (b) 90 min after vehicle (i.p.) or Nor-BNI (5 mg kg^{-1}, i.p.) administration to rats pretreated with saline or U-50,488H for 4 days.

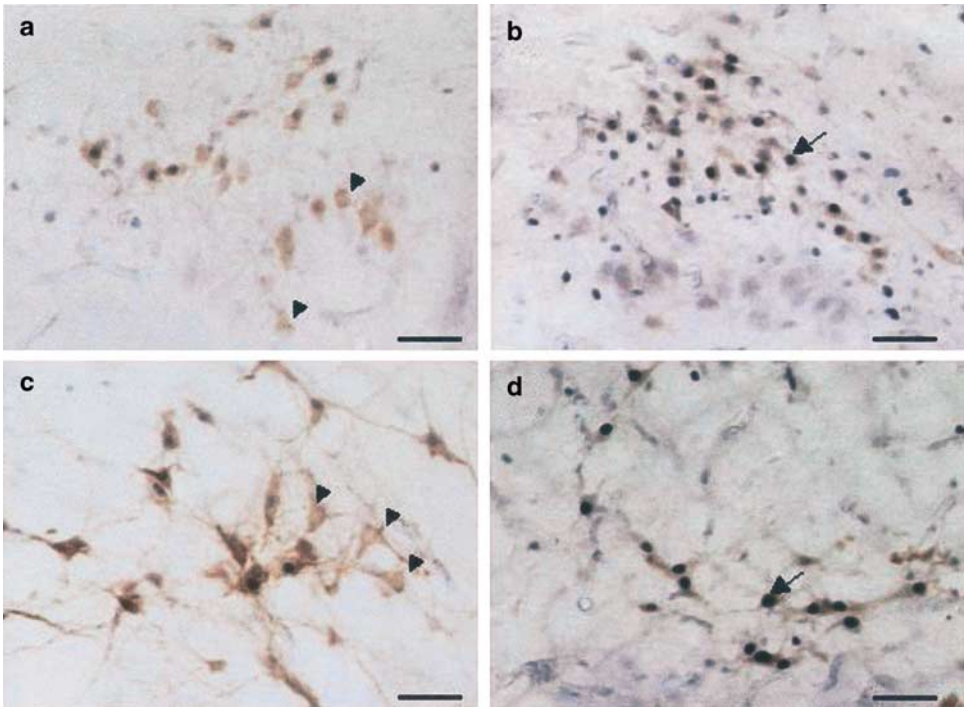

Figure 5 Representative photomicrographs of double-labelled cells in the NTS-A_2 (a, b) and VLM-A_1 (c, d). Double immunohistochemical staining for Fos (blue-dark nuclei) and TH (brown cytoplasm) in sections from acute saline (a, c) and U-50,488H (b, d) injected rats. Arrowheads indicate Fos-negative catecholaminergic neurons in the control groups. Arrows indicate Fos$^+$/TH$^+$ neurons. (*Scale bar*) 50 μm.

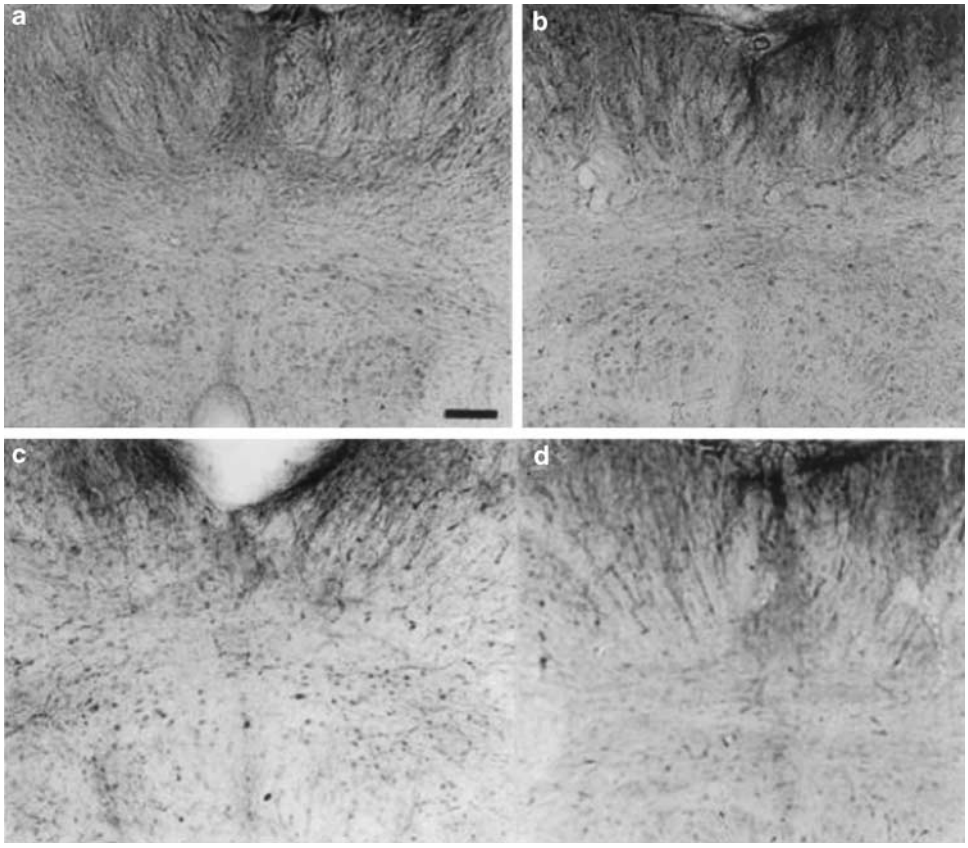

Figure 7 Representative photomicrographs of Fos-IR in the NTS-A_2 cell group, 90 min after vehicle (i.p.) or Nor-BNI (5 mg kg^{-1}, i.p.) administration to rats pretreated with saline or U-50,488H (15 mg kg^{-1}, i.p.) (a) saline + vehicle, (b) saline + Nor-BNI, (c) U-50,488H + vehicle and (d) U-50,488H + Nor-BNI. (*Scale bar*) 87 μm.

techniques. However, as has been proposed previously (Li & Sawchenko, 1998; Dayas *et al*., 1999), the Fos qexpression within the medial PVN can be taken as an index of the HPA axis activity and also as an indicator of the degree of activation of the medial parvocellular cell populations.

The present data also demonstrate that tolerance develops towards the activation of Fos protein induced by U-50,488H in the PVN and the NTS and VLM. Our results are consistent with previous findings, using immunocytochemical staining of Fos, showing that chronic exposure to morphine attenuates the cellular responsiveness to this opioid agonist in hypothalamic nuclei (Chang *et al*., 1996; Laorden *et al*., 2002). In addition, it is known that chronic treatment with U-50,488H results in a high degree of tolerance to the different neuroendocrine effects of the opioid at hypothalamic level, including HPA axis and NA turnover activation (Ignar & Kuhn, 1990; Laorden & Milanés, 2000). Taken together, these results could indicate that the alteration in transcription factors after opioids could be involved in the cellular adaptive changes in the hypothalamic neurosecretory neurons that control the HPA axis activity.

Most studies on opioid dependence have focused on μ-agonists, and only a few studies have been performed on the effects of κ-agonists, especially at hypothalamic level (Ignar & Kuhn, 1990; Laorden & Milanés, 2000), a brain area that regulates endocrine responses and participates in opioid dependence (Maldonado *et al*., 1992; Nestler & Aghajanian, 1997). Interestingly, present data show, for the first time, that in contrast to morphine, U-50,488H withdrawal was not accompanied by changes in Fos-IR in the PVN. The regulation of c-*fos* expression in other nuclei or areas different from PVN after opioid withdrawal has not been investigated thoroughly and there are no studies about the regulation of Fos expression after U-50,488H withdrawal. Noradrenergic involvement in the activity of the HPA axis in response to morphine dependence, primarily via secretion of NA, has recently been proposed. Thus, it has been shown that morphine withdrawal excitates catecholaminergic NTS/VLM neurons, which project to the PVN, to participate directly in the activation of parvocellular neurons (Laorden *et al*., 2000b, 2002). In contrast to morphine withdrawal, present results show that U-50,488H withdrawal did not induce changes in expression of Fos in the NTS or in the VLM. These findings seem to reveal that U-50,488H withdrawal did not activate neurons in the A_1 and A_2 cell groups.

Previous studies from our laboratory demonstrated that administration of κ-antagonists to U-50,488H tolerant rats did not increase catecholaminergic activity in the PVN and did not modify the HPA axis activity (Laorden & Milanés, 2000). These data clearly indicate that κ-opioid receptor is not involved in the development of dependence on opioid-induced neuroendocrine actions

in rats. In agreement with previous data (Ignar & Kuhn, 1990; Milanés *et al*., 1991; Laorden & Milanés, 2000), our results also demonstrated that, unlike morphine withdrawal, U-50,488H withdrawal does not induce either behavioural or vegetative signs of abstinence syndrome, suggesting that, under the conditions of the present study, the κ-opioid receptor is not involved in the development of physical dependence.

In summary, our results show that acute activation of κ-opioid receptor by U-50,488H triggers the expression of c-*fos* in parvocellular region of the PVN, which confirms that opioids activate neurosecretory neurons implicated in the activity of the HPA axis. This effect was accompanied by an increase in Fos-IR in the NTS-A_2 and VLM-A_1 noradrenergic cell groups. Furthermore, NTS-A_2 and VLM-A_1 exhibited significant increases in the percentage of TH-containing neurons expressing Fos in response to acute U-50,488H administration, indicating an activation of catecholaminergic neurons in both areas. In addition, the present data demonstrate that tolerance develops towards increasing c-*fos* expression induced by U-50,488H. By contrast to morphine, U-50,488H withdrawal was not accompanied by modifications in Fos expression in the PVN or in the noradrenergic neuronal groups in the brainstem. Since opioid inducing transcription of c-*fos* may be involved in the dependence-inducing properties of opioids (Blendy & Maldonado, 1998), the present data suggest that κ-opioid receptors are not involved in the molecular adaptive mechanisms responsible for the development of dependence on opioid-induced neuroendocrine actions at the PVN level and that different molecular mechanisms underlie chronic effects of μ- and κ-agonists in this nucleus.

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