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ORIGINAL INVESTIGATION

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Effect of the antidepressant nefazodone on the density of cells expressing mu-opioid receptors in discrete brain areas processing sensory and affective dimensions of pain

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Abstract *Rationale:* The principal use of antidepressants is in the treatment of depression and affective disorders. Antidepressants have also been used as an adjuvant to analgesics in pain treatment. However, in chronic treatment, their antinociceptive and antidepressive effects coexist simultaneously. Antidepressants can interact with the opioid system, which is also involved in regulating nociceptive processing and affective state. Chronic antidepressants could act by increasing mu-opioid receptor expression in many brain areas involved in the regulation of nociception and affective state. *Objectives:* The aim of this study was to evaluate the antinociceptive and antidepressant-like effects and the possible variations in mu-opioid receptor expression induced by a chronic nefazodone treatment in brain areas related to pain and affective state. *Methods:* Wistar rats were chronically treated with nefazodone (10 and 25 mg/kg IP, twice a day, for 14 days). Twelve hours after the last day 14 dose of nefazodone, a tail-flick test was performed. After the administration of a daily dose of nefazodone, Porsolt's test was carried out 12 h after last dose. Two hours after completion of 14 days treatment, other animals were processed for mu-opioid receptor immunocytochemistry using polyclonal antisera raised in rabbits. Several brain regions were analyzed: the frontal and cingulate cortex, the dorsal raphe nucleus and the periaqueductal gray. *Results:* Chronic nefazodone treatment induced a significant increase in tail-flick latency and a significant decrease in immobility time at total doses of 20 and 50 mg/kg per day ($P<0.05$). In treated animals, the density of neural cells immunostained for mu-opioid receptor in the frontal and cingulate cortices, dorsal raphe nucleus and periaqueductal gray had increased after chronic nefazodone compared to controls. *Conclusion:* Therefore, chronic nefazodone induces antinociceptive and antidepressant-like effects in rats and increases mu-opioid receptor expression in brain areas related to pain and affective state. These results suggest that antidepressants could be effective on somatic and affective dimensions of pain and this action could be related to its influence on the opioid system.

Keywords Nefazodone · Mu-opioid receptor · Rat · Antidepressant · Affective dimension · Pain

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Introduction

Pain sensation can be viewed as having three aspects: sensory, hedonic and affective-motivational. The sensory dimension concerns the localization, size, intensity and nature of somatosensory stimuli. The hedonic aspect concerns the unpleasantness of the sensation, while the affective-motivational aspect produces the desire (motivation) to terminate, reduce or escape from the experience of noxious stimuli (Sewards and Sewards 2002). Moreover, there is evidence that parallel spinal pathways distribute information to brain circuits that are concerned with either the sensory or affective qualities of pain (Price 2000).

These pain pathways terminate in discrete brain areas that monitor the sensory and affective qualities of the initiating stimulus and show remarkable plasticity (Rosso et al. 2003). These areas include the somatosensory cortices, which are thought to be concerned with the sensory qualities of pain, and other cortical areas such as the cingulate cortex and insula (Singer et al. 2004) and the prefrontal cortex, which are more closely aligned with affective-motivational aspects of pain. Therefore, changes in neuronal structure, connections between neurons and

alterations in the quantity and properties of neurotransmitters and receptors can ultimately result in increased or decreased functional activity of the neurons in the pain pathway, resulting ultimately in different perceptions, sensation and emotional responses to pain (Stucky et al. 2001).

Antidepressants are widely used in clinical practice to treat several pain processes (Carter and Sullivan 2002) both in depressed (Saper et al. 2001) and in non-depressed patients (Bendtsen and Jensen 2000; Pernia et al. 2000; Schreiber et al. 2001). Moreover, antidepressants are used in monotherapy or in association with opiates (Saper et al. 2001; Schreiber et al. 2001; Goldstein 2002). Although antidepressants were originally used in chronic pain conditions because those with chronic pain also become depressed (Sindrup 1997), there is now considerable evidence, mainly deriving from basic research, relating the intrinsic pharmacological action of these compounds (i.e. the inhibition of the reuptake of noradrenaline and serotonin) with the main pathways regulating endogenous pain processes, i.e. the activation of the monoaminergic descending systems from the brainstem to the spinal cord.

The main serotonergic nuclei thought to be involved in pain modulation are the nucleus raphe magnus and the nucleus raphe dorsalis. The former project, directly or indirectly, to the dorsal horn (Sorkin et al. 1993), while the latter is thought also to project to the forebrain area, thus probably contributing to the affective dimensions of pain as mentioned above (Wang and Nakai 1994). However, it is not know whether the effectiveness of chronic treatment with antidepressants as co-analgesics is due to a direct effect on the nociceptive pathways or whether it results solely from their effect on affective state. In this study, we use nefazodone because it is a serotonergic antidepressant acting as a selective serotonin reuptake inhibitor and 5-HT_2 receptor antagonist. In a previous study, it had not shown antinociceptive effects in tail-flick after acute administration in mice, but facilitated morphine analgesia (Pick et al. 1992). However, there are no data regarding an antinociceptive effect of nefazodone in rats.

It is difficult to determine the effectiveness of an antidepressant treatment on the affective dimension of pain in animals, and it is therefore necessary to use an indirect method to evaluate it. In this sense, we can measure the effectiveness of an antidepressant treatment in animal models of pain and depression at the same time. In order to determine the coexistence of the antinociceptive and antidepressant-like effects of chronic treatment with antidepressants, we need to carry out both tests in the same animal with a minimal time interval between them. In addition, animal models and protocol used must not induce any interference on the behavioral response of the animal. Thus, we have chosen tail-flick as a nociceptive test, and Porsolt's test as a depression test, to determine the effectiveness of chronic antidepressant treatment. In addition, we could evaluate the influence of this treatment on brain areas processing somatic and affective dimensions of pain, measuring variations on mu-opioid receptor expression in these areas as previously described for other antidepressants as imipramine and fluoxetine (de Gandarias et al. 1998, 1999).

The aim of this study was to evaluate the antinociceptive and antidepressant-like effectiveness of chronic antidepressant treatment with nefazodone using tail-flick and Porsolt's tests, and its influence on the expression of mu-opioid receptors in cells of different brain areas related to both somatosensory and affective dimensions of pain using immunocytochemistry techniques to stain mu-opioid receptors.

Materials and methods

Experimental animals

The experimental protocols were reviewed and approved by the Local Committee for Animal Experimentation of the Faculty of Medicine at the University of Cádiz (License number 079604). Animal care complied with that stipulated by the local committee and conformed to the guidelines on the study of pain in awake animals established by the International Association for the Study of Pain (Zimmermann 1983).

Experiments were performed using albino male Wistar rats (225–250 g). All the animals were provided by the "Servicio de Experimentación y Producción Animal" (SEPA) at the University of Cádiz. Animals were maintained under standard conditions: 12 h light/dark schedule (lights on at 0800 hours) with ad libitum food and water and at constant temperature (21±1°C). All experimental procedures were performed in the light phase of the light/dark cycle by an experimenter who was blind to drug treatment.

Drugs

Nefazodone HCl (NFZ) (Bristol-Myers-Squibb, Spain) was dissolved in saline (0.9% NaCl). Nefazodone was administered IP at a total daily dose of 20 and 50 mg/kg (10 and 25 mg/kg/twice a day) using an injection volume of 1 ml/kg.

The anesthetic equithesin is a non-commercial solution made in our laboratory. It was prepared as follows: chloral hydrate (21.25 g) diluted in absolute ethanol (49.4 ml) +nembutal (81 ml)+propylene glycol (198 ml) +magnesium sulphate (10.64 g)+distilled water to make 500 ml. It was used at a dose of 0.2 ml/100 g body weight.

Experimental protocol

Chronic nefazodone treatment was administered twice a day for 2 weeks. At 12 h after the last dose of nefazodone, on day 15, a tail-flick test was performed. Then a new daily dose of nefazodone was administered and 12 h later Porsolt's test was carried out. Other animals were

processed, 2 h after completion of 14 days treatment, for mu-opioid receptor immunocytochemistry.

Tail-flick procedure

The antinociceptive responses were determined by measuring the time required to respond to a painful thermal stimulus (D'Amour and Smith 1941). An automated tail-flick analgesimeter (Ugo Basile 7360) was used to assess nociceptive responses to thermal stimuli in rats. In each trial, the rat was restrained on the instrument's upper panel in such a way that its tail, placed over a flush mounted window, received the IR energy. The time in seconds from initial heat source activation until tail withdrawal was recorded. Three latencies were measured per rat with a 5-min interval at different levels of rat tail (the base, the middle and the tip). The mean of three measurements was used for each experimental animal and was taken to be the tail withdrawal latency. To avoid excessive suffering of animals, a cut-off was set at 10 s.

Porsolt's test procedure

The forced swimming test (Porsolt et al. 1978) was performed without a previous adaptation period 24 h before. Briefly, rats were individually forced to swim inside vertical Plexiglas cylinders (height: 50 cm; diameter: 20 cm) containing 15 cm of water maintained at 25° C, for 5 min. The rat was judged to be immobile whenever it remained floating passively in the water in a slightly hunched but upright position, its head just above the surface.

Immunocytochemistry studies

The immunocytochemistry studies have been conducted as previously described by De Gandarias et al. (1998). Two hours after the last treatment, the animals were anaesthetized intraperitoneally with equithesin (2 ml/kg), and were perfused transcardially under deep anaesthesia with saline plus 50 mM phosphate buffer, pH 7.4, followed by 4% paraformaldehyde. The brains were removed, cut into smaller pieces and then immersed in the same fixative medium overnight. They were stored for 2 days in 0.1 M phosphate buffer containing 30% sucrose at 4°C. Sections (50 μm) were cut using a cryostatic microtome with a stereotaxic atlas guide and immunostained for mu-opioid receptor with polyclonal antisera raised in rabbits. The antigens were detected by the avidin-peroxidase technique using 3,3′-diaminobenzidine as chromogen. Following reduction of endogenous peroxidases with 1% hydrogen peroxide and blocking of non-specific background staining with 5% normal goat serum (NGS), the sections were incubated with the following immunoreagents: (1) primary antiserum: rabbit anti mu-opioid receptor (Chemicon International, Inc., Temecula, Calif., USA), a commercially obtained polyclonal antibody raised in rabbits and directed against mu-opioid receptor third extracellular loop peptide (dilution 1:1.000); (2) goat anti-rabbit immunoglobulin: goat anti-rabbit biotinylated (Chemicon International, Inc.), dilution 1:200; (3) avidin-peroxidase complex: strept ABC complex HRP (Dako A/S, Glostrup, Denmark), dilution 1:300; (4) chromogen: 3,3′-diaminobenzidine (Sigma-Aldrich Química SA), 0.3 mg/ml in 0.2 M Tris HCl buffer containing 0.03% hydrogen peroxide. Each step was followed by an appropriate wash in triplicate in phosphate buffer saline and 0.3% Triton X-100 was used. Sections were carefully extended, dehydrated, mounted and examined with an Olympus BX50F optic microscope (Olympus Optical Co. Ltd, Japan). Several brain regions were analyzed: frontal and cingulate cortex, periaqueductal gray and dorsal raphe nucleus. A Leica image analysis system (Quantimet 500 MC; Leica España SA, Barcelona, Spain) was used to obtain digitized images from brain slices and to trace a target area. To assess the density of positively stained neural cells, counts were manually made in a particular area that was previously determined by tracing contours with a cursor (target area: 4×104 m^2). At least six random counts of positively immunostained neural cells for a particular area were made in every section analyzed, and no fewer than ten sections were selected in each rat (n=12) and brain region.

Statistical analysis

The results obtained from the tail-flick and Porsolt tests are expressed as the mean±SEM of the tail withdrawal latency and immobility time, respectively. For statistical analysis, individual treatment effects were analyzed using the Student Newman–Keuls test following significant main effects of treatment by one-way ANOVA. The level of significance was $P<0.05$.

The results obtained from immunocytochemistry studies were taken as the number of positively immunostained cells for mu-opioid receptors in 4×10^{-2} mm^2 of nervous tissue, in treated animals and controls (mean±SEM). Differences between means were calculated by the Student t-test.

Results

Antinociceptive studies

One-way ANOVA showed a significant effect of nefazodone treatment [$F(2, 44)=6.89$, $P<0.005$]. Post-hoc Newman–Keuls comparisons showed a significant increase in the nociceptive threshold in rats receiving chronic nefazodone compared with saline-treated rats (NFZ 20=4.66±0.26 versus saline=3.66±0.17, $P<0.05$; NFZ 50=4.60±0.17 versus saline=3.66±0.17, $P<0.05$) (Fig. 1).

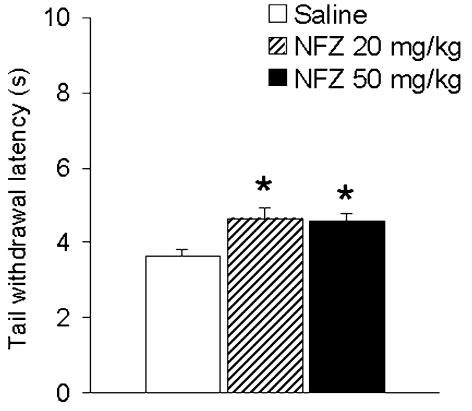

Fig. 1 Effect of chronic nefazodone treatment in tail-flick withdrawal latency (n=15–16; * P<0.05 versus saline)

Porsolt's test

One-way ANOVA showed a significant effect of nefazodone treatment [$F(2, 32)=17.87$, $P<0.001$]. Post-hoc Newman–Keuls comparisons showed a significant decrease in immobility time in rats receiving chronic nefazodone compared with saline-treated rats (NFZ 20=28.16±5.75 versus saline=58.90±4.00, $P<0.05$; NFZ 50=19.16±4.50 versus saline=58.90±4.00, $P<0.05$) (Fig. 2).

Immunocytochemical studies

Utilization of a specific polyclonal antibody against mu-opioid receptors showed the presence of abundant round cells intensely immunostained for mu-opioid receptors densely localized in the clusters of the striatum, cerebral cortex and subcallosal region. We found an increase in the density of neural cells immunostained for mu-opioid receptor in the frontal (NFZ 20=110.45±2.15 versus saline=79.35±1.87; $t=-10.91$, df 94, $P<0.001$) and cingulate (NFZ 20=98.66±1.11 versus saline=83.18±1.06; $t=-10.02$, df 94, $P<0.001$) cortices, dorsal raphe nucleus (NFZ 20=60.72±0.86 versus saline=41.69±0.40; $t=-4.11$, df 94, $P<0.01$) and periaqueductal gray (NFZ 20=126.22 ±3.17 versus saline=95.52±1.41; $t=-8.84$, df 94, $P<0.001$) after chronic nefazodone treatment, compared to controls (Figs. 3, 4).

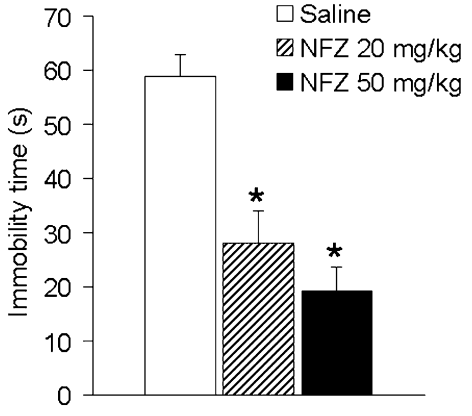

Fig. 2 Effect of chronic nefazodone treatment in immobility time response in rats (n=11–12; * P<0.05 versus saline)

Discussion

These results show that chronic nefazodone treatment displays antinociceptive and antidepressant-like effects, as measured by two behavioral tests. Moreover, it induces modifications of mu-receptor expression in the brain areas studied, leading to a significant increase in the number of neural cells expressing this receptor.

Regarding the antinociceptive effect of antidepressants in experimental pain in animals, this has been demonstrated mainly using acute treatments (Singh et al. 2001; Yokogawa et al. 2002; Zarrindast and Sahebgharani 2002). Among antidepressants, selective serotonin reuptake inhibitors have not shown a clear antinociceptive effect (Dirksen et al. 1998; Singh et al. 2001). Nefazodone is a serotonergic agent acting as a serotonin reuptake inhibitor and as a 5-HT_2 receptor antagonist (Bonhomme and Esposito 1998). In this sense, acute nefazodone treatment has shown an antinociceptive effect in the hot-plate test in mice but not in the tail-flick test (Pick et al. 1992), whereas there were no data from chronic treatment studies. However, our results with chronic treatment show a significant increase in pain threshold that was not related to dose.

In addition, a few studies have examined the effects of chronic administration of antidepressants in animal models of pain. Chronic administration of antidepressants for 28 days has shown a weak antinociceptive effect in the formalin test and no antinociceptive effect in the tail-flick test in rats (Ortega-Alvaro et al. 1994, 1997). While other studies using neuropathic pain models reported an antinociceptive effect with several antidepressants (Ardid and Guilbaud 1992; Lang et al. 1996; Esser et al. 2001). In this study, the tail-flick test was performed 12 h after last dose of nefazodone, when plasma levels had risen above the minimal level. The weak but significant antinociceptive effect displayed in animals treated with nefazodone

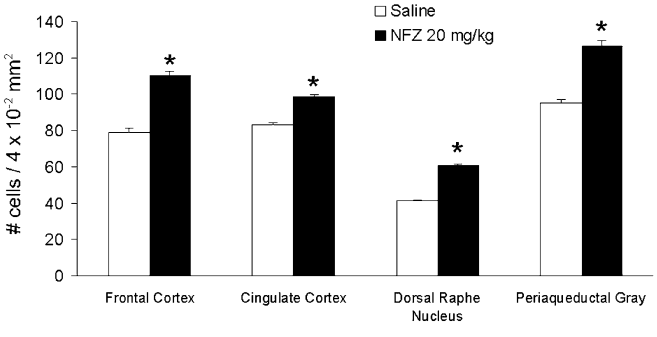

Fig. 3 Graphic representation of variations in the density of neural cells immunostained for mu-opioid receptors in the selected brain regions, expressed in cells/4×10^{-2} mm^2, in saline-treated and nefazodone-treated rats

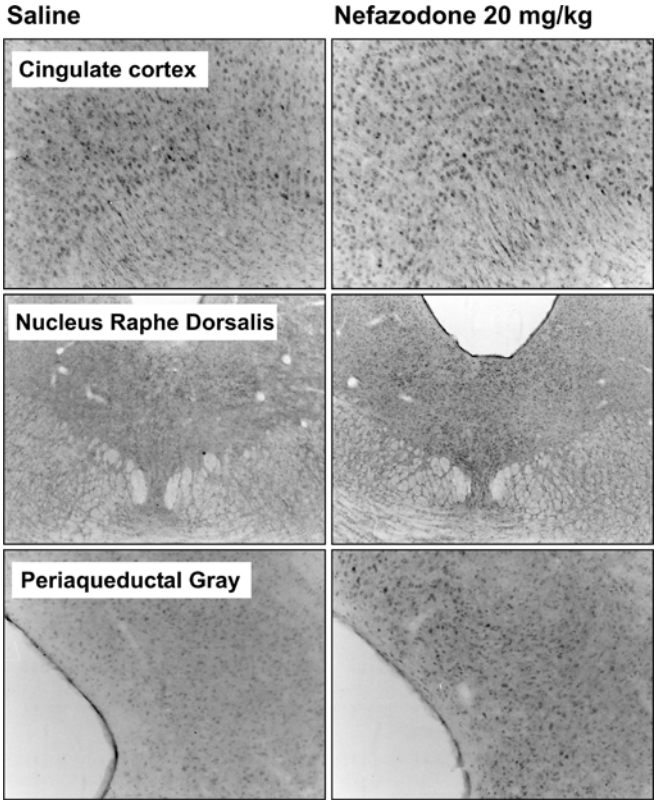

Fig. 4 Light micrographs showing mu-opioid receptor immunostaining in saline-treated rats (*left*) and nefazodone-treated rats (*right*). Abundant stained neuronal perykaria can be seen in the cingulate cortex, dorsal raphe nucleus and periaqueductal gray (×10)

should be taken into account. In fact, we have measured the variation of pain threshold induced by chronic nefazodone treatment. Thus, after administration of a new dose of nefazodone, it would be expected that a more potent antinociceptive effect could be achieved in tail-flick test.

The antidepressant-like effect showed by chronic nefazodone treatment confirms its principal clinical indication. Similarly, chronic 3-week treatment with imipramine and desipramine had a significant immobility-reducing effect in Porsolt's test, whereas the effect of amitriptyline was not significant and citalopram had no effect (Harro et al. 1997). Other positive results have been obtained with chronic imipramine (Duncan et al. 1998; Harvey et al. 2002) and fluoxetine (Duncan et al. 1998) treatment. Moreover, minaprine administration for 10 days reduced immobility time in Porsolt's test (Cabib et al. 1995).

Since nefazodone has shown an antinociceptive effect in these animals and, at the same time, an antidepressant-like effect, we could suggest that nefazodone treatment can act on the affective dimension of pain in a similar way to its action on sensory dimension (nociception).

Regarding the effect of chronic nefazodone treatment on the density of neural cells expressing mu-opioid receptors, there was a significant increase in all brain areas studied. Mu-opioid receptors are widely distributed throughout the central nervous system, mainly in the basal ganglia, limbic structures and cortical regions (Kaneko et al. 1995; Ding et al. 1996). This agrees with our results showing the presence of abundant stained cells in the frontal and cingulate, periaqueductal gray and dorsal raphe nucleus in control rats using immunocytochemical methods (Maneckjee et al. 1988; Gioannini et al. 1993). Since enkephalins may act as neuromodulators in the central nervous system and mediate pleasure, reward and emotions in rodents through their action on opioid receptors (Belluzzi and Stein 1977), we focused on the effects of in vivo nefazodone treatment on mu-opioid receptor expression in rat brain. Chronic nefazodone treatment generated an increase in the density of neurons expressing mu-opioid receptors in several regions, including anterior cingulate cortex, suggesting that the pharmacological action of nefazodone has an opioid-mediated neuroanatomical substrate with a direct involvement of brain areas related to the sensory and affective dimensions of pain. In this sense, subjective reduction of pain associated with opioid analgesia in human is associated with increased activity in rostral anterior cingulate cortex and right anterior insula (Petrovic et al. 2002). Therefore, we could suggest that nefazodone might affect affective dimension of pain through its action on the anterior cingulate cortex, and its antidepressant-like effect observed in Porsolt's test may serve as a reflect of this action on animal behavior. It is evident that we cannot establish a direct relationship between changes in mu-opioid receptor expression and modifications of affective state in these animals.

The opioid system is involved in the mechanisms underlying the physiopathology of depression and the action of antidepressants. Several behavioral studies have demonstrated an antidepressant-like effect of opioid peptides (Tejedor-Real et al. 1995; Kita et al. 1997) and opiates (Besson et al. 1996; Rojas-Corrales et al. 1998, 2002). In human, antidepressants require 2 weeks to elicit their therapeutic effect, although their neuronal biochemical effects begin a few hours after the first administration. This suggests that some adaptative mechanisms are "turned on" by them and it has been postulated that opioid receptors could participate in these mechanisms (Vilpoux et al. 2002). However, several studies in rats have shown contradictory effects of chronic antidepressant treatments on various components of the endogenous opioid system (De Felipe et al. 1985; Hamon et al. 1987; Kurumaji et al. 1988). With regard to opioid receptor, there have been reports of negative (Reisine and Soubrie 1982; Hamon et al. 1987), positive (Antkiewicz-Michaluk et al. 1984; Hamon et al. 1987) and null (Reisine and Soubrie 1982; Stengaard-Pedersen and Schou 1986; Hamon et al. 1987) effects of chronic antidepressant treatment on opioid receptor binding, perhaps due to differences in the antidepressants, experimental protocols and techniques used. Recently, Vilpoux et al. (2002) performed a systematic comparison of the consequences of chronic treatment with three new-generation non-tricyclic antidepressants (paroxetine, reboxetine and moclobemide)

on mu-opioid receptor binding in brain areas related mainly to the limbic system. They reported some increases in mu-opioid binding without any homogeneity in brain localization and a temporal lapse of treatment. After 4 days of treatment, paroxetine had increased binding in the cingulate cortex, whereas reboxetine had induced a decrease in the dorsal raphe nucleus. At the end of week 3, paroxetine and reboxetine had not induced any significant changes in mu-opioid binding in either the frontal and cingulate cortices or the dorsal raphe nucleus. These authors suggest that changes in mu-opioid receptors do not constitute a common feature of the mode of action of antidepressants (Vilpoux et al. 2002).

In contrast to previous works, we have used immunocytochemistry techniques to evaluate mu-opioid receptor expression because they provide information on localization and density of cells expressing it. In addition, this technique detects all mu-opioid receptors, including those in the interior of cells. However, this technique does not give us an idea of the functionality of these receptors.

Previously, using the same immunocytochemical protocol as ours, de Gandarias et al. (1998, 1999) demonstrated a common pattern of change in mu-opioid receptor expression after 14 days of treatment with imipramine and fluoxetine. A similar pattern was observed with nefazodone, but we chose cortical areas related to cognitive and emotional dimensions of pain and two nuclei (one serotonergic, i.e. the dorsal raphe, and one opioidergic, i.e. the periaqueductal gray) closely related with the descendent inhibitory pathway of pain (Millan 2002).

We did not assess the possible functionality of these increases in the number of cells expressing mu-receptors in the brain areas studied. For this purpose, we would now need to determine the effect of local administration of opiates in the areas studied.

In conclusion, chronic treatment with a serotonergic antidepressant like nefazodone induces antinociceptive and antidepressant-like effects in rats and at the same time produces an increase in the number of neural cells expressing mu-opioid receptors in brain areas related to the sensory and affective dimensions of pain. These results suggest that antidepressants could be effective on somatic and affective dimensions of pain and this action could be related to its influence on the opioid system.

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