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PERIPHERAL AND SPINAL 5-HT RECEPTORS PARTICIPATE IN THE PRONOCICEPTIVE AND ANTINOCICEPTIVE EFFECTS OF FLUOXETINE IN RATS

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Abstract—The role of 5-HT receptors in fluoxetine-induced nociception and antinociception in rats was assessed. Formalin produced a typical pattern of flinching and licking/lifting behaviors. Local peripheral ipsilateral, but not contralateral, pre-treatment with fluoxetine (0.3–3 nmol/paw) increased in a dose-dependent fashion 0.5% formalin-induced nociception. In contrast, intrathecal pretreatment with fluoxetine (0.3–3 nmol/rat) prevented nociception induced by formalin. The peripheral pronociceptive effect of fluoxetine was prevented by the 5-HT_{2A} (ketanserin, 3–10 pmol/paw), 5-HT_{2B} (3-(2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl)-2,4(1H,3H)-quinazolinedione (+) tartrate, RS-127445, 3–10 pmol/paw), 5-HT_{2C} (8-[5-(2,4-dimethoxy-5-(4-trifluoromethylphenylsulphonamido) phenyl-5-oxopentyl]1,3,8-triazaspiro[4.5] decane-2,4-dione hydrochloride, RS-102221, 3–10 pmol/paw), 5-HT_3 (ondansetron, 3–10 nmol/paw), 5-HT_4 ([1-[2-methylsulphonylamino ethyl]-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate, GR-113808, 3–100 fmol/paw), 5-HT_6 (4-iodo-N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]benzene-sulfonamide hydrochloride, SB-258585, 3–10 pmol/paw) and 5-HT_7 ((R)-3-(2-(2-(4-methylpiperidin-1-yl) ethyl) pyrrolidine-1-sulfonyl) phenol hydrochloride, SB-269970, 0.3–1 nmol/paw), but not by the 5-HT_{1A} (N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide maleate, WAY-100635, 0.3–1 nmol/paw), 5-$HT_{1B/1D}$ (N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1'-biphenyl-4-carboxamide hydrochloride hydrate, GR-127935, 0.3–1 nmol/paw), 5-HT_1B (1'-methyl-5-[[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydrospiro[furo[2,3-*f*]indole-3,4'-piperidine hydrochloride, SB-224289, 0.3–1 nmol/paw), 5-HT1D (4-(3-chlorophenyl)-α-(diphenylmethyl)-1-piperazineethanol hydrochloride, BRL-15572, 0.3–1 nmol/paw) nor 5-HT_{5A} ((*N*-[2-(dimethylamino)ethyl]-*N*-[[4'-[[(2-phenylethyl)amino]methyl] [1,1'-biphenyl]-4-yl]methyl]cyclopentanepropanamide dihydrochloride, SB-699551, 1–3 nmol/paw), receptor antagonists. In marked contrast, the spinal antinociceptive effect of fluoxetine was prevented by the 5-HT_{1A} (WAY-100635, 0.3–1 nmol/rat), 5-$HT_{1B/1D}$ (GR-127935, 0.3–1 nmol/rat), 5-HT_{1B} (SB-224289, 0.3–1 nmol/rat), 5-HT_{1D} (BRL-15572, 0.3–1 nmol/rat) and 5-HT_{5A} (SB-699551, 1–3 nmol/rat), but not by the 5-HT_{2A} (ketanserin, 3–10 pmol/rat), 5-HT_{2B} (RS-127445, 3–10 pmol/rat), 5-HT_{2C} (RS-102221, 3–10 pmol/rat), 5-HT_3 (ondansetron, 3–10 nmol/rat), 5-HT_4 (GR-113808, 3–100 fmol/rat), 5-HT_6 (SB-258585, 3–10 pmol/rat) nor 5-HT_7 (SB-269970, 0.3–1 nmol/rat), receptor antagonists. These results suggest that fluoxetine produces nociception at the periphery by activating peripheral 5-$HT_{2A/2B/2C/3/4/6/7}$ receptors. In addition, intrathecal fluoxetine produces antinociception by activation of spinal 5-$HT_{1A/1B/1D/5A}$ receptors.

Key words: fluoxetine, formalin test, mechanism of action, nociception, 5-HT receptors.

INTRODUCTION

Serotonergic descending pathway from rostral ventromedial medulla (RVM) to the dorsal horn is crucial to spinal nociceptive processing (Millan, 2002; Suzuki et al., 2004; Heinricher et al., 2009). Once released 5-hydroxytryptamine (5-HT) can exert facilitatory (Bee and Dickenson, 2007; Wei et al., 2010) or inhibitory (Zhao et al., 2007; Braz and Basbaum, 2008) influences onto dorsal horn neurons depending on the spinal 5-HT receptor subtype activated and on the cellular distribution of such receptors (Sommer, 2006). For instance, intrathecal injection of 5-HT produces antinociception (Yaksh and Wilson, 1979) while

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Abbreviations: ANOVA, one-way analysis of variance; AUC, area under the number of flinches against time curves; 5-HT, 5-hydroxytryptamine; BRL-15572, 4-(3-chlorophenyl)-α-(diphenylmethyl)-1-piperazineethanol hydrochloride; DRG, dorsal root ganglion; GR-113808, [1-[2-methylsulphonylamino ethyl]-4-piperidinyl] methyl 1-methyl-1H-indole-3-carboxylate; GR-127935, N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1′-biphenyl-4-carboxamide hydrochloride hydrate; RS-102221, 8-[5-(2,4-dimethoxy-5-(4-trifluoromethylphenyl sulphonamido)phenyl-5-oxopentyl]1,3,8-triazaspiro[4.5] decane-2,4-dione hydrochloride; RS-127445, 3-(2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl)-2,4(1H,3H)-quinazolinedione(+)tartrate; SB-224289, 1′-methyl-5-[[2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl) biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydrospiro[furo[2,3-*f*]indole-3,4′-piperidine hydrochloride; SB-699551, (*N*-[2-(dimethylamino)ethyl]-*N*-[[4′-[[(2-phenylethyl)amino]methyl][1,1′-biphenyl]-4-yl]methyl]cyclopentanepropanamide dihydrochloride; SB-258585, 4-iodo-N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]benzene-sulfonamide hydrochloride; SB-269970, (R)-3-(2-(2-(4-methylpiperidin-1-yl)ethyl)pyrrolidine-1-sulfonyl)phenol hydrochloride; SSRIs, selective serotonin reuptake inhibitors; WAY-100635, N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexane-carboxamide maleate.

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depletion of 5-HT reduces the antinociceptive effect of morphine (Sawynok and Reid, 1989) suggesting an inhibitory role for the descending serotonergic pathway. In contrast, depletion of spinal 5-HT reduces formalin-induced nociception (Svensson et al., 2006; Wei et al., 2010) indicating a facilitatory role. All 5-HT receptor subtypes (5-$HT_{1–7}$) are expressed in the dorsal root ganglion (DRG) and spinal dorsal horn (Pierce et al., 1996; Wu et al., 2001; Doly et al., 2004; Liu et al., 2005). Previous studies have shown that activation of the spinal 5-HT_{1A} (Mjellem et al., 1992; Oyama et al., 1996; Jeong et al., 2012), 5-HT_{1B} (Jeong et al., 2004; Liu et al., 2007) and 5-$HT_{1B/1D/1F}$ (Nikai et al., 2008) receptors attenuates nociception in models of inflammatory pain. Contrariwise, there is evidence that activation of spinal 5-$HT_{2/3/6/7}$ receptors (Oyama et al., 1996; Kjørsvik et al., 2001; Sasaki et al., 2001; Rocha-González et al., 2005; Castañeda-Corral et al., 2009) increases formalin-induced nociception.

Selective serotonin reuptake inhibitors (SSRIs), as fluoxetine, have been used as a first-line therapy for treating chronic pain in humans (Sindrup and Jensen, 1999; Crowell et al., 2004). Systemic administration of fluoxetine reduces nociception in inflammatory and neuropathic pain models (Nayebi et al., 2001; Singh et al., 2001; Pedersen et al., 2005; LaBuda and Little, 2005; Leventhal et al., 2007; Sikka et al., 2011). Furthermore, it has been reported that local peripheral injection of fluoxetine reduces formalin-induced nociceptive behavior while it increases paw volume (Sawynok et al., 1999). Fluoxetine exhibits high affinity for the 5-HT transporter (Owens et al., 1997; Tatsumi et al., 1997) and it blocks 5-HT reuptake, release and synthesis as well as neuronal discharge (Hjorth and Auerbach, 1994; Barton and Hutson, 1999). The antinociceptive effect of fluoxetine has been attributed to these actions prolonging the inhibitory actions of 5-HT on the spinal cord neurons involved in transmitting/modulating pain (Basbaum and Fields, 1978; Yaksh and Wilson, 1979). However, systemic fluoxetine can increase 5-HT levels in central (Beyer and Cremers, 2008; Nagayasu et al., 2010) as well as in peripheral sites (Weihe and Eiden, 2000; Bianchi et al., 2002; Vega and Rudolph, 2002; O'Connell et al., 2006; Mercado and Kilic, 2010). Since accumulation of 5-HT at the periphery and spinal sites may lead to different effects by activating several 5-HT receptors, the present study investigated the participation of peripheral and spinal 5-HT receptors in the effects of fluoxetine in formalin-induced acute nociception and long-lasting allodynia and hyperalgesia.

EXPERIMENTAL PROCEDURES

Animals

Female Wistar rats aged 8–10 weeks (weight range 180–220 g) from our own breeding facilities were used in this study. Animals were housed in a controlled environment with temperature maintained at 22 °C and a 12-h light/dark cycle. They had free access to food and drinking water before experiments. All experiments were in compliance with the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1996). Protocol was approved by our Institutional Animal Care and Use Committee (Cinvestav, México City, Protocol 455-09). Efforts were made to minimize the number of animals used and their suffering.

Spinal surgery

Rats underwent surgery for insertion of a spinal catheter for drug administration 5 days prior to formalin injection. Animals were anesthetized with a ketamine/xylazine mixture (45/12 mg/kg, i.p.), placed in a stereotaxic head holder, and the atlanto-occipital membrane was exposed (LoPachin et al., 1981). The membrane was pierced, and a PE-10 catheter (8 cm) was introduced intrathecally to the level of the thoracolumbar junction after which the wound was sutured. Rats were allowed to recover from surgery for at least five days in individualized cages before use. Animals showing any sign of motor impairment were euthanized in a CO_2 chamber.

Measurement of acute nociceptive activity

Antinociception was assessed using the formalin test described by Dubuisson and Dennis, 1977 with some modifications (Rocha-González et al., 2005). Briefly, rats were placed in open clear acrylic cylinders for 30 min to allow them to acclimate to their surroundings. Then, they were removed for formalin injection. Rats were gently restrained while the dorsum of the hind paw was injected with 50 μL of diluted formalin (0.5%) into the dorsal surface of the right hind paw with a 30-gauge needle. The animals were returned to the chambers and nociceptive behavior was observed immediately after formalin injection. Mirrors were placed in each cylinder to enable unhindered observation. Nociceptive behavior was quantified as the number of flinches of the injected paw/hindquarters during 1-min periods every 5 min, up to 60 min after injection (Wheeler-Aceto et al., 1990). Flinching was readily discriminated and was characterized as rapid and brief withdrawal, or as flexing of the injected paw/hindquarters. We decided to evaluate flinching because it is a simple and reliable parameter of pain behavior and one producing high scores (Wheeler-Aceto et al., 1990; Abbott et al., 1995). Formalin-induced flinching behavior was biphasic (Wheeler-Aceto et al., 1990; Rocha-González et al., 2005). The initial acute phase (0–10 min) was followed by a relatively short quiescent period, which was then followed by a prolonged tonic response (15–60 min). At the end of the experiment the rats were sacrificed in a CO_2 chamber.

Measurement of secondary allodynia and hyperalgesia

Rats were briefly immobilized to get open access to the right hind limb. Then, they received a s.c. injection of

saline solution or formalin (0.5%, 50 μL) into the dorsal surface of the hind paw using a 30-gauge needle (Fu et al., 2001). The sensitization induced by formalin was tested at 0, 1, 3, 6, 9 and 12 days after injection. The sixth day was chosen to evaluate nociceptive behaviors for subsequent experiments because secondary allodynia and hyperalgesia were already established at this time without evidence of tissue damage (Godínez-Chaparro et al., 2011). Rats were sacrificed in a CO_2 chamber at the end of the experiment.

Secondary mechanical allodynia and hyperalgesia were assessed as previously reported (Valencia-de Ita et al., 2006; Ambriz-Tututi et al., 2011). Briefly, rats were placed in testing cages with a wire mesh bottom and allowed to acclimate for 30 min. Baseline measurements were recorded first. Two von Frey filaments (Stoelting Co., Wood Dale, IL, USA, bending forces of 10 mN (1 g) and 250 mN (26 g) were applied ten times in each testing set at the base of the third toe on the plantar surface on both paws. Three trials were completed to get an average of the number of paw withdrawal responses. Under normal conditions, a force of 10 mN neither activates cutaneous nociceptors (Leem et al., 1993) nor causes paw withdrawal in normal animals. Accordingly, the occurrence of responses to the 10 mN filament was indicative of allodynia. On the other hand, a force of 250 mN or higher is considered a noxious stimulus, and hyperalgesia occurred when there was an increased response to the 250 mN filament. Allodynia and hyperalgesia were considered secondary as stimulation with the von Frey filaments was applied in a site different from that of the formalin injection.

Drugs

WAY-100635 (N-[2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl]-N-2-pyridinylcyclohexanecarboxamide maleate), GR-113808 ([1-[2-methylsulphonylamino ethyl]-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate), GR-127935 (N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1′-biphenyl -4-carboxamide hydrochloride hydrate), RS-127445 ((3-(2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl)-2,4(1H,3H)-quinazolinedione(+)tartrate), ondansetron, ketanserin and fluoxetine were purchased from Sigma–Aldrich (St. Louis, MO, USA).

SB-224289 (1′-methyl-5-[[2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydrospiro[furo[2,3-*f*]indole-3,4′-piperidine hydrochloride), BRL-15572 (4-(3-chlorophenyl)-α-(diphenylmethyl)-1-piperazineethanol hydrochloride), RS-102221 (8-[5-(2,4-dimethoxy-5-(4-trifluoromethylphenylsulphonamido) phenyl-5-oxopentyl]1,3,8-triazaspiro[4.5]decane-2,4-dione hydrochloride), SB-699551 (*N*-[2-(dimethylamino)ethyl]-*N*-[[4′-[[(2-phenylethyl)amino]methyl][1,1′-biphenyl]-4-yl] methyl]cyclopentanepropanamide dihydrochloride), SB-258585 (4-iodo-N-[4-methoxy-3-(4-methyl-1-piperazinyl) phenyl]benzene-sulfonamide hydrochloride) and SB-269970 ((R)-3-(2-(2-(4-methylpiperidin-1-yl) ethyl) pyrrolidine-1-sulfonyl) phenol hydrochloride) were purchased from Tocris (Elisville, MO, USA).

Formaldehyde (37%) was purchased from Merck Mexico, S.A. (Naucalpan, Mexico). Formaldehyde was dissolved in saline. Fluoxetine, ondansetron WAY-100635, ketanserin, GR-113808, GR-127935, SB-224 289, BRL-15572, SB-699551, SB-258585, SB-269970, RS-127445 and RS-102221 were dissolved in 1% dimethyl sulfoxide (DMSO).

Materials

The serotonergic drugs tested were selected based on relevant selectivity and efficacy (Table 1). We used fluoxetine (5-HT reuptake inhibitor (Tatsumi et al., 1997)). In addition, the following selective 5-HT receptor antagonists were tested: (i) WAY-100635 (selective 5-HT_{1A} receptor antagonist (Laporte et al., 1994; Forster et al., 1995)), GR-127935 (selective 5-$HT_{1B/1D}$ receptor antagonist (Skingle et al., 1995)), SB-224289 (selective 5-HT_{1B} receptor antagonist (Gaster et al., 1998; Selkirk et al., 1998)) and BRL-15572 (selective 5-HT_{1D} receptor antagonist (Price et al., 1997; Hagan et al., 1997)); (ii) ketanserin (5-HT_{2A} receptor antagonist (Knight et al., 2004)), RS-127445 (5-HT_{2B} receptor antagonist

Table 1. Binding affinity constants (pK_i) of fluoxetine and 5-HT receptor antagonists used in this study

Drugs	5-HT_{1A}	5-HT_{1B}	5-HT_{1D}	5-HT_{2A}	5-HT_{2B}	5-HT_{2C}	5-HT_3	5-HT_4	5-HT_{5A}	5-HT_6	5-HT_7
5-HT reuptake inhibitor											
Fluoxetine	–	–	–	6.5	5.3	7.3	–	–	–	5.8	–
Antagonists											
WAY-100635	**9.2**	5.1	5.6	–	–	–	–	–	–	–	–
GR-127935	7.2	**9.2**	**8.6**	–	–	7.0	–	–	–	–	–
SB-224289	5.5	**8.6**	6.3	5.3	5.9	6.2	–	–	–	–	–
BRL-15572	7.7	6.1	**7.9**	6.6	7.4	6.2	–	–	–	5.9	6.3
Ketanserin	–	–	–	**9.0**	5.4	–	–	–	–	–	6.7
RS-127445	–	–	–	6.0	**9.5**	6.3	–	–	–	–	–
RS-102221	–	–	–	6.8	–	**8.4**	–	–	–	–	–
Ondansetron	–	–	–	–	–	–	**8.3**	–	–	–	–
GR-113808	–	–	–	–	–	–	–	**9.4**	–	–	–
SB-699551	–	–	–	–	–	–	–	–	**8.2**	–	–
SB-258585	6.6	6.4	6.4	5.9	6.2	6.0	–	5.3	–	**8.3**	5.6
SB-269970	< 5	6.0	5.8	< 5	5	< 5	–	5.9	7.2	5.2	**8.9**

(Bonhaus et al., 1999; Knight et al., 2004)) and RS-102221 (selective 5-HT_{2C} receptor antagonist (Bonhaus et al., 1997; Knight et al., 2004)); (iii) ondansetron (selective 5-HT_3 receptor antagonist (Butler et al., 1988)); (iv) GR-113808 (selective 5-HT_4 receptor antagonist (Waeber et al., 1993; Gale et al., 1994)); (v) SB-699551 (selective 5-HT_{5A} receptor antagonist (Thomas et al., 2006)); (vi) SB-258585 (selective 5-HT_6 receptor antagonist (Hirst et al., 2000)); and (vii) SB-269970 (selective 5-HT_7 receptor antagonist (Thomas et al., 2000)).

Experimental design

We assessed first the effect of the local peripheral and intrathecal administration of fluoxetine in rats submitted to the formalin test. For this purpose, rats were pre-treated with increasing doses of fluoxetine into the paw (0.3, 1 and 3 nmol/paw) or at the intrathecal space (0.3, 1 and 3 nmol/rat).

In order to assess the possible participation of the peripheral 5-HT receptors in the fluoxetine-induced pronociceptive effects, rats were treated with the selective 5-HT_{1A} (WAY-100635, 0.3–1 nmol/paw), 5-$HT_{1B/1D}$ (GR-127935, 0.3–1 nmol/paw), 5-HT_{1B} (SB-224289, 0.3–1 nmol/paw), 5-HT_{1D} (BRL-15572, 0.3–1 nmol/paw), 5-HT_{2A} (ketanserin, 3–10 pmol/paw), 5-HT_{2B} (RS-127445, 3–10 pmol/paw), 5-HT_{2C} (RS-102221, 3–10 pmol/paw), 5-HT_3 (ondansetron, 3–10 nmol/paw), 5-HT_4 (GR-113808, 3–100 fmol/paw), 5-HT_{5A} (SB-699551, 1–3 nmol/paw) 5-HT_6 (SB-258585, 3–10 nmol/paw) and 5-HT_7 (SB-269970, 0.3–1 nmol/paw) receptor antagonists 10 min before local peripheral administration of fluoxetine (1 nmol/paw) which was injected 10 min before the formalin injection. To confirm the effects of locally injected fluoxetine, we pre-treated (10 min before) a separate group with the greatest dose of fluoxetine tested into the contralateral paw. In addition, to assess the effect of vehicle, animals were injected with saline or 1% DMSO 10 min before fluoxetine. Drugs were injected dissolved in 50 μL.

To investigate the possible participation of spinal 5-HT receptors in the fluoxetine-induced antinociceptive effects, rats were intrathecally treated with the selective 5-HT_{1A} (WAY-100635, 0.3–1 nmol/rat), 5-$HT_{1B/1D}$ (GR-127935, 0.3–1 nmol/rat), 5-HT_{1B} (SB-224289, 0.3–1 nmol/rat), 5-HT_{1D} (BRL-15572, 0.3–1 nmol/rat), 5-HT_{2A} (ketanserin, 3–10 pmol/rat), 5-HT_{2B} (RS-127445, 3–10 pmol/rat), 5-HT_{2C} (RS-102221, 3–10 pmol/rat), 5-HT_3 (ondansetron, 3–10 nmol/rat), 5-HT_4 (GR-113808, 3–100 fmol/rat), 5-HT_{5A} (SB-699551, 1–3 nmol/rat) 5-HT_6 (SB-258585, 3–10 pmol/rat) and 5-HT_7 (SB-269970, 0.3–1 nmol/rat) receptor antagonists 10 min before intrathecal administration of fluoxetine (3 nmol/rat) which was injected 10 min before the formalin injection. We designed the experiments to use doses of antagonists that *per se* do not modify formalin-induced nociception but that are able to block activation of their receptors as previously demonstrated for the 5-HT_6 (Castañeda-Corral et al., 2009) and 5-HT_7 (Rocha-González et al., 2005) receptors in acute pain and for 5-$HT_{3/4/6/7}$ receptors (Godínez-Chaparro et al., 2011; Bravo-Hernández et al., 2012) in chronic pain. Drugs were injected dissolved in 10 μL followed by 10 μL of 0.9% saline to flush the catheter. In all cases, doses of drugs were selected based on pilot experiments and formalin-induced nociception was determined as described above. The observer was unaware of the treatment given to each animal.

Finally, to investigate the effects of fluoxetine in chronic nociception, formalin-induced long-lasting secondary allodynia and hyperalgesia were determined after local peripheral and intrathecal administration of fluoxetine.

Data and statistical analysis

In all cases data are the mean (n = 6) ± S.E.M. Curves were constructed plotting the number of flinches as a function of time. The area under the number of flinches against time curves (AUC) was calculated by the trapezoidal rule. We used the AUC considering three points for the first phase (0, 5 and 10 min) and 10 points

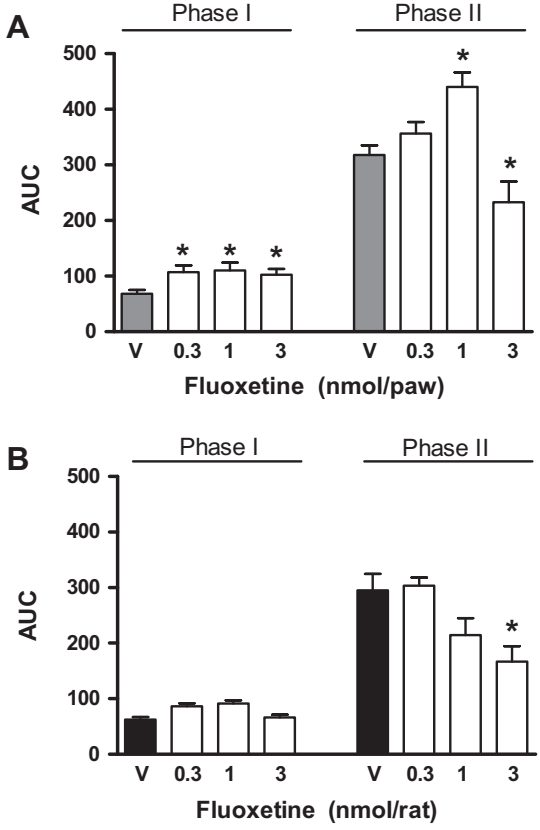

Fig. 1. Effect of the local peripheral (A) and intrathecal (B) pre-treatment (−10 min) with fluoxetine in rats submitted to the formalin test. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean (n = 6) ± S.E.M. Rats were pretreated with vehicle (V) or increasing doses of fluoxetine and 0.5% formalin. Note that local peripheral fluoxetine increased formalin-induced nociception during phases 1 and 2 (A) whereas that intrathecal injection produced antinociception (B). $^*P < 0.05$ vs V group, by one-way ANOVA followed by the Student–Newman–Keuls' test.

for the second phase (15, 20, 25, 30, 25, 40, 45, 50, 55 and 60 min).

One-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls' test was used to compare differences between treatments. Differences were considered significant when $P < 0.05$.

RESULTS

Subcutaneous 0.5% formalin injection into the right hind paw produced a pattern of flinching behavior characterized by a biphasic time course (data not shown). Phase 1 of the nociceptive response began immediately after formalin administration and then declined gradually in approximately 10 min. Phase 2 began about 15 min after formalin administration and lasted about 1 h (Wheeler-Aceto et al., 1990; Rocha-González et al., 2005).

Local peripheral ipsilateral injection of fluoxetine (0.3–1 nmol/paw) significantly increased 0.5% formalin-induced flinching behavior during both phases in the rats ($P < 0.05$) (Fig. 1A). In contrast, a greater dose of fluoxetine (3 nmol/paw) prevented formalin-induced phase 2 nociception (Fig. 1A). Furthermore, intrathecal administration of fluoxetine significantly prevented ($P < 0.05$) phase 2 nociception induced by the formalin injection (Fig. 1B).

Local peripheral injection of the selective 5-HT_{1A} (WAY-100635, 0.3–1 nmol/paw, Fig. 2A), 5-$HT_{1B/1D}$ (GR-127935, 0.3–1 nmol/paw, Fig. 2C), 5-HT_{1B} (SB-224289, 0.3–1 nmol/paw, Fig. 3A), 5-HT_{1D} (BRL-15572, 0.3–1 nmol/paw, Fig. 3C) or 5-HT_{5A} (SB-699551, 1–3 nmol/paw, Fig. 6A) receptor antagonists did not modify the pronociceptive effect induced by fluoxetine. In marked contrast, local peripheral administration of the selective 5-HT_{2A} (ketanserin, 3–10 pmol/paw, Fig. 4A), 5-HT_{2B} (RS-127445, 3–10 pmol/paw, Fig. 4C), 5-HT_{2C} (RS-102221, 3–10 pmol/paw, Fig. 4E), 5-HT_3 (ondansetron, 3–10 nmol/paw, Fig. 5A), 5-HT_4 (GR-113808, 3–100 fmol/paw, Fig. 5C), 5-HT_6 (SB-258585,

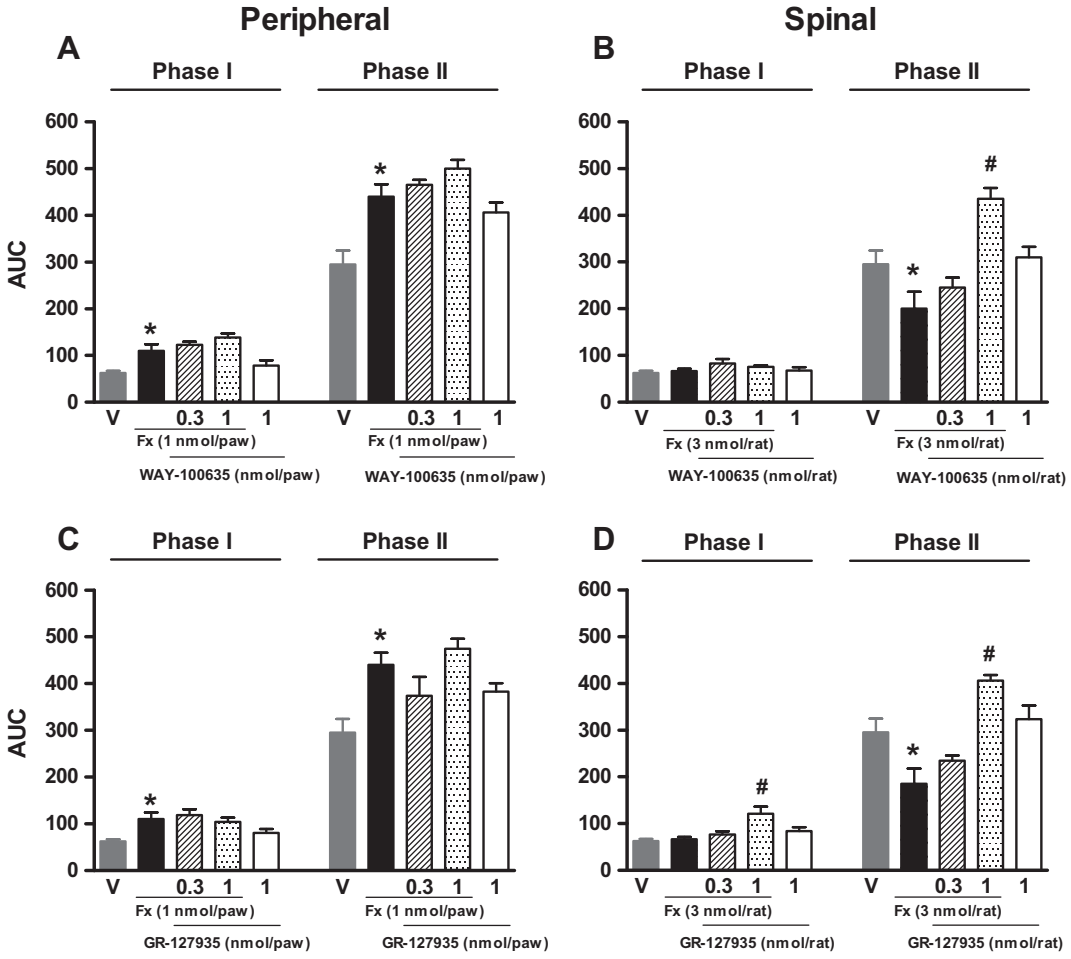

Fig. 2. Effect of peripheral (A and C) and intrathecal (B and D) pre-treatment with the 5-HT_{1A} (WAY-100635) and 5-$HT_{1B/1D}$ (GR-127935) receptor antagonists on the pronociceptive effect produced by fluoxetine (Fx) in rats submitted to the 0.5% formalin test. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean ($n = 6$) ± S.E.M. Rats were pretreated with vehicle (V), fluoxetine alone or co-administered with antagonists and 0.5% formalin. Note that local peripheral fluoxetine increased formalin-induced nociception during phases 1 and 2 whereas that peripheral injection of WAY-100635 and GR-127935 did not modify the pronociceptive effect induced by fluoxetine. In contrast, intrathecal injection of fluoxetine produced antinociception and WAY-100635 and GR-127935 prevented the spinal antinociceptive effect induced by fluoxetine. $^{*}P < 0.05$ vs V group, and $^{\#}P < 0.05$ vs fluoxetine group, by one-way ANOVA followed by the Student–Newman–Keuls' test.

3–10 pmol/paw, Fig. 7A) or 5-HT$_7$ (SB-269970, 0.3–1 nmol/paw, Fig. 7C) receptor antagonists significantly prevented ($P < 0.05$) the pronociceptive effect induced by fluoxetine (1 nmol/paw) in rats submitted to the 0.5% formalin test. Of note, ondansetron prevented the pronociceptive effect of fluoxetine along with that induced by formalin (Fig. 5A).

On the other hand, intrathecal injection of the selective 5-HT$_{1A}$ (WAY-100635, 0.3–1 nmol/rat, Fig. 2B), 5-HT$_{1B/1D}$ (GR-127935, 0.3–1 nmol/rat, Fig. 2D), 5-HT$_{1B}$ (SB-224289, 0.3–1 nmol/rat, Fig. 3B), 5-HT$_{1D}$ (BRL-15572, 0.3–1 nmol/rat, Fig. 3D) and 5-HT$_{5A}$ (SB-699551, 1–3 nmol/rat, Fig. 6B), but not the 5-HT$_{2A}$ (ketanserin, 3–101 pmol/rat, Fig. 4B), 5-HT$_{2B}$ (RS-127445, 3–10 pmol/rat, Fig. 4D), 5-HT$_{2C}$ (RS-102221, 3–10 pmol/rat, Fig. 4F), 5-HT$_3$ (ondansetron, 3–10 nmol/rat, Fig. 5B), 5-HT$_4$ (GR-113808, 3–100 fmol/rat, Fig. 5D), 5-HT$_6$ (SB-258585, 3–10 pmol/rat, Fig. 7B) nor 5-HT$_7$ (SB-269970, 0.3–1 nmol/rat, Fig. 7D), receptor antagonists significantly prevented ($P < 0.05$) the antinociceptive effect induced by intrathecal fluoxetine (3 nmol) in rats submitted to the 0.5% formalin test. Interestingly, this effect was prevented at a level beyond than that induced by formalin. At the doses used, local peripheral or intrathecal injection of the 5-HT receptor antagonists *per se* did not affect formalin-induced nociception (Figs. 2–7).

Besides acute nociception, formalin administration produced bilateral long-lasting secondary allodynia and hyperalgesia on the 6th day after injection (Fig. 8). Local peripheral pretreatment with fluoxetine (1 nmol/paw) significantly increased secondary allodynia and hyperalgesia in both paws (Fig. 8A, C). In contrast, intrathecal administration of fluoxetine (3 nmol/rat) prevented formalin-induced secondary allodynia and hyperalgesia (Fig. 8B, D).

DISCUSSION

We found that local peripheral injection of fluoxetine increased while intrathecal administration reduced formalin-induced nociception. Previous studies have

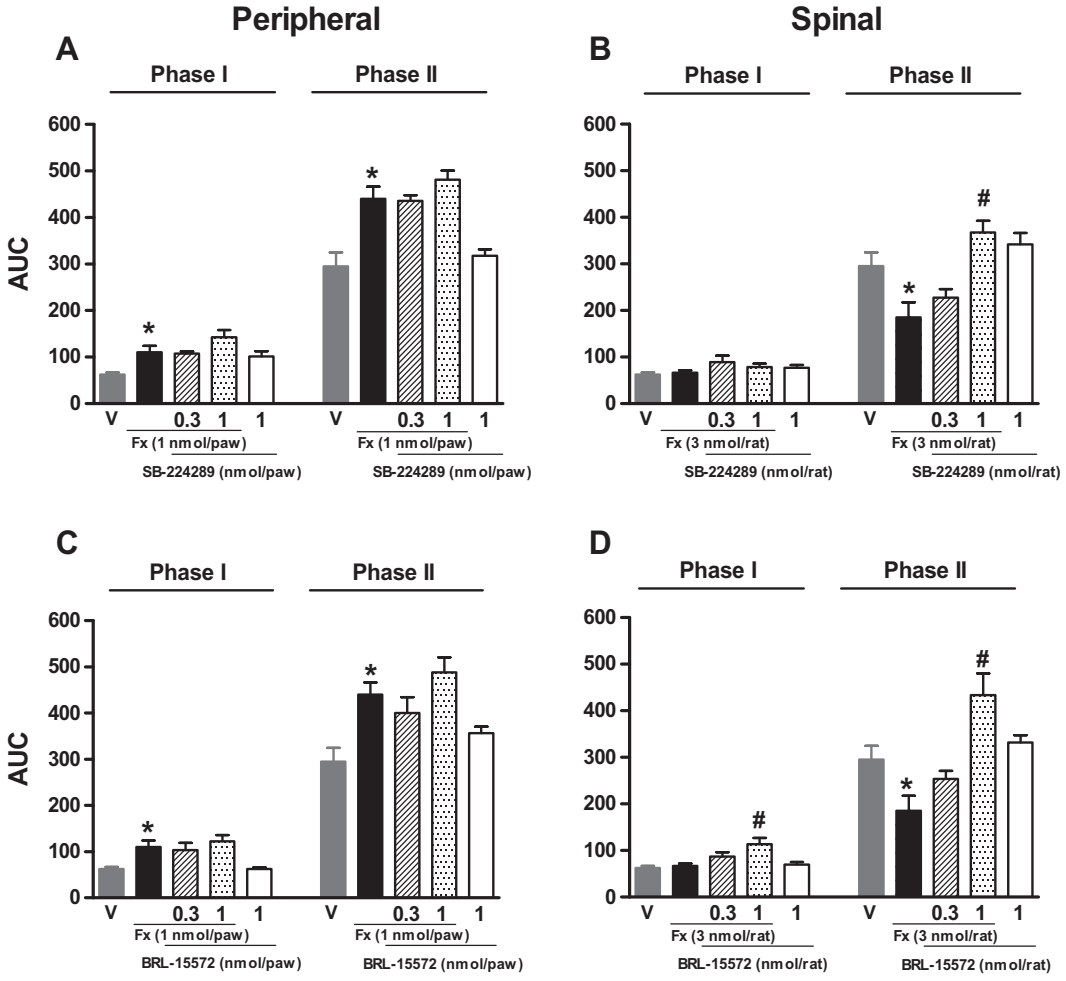

Fig. 3. Effect of peripheral (A and C) and intrathecal (B and D) pre-treatment with the 5-HT$_{1B}$ (SB-224289) and 5-HT$_{1D}$ (BRL-15572) receptor antagonists on the pronociceptive effect produced by fluoxetine (Fx) in rats submitted to the 0.5% formalin test. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean ($n = 6$) ± S.E.M. Rats were pretreated with vehicle (V), fluoxetine alone or co-administered with antagonists and 0.5% formalin. Note that local peripheral fluoxetine increased formalin-induced nociception during phases 1 and 2 whereas that peripheral injection of SB-224289 and BRL-15572 did not modify the pronociceptive effect induced by fluoxetine. In contrast, intrathecal injection of fluoxetine produced antinociception and SB-224289 and BRL-15572 prevented the spinal antinociceptive effect induced by fluoxetine. $^*P < 0.05$ vs V group, and $^\#P < 0.05$ vs fluoxetine group, by one-way ANOVA followed by the Student–Newman–Keuls' test.

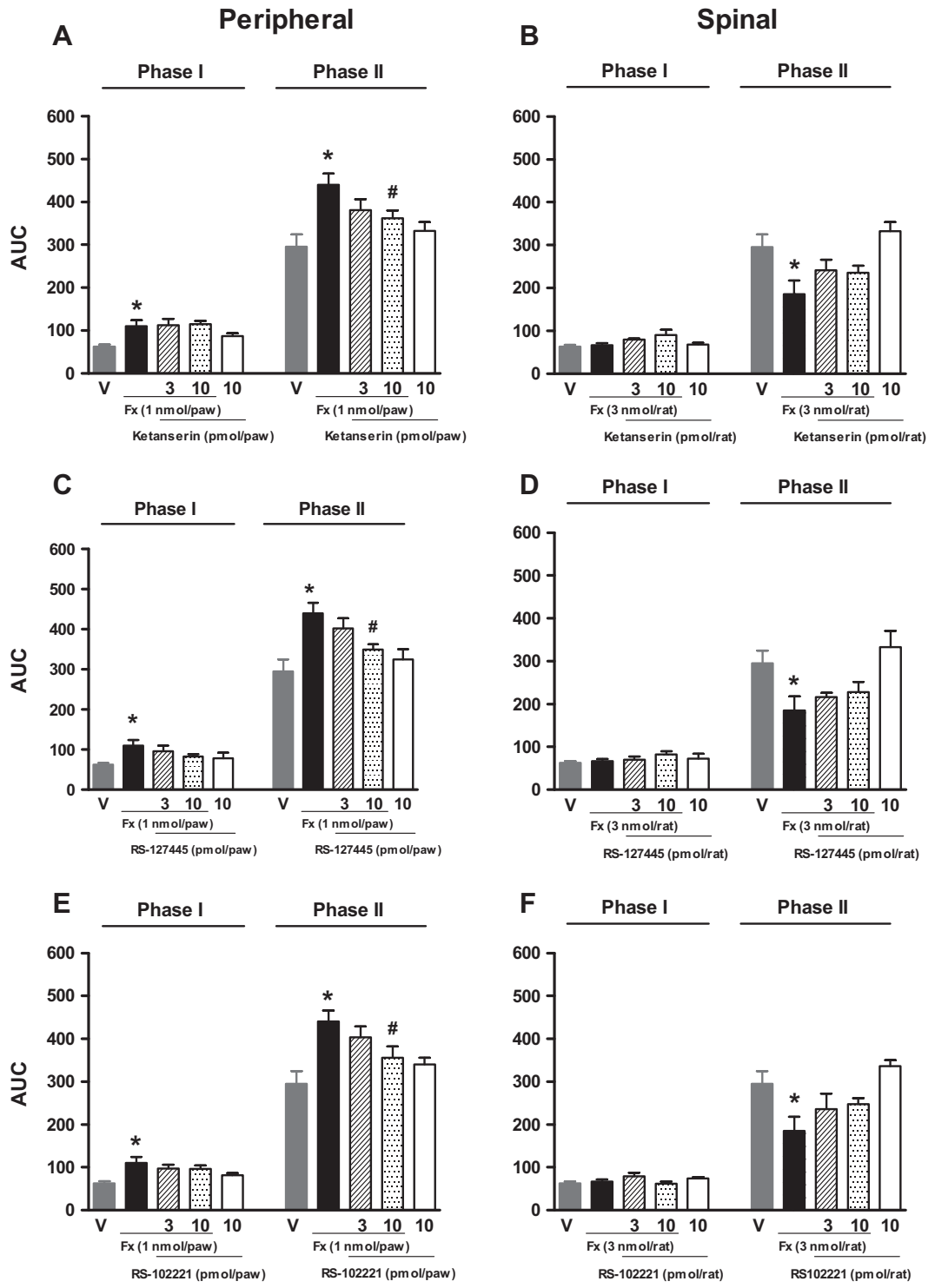

Fig. 4. Effect of peripheral (A, C, and E) and intrathecal (B, D and F) pre-treatment with the 5-HT_{2A} (ketanserin), 5-HT_{2B} (RS-127445) and 5-HT_{2C} (RS-102221) receptor antagonists in fluoxetine (Fx)-induced pronociception in rats submitted to the 0.5% formalin test. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean ($n = 6$) ± S.E.M. Rats were pretreated with vehicle (V), fluoxetine alone or co-administered with antagonists and 0.5% formalin. Note that local peripheral fluoxetine increased formalin-induced nociception during phases 1 and 2 whereas that peripheral injection of ketanserin, RS-127445 and RS-102221 partially prevented the pronociceptive effect induced by fluoxetine during phase 2. In contrast, intrathecal injection of fluoxetine prevented formalin-induced nociception and ketanserin, RS-127445 and RS-102221 did not modify the antinociceptive effect induced by fluoxetine during phase 2. $^{*}P < 0.05$ vs V group, and $^{\#}P < 0.05$ vs flouxetine group, by one-way ANOVA followed by the Student–Newman–Keuls' test.

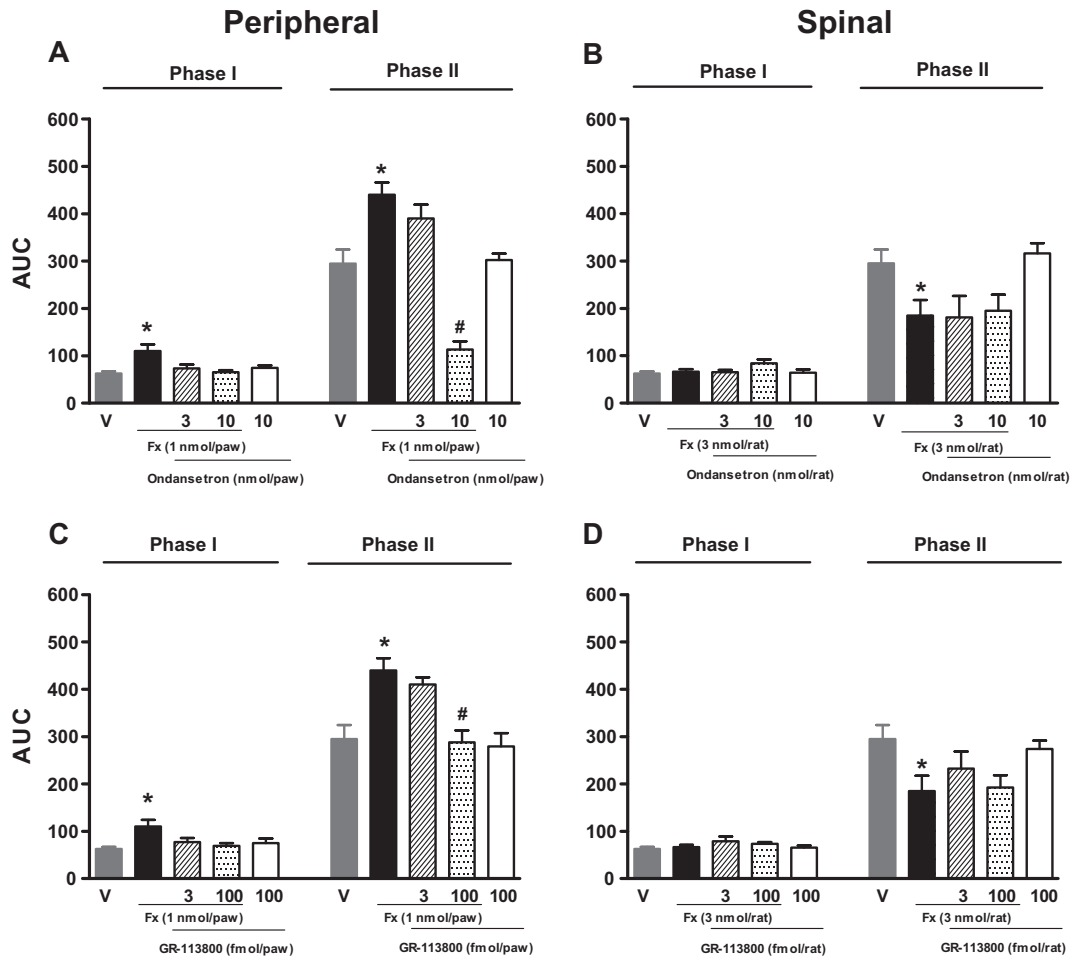

Fig. 5. Effect of peripheral (A and C) and intrathecal (B and D) pre-treatment with the 5-HT_3 (ondansetron) and 5-HT_4 (GR-113808) receptor antagonists on the pronociceptive effect produced by fluoxetine (Fx) in rats submitted to the 0.5% formalin test. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean (n = 6) ± S.E.M. Rats were pretreated with vehicle (V), fluoxetine alone or co-administered with antagonists and 0.5% formalin. Note that local peripheral fluoxetine increased formalin-induced nociception during phases 1 and 2 whereas that peripheral injection of ondansetron and GR-113808 prevented the pronociceptive effect induced by fluoxetine. In contrast, intrathecal injection of fluoxetine produced antinociception and ondansetron and GR-113808 did not modify spinal antinociceptive effect induced by fluoxetine. $^{*}P < 0.05$ vs V group, and $^{\#}P < 0.05$ vs fluoxetine group, by one-way ANOVA followed by the Student–Newman–Keuls' test.

reported that systemic (Singh et al., 2001; Nayebi et al., 2001; Pedersen et al., 2005) and intrathecal (Sawynok and Reid, 2001) administration of fluoxetine partially attenuate formalin-induced nociception. Thus, our study agrees with these observations and indicates that systemic/intrathecal administration of fluoxetine produces antinociception in the formalin test. At the periphery, lower doses of fluoxetine (0.3–1 nmol/paw) enhanced while a greater dose (3 nmol/paw) prevented the pronociceptive effect of formalin. Our study partially agrees with that of Sawynok et al. (1999) which reported that local injection of fluoxetine (30–300 nmol/paw) reduces flinching behavior and increases paw volume in rats submitted to the formalin test. In contrast, we observed an antinociceptive effect. Differences could be due to fluoxetine doses (1 versus 300 nmol/paw). Since fluoxetine is a high affinity (K_i 3×10^{-9} M) inhibitor of the serotonin transporter in the central nervous system (Tatsumi et al., 1997), and taking into account that this transporter is present in platelets (Mercado and Kilic, 2010), dendritic (O'Connell et al., 2006) and mast cells (Vega and Rudolph, 2002), we hypothesized that its pronociceptive and antinociceptive effect may be due to the actions of 5-HT on its peripheral receptors, as previously suggested for the inflammatory effect of fluoxetine (Sawynok et al., 1999). Accordingly, we found that ketanserin (5-HT_{2A} receptor antagonist (Knight et al., 2004)), RS-127445 (5-HT_{2B} receptor antagonist (Bonhaus et al., 1997, 1999; Knight et al., 2004)), RS-102221 (5-HT_{2C} receptor antagonist (Bonhaus et al., 1997; Knight et al., 2004)), ondansetron (5-HT_3 antagonist (Butler et al., 1988)), GR-113808 (5-HT_4 receptor antagonist (Waeber et al., 1993; Gale et al., 1994)), SB-258585 (5-HT_6 receptor antagonist (Hirst et al., 2000)) and SB-269970 (5-HT_7 receptor antagonist (Thomas et al., 2000)) prevented the pronociceptive effect of fluoxetine in the formalin test. In contrast, WAY-100635 (5-HT_{1A} receptor antagonist (Laporte et al., 1994; Forster et al., 1995)), GR-127935 (5-$HT_{1B/1D}$ receptor antagonist (Skingle et al., 1995)),

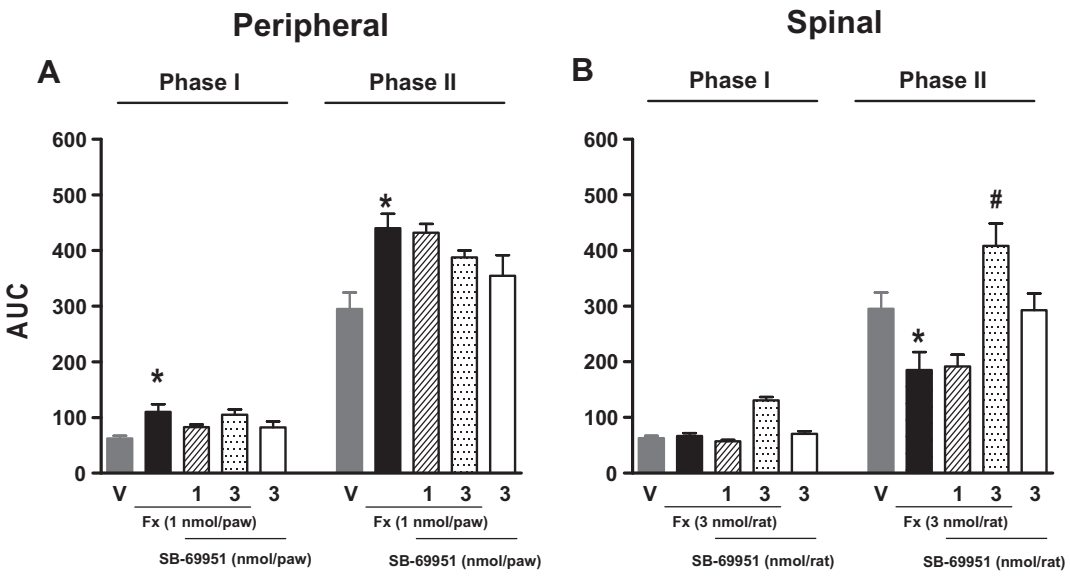

Fig. 6. Effect of peripheral (A) and intrathecal (B) pre-treatment with the 5-HT$_5$ (SB-69951) receptor antagonist on the pronociceptive effect produced by fluoxetine (Fx) in rats submitted to the 0.5% formalin test. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean (n = 6) ± S.E.M. Rats were pretreated with vehicle (V), fluoxetine alone or co-administered with antagonists and 0.5% formalin. Note that local peripheral fluoxetine increased formalin-induced nociception during phases 1 and 2 whereas that peripheral injection of SB-69951 did not modify the pronociceptive effect induced by fluoxetine. In contrast, intrathecal injection of fluoxetine produced antinociception and SB-69951 prevented the spinal antinociceptive effect induced by fluoxetine. $^{*}P < 0.05$ vs V group, and $^{\#}P < 0.05$ vs fluoxetine group, by one-way ANOVA followed by the Student–Newman–Keuls' test.

SB-224289 (5-HT$_{1B}$ receptor antagonist (Gaster et al., 1998; Selkirk et al., 1998)), BRL-15572 (5-HT$_{1D}$ receptor antagonist (Price et al., 1997; Hagan et al., 1997)) and SB-699551 (5-HT$_{5A}$ receptor antagonist (Thomas et al., 2006)) did not affect the pronociceptive effect of fluoxetine. Based on the antagonists' affinity (Table 1), our data suggest that fluoxetine promotes nociception indirectly at the periphery by releasing 5-HT which then activates 5-HT$_{2A/2B/2C/3/4/6/7}$ receptors. In support of this, formalin injection leads to local 5-HT release (Parada et al., 2001; Nakajima et al., 2009; Godínez-Chaparro et al., 2011) which acting *via* 5-HT$_{2A}$ (Tokunaga et al., 1998; Obata et al., 2000; Nakajima et al., 2009), 5-HT$_{2B}$ (Lin et al., 2011; Cervantes-Durán et al., 2012), 5-HT$_{2C}$ (Nakajima et al., 2009), 5-HT$_3$ (Sufka et al., 1992; Doak and Sawynok, 1997), 5-HT$_4$ (Doak and Sawynok, 1997), 5-HT$_6$ (Castañeda-Corral et al., 2009) and 5-HT$_7$ (Rocha-González et al., 2005; Brenchat et al., 2012) receptors leads to development of thermal hyperalgesia or to the increase of formalin-induced nociceptive behaviors suggesting that stimulation of peripheral 5-HT$_{2A/2B/2C/3/4/6/7}$ receptors plays a role in the nociception induced by 5-HT/formalin. Our data resemble these observations and suggest that fluoxetine avoids 5-HT reuptake at the periphery allowing it to act on its receptors to promote nociception. Reinforcing this, the mast cell degranulator compound 48/80 and membrane stabilizer cromoglycate prevent flinching behavior induced by formalin (Godínez-Chaparro et al., 2011). Collectively, these data suggest that formalin injection leads to 5-HT release from mast cells which then activate peripheral 5-HT$_{2/3/4/6/7}$ receptors.

The observation that 5-HT$_{1A/1B/1D}$ receptor antagonists do not prevent the pronociceptive effect of fluoxetine may be due to: (i) the greater affinity of 5-HT for the stimulatory (in affinity order: 5-HT$_7$ > 5-HT$_4$ > 5-HT$_{2B}$ > 5-HT$_6$ > 5-HT$_{2C}$) than for inhibitory (5-HT$_1$ and 5-HT$_5$) receptors (Barnes and Sharp, 1999; Rocha-González et al., 2005); (ii) activation of peripheral 5-HT$_7$ receptors leads to nociception (Rocha-González et al., 2005); and (iii) SSRIs have an excitatory effect on neuronal activity mediated by 5-HT$_7$ receptors (Bosker et al., 2009). In contrast, the peripheral antinociceptive effect of fluoxetine could be due to a greater accumulation of 5-HT that may lead to activation of antinociceptive, instead of pronociceptive, 5-HT receptors. Of note, ondansetron prevented the pronociceptive effect of fluoxetine to a level lower than that induced by formalin. A similar effect was observed when formalin was co-injected with exogenous 5-HT (Bravo-Hernández et al., 2012). In contrast, when formalin was co-injected with the 5-HT$_3$ receptor agonist m-CPBG, ondansetron was only able to block the pronociceptive effect of the agonist but not that induced by formalin. Speculating, the observed effect may represent a synergic action between the blockade of the pronociceptive effects of endogenous or exogenous 5-HT on peripheral 5-HT$_3$ receptors following formalin injection, by ondansetron, and activation of 5-HT$_{1/5}$ receptors. Since the formalin-induced nociceptive responses are reduced ~45% in 5-HT$_{3A}^{-/-}$ mice (Zeitz et al., 2002; Kayser et al., 2007), our data would suggest that activation of antinociceptive receptors (5-HT$_{1/5}$) by 5-HT contributes to the observed effect. Unlike several studies (Ali et al., 1996; Green et al.,

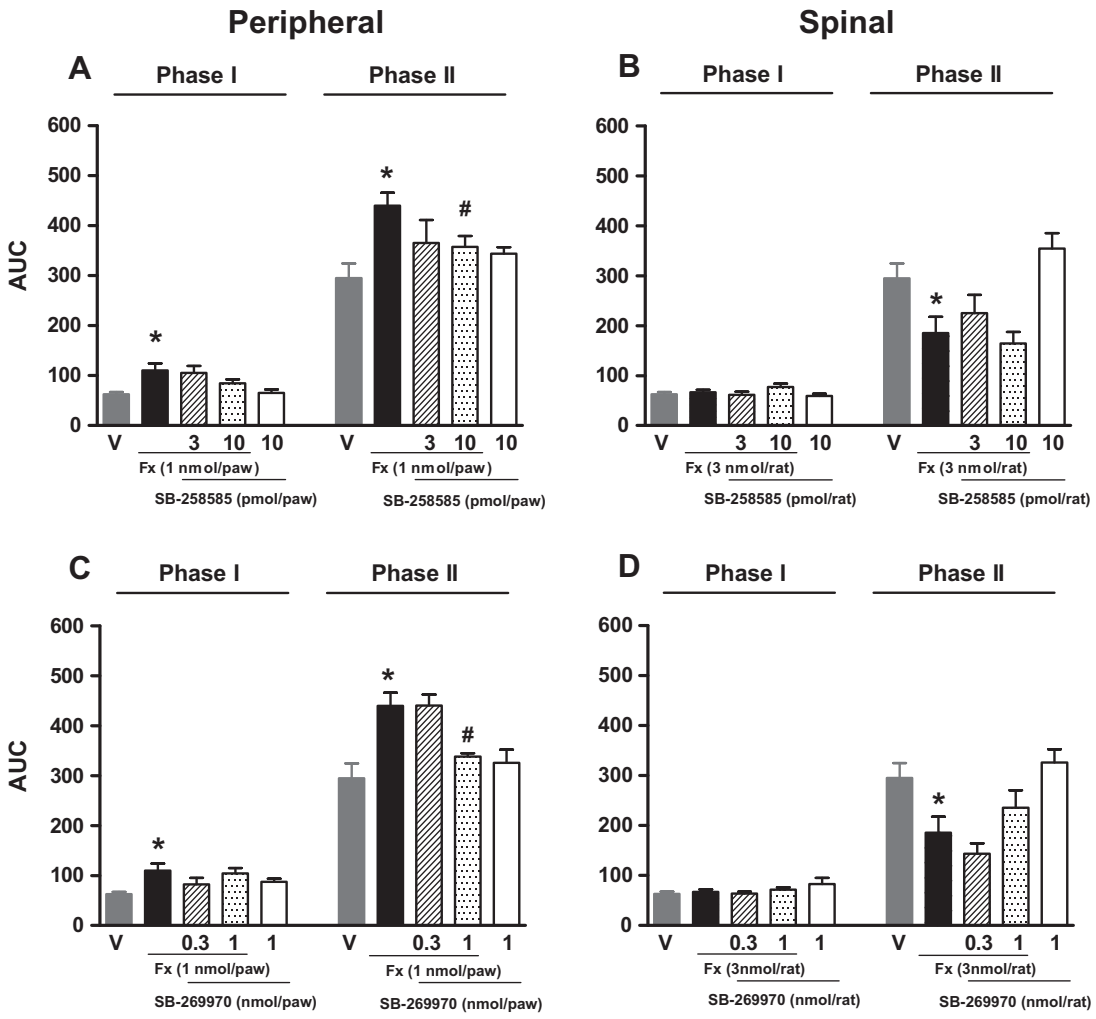

Fig. 7. Effect of peripheral (A and C) and intrathecal (B and D) pre-treatment with the 5-HT$_6$ (SB-258585), and 5-HT$_7$ (SB-269970) receptor antagonists on the pronociceptive effect produced by fluoxetine (Fx) in rats submitted to the 0.5% formalin test. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean (n = 6) ± S.E.M. Rats were pretreated with vehicle (V), fluoxetine alone or co-administered with antagonists and 0.5% formalin. Note that local peripheral fluoxetine increased formalin-induced nociception during phases 1 and 2 whereas that peripheral injection of SB-258585 and SB-269970 prevented the pronociceptive effect induced by fluoxetine. In contrast, intrathecal injection of fluoxetine produced antinociception and SB-258585 and SB-269970 did not modify spinal antinociceptive effect induced by fluoxetine. $^{*}P < 0.05$ vs V group, and $^{\#}P < 0.05$ vs fluoxetine group, by one-way ANOVA followed by the Student–Newman–Keuls' test.

2000; Svensson et al., 2006), local peripheral or intrathecal injection of ondansetron did not affect formalin-induced nociception. Difference could be due to the lower doses used in our study (10 nmol).

Contrary to the periphery, intrathecal injection of WAY-100635, GR-127935, SB-224289, BRL-15572 and SB-699551, but not ketanserin, RS-127445, RS-102221, ondansetron, GR-113808, SB-258585 nor SB-269970, prevented fluoxetine-induced acute antinociception in the formalin test. Data suggest that fluoxetine-induced acute accumulation of 5-HT in the spinal cord activates 5-HT$_{1A/1B/1D/5A}$ receptors which in turn reduces nociception. In support of this, formalin injection or complete Freund's Adjuvant increases 5-HT content in the spinal cord of rats (Godefroy et al., 1987; Svensson et al., 2006) or spinal nerve injury (Wei et al., 2010; Marshall et al., 2012). Fluoxetine would increase the 5-HT content by avoiding reuptake (Tatsumi et al., 1997). Of note WAY-100635 (Fig. 2B), GR-127935 (Fig. 2D), SB-224289 (Fig. 3B), BRL-15572 (Fig. 3D) and SB-699551 (Fig. 6B) blocked the antinociceptive effect of fluoxetine to a level greater than that produced by formalin. The explanation of this phenomenon is unclear. However, it is likely that blockade of antinociceptive receptors (5-HT$_{1A/1B/1D/5A}$) by their respective antagonists may lead to a pronociceptive effect by 5-HT through pronociceptive receptors (5-HT$_{2/3/4/6/7}$). Our study agrees with previous observations that activation of spinal 5-HT$_{1A}$ receptors leads to antinociception (Mjellem et al., 1992; Oyama et al., 1996; Jeong et al., 2012). Spinal activation of 5-HT$_{1B}$ receptors has produced conflicting results (Mjellem et al., 1992; Zhang et al., 2001). Regarding 5-HT$_{1D}$ receptors, there is evidence that the spinal 5-HT$_{1B/1D/1F}$ agonist sumatriptan reduces nociception (Nikai et al., 2008; Zhang et al., 2001). The participation

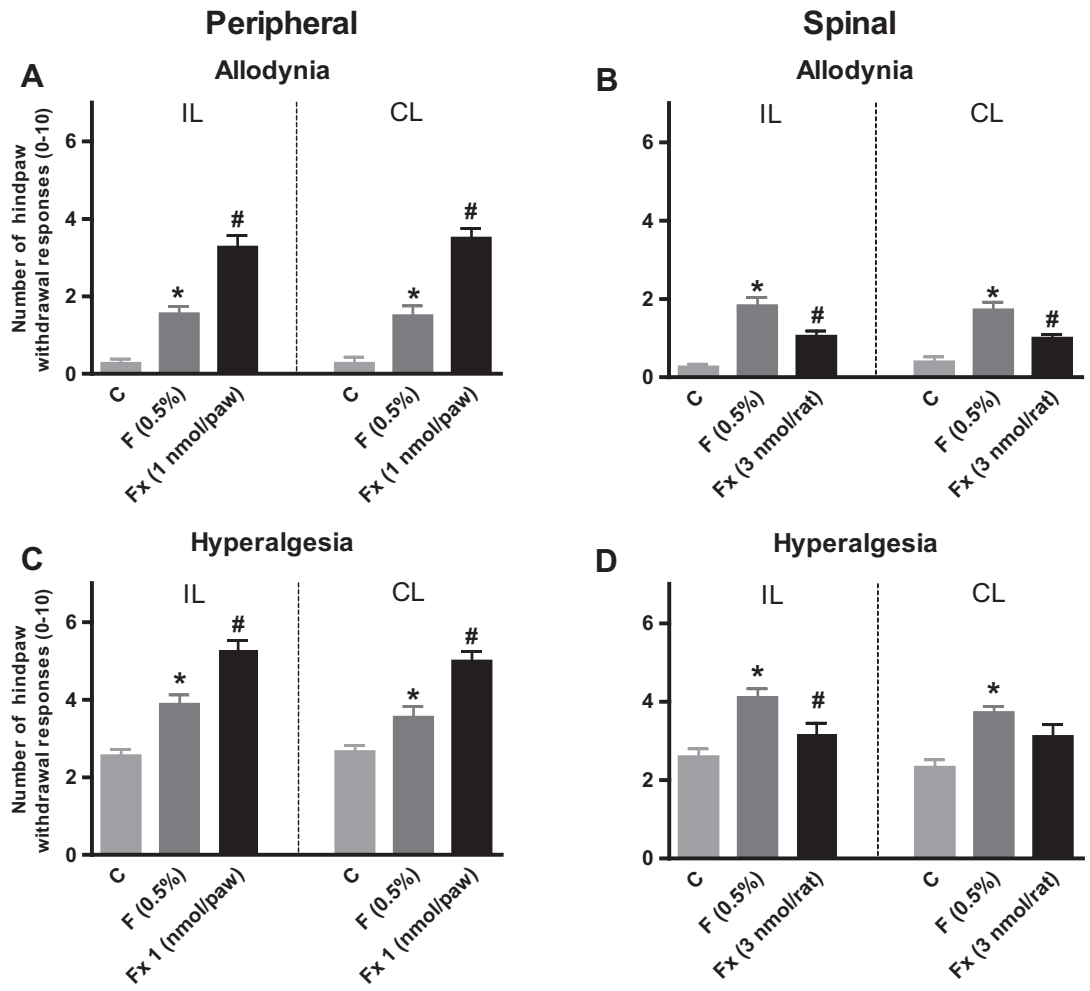

Fig. 8. Effect of peripheral and intrathecal pre-treatment with fluoxetine (Fx) in 0.5% formalin-induced secondary mechanical allodynia (panels A and C) and hyperalgesia (panels B and D) in rats. Data are expressed as the paw withdrawal ipsilateral (IL) and contralateral (CL) responses to the applications of von Frey filaments (10 and 250 mN) to the plantar surface of rat paws before (control, C) and after formalin (F) injection. $^{*}P < 0.05$ vs C group and $^{\#}P < 0.05$ vs F group, by one-way ANOVA followed by the Student–Newman–Keuls' test.

of spinal 5-HT_{5A} receptors in pain is unknown. 5-HT_{5A} receptors are present in post-synaptic neurons of laminae I–II and at a lesser extent in laminae III–IV of the dorsal horn (Doly et al., 2004). This localization suggests that 5-HT_{5A} receptors participate in the control of nociception exerting an inhibitory effect (Doly et al., 2004). Our results confirm for the first time this suggestion. These data strongly support our suggestion that fluoxetine produces antinociception by accumulation of 5-HT in the dorsal spinal cord which in turn activates spinal 5-$HT_{1A/1B/1D/5A}$ receptors. Reinforcing this, 5-$HT_{1A/1B/1D/5A}$ receptors are found in the dorsal root ganglia and spinal cord (Pierce et al., 1996; Chen et al., 1998; Doly et al., 2004) and are negatively coupled to adenylyl cyclase (Francken et al., 1998; Barnes and Sharp, 1999).

Since fluoxetine is a 5-HT reuptake inhibitor that produces its central antinociceptive effect through the actions of 5-HT on 5-$HT_{1/5}$ receptors, we could speculate that other drugs that share the same mechanism of action (such as tricyclic anti-depressant drugs, selective and non-selective 5-HT reuptake inhibitors) would also active these receptors. There are experiments running in our laboratory to demonstrate this possibility.

Data suggest that fluoxetine produces its effects by indirect activation of several 5-HT receptors. However, fluoxetine may act like a low affinity antagonist at 5-HT_{2C} receptors (Table 1). Thus, we cannot discharge that it may exert part of its antinociceptive effect directly binding some of them. As with the acute effects, local peripheral injection of fluoxetine increased whereas that intrathecal administration prevented formalin-induced long-lasting secondary allodynia and hyperalgesia. These data suggest that fluoxetine acts on the same receptors to produce its acute and chronic effects. However, it is important to state that our study does not predict the effect of fluoxetine after chronic use.

CONCLUSION

Our data indicate that local peripheral injection of fluoxetine increases formalin-induced nociception

whereas that intrathecal injection of this drug prevents it. The pronociceptive effect of fluoxetine seems to result from activation of peripheral 5-$HT_{2A/2B/2C/3/4/6/7}$, but not of 5-$HT_{1A/1B/1D/5A}$, receptors. In contrast, its spinal antinociceptive effect seems to result from activation of 5-$HT_{1A/1B/1D/5A}$, but not of 5-$HT_{2A/2B/2C/3/4/6/7}$, receptors. Spinal activation of 5-HT_{5A} receptors could be a therapeutic target to treat inflammatory pain.

CONFLICT OF INTEREST

The authors state no conflicts of interest.

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