Pharmacology, Biochemistry and Behavior 91 (2009) 441–446

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Intracerebroventricular fluvoxamine administration inhibited pain behavior but increased Fos expression in affective pain pathways

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ARTICLE INFO

Article history:
Received 11 December 2007
Received in revised form 19 August 2008
Accepted 27 August 2008
Available online 12 September 2008

Keywords:
Prefrontal cortex
Amygdala
Nociception

ABSTRACT

Anti-nociceptive effects of fluvoxamine, administered by intracerebroventricular (i.c.v.) injection, include inhibited pain behavior in both formalin-induced acute pain ($p<0.05$–0.01) and sciatic nerve ligation-allodynia ($p<0.03$). A 5-HT_1 receptor antagonist (WAY-100635) and a 5-HT_2 receptor antagonist (ketanserin), injected i.c.v., induced hyperalgesia and inhibited fluvoxamine's anti-nociceptive effects. We also investigated how fluvoxamine affects neural activities in brain areas involved in affectional pain using Fos-like protein immunohistochemistry. The acute pain and allodynia increased Fos-positive cells in the prefrontal cortex (PFC), basolateral nucleus (BL) and central nucleus of the amygdala (Ce), indicating that these areas are involved in pain processing. Fluvoxamine did not block the Fos expression, though it did produce anti-nociception. Moreover, fluvoxamine alone increased Fos in the BL and PFC. Ketanserin did not decrease the Fos expression induced by fluvoxamine. The results indicated that 5-HT_2 receptor activities participate minimally in Fos induction by fluvoxamine in the PFC and BL. In contrast, WAY-100635 affected the Fos expression produced by fluvoxamine. In the portion of the brain with affectional pain pathways, 5-HT_1 receptor activities induced anti-nociceptive effects and decreased Fos expression with fluvoxamine, while 5-HT_2 receptor activation affected to anti-nociceptive effects but did not induce Fos expression.

1. Introduction

Anti-nociceptive effects of antidepressants on opiate insensitive intractable pain (Blumer et al., 1980; Evans, 1985; France et al., 1984; Gordon and Love 2004; Merskey and Hester, 1972; Silverstrini and Lisciani, 1973) were discovered to have an unpredicted advantage. Serotonin-selective reuptake inhibitors (SSRI), which are prescribed for anxiety based on their anxiolytic action (Izumi et al., 2006; Jensen et al., 1999; Nemoto et al., 2003), have also been used for anti-nociception in treating inflammatory (Jones et al., 2005; Petitto et al., 1992; Rani et al., 1996), chronic (Goldstein et al., 2004; Jung et al., 1997; Taylor and Rowbotham, 1996) and neuropathic pain (Mattia and Coluzzi, 2003; Shimodozono et al., 2002; Sumpton and Moulin, 2001; Wilson, 1999). We previously reported that 5-HT_{1A} and 5-HT_3 antagonists impaired anti-nociceptive effects induced by antidepressants in formalin tests (Zhang et al., 2004a). Acute SSRI administration increases brain extracellular serotonin (Bundgaard et al., 2006) and the anti-nociceptive effect of SSRI was considered to be induced via 5-HT receptor activities (Bonnefont et al., 2005).

Lesions of prefrontal cortex (PFC) impaired pain conditioned avoidance responses (Gao et al., 2004), suggesting that the areas related to pain are responsible for the affectional–emotional component of pain. The PFC has been regarded as the center of the medial pain system (Price, 2000; Vogt, 1993). Human brain imaging analysis by fMRI (Büchel et al., 2002; Buffington et al., 2005; Rainville et al., 1997) indicated that PFC neural activities mediate the affectional dimension of pain. The amygdala also plays a role in pain and fear conditioned avoidance tests (Gao et al., 2004). In pain processing, nociceptive responses elicited by peripheral noxious stimulation were modulated by amygdala stimulation (Mena et al., 1995). The PFC and amygdala have numerous reciprocal connections (Cassell and Wright, 1986; Bacon et al., 1996; McDonald, 1998). High frequency stimulation (HFS) delivered to basolateral amygdala induced long-term potentiation (LTP)-like effects in the PFC (Maroun and Richter-Levin, 2003). HFS delivered to the amygdala inhibited nociceptive responses in PFC, suggesting that the amygdala modulates PFC nociceptive responses (Izawa and Kawakami, 2006). Moreover, SSRIs affect neuronal activities in the PFC (Gronier and Rasmussen, 2003; Ohashi et al., 2002) and the amygdala (Veening et al., 1998). These brain areas, both of which participate in pain and emotion, may be involved in the affectional dimension of pain and both are possible fluvoxamine target areas.

Nociceptive stimulation induced Fos expressions in various brain areas, including the PFC (Zhang et al., 2004b) and the amygdala (Nakagawa et al., 2003). Anti-nociceptive effects of fluvoxamine were anticipated to block Fos increases in these brain areas. Previous

Abbreviations: BL, basolateral nucleus of the amygdala; Ce, central nucleus of the amygdala; i.c.v., intracerebroventricular; PFC, prefrontal cortex; SSRI, selective serotonin reuptake inhibitor.

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doi:10.1016/j.pbb.2008.08.029

studies found, however, that administration of SSRI alone increased Fos expression in various brain areas (Morelli et al., 1999) including the PFC (Jongsma et al., 2002). We attempted to identify the anti-nociceptive effects of fluvoxamine on Fos expression in the PFC and amygdala in acute pain and allodynia.

Many clinical reports have described depressive symptoms and chronic pain as often being seen together (Clark and Treisman, 2004; Lépine and Briley, 2004), though the nature of this relationship between the two remains unclear. Serotonin and dopamine levels in the amygdala and PFC rise in response to psychological stress (Kawahara et al., 1993; Yokoyama et al., 2005). Moreover, anticipatory anxiety induced by pain involves the PFC (Simpson et al., 2001) and the amygdala (Narita et al., 2006), suggesting that these brain areas may be key to elucidating the connection between pain and depression.

2. Methods

2.1. Animals

All experiments conformed to the guidelines for animal care of the Animal Experiments Committee of Tokyo Women's Medical University. Male Slc–ddY mice (25–30 g, Sankyo Lab Co) were kept on a 12-h light, 12-h dark cycle under constant temperature and humidity for one week before the experiments. Experiments were performed during the light phase of the light–dark cycle.

2.2. Intracerebroventricular (i.c.v.) injection

We used the i.c.v. route to inject a precise dose of fluvoxamine into the brain. Effects of fluvoxamine on the supra-spinal level can be clearly observed with i.c.v. injection methods (Fu et al., 2006; Pan et al., 2007). Direct effects of SSRI on the brain activities, which were our main interest, would be more conspicuous than other administration methods. We detected precisely peak expression of Fos in the brain by acute i.c.v. injections. The original i.c.v. injection method in the conscious mouse was reported by Haley in 1957 (Haley and McCromick, 1957). The procedure did not affect tail-flick responses (Harris et al., 1975) and has thus been regarded as a useful tool in pain and other behavioral experiments (Herman, 1975; Laursen and Belknap, 1986; Pan et al., 2007; Sánchez-Blázquez et al., 1995). We confirmed that i.c.v. saline injection had no effects on behavior or motor system prior to these experiments (Zhang et al., 2004a). Drugs were injected transcutaneously with a micro-syringe (Hamilton, with a 31 G needle) into the cerebral ventricle (Target point is 2 mm lateral to the midline, 1.5–2.0 mm posterior to the Bregma) under halothane anesthesia. The mice recovered from anesthesia and showed normal behavior within 10 min after injection.

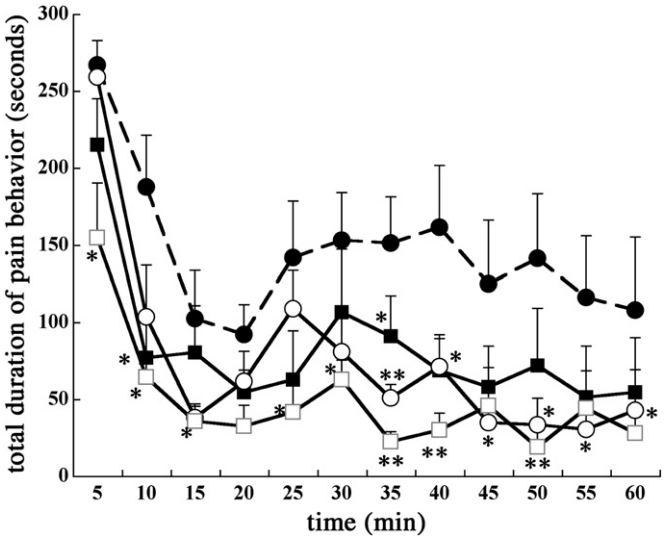

Fig. 1. Formalin tests. A fluvoxamine dose of 2 μg (solid squares, $n=7$), 6 μg (open circles, $n=7$) or 12 μg (open squares, $n=7$) was injected i.c.v. The 6 and 12 μg fluvoxamine doses clearly reduced pain responses as compared to the control, saline injection (solid circles). The largest fluvoxamine dose (12 μg) inhibited the first phase of responses (5 min) as well as the second phase.

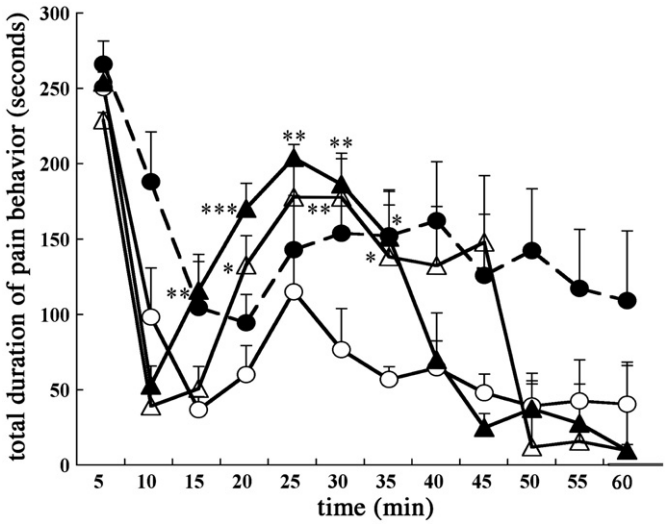

Fig. 2. 5-HT receptor antagonists affect anti-nociceptive action of fluvoxamine. Results of formalin tests. Solid circles: untreated control, Open circles: 6 μg fluvoxamine (i.c.v.). Open triangles: WAY-100635(5-HT_1 receptor antagonist, $n=5$), Solid triangles: Ketanserin (5-HT_2 receptor antagonist, $n=5$). 5-HT receptor antagonists reduced anti-nociceptive effects of fluvoxamine. Hyperalgesia was observed from 20 to 30 min. WAY-100635 (or Ketanserin) versus fluvoxamine.

2.3. Drug application

On the day of the experiments, the mice were allowed to move freely in the observation box for 1 h before injection. Fluvoxamine (2 μg, 6 μg, 12 μg, $n=7$ for each group) dissolved 0.9% saline (6 μl) or 6 μl of saline was injected transcutaneously with a micro-syringe into the cerebral ventricle. Prior to these experiments, we examined the motor functions of mice under i.c.v. fluvoxamine (6 μg) conditions, using rota-rod tests ($n=10$). There were no significant differences between pre and post-i.c.v. fluvoxamine injections results. Ketanserin (6 μg in 0.9% saline, SIGMA, $n=5$) or WAY1000635 (6 μg in 0.9% saline,

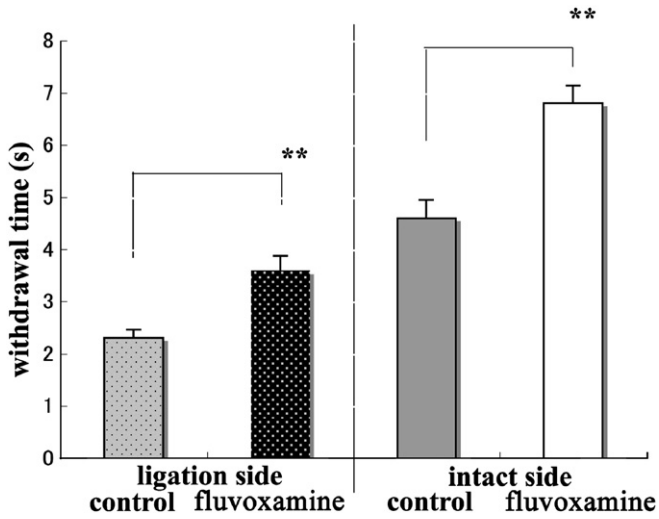

Fig. 3. Plantar tests in sciatic nerve ligation-allodynia. Fluvoxamine (i.c.v.) increases withdrawal duration on plantar tests of both the ligated and the intact side. ($n=6$), $^{**}p<0.01$.

Table 1
Number of Fos-positive cells

Groups ($n=10$)	No treatment of control	Formalin tests	ACF	ACF+formalin tests	Fluvoxamine	Fluvoxamine+formalin tests
Basolateral nucleus amygdala	7.5±0.9	45.2±4.7***	7.6±0.7	45.3±3.4^{+++}	43.1±3.3***	53.3±4.7^{+}
Central nucleus amygdala	7.1±1.2	14.5±0.93**	10.2±1.2	64±5.4^{+++}	9.7±1.3	36.3±4.2^{+++}
Prefrontal cortex	10.3±3.1	67.3±2.8***	28.2±0.9***	75.6±4^{+++}	40.3±3.2***	51.5±3.3^{+}

Asterisks (*) represent statistically significant differences from the untreated control value. Crosses (+) represent statistically significant differences from the Fos-positive cell numbers on preformalin tests. All data were obtained from 10 animals in each group. Statistical significance was calculated using ANOVA post-hoc (Fisher PLSD). Data are mean ± SEM.

SIGMA, $n=5$) +6 μg fluvoxamine (total 6 μl) was injected i.c.v. by the same methods as described above. After 1 h of observation, the experiments were performed. At the end of the experiments, the animals were deeply anesthetized with barbiturate (i.p. 60 mg/kg) for euthanasia and injected with cresylviolet at the site of the first injection, in order to evaluate the effects of successive i.c.v. injections. All data obtained in this study were from mice undergoing successive injections into the ventricle. Fluvoxamine was provided by Solvay Pharmaceuticals (Netherlands).

2.4. Formalin test

One hour after injection of a drug or saline, 30 μl of 0.5% formalin were injected into the plantar portion of the hindpaw. Lifting of the injected hindpaw was assessed as a noxious sign. Total elevation time was recorded during a 5 min period. Data were recorded for 50 min after injection. The mice were placed in a transparent observation box (23×23×23 cm, Plexiglas) with a mirror, which was set at a 30-degree angle under the box for the formalin test, for 1 h for each of the three days prior to the day of the experiment.

2.5. Sciatic nerve ligation, plantar test and Fos

Before the nerve ligation, withdrawal latencies of infrared plantar stimulation were measured on both sides. We obtained intact control animal data in advance ($n=6$, 6 trials for each of the animals). All withdrawal latencies before nerve ligation were within the control range. The right side of the sciatic nerve was exposed under anesthesia (barbiturate i.p. 40 mg/kg) and half of the nerve bundle was ligatured with silk braid. One week after the operation, withdrawal latencies were obtained from both sides. One hour after fluvoxamine injection, plantar tests were performed.

2.6. Statistical analysis

Statistical significance was calculated using ANOVA post-hoc (Fisher PLSD or Scheffé) tests with the appropriate software (Statview, SAS Institute Inc.). *$p<0.05$, **$p<0.01$, ***$p<0.001$ (in the figures).

2.7. Histological examination

We counted the number of Fos-positive cells in the contralateral basolateral nucleus (BL), PFC and the central nucleus (Ce) (Paxinos and Franklin, 2001) in six experimental groups for the formalin test experiments. Sampling areas were circles 300 μm in diameter. The six groups were (a) untreated control, (b) formalin tests, (c) saline (i.c.v.), (d) saline (i.c.v.)+formalin tests, (e) fluvoxamine (i.c.v.) and (f) fluvoxamine (i.c.v.)+formalin tests (data were obtained for 10 animals in each group). For experiments with 5-HT receptor antagonists, there were 5 animals in each group (WAY-100635+fluvoxamine group and ketanserin+fluvoxamine group). We also examined Fos expression in a nerve ligation-allodynia model ($n=7$ mice). For the allodynia experiments, we counted Fos-positive cells on both sides. All mice were perfused with 4% formalin for Fos staining after the tests or 1 h after injection of the drugs. The brains were left in 10% sucrose over night and then stored frozen. The frozen brain tissues were sectioned at 25 μm (Kryostat CM1850, Leica) and incubated with anti-c-Fos antibody (10,000×, Ab-5, Oncogene, CatnoPC38) over night at 4 °C.

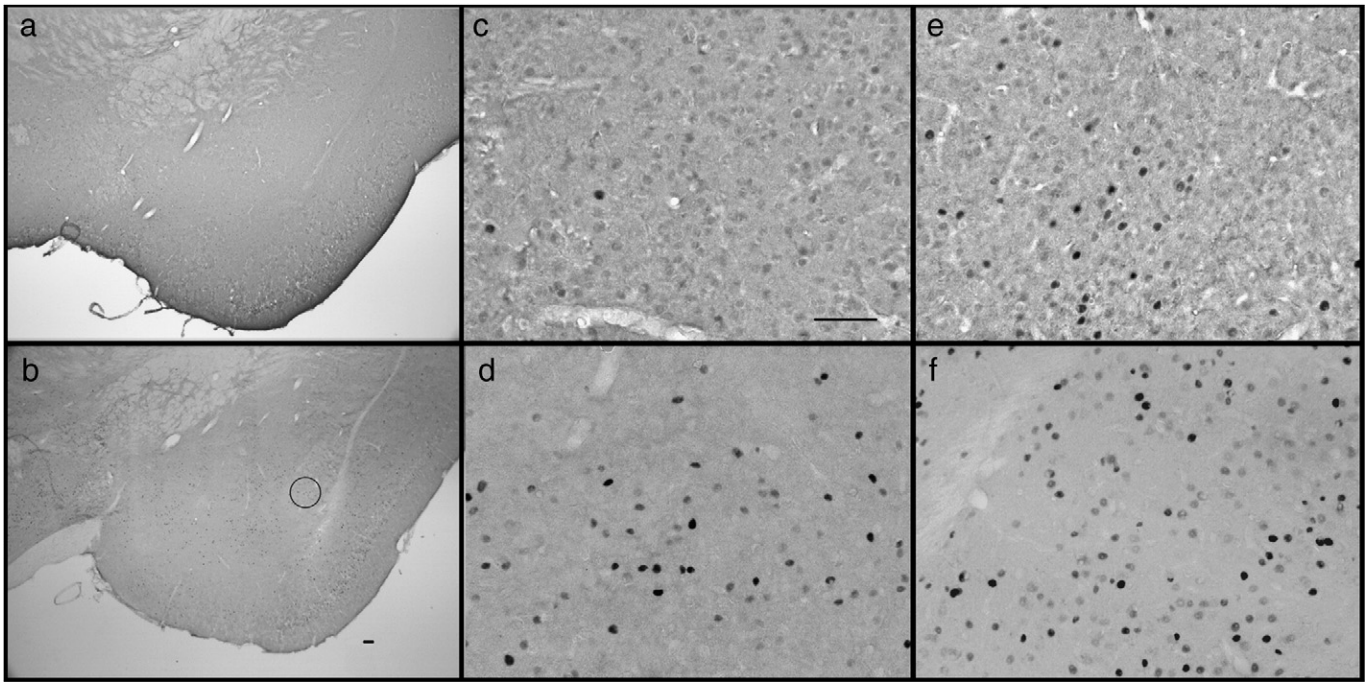

Fig. 4. Typical Fos expressions in the basolateral nucleus of the amygdala (BL). a and c: untreated control, b and d: formalin test, e: i.c.v. fluvoxamine, f: i.c.v. fluvoxamine+formalin test, Bars represent 100 μm, Circles 300 μm, in diameter.

Table 2
Fluvoxamine alone and 5-HT antagonists

Groups	No treatment of control (n=10)	Fluvoxamine (n=10)	Fluvoxamine+ WAY100635 (n=5)	Fluvoxamine+ ketanserin (n=5)
Basolateral nucleus	7.5±0.9	43.1±3.3	66.2±7**	49.1±4.3
Prefrontal cortex	10.3±3.1	40.3±3.2	103.5±3.6**	50.1±6.5

ANOVA post-hoc (Scheffé) tests, statistical significance between fluvoxamine alone and 5-HT antagonists **p<0.001, **p<0.01.

Slices were stained with 0.05% DAB and 0.01% H_2O_2. Double-staining with anti-c-Fos antibody and anti-5-TH_2 receptor antibody (500× ImmunoStar, Catno24288) was performed in control (n=5) and fluvoxamine injected animals (n=5). After Fos staining using the same methods as described above, slices were incubated with anti-5-TH_2 receptor antibody and stained with Vector SG (Vector Lab). Double-label immunohistochemistry results were obtained using the methods of Janusonis and Fite (2001).

Data were obtained from three slices from each animal and the mean number of Fos-positive cells in the left hemisphere was calculated for 10 (or 5) animals from each group. The statistical significance of differences among the groups was determined using ANOVA and post-hoc tests (Fisher PLDS).

3. Results

3.1. Anti-nociceptive effects of fluvoxamine

The i.c.v. injection of fluvoxamine (2 μg, 6 μg and 12 μg) attenuated noxious responses induced by subcutaneous formalin injections as compared to the saline vehicle (Fig. 1, solid circles). Injection of 6 or 12 μg of fluvoxamine (Fig. 1, open circles and open squares) clearly attenuated pain behavior at 5, 10 and 15 min (the first phase, p<0.05) and the late phase was also inhibited at 30, 35, 40, 45, 50 and 55 min (p<0.05 or 0.01). Even a small dose (2 μg, Fig. 1 solid squares) of fluvoxamine produced statistically significant inhibitory effects on pain behavior but only at 10, 35 and 40 min (p<0.05). All subsequent experiments were performed with 6 μg of fluvoxamine.

3.2. 5-HT receptor subtypes and anti-nociceptive effects of fluvoxamine

5-HT_1 and 5-HT_2 receptor antagonists blocked anti-nociceptive effects of fluvoxamine on formalin tests. WAY-100635, a 5-HT_1 receptor antagonist, injected i.c.v. with fluvoxamine (6 μg) inhibited the anti-nociceptive effects of fluvoxamine (Fig. 2). At 20 min (p<0.02 versus a single injection of fluvoxamine), 25 min (p<0.05), 30 min (p<0.01) and 35 min (p<0.05) on formalin tests, WAY-100635 inhibited the anti-nociceptive effects of fluvoxamine. At 20, 25 and 30 min especially, hyperalgesia was observed (p<0.05–0.001, versus control values). Mice walked restlessly around in the observation box and frequently reared. Some animals showed rearing behaviors more than 40 times during the 5 min observation period. Ketanserin, a 5-HT_2 receptor antagonist, also inhibited the anti-nociceptive effects of fluvoxamine at 15 min (p<0.01), 20 min (p<0.003), 25 min (p<0.01), 30 min (p<0.005) and 35 min (p<0.03). Ketanserin produced hyperalgesia at 20 (p<0.01), 25(p<0.01) and 30 (p<0.01) min on formalin tests, as had WAY-100635. However, ketanserin did not induce restless behavior.

3.3. Sciatic nerve ligation-allodynia model and fluvoxamine

The mean withdrawal latency for plantar tests in intact animals was 3.6±0.19 s. One week after sciatic nerve ligation, the mean withdrawal latency on the ipsilateral side decreased from 3.6±0.2 to 2.3±0.16. Prior to ligation, withdrawal latency on the contralateral side was 4.6±0.3, not significantly different from the control (3.6± 0.2 s). Fluvoxamine (i.c.v.) significantly increased latency bilaterally (Fig. 3). The mean latency on the ligated side increased to 3.6±0.3 s (p<0.001), the same as that of intact animals. Even on the contralateral side, withdrawal latency was significantly increased as compared to the value of intact animals (6.8±0.34 s, p<0.001).

3.4. Fos-positive cells in the PFC and the amygdala

In three brain areas, Fos-positive cells were observed under control conditions. Mean numbers of Fos-positive cells in the PFC, BL and Ce were 10.3±3.1, 7.3±0.9, and 7.1±1.2, respectively (mean±SEM, Table 1). Formalin tests showed significantly increased Fos expressions in the BL (45.2±4.7, p<0.001), PFC (67.3±2.8, p<0.001) and Ce (14.5±0.93, p<0.01). Saline (i.c.v.) had no effects on the Fos expression in formalin tests. Fluvoxamine (i.c.v.) alone increased Fos-positive cells (Table 1) in the PFC (40.3±3.2, p<0.001) and BL (43.1±3.3, p<0.001, Fig. 4). WAY-100635 injected with fluvoxamine enhanced the Fos expression induced by fluvoxamine. Fos-positive cells increased from 40.3 to 103.5 (p<0.001) in the PFC and from 43.1 to 66.2 (p<0.01) in the BL (Table 2). In contrast, ketanserin with fluvoxamine had no significant effect on Fos expression in the PFC (50.1±6.5) or the BL (49.1±4.3). Double-label immunohistochemistry with anti-c-Fos and anti-5-TH_2 receptor antibodies showed a small number of double-positive neurons in the PFC (Fig. 5a) and BL

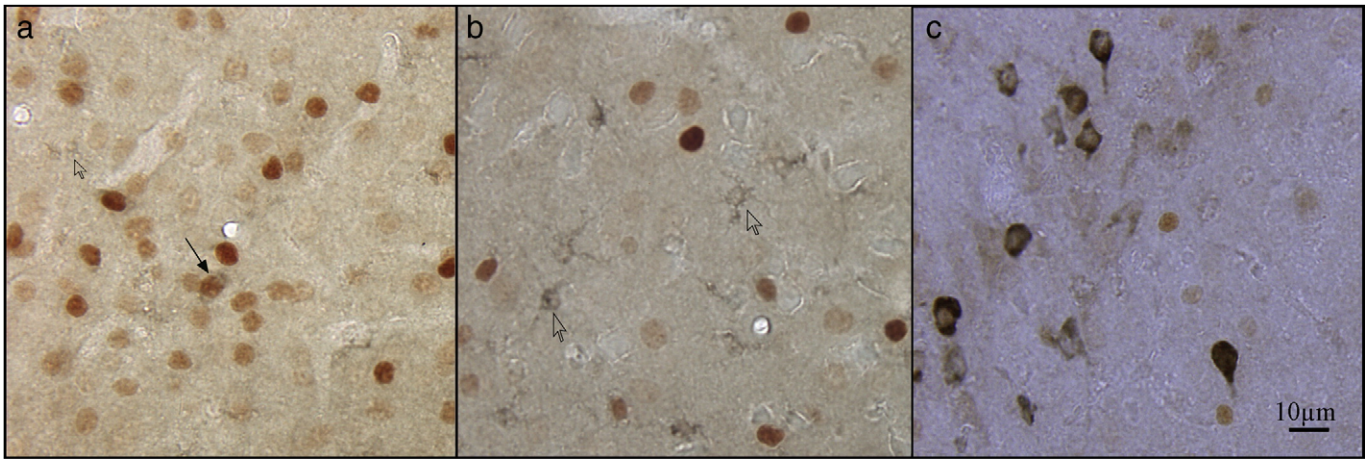

Fig. 5. Double-staining of 5-HT_2 receptor and Fos. After fluvoxamine 6 μg (i.c.v.), Fos expression increased in the PFC (a) and BL (b). Few double-positive cells (arrow) were observed and there were 5-HT_2 receptor-positive cells only in the deep layer of the PFC (c). In the BL, 5-HT_2 receptor-positive cells (blank arrows) were present but double-positive cells were also scarcely observed.

Table 3
Numbers of Fos-positive cells in the allodynia model

Groups (n=7)	Nerve ligation ipsi side	Nerve ligation contra side	Fluvoxamine+Nerve ligation ipsi side	Fluvoxamine+Nerve ligation contra side
Basolateral nucleus	9.2±1.0	24±1.6***	12±0.9	49.83±8.6***
Prefrontal cortex	121.3±6.9	146.4±6.75***	84.1±4.7	147.6±4.6***

Asterisks (*) represent statistically significant differences between the ipsilateral and contralateral sides. Statistical significance was calculated using ANOVA post-hoc (Fisher PLSD) test.
***$p<0.001$.

(Fig. 5b). Cells positive only for the 5-TH$_2$ receptor were limited to the deep layer of the PFC (Fig. 5c). In the BL, in which cells are small and have a characteristic shape, 5-TH$_2$ receptor-positive cells could be interneurons.

3.5. Fos expression in sciatic nerve ligation-allodynia

We examined Fos expressions in Fos-positive cells of the BL and PFC in allodynia model mice. Nerve ligation increased Fos expression in the contralateral BL (24±1.6, Table 3) as compared to that in the ipsilateral BL (9.2±1.0, $p<0.01$). In the PFC, sciatic nerve ligation bilaterally increased Fos-positive cells. Fluvoxamine did not inhibit Fos expression in either the PFC (147.6±4.6) or the BL (49.83±8.6, Table 3).

4. Discussion

4.1. Anti-nociceptive effects of fluvoxamine

All three doses of fluvoxamine induced anti-nociceptive effects. The 6 and 12 μg fluvoxamine doses produced marked inhibition of nociceptive responses. The first phase of formalin tests was inhibited by fluvoxamine, suggesting that fluvoxamine affects pain processing at the spinal level. Fluvoxamine may activate descending anti-nociceptive pathways by increasing the brain stem serotonin level. In allodynia models, fluvoxamine reduced plantar test latencies bilaterally, indicating effects on chronic (ipsilateral-allodynia) and acute (contralateral-heat) pain. The results of our behavioral experiments with WAY-100635 and ketanserin support the concept that SSRI produces 5-HT$_1$ and 5-HT$_2$ receptor mediated anti-nociceptive effects (Anjaneyulu and Chopra, 2004; Honda et al., 2006). SSRI increased serotonin levels in the brain (Bundgaard et al., 2006), thereby altering the effects of fluvoxamine depending on the locations of 5-HT receptor subtypes in different brain areas.

4.2. Fluvoxamine and Fos

Acute (formalin injection) and chronic (sciatic nerve ligation) pain stimulation increased Fos expression in the PFC as in our previous study (Zhang et al., 2004a). In the BL, acute pain increased extracellular glutamate (Silva and Hernández, 2007), indicating that nociceptive stimulation elicited neuronal activities in the BL. Both brain areas, in response to pain, showed induction of Fos expression. Given the reciprocal projections between the PFC and BL, theses areas may act synergistically to modify pain.

A high extracellular serotonin concentration produced by fluvoxamine may activate 5-HT$_1$ receptors. It may also potentiate other 5-HT receptor subtypes. The overall balance of 5-HT receptor subtype activities may account for the density of Fos expression. The 5-HT$_2$ receptor antagonist, ketanserin, inhibited behavioral anti-nociceptive responses, while not changing the number of Fos-positive cells in the PFC and BL. Double-staining (5-HT$_2$ receptor and Fos antibodies) immunohistochemical experiments revealed a few double-positive neurons in the PFC and BL. Fos or 5-HT$_2$ receptor-positive neurons were seen in different layers of the PFC, while staining for Fos and 5-HT$_2$ receptors differed among BL cell types. These results revealed 5-HT$_2$ receptors to be minimally, if at all, involved in the neuronal excitation produced by fluvoxamine, which induced Fos expression. Fos was expressed bilaterally in allodynia models, supporting the concept of bilateral receptor fields in the PFC (Sikes and Vogt, 1992). The contralateral side, however, showed more projections than the ipsilateral side in our experiments. The density of Fos expression on the contralateral side of the BL was also clearly higher than that on the ipsilateral side. Fluvoxamine (i.c.v.) alone increased Fos in allodynia animals as well as that in intact animals on the contralateral side of the BL and the bilateral PFC.

The results of our Fos experiments suggest that fluvoxamine may not directly affect Ce, though the Ce has also been reported to receive nociceptive projections (Bernard et al., 1996) from lamina I of the spinal cord (Jasmin et al., 1997). Multi-projections from the Ce to other brain areas indicate that Ce involvement in the central fear and anxiety system (Davis, 2001). However, unconditioned stimuli, while increasing the extracellular serotonin and dopamine levels in the BL, had no effect on the Ce (Macedo et al., 2005). Increased serotonin levels produced by fluvoxamine in the BL may also enhance the neuronal excitability of the Ce, receiving inter-amygdala fibers from the BL. This may explain the increased Fos expression on formalin tests with i.c.v. fluvoxamine. Saline or fluvoxamine injection may change cell excitability via dopamine or serotonin levels in the amygdala (Aggleton and Saunders, 2000) and affect Fos expression in the Ce. However, more experiments are needed to assess this hypothesis.

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