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Anxiolytic-like effect of milnacipran in the four-plate test in mice: Mechanism of action

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Abstract

Milnacipran is a serotonin/noradrenaline reuptake inhibitor (SNRI) which has not yet been systematically studied preclinically or clinically for the treatment of anxiety disorders. In the four-plate test (FPT) which is known to predict anxiolytic-like activity in mice, milnacipran (4, 8, 16 and 32 mg/kg) demonstrated strong anti-punishment effects following acute administration. The anxiolytic-like effect of milnacipran was not reversed by the selective $GABA_A$ receptor antagonist, flumazenil (2 and 4 mg/kg), the selective α_1-adrenoceptor antagonist, prazosin (0.5 and 2 mg/kg), the selective α_2-adrenoceptor antagonist, idazoxan (1 and 4 mg/kg) or the selective 5-HT_{2B} receptor antagonist, SB 206553 (0.1 and 1 mg/kg). In contrast, the selective 5-HT_{2A} receptor antagonist, SR 46349B (0.1 and 1 mg/kg), and the non-selective 5-HT_2 receptor antagonist, ketanserin (0.125 and 0.5 mg/kg), completely abolished the anxiolytic-like effect of milnacipran in FPT. Neurochemical depletion of NA or 5-HT completely abolished the activity of milnacipran.

These results strongly suggest that activation of 5-HT_{2A} receptors is critically involved in the anxiolytic activity of milnacipran. On the other hand the lack of activity of milnacipran after depletion of NA or 5-HT is consistent with milnacipran acting on the locus coeruleus to induce 5-HT release. The present data suggest a strong connection between 5-HT_{2A} receptors and NA neurotransmission.

Keywords: Milnacipran; Anxiety; Four-plate test; 5-HT_{2A} receptors; Noradrenaline

1. Introduction

Over the last 10 years, growing evidence suggests that antidepressants including the selective serotonin/noradrenaline reuptake inhibitor (SNRI) milnacipran constitute a novel class of drugs acting in anxiety disorders, but their exact mechanism of action remains unclear (Bourin and Hascoët, 2001).

Milnacipran is an SNRI (Lambert and Bourin, 2002) which has not yet been systematically studied preclinically or in humans for the treatment of anxiety disorders. Milnacipran simultaneously inhibits 5-HT and NA reuptake. In contrast to venlafaxine, the reuptake inhibition of both monoamines is more balanced (Bourin, 1999). Although reuptake inhibition is dose-dependent the balance between NA and 5-HT reuptake blockade remains constant. In a previous paper we studied the activity of a wide range of antidepressants including milnacipran on the four-plate test (FPT) which is known to predict anxiolytic-like activity in mice (Hascoët et al., 2000). The number of punished crossings was dramatically increased by the SSRIs citalopram, fluvoxamine and paroxetine but not fluoxetine. The SNRIs, milnacipran and venlafaxine, also demonstrated strong anti-punishment effects following acute administration. In contrast, the specific NA reuptake inhibitors, desipramine and maprotiline, and the atypical antidepressant, trazodone, enhanced freezing behaviour suggesting anxiogenic-like behaviour. It was concluded that, in the FPT, a model based on spontaneous responses, where animals are exposed to an aversive environment from which they can only escape by being motionless, this kind of behaviour might be related to anticipatory anxiety. In this

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situation, antidepressants acting acutely on 5-HT transmission possessed clear anxiolytic-like effects but the balance between the two transmitters, 5-HT and NA, seemed to be a crucial factor in the anxiolytic-like effect (Hascoët et al., 2000).

In a recent paper, (Nic Dhonnchadha et al., 2003a) we hypothesised that the FPT and the elevated plus maze (EPM) generated different kinds of anxiety. Indeed, the injection of benzodiazepines into the amygdala or lesions of the amygdala are without effect in the plus maze possibly implicating the peri-aqueductal gray area (PAG) in the kind of anxiety generated by exposure to the mouse EPM. (Gonzalez et al., 1996; Menard and Treit, 1996). The activation of central 5-HT_2 receptors resulting in anxiolytic-like effects in some regions of the brain (e.g. medial amygdala) while anxiogenic effects in others (e.g. dorsal hippocampus) also lends to support to this speculation (Menard and Treit, 1999). Using three mice models of anxiety in mice we have shown that DOI, a 5-HT_2 receptor agonist, induced anxiolytic-like effect in the FPT and the EPM (Nic Dhonnchadha et al., 2003a) and these effects are likely to be 5-HT_{2A} receptor-mediated in both tests (Nic Dhonnchadha et al., 2003b). Even though DOI possesses affinity for all three 5-HT_2 receptor subtypes, it is nevertheless considered as one of the most selective 5-HT_{2A} agonists available (Porter et al., 1999).

Recently, we have demonstrated that direct binding to 5-HT_2 receptors seems to be implicated in the acute effect of antidepressants, including the selective reuptake inhibitor (SSRI), paroxetine, and the serotonin noradrenaline reuptake inhibitor (SNRI), venlafaxine, in the FPT (Nic Dhonnchadha et al., 2005) and that the 5-HT_{2A} receptor subtype seemed to be implicated.

The aim of the present study was to investigate the mechanism of action of milnacipran in the FPT after acute administration. Experiments using antagonists were performed to determine the role of the GABAergic, noradrenergic and serotonergic systems by combining available antagonists selective for each system. Flumazenil was expected to antagonise the anxiolytic-like effect of milnacipran, if it acts via a GABAergic mechanism. Two subtypes of noradrenergic receptors were investigated, the α_1 and α_2 receptor subtypes using the antagonists prazosin and idazoxan, respectively. Regarding the serotoninergic system, the 5-HT_{2A} ligand SR 46349B was used as the antagonist, the 5-HT_{2B} receptor antagonist SB 206553 was chosen even though it possesses some affinity for the 5-HT_{2C} receptor since a more selective antagonist is not yet available. The mixed 5-$HT_{2A/C}$ antagonist ketanserin was also studied. Doses of the antagonists that were inactive by themselves were selected based on preliminary experiments including a spontaneous locomotor activity test (Boissier and Simon, 1965) and one or two animal models of anxiety (FPT or EPM) with large range doses of the compounds. The dose–effect of SR 46349B, SB 206553 and ketanserin have been previously evaluated on locomotion and anxiolytic-like activity (Nic Dhonnchadha et al., 2003a). Flumazenil was found to have no effect in the same tests (Clenet et al., 2004). The dose–effect of prazosin and idazoxan was determined just before the interaction studies.

The activity of milnacipran in the FPT was also investigated after depletion of NA or 5-HT by specific neurochemical lesion using DSP-4 and *p*-CPA.

2. Materials and methods

2.1. Animals

Male Swiss mice (4 weeks old) were purchased from R. Janvier (Le Genest, France). Their average body weight on the day of the study was 20±2 g. These animals were housed in groups of 18 per cage (40 cm × 28 cm × 17 cm), at a constant temperature (20 °C), and a standard light cycle (lights on between 0700 h and 1900 h). The animals had free access to food and water.

2.2. Drugs

Milnacipran (Laboratoire Pierre Fabre), benzodiazepine receptor antagonist: flumazenil (RO 15-178) (Hoffmann-La Roche SA). 5-HT_2 ligands: SR 46349B [2-propen-1-one, 1-(2-fluorophenyl)-3-(4-hydroxyphenyl)-*O*-[2-(dimethylamino) ethyl] oxime] (Sanofi Recherche, France), SB 206553 hydrochloride (-3-pyridinyl-3,5-dihydro-5-methyl-benzo[1,2-*b*:4,5-*b*′]dipyrrole-1[2*H*]carboxamide hydrochloride) (Sigma, France) ketanserin tartrate (Tocris, Fisher Bioblock scientific France). Noradrenergic ligands: prazosin (Tocris, Fisher Bioblock Scientific France) and idazoxan (Tocris; Fisher Bioblock scientific France). *p*-Chlorophenylalanine (*p*-CPA) (RBI, Sigma-Aldrich chimie France), DSP-4 HCl (RBI, Sigma-Aldrich chimie France). Alprazolam (Tocris, Fisher Bioblock Scientific France).

All drugs were dissolved in a 5% concentration of Tween 80. All drugs or vehicle were administered i.p. in a volume of 0.5 ml/20 g of body weight. Control animals received vehicle only.

2.2.1. Locomotor activity test (Boissier and Simon, 1965)

The spontaneous activity of naive animals was recorded using a photoelectric actimeter (OSYS). This apparatus consists of a transparent cage from which the animal's activity is measured by light beams connected to a photoelectric cell. The total number of horizontal cage crossings was recorded over a period of 10 min. The actimeter test was performed independently of the FPT in order to examine the effect of drugs on spontaneous locomotor activity of mice.

2.2.2. The "four-plate" test (FPT) (Aron et al., 1971)

This apparatus consists of a cage (18 cm × 25 cm × 16) floored by four identical rectangular metal plates (8

cm × 11 cm) separated from one another by a gap of 4 mm. The plates are connected to a device that generated electric shocks (0.6 mA, 0.5 s). Following a 15-s habituation period, the animal is subjected to an electric shock triggered by a foot pedal when crossing from one plate to another. The number of crossings is recorded during a 1-min test period.

2.2.3. Neurochemical assays

The method is adapted from the assay described by Baker et al. (1987). Briefly, mice were killed by cervical dislocation without anaesthesia. The brain was rapidly removed from the cranium and dissected on a cooled aluminium apparatus. The brain sections (cortex, striatum, hippocampus, hypothalamus) were weighed and disrupted by sonication in 600 μl of an acid solution (8.8 mg of ascorbic acid and 122 mg of EDTA in 1000 ml of perchloric acid 0.1 M). After sonication, the solution was centrifuged at 12,000×*g* for 10 min at +4 °C. The supernatant was stored at −80 °C before use. The concentrations of noradrenaline (NA), serotonin (5-HT) and dopamine (DA) were measured in the supernatant by high performance liquid chromatography with electrochemical detection [Decade amperometric detector (Leiden, The Netherlands) with an electrochemical Antec Leyden model VT-03 Flow cell (Zolterwoude, The Netherlands)]. The chromatographic conditions were (i) a C18 column (Nucleosil, 5 μm particle size, 15 cm, Colochrom, Gagny, France) in a column heater (+45 °C), (ii) an oxidation potential for amperometric detection of 0.48 V, (iii) a mobile phase composed of 4.2 g/L of citric acid monohydrate, 6.8 g/L of sodium acetate trihydrate, 0.8 g/L of octanesulphonic acid sodium salt, 0.05 g/L of EDTA, 0.02% (v/v) dibutyl amine, 7%(v/v) methyl alcohol (iv) a rate flow of 1.6 ml/min, and (v) a total run time of 25 min. With these chromatographic conditions detection limits were 3×10^{-5} mg/L for NA, 7.5×10^{-5} mg/L for DA and 16.7×10^{-5} mg/L for 5-HT in the supernatant. The concentrations in brain tissues of NA, 5-HT and DA are calculated from supernatant concentrations, the volume of the acid solution used to dilute brain sections and the weight of these brain sections. Results are expressed as ng of NA, 5-HT, or DA per g of brain section tissue.

2.3. Experimental protocol

Dose–effect relationship of milnacipran was determined after acute administration in the FPT, 30 min before testing. The doses of 4 to 32 mg/kg were investigated together with a vehicle control group and alprazolam 0.25 mg/kg as internal control. Two inactive doses of the antagonists (45 min before testing) were co-administered with active doses of milnacipran (30 min before the test) in the FPT. For each experiment, all doses of antagonists used in this study were studied alone and in combination with milnacipran, alprazolam (0.25 mg/kg) was used as a positive control.

Serotonergic lesion was carried out using the 5-HT synthesis inhibitor *p*-CPA (300 mg/kg) administered intraperitoneally 72 h, 48 h and 24 h before the test (Artaiz et al., 1998; Dursun and Handley, 1993), milnacipran was administered 30 min before testing. The extent of depletion was determined in vehicle control using HPLC by comparing concentration of NA, 5-HT and DA in vehicle-treated depleted group and vehicle-treated non-depleted group.

Lesion of the noradrenergic system was achieved by systemic pre-treatment with the selective noradrenergic neurotoxin, DSP-4 (50 mg/kg) administered intraperitoneally 168 h before the test (Yu et al., 1994), milnacipran was administered 30 min before testing. The extent of depletion was determined in vehicle control using HPLC by comparing concentration of NA, 5-HT and DA in vehicle-treated depleted group and vehicle-treated non-depleted group.

2.4. Statistics

2.4.1. Behavioural studies

The mean number of responses for each group and for each test was calculated, and the final results expressed as a mean (with standard error of the mean in parentheses).

Statistical comparisons were performed initially via an one-way analysis of variance (ANOVA) for independent groups, after verifying the normality of distribution by a Kolmogorof–Smirov non-parametric test. If any statistical change was observed, data was further analysed using post-hoc comparisons, with a Dunnett's test, to detect eventual differences between control and treated groups. Data was deemed significant when $p < 0.05$. The effects of alprazolam, included as internal standards in the anxiety model, were compared to the control group via a Student's *t*-test ($p < 0.05$). For interaction and association studies a two-way ANOVA (pre-treatment × treatment) was employed for global analysis purposes. If the ANOVA showed a significant difference between groups ($p < 0.05$), a Sidak post-hoc comparison test was performed to compare the effects of pre-treatment on treatment administered.

2.4.2. Neurochemical studies

The mean concentration of monoamines was calculated, and the final results expressed as a mean (with standard error of the mean in parentheses) and percentage residual from controls. Statistical comparisons were performed initially via a one-way analysis of variance (ANOVA) for independent groups, after verifying the normality of distribution by a Kolmogorof–Smirov non-parametric test. If any statistical change was observed, data was further analysed using post-hoc comparisons, with a Fisher test, to detect eventual differences between control and *p*-CPA or DSP-4 treated groups. Data was deemed significant when $p < 0.05$.

Table 1
Effects of acute administration of compounds injected i.p. 30 min before the test on mouse spontaneous locomotor activity in the actimeter test

Compounds	Dose (mg/kg)											
	Vehicle	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32
Ketanserin	100%	103%	105%	106%	95%	87%	53%***	37%***				
SR 46349B	100%	109%	104%	90%	111%	86%	78%	83%				
SB 206553	100%	110%	111%	112%	111%	100%	112%	114%	116%			
Flumazenil	100%	99.2%	120.3%	103.3%	90.1%	93.2%						
Idazoxan	100%			114%	122%*	125%*	120%	90%	112%	110%		
Prazosin	100%			74*%	65%*	51%**	38%**	21%***	36%**			
Milnacipran	100%								103.6%	105.8%	101.5%	93.8%

All data are given as a percentage of the value observed in control animals ($n=10$). Significantly different from relative control group, *$p=0.05$, **$p=0.01$ and ***$p=0.001$, determined by one-way ANOVA followed by a Dunnett's test.

The ethical rules of the French Ministry of Agriculture for experiments with laboratory animals (no. 87.848) were followed at all times.

3. Results

3.1. Effects of compounds in the actimeter test

Ketanserin (1 and 2 mg/kg) and prazosin (0.125 to 4 mg/kg) significantly reduced spontaneous locomotor activity of mice. Idazoxan (0.25 and 0.5 mg/kg) increased locomotor activity of mice. From the dose ranges of all compounds tested, non-psycho-stimulant doses for subsequent anxiety tests were chosen (Table 1).

3.2. Effects of milnacipran in the FPT

Milnacipran (4–32 mg/kg) significantly increased the number of shocks accepted by mice, [$F_{(5,54)}=6.943$, $p<0.001$]. The maximum effect was observed for the dose of 8 mg/kg with 8.2±0.6 punished crossings ($p<0.01$). Alprazolam (0.25 mg/kg) used as internal control dramatically increased the number of punished crossings accepted by mice (9±0.6 versus 4±0.3 for vehicle control) ($p<0.001$). The active doses of 8 and 16 mg/kg milnacipran were chosen for interaction studies (Fig. 1).

3.3. Effect of flumazenil on the anxiolytic-like effect of alprazolam in the FPT

Flumazenil was used at the doses of 2 and 8 mg/kg. At these doses it had no effect per se on the number of punished passages ($p>0.05$) in the FPT. Alprazolam (0.25 mg/kg) significantly increased the number of punished passages in comparison with vehicle controls ($p<0.001$) [$F_{(1,54)}=39.808$, $p<0.001$]. Flumazenil (2 and 8 mg/kg) significantly antagonized the anxiolytic like-effect of alprazolam treated mice ($p<0.001$ for both doses) [$F_{(2,54)}=6.084$, $p=0.004$] (Table 2a).

3.4. Effect of flumazenil on milnacipran anxiolytic-like effect in the FPT

Flumazenil was used at the doses of 2 and 4 mg/kg. In the FPT, the doses of 8 and 16 mg/kg of milnacipran significantly increased the number of punished crossings in

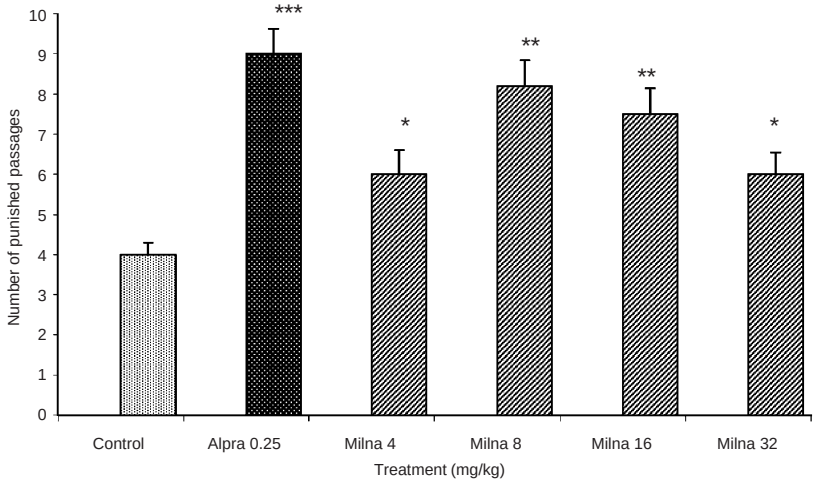

Fig. 1. Effects of acute administration of milnacipran (Milna), i.p. 30 min in the FPT. The results are cited as means±S.E.M. ($n=10$). Statistical analysis was performed by a one-way ANOVA followed by a Dunnett's test for comparisons between treated groups and control group: *** ($p<0.001$), ** ($p<0.01$), * ($p<0.05$). A Student's t-test was used for statistical analysis between the alprazolam (Alpra) group and control group: *** ($p<0.001$).

Table 2a
Effects of acute administration of flumazenil, (i.p. 45 min pre-test) and alprazolam or milnacipran (i.p. 30 min pre-test) in the FPT

Antagonist (mg/kg)	Antidepressant	Punished passages
Vehicle	Vehicle	3.75±0.3
Vehicle	Alprazolam 0.25	7.25±0.7***
Flumazenil 2	Vehicle	4±0.3
Flumazenil 8	Vehicle	3.67±0.3
Flumazenil 2	Alprazolam 0.25	5.08±0.4†††
Flumazenil 8	Alprazolam 0.25	5.67±0.8†††
Vehicle	Vehicle	4.3±0.3
Vehicle	Milnacipran 8	5.8±0.4*
Vehicle	Milnacipran 16	6.0±0.4**
Flumazenil 2	Vehicle	4.2±0.4
Flumazenil 4	Vehicle	4.7±0.3
Flumazenil 2	Milnacipran 8	6.2±0.2
Flumazenil 4	Milnacipran 4	6.1±0.2
Flumazenil 2	Milnacipran 16	6.3±0.4
Flumazenil 4	Milnacipran 16	6.1±0.2
Vehicle	Alprazolam 0.25	7.1±0.4***

The data are cited as means±S.E.M., ($n=10$). Statistical analysis was performed by a one-way ANOVA followed by a Dunnett's test for comparisons between alprazolam or milnacipran treated groups and vehicle control group: ***($p<0.001$), ** ($p<0.01$), * ($p<0.05$).
For interaction with flumazenil, statistical analysis was performed by a two-way ANOVA followed by a Sidak test [†††($p<0.001$), ††($p<0.01$), †($p<0.05$), versus appropriate control group].

comparison with vehicle controls ($p<0.05$ and $p<0.01$, respectively) [$F_{(9,90)}=7.747$, $p<0.001$].

Flumazenil (2 and 4 mg/kg) had no effect alone on the number of punishments accepted by mice ($p>0.05$, after a post-hoc analysis) in comparison with control group. Flumazenil, at any doses, failed to antagonise milnacipran anxiolytic-like effect in the FPT ($p>0.05$ for both doses) [$F_{(4,90)}=2.322$, $p=0.063$]. The positive control alprazolam increased the number of punished crossing as compared with vehicle control ($p<0.001$) (Table 2a).

3.5. Noradrenergic system: effect of prazosin and idazoxan on milnacipran anxiolytic-like effects in the FPT

First, the effect–dose of prazosin and idazoxan was determined in the FPT after acute administration. For a dose range from 0.25 mg/kg to 8 mg/kg neither idazoxan nor prazosin showed any anxiolytic-like effect in the FPT (results not shown).

3.5.1. Prazosin

In the FPT, the doses of 8 and 16 mg/kg of milnacipran significantly increased the number of punished crossings when compared with vehicle controls ($p<0.05$ and $p<0.01$, respectively) [$F_{(9,90)}=11.18$, $p<0.001$]. The positive control alprazolam dramatically increased the number of punished crossing as compared with vehicle control ($p<0.001$). Prazosin alone (0.5 and 2 mg/kg) had no effect on the number of punishments accepted by mice ($p>0.05$) after post-hoc analysis with control group. The co-administration of prazosin (0.5 and 2 mg/kg) with milnacipran (8 and 16 mg/kg) failed to alter the anti-punishment effect of milnacipran. The association of milnacipran (16 mg/kg) and prazosin (2 mg/kg) resulted in a weak but non-significant decreased of punished crossing ($p>0.05$, after a post-hoc analysis) [$F_{(4,90)}=3.733$, $p=0.008$].

3.5.2. Idazoxan

In the FPT, the doses of 8 and 16 mg/kg of milnacipran significantly increased the number of punished crossings in comparison with vehicle controls ($p<0.05$ and $p<0.01$, respectively) [$F_{(9,90)}=14.54$, $p<0.001$]. The positive control alprazolam dramatically increased the number of punished crossing as compared with vehicle control ($p<0.001$). Idazoxan (1 and 4 mg/kg) had no effect on the number of punishments accepted by mice ($p>0.05$, after post-hoc analysis) with control group. Neither of the two doses of milnacipran were antagonised by idazoxan at the doses of 1 and 4 mg/kg ($p>0.05$, after a post-hoc analysis) [$F_{(4,90)}=2.507$, $p=0.047$] (Table 2b).

Table 2b
Effects of acute administration of antagonists, (i.p. 45 min pre-test) and milnacipran, (i.p. 30 min pre-test) in the FPT

Antagonists (mg/kg)	Antidepressant	Punished passages
Vehicle	Vehicle	4.8±0.2
Vehicle	Milnacipran 8	7.1±0.7*
Vehicle	Milnacipran 16	7.7±0.7**
Prazosin 0.5	Vehicle	3.9±0.3
Prazosin 2	Vehicle	4.6±0.3
Prazosin 0.5	Milnacipran 8	6.3±0.4
Prazosin 2	Milnacipran 8	8.4±0.6
Prazosin 0.5	Milnacipran 16	7.0±0.8
Prazosin 2	Milnacipran 16	5.4±0.4
Vehicle	Alprazolam 0.25	9.9±0.7***
Vehicle	Vehicle	4.4±0.2
Vehicle	Milnacipran 8	6.3±0.6*
Vehicle	Milnacipran 16	6.8±0.5**
Idazoxan 1	Vehicle	4.2±0.2
Idazoxan 4	Vehicle	4.2±0.2
Idazoxan 1	Milnacipran 8	5.9±0.3
Idazoxan 4	Milnacipran 8	5.4±0.3
Idazoxan 1	Milnacipran 16	7.0±0.5
Idazoxan 4	Milnacipran 16	5.7±0.5
Vehicle	Alprazolam 0.25	9.5±0.6***
Vehicle	Vehicle	4.3±0.3
Vehicle	Milnacipran 8	6.3±0.3**
Vehicle	Milnacipran 16	6.2±0.4**
SB 206553 0.1	Vehicle	4.6±0.2
SB 206553 1	Vehicle	5.0±0.1
SB 206553 0.1	Milnacipran 8	5.7±0.4
SB 206553 1	Milnacipran 8	5.5±0.6
SB 206553 0.1	Milnacipran 16	5.0±0.3
SB 206553 1	Milnacipran 16	5.0±0.3
Vehicle	Alprazolam 0.25	6.9±0.5***

The data are cited as means±S.E.M., ($n=10$). Statistical analysis was performed by a one-way ANOVA followed by a Dunnett's test for comparisons between milnacipran treated groups and vehicle control group: ***($p<0.001$), **($p<0.01$), *($p<0.05$). A Student's t-test was used for statistical analysis between the alprazolam group and control group: ***($p<0.001$).
For interaction with selective antagonists, no significant difference between groups was observed [two-way ANOVA].

3.6. Serotoninergic system: effect of 5-HT_2 receptor antagonists on milnacipran anxiolytic-like effects in the FPT

3.6.1. 5-HT_{2A} receptor antagonist: SR 46349B

In the FPT, the dose of 16 mg/kg of milnacipran significantly increased the number of punished crossings when compared with vehicle controls ($p<0.01$) [$F_{(9,90)}=9.44$, $p<0.001$]. On the day of experiment the lowest dose of milnacipran weakly increased the number of punished crossing but the result did not reach statistical significance ($p=0.11$). The positive control alprazolam increased the number of punished crossing as compared with vehicle control ($p<0.001$). SR 46349B (0.1 and 1 mg/kg) alone had no effect on the number of punishments accepted by mice ($p>0.05$, after post-hoc analysis) in comparison with control group. Pre-treatment of SR 46349B (0.1 and 1 mg/kg) had no effect on the anti-punishment action of milnacipran (8 mg/kg) in FPT ($p>0.05$, after a post-hoc analysis). This might be due to the weak effect of milnacipran 8 mg/kg on the day of experiment. On the other hand, the pre-treatment of SR 46349B (0.1 and 1 mg/kg) did antagonise the anti-punishment effect of milnacipran 16 mg/kg ($p<0.05$) [$F_{(4,90)}=3.688$, $p=0.008$] (Fig. 2).

3.6.2. 5-HT_{2B} receptor antagonist: SB 206553

In the FPT, the doses of 8 and 16 mg/kg of milnacipran significantly increased the number of punished crossings when compared with vehicle controls ($p<0.01$) [$F_{(9,90)}=5.02$, $p<0.001$]. The positive control alprazolam increased the number of punished crossing as compared with vehicle control ($p<0.001$). SB 206553 (0.1 and 1 mg/kg) alone had no effect on the number of punishments accepted by mice ($p>0.05$) after post-hoc analysis with control group. Neither of the two doses of milnacipran were antagonised by SB 206553 at the doses of 0.1 and 1 mg/kg [$F_{(4,90)}=2.189$, $p=0.078$] (Tables 2a and 2b).

3.6.3. The mixed 5-HT_2 receptor antagonist ketanserin

In the four-plate test the doses of 8 and 16 mg/kg of milnacipran significantly increased the number of punished crossings when compared with vehicle controls ($p<0.05$ and $p<0.01$, respectively) [$F_{(9,90)}=20.22$, $p<0.001$]. The positive control alprazolam increased the number of punished crossing as compared with vehicle control ($p<0.001$). Ketanserin (0.125 and 0.5 mg/kg) alone had no effect on the number of punishments accepted by mice ($p>0.05$) after post-hoc analysis with control group. Both doses of ketanserin (0.125 and 0.5 mg/kg) strongly antagonised the anxiolytic-like effect of milnacipran at the doses of 8 and 16 mg/kg ($p<0.001$) [$F_{(4,90)}=8.332$, $p<0.001$] (Fig. 3).

3.7. Neurochemical analysis

3.7.1. Effect of DSP-4 and p-CPA on noradrenaline concentrations

The noradrenaline levels were decreased in the hippocampus ($p<0.05$) and the cortex ($p<0.05$) following DSP-4 administration, but not affected by *p*-CPA (Table 3).

3.7.2. Effect of DSP-4 and p-CPA on serotonin concentrations

The serotonin concentrations were reduced in the hypothalamus, hipoccampus, striatum and the cortex following *p*-CPA administration ($p<0.05$). 5-HT concentration was also decreased in DSP-4 treated mice in the hypothalamus ($p<0.05$) (Table 4).

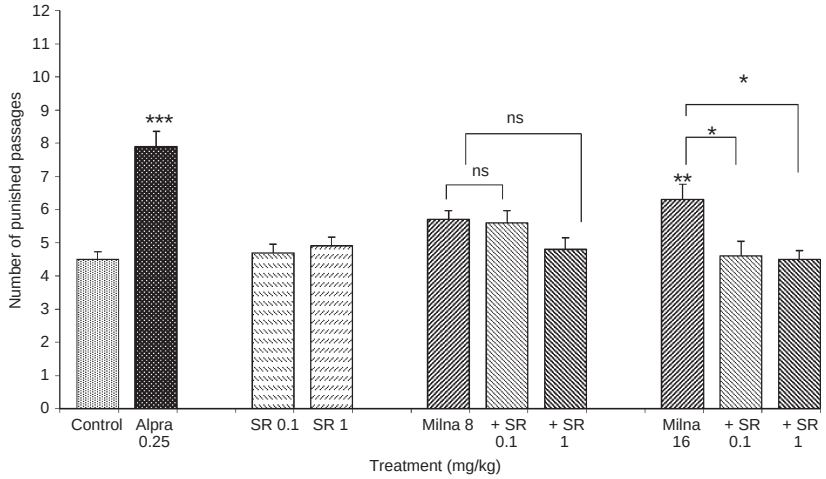

Fig. 2. Effects of acute administration of SR 46349B (SR) (i.p. 45 min pre-test) and milnacipran (Milna) (i.p. 30 min pre-test), on the number of punishments accepted by mice in the FPT. The data are cited as means±S.E.M. ($n=10$). Statistical analysis was performed by a one-way ANOVA followed by a Dunnett's test for comparisons between milnacipran treated groups and vehicle control group: *** ($p<0.001$), ** ($p<0.01$), * ($p<0.05$). For interaction studies, statistical analysis was performed by a two-way ANOVA followed by a Sidak test [*** ($p<0.001$), ** ($p<0.01$), * ($p<0.05$), versus appropriate control group]. A Student's *t*-test was used for statistical analysis between the alprazolam (Alpra) group and control group: *** ($p<0.001$).

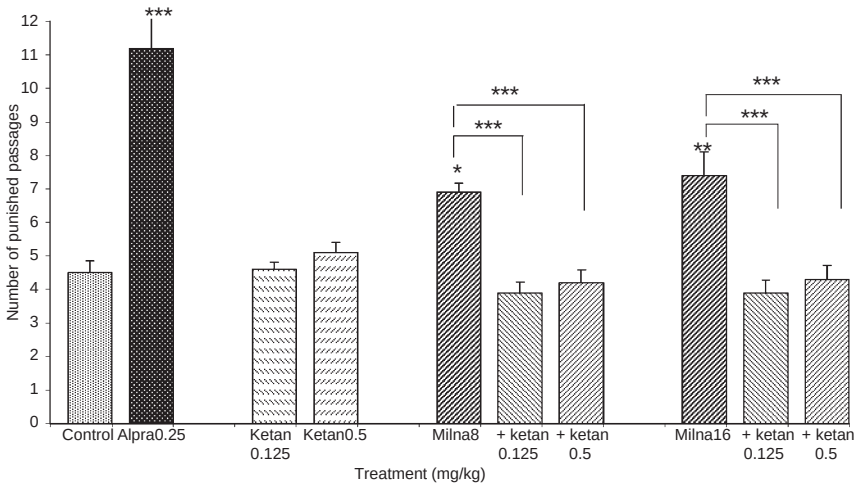

Fig. 3. Effects of acute administration of ketanserin (Ketan) (i.p. 45 min pre-test) and milnacipran (Milna) (i.p. 30 min pre-test), on the number of punishments accepted by mice in the FPT. The data are cited as means ± S.E.M. ($n=10$). Statistical analysis was performed by a one-way ANOVA followed by a Dunnett's test for comparisons between milnacipran treated groups and vehicle control group: *** ($p<0.001$), ** ($p<0.01$), * ($p<0.05$). For interaction studies, statistical analysis was performed by a two-way ANOVA followed by a Sidak test [*** ($p<0.001$), ** ($p<0.01$), * ($p<0.05$), versus appropriate control group]. A Student's *t*-test was used for statistical analysis between the alprazolam (Alpra) group and control group: *** ($p<0.001$).

3.7.3. Effect of DSP-4 and p-CPA on dopamine concentrations

The dopamine levels were affected neither by DSP-4 nor by *p*-CPA administration (Table 5).

3.8. Behavioural analysis

3.8.1. Effect of DSP-4 on milnacipran anxiolytic-like activity in the FPT

Milnacipran at the doses of 8 and 16 mg/kg dramatically increased the number of shocks accepted by naive mice (non-depleted) [$F_{(5,54)}=6.943$, $p<0.001$].

Alprazolam (0.25 mg/kg), used as a positive control, dramatically increased the number of shocks accepted by mice ($p<0.001$). DSP-4 treatment had no effect on the number of punishments accepted by mice ($p>0.05$, after post-hoc analysis) in comparison with control group. DSP-4 treatment totally abolished the anxiolytic-like effect of milnacipran (8 and 16 mg/kg) (Fig. 4).

3.8.2. Effect of p-CPA on milnacipran anxiolytic like activity in the FPT

Milnacipran at the doses of 8 and 16 mg/kg dramatically increased the number of shocks accepted by mice [$F_{(5,54)}=8.54$, $p<0.001$].

Alprazolam (0.25 mg/kg), used as positive control, dramatically increased the number of shocks accepted by mice (8.6 ± 0.6 versus 4.2 ± 0.3 for vehicle control) ($p<0.001$). *p*-CPA treatment had no effect on the number of punishments accepted by mice ($p>0.05$, after post-hoc analysis) in comparison with control group. *p*-CPA treatment totally abolished the anxiolytic-like effect of milnacipran (8 and 16 mg/kg) [$F_{(9,90)}=12.62$, $p<0.001$] (Fig. 5).

4. Discussion

Even though it is now well established that 5-HT selective antidepressants possess anxiolytic properties, their

Table 3
Effect of DSP-4 and *p*-CPA on noradrenaline concentrations

	Hypothalamus ng/g ± S.E.M. (% residual)		Hippocampus ng/g ± S.E.M. (% residual)		Striatum ng/g ± S.E.M. (% residual)		Cortex ng/g ± S.E.M. (% residual)	
Controls	1614.3 ± 52.6		346.8 ± 9.3		153.6 ± 9		227.6 ± 6.1	
DSP-4	1666.5 ± 89.2		160.0 ± 27.7		178.8 ± 26.7		140.9 ± 19.1	
	103.2	NS	46.1	$p \leq 0.05$	116.4	NS	61.9	$p \leq 0.05$
p-CPA	1693.7 ± 98.4		345.7 ± 16		163.3 ± 14		252.0 ± 8.6	
	104.9	NS	99.7	NS	106.3	NS	110.7	NS

Results are expressed as mean of concentration ng/g tissue ± S.E.M. and % residual from controls. Statistical analysis was performed by a one-way ANOVA followed by a Fisher test for comparisons between *p*-CPA or DSP-4 groups and vehicle control group.
Statistic: $p \leq 0.05$. NS, non-significant.

Table 4
Effect of DSP4 and *p*-CPA on serotonin concentrations

	Hypothalamus ng/g tissue ± S.E.M. (% residual)		Hippocampus ng/g tissue ± S.E.M. (% residual)		Striatum ng/g tissue ± S.E.M. (% residual)		Cortex ng/g tissue ± S.E.M. (% residual)	
Controls	1165 ± 41.9		577.7 ± 26		420.9 ± 28.3		507 ± 11.6	
DSP-4	980.1 ± 68.4		552.8 ± 30.7		390.6 ± 42.7		540.7 ± 20.5	
	84.1	$p \leq 0.05$	95.7	NS	92.8	NS	106.6	NS
p-CPA	628.3 ± 45.2		288.9 ± 22.5		255.6 ± 17.7		298.4 ± 16.8	
	53.9	$p \leq 0.05$	50.0	$p \leq 0.05$	60.7	$p \leq 0.05$	58.9	$p \leq 0.05$

Results are expressed as mean of concentration ng/g tissue ± S.E.M. and % residual from controls. Statistical analysis was performed by a one-way ANOVA followed by a Fisher test for comparisons between *p*-CPA or DSP-4 groups and vehicle control group.
Statistic: $p \leq 0.05$. NS, non-significant.

underlying neuropharmacological mechanism of action is still not understood (Zohar and Westenberg, 2000; Bourin et al., 2002; Lambert and Bourin, 2002; Vaswani et al., 2003; Nemeroff, 2003). Here, we examined the effects of the mixed 5-HT/NA reuptake inhibitor (SNRI) milnacipran in the mouse four-plate test (FPT). In synaptosomes from rat cerebral cortex, the ratio of IC50 of 5-HT uptake/IC50 of NA uptake was 0.95, showing that milnacipran inhibits both 5-HT and NA uptake with similar potency with no affinity for the dopaminergic (DA) transporter (Mochizuki et al., 2002). Furthermore, milnacipran was devoid of any affinity ($K_i > 10{,}000$) for 5-HT receptors including the 5-HT_2 subtypes and noradrenergic receptors, especially the α_1 and α_2 receptor subtypes (Mochizuki et al., 2002). Milnacipran administered intraperitoneally at the doses of 4 to 32 mg/kg potently increased the number of punished passages accepted by mice in the FPT paradigm with a maximum effect seen for the dose of 8 mg/kg. These results are in accordance with a previous study (Hascoët et al., 2000) that demonstrated strong anxiolytic-like effect of both SSRIs (excepting fluoxetine) and SNRIs including milnacipran in the FPT. In the conditioned fear stress model of anxiety in the rat, milnacipran decreased freezing behaviour indicating anxiolytic like effect for doses of 10 and 60 mg/kg p.o. (Mochizuki et al., 2002) and for lower doses from 0.5 to 4 mg/kg i.p. in mice (Miyamoto et al., 2002).

First, we have evaluated the implication of the BZD/GABA system in the activity observed in the FPT. The effect of milnacipran was not antagonised by antagonism of BZD receptors flumazenil proving that the BZD/GABA system was not implicated in the mechanism of action of this drug on the FPT. In the same test, however, flumazenil totally antagonised the anti-punished behaviour induced by alprazolam.

One aim of the present article was to examine the potential role of α-adrenoreceptors in the anxiolytic-like effects of milnacipran in the FPT. Excitatory α_1-adrenoreceptors are expressed in the dorsal raphe nucleus where they modulate the activity of ascending serotoninergic neurones (Adell and Artigas, 1999). α_2-Autoreceptors act as the inhibitory somatodendritic and presynaptic autoreceptor at noradrenergic afferents from the locus coeruleus, as well as heteroreceptors on serotoninergic neurones in the prefrontal cortex and hippocampus. Blockade of α_2-adrenoceptors at noradrenergic afferents in the dorsal raphe nucleus has been shown to enhance the firing rate and 5-HT release at the terminal areas (Weikop et al., 2004). In view of the reports that α_2-adrenoceptors are strongly implicated in the modulation of not only noradrenergic but also serotoninergic and dopaminergic neurotransmission, it was of interest to further investigate the effects of the prototypical α_2-adrenoceptor antagonist, idazoxan, following the administration of milnacipran. In addition, the α_1-adrenoceptor antagonist prazosin was used to evaluate the regulatory role of α_1-receptors.

Neither blockade of α_1- nor α_2-noradrenergic receptor subtypes modified the effect of milnacipran, suggesting that these receptors do not participate in the milnacipran anxiolytic-like effect in the FPT.

The implication of α_2-adrenoreceptors in anxiety has been demonstrated but there is still considerable confusion

Table 5
Effect of DSP-4 and *p*-CPA on dopamine concentrations

	Hypothalamus ng/g tissue ± S.E.M. (% residual)		Hippocampus ng/g tissue ± S.E.M. (% residual)		Striatum ng/g tissue ± S.E.M. (% residual)		Cortex ng/g tissue ± S.E.M. (% residual)	
Controls	313.4 ± 11.2		Undetected		5720.84 ± 144.7		599.65 ± 22.0	
DSP-4	377.2 ± 24.3		Undetected		5260 ± 185.0		667.09 ± 36.6	
	120.3	NS	–	–	91.95	NS	61.9	NS
p-CPA	349.7 ± 32.1		Undetected		5885.36 ± 211.0		657.72 ± 34.32	
	111.6	NS	–	–	102.88	NS	109.68	NS

Results are expressed as mean of concentration ng/g tissue ± S.E.M. and % residual from controls. Statistical analysis was performed by a one-way ANOVA followed by a Fisher test for comparisons between *p*-CPA or DSP-4 groups and vehicle control group.
Statistic: $p \leq 0.05$. NS, non-significant.

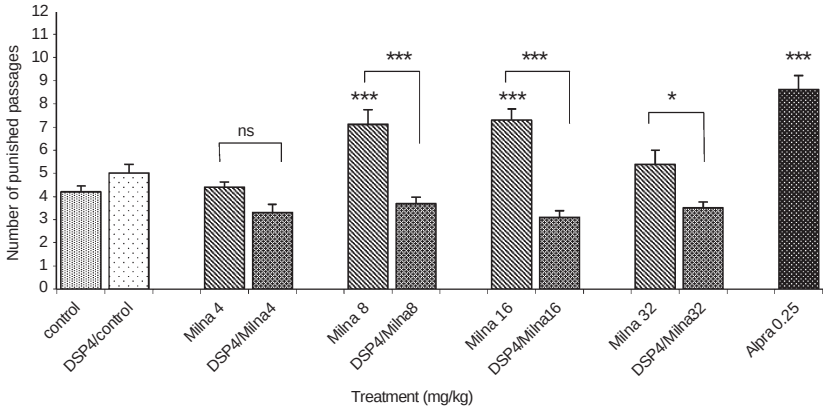

Fig. 4. Effects of DSP-4 pre-treatment on milnacipran (Milna) acute administration, i.p. 30 min in the FPT. The results are given as means ± S.E.M. ($n = 10$). Statistical analysis was performed by a one-way ANOVA followed by a Dunnett's test for comparisons between treated groups and control group: *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$).

as to the effects of agonists or antagonist (Millan, 2003). The α_2-adrenoceptor antagonist yohimbine elicited anxiety in man (Stine et al., 2002). In the FPT, however, no effect was detected even after administration of a large dose range (0.25 to 8 mg/kg i.p) of idazoxan (results not shown). On the other hand, a strong anxiogenic action of yohimbine has been found in many but not all animal models of anxiety (Cole et al., 1995). One hypothesis is that such actions might reflect an induction of NA and DA release in corticolimbic structures (Millan et al., 2000) with a suppressive influence upon the activity of GABAergic neurones. Here, however, as shown by the lack of effect of flumazenil, the GABAergic system does not seem to be implicated. Recently, a combined treatment with idazoxan and venlafaxine has been found to cause an enhancement of NA and DA levels in the prefrontal cortex and hippocampus compared to rats only treated by venlafaxine. However, no elevation of the 5-HT level was found following idazoxan and venlafaxine or duloxepine administration, suggesting that the inhibition of α_2-adrenoreceptors had no enhancing effect on SNRI-induced accumulation of extracellular 5-HT in either structure (Weikop et al., 2004; Gobert et al., 1997). In the present study the lack of effect of idazoxan on the anxiolytic-like effect of milnacipran suggests that either direct blockade of α_2-adrenoceptors is not sufficient to counteract milnacipran's activity in the FPT or in view that idazoxan also possesses some activity at α_1 receptors, it might exert activity via blockade of postsynaptic α_1-adrenoceptors (Millan, 2003).

Prazosin (0.25 to 8 mg/kg) was found to have no action in the four-plate test and did not modify the anxiolytic-like effect of milnacipran in the present study or the effects of venlafaxine (unpublished results). The implication of α_1-adrenoceptors needs further evaluation. Nevertheless despite limited and contradictory evidence for a role of α_1-adrenoceptors in anxiety, the balance of evidence favours an anxiogenic-like action following α_1-adrenoceptor activation (Cecchi et al., 2002; Millan, 2003).

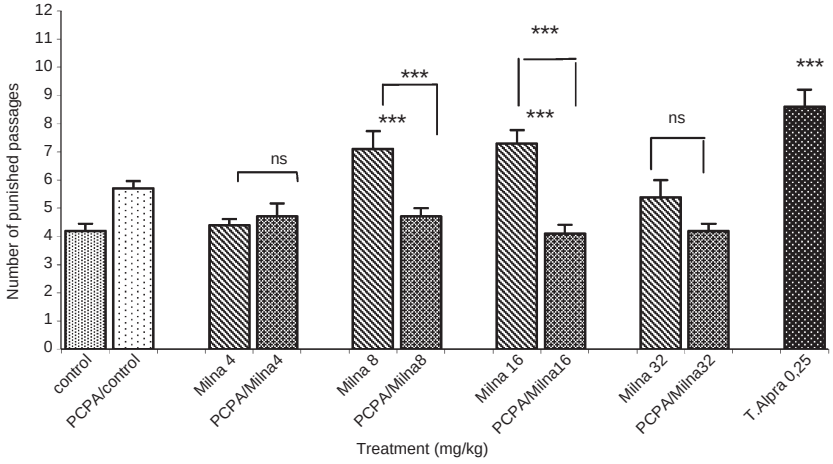

Fig. 5. Effects of *p*-CPA pre-treatment on milnacipran (Milna) acute administration, i.p. 30 min in the FPT. The results are given as means ± S.E.M. ($n = 10$). Statistical analysis was performed by a one-way ANOVA followed by a Dunnett's test for comparisons between treated groups and control group: *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$).

As stated above it does not seem that α-adrenoceptors participate directly in milnacipran anxiolytic-like effect. However, it would be of great interest to evaluate the effect of a direct agonist at the α_2-autoreceptor on milnacipran anxiolytic-like effect in the FPT. This would be expected to decrease 5-HT efflux through an α_2-adrenoceptor-mediated mechanism.

The effects of milnacipran (8 and 16 mg/kg) were not reversed by the selective 5-$HT_{2B/2C}$ receptor antagonist SB 206553 (0.1 and 1 mg/kg), at doses which lack any effect when administered alone. In contrast, the selective 5-HT_{2A} receptor antagonist, SR 46349B (1 mg/kg), completely abolished the milnacipran-induced increase in punished crossings. In addition, the non-selective 5-HT_2 antagonist, ketanserin, totally abolished the anti-punishment activity of milnacipran in the FPT at all doses studied.

Preliminary research implicating the 5-HT_2 receptors in anxiety utilised non-selective 5-$HT_{2A/2C}$ receptor antagonists (the majority of studies involving ritanserin or ketanserin). Few studies have examined the effects of selective 5-HT_{2A} receptor agonists, due to the lack of suitable ligands and the interest in 5-HT_{2A} receptor blockade. It has been suggested that the burying of glass marbles by mice could constitute a useful test for anxiolytic activity since it was differentially inhibited by a variety of anxiolytics (Broekkamp et al., 1986). The 5-$HT_{2A/2C}$ receptor agonists, TFMPP (1-(3-trifluoromethylphenyl)piperazine), and DOI selectively reduced burying at doses not affecting locomotor activity (Njungé and Handley, 1991). Subsequent studies revealed opposing effects of DOI; no influence in the mouse EPM (Rodgers et al., 1995) and both anxiolytic and anxiogenic-like effects in another study depending on the mouse strain and dose of DOI administered (Onaivi et al., 1995).

Enduring changes following stress are of particular importance to the development of pathological responses and thus it is interesting that long-lasting changes in 5-HT_{2A} receptors were found after a single exposure to stress (Stanford, 1996). Of the various compounds with antagonist properties at the 5-HT_{2A} receptor that have been tested clinically, serazepine (CGS-15040A) showed efficacy in a multicenter trial in GAD (Jones and Blackburn, 2002). Among the accepted effective anxiolytic treatments, buspirone has been shown to markedly increase 5-HT_{2A} receptor mRNA levels in various brain areas. This was accompanied by a significant increase in the level of 5-HT_{2A} receptor binding sites in all sub-hippocampal regions. These results demonstrated that chronic buspirone treatment differentially regulates 5-HT_{1A} and 5-HT_{2A} receptor mRNA as well as their expression in various regions of the hippocampus (Chen et al., 1995) which could implicate this receptor subtype in its anxiolytic properties. Equally, it has been suggested that decreased serotonin metabolism and characteristic distribution of 5-HT_{2A} receptors can underlie the expression of genetic predisposition to anxiety (Popova et al., 1996).

Clinical studies have demonstrated SSRI-induced anxiety and even occasional panic attacks at initiation of SSRI treatment, a phenomenon suggested to be mediated by stimulation of 5-HT_2 receptors in the serotonin pathway that projects to the hippocampus and limbic cortex. Equally the 5-HT_{2A} receptor antagonist properties are believed to enhance antidepressant and anti-anxiety activities of many antidepressants (Szabo and Blier, 2002). In contrast, in a previous study (Nic Dhonnchadha et al., 2003a,b), we have observed an anxiolytic-like action of 5-$HT_{2A/2B}$ receptor agonists in the FPT. Different brain areas may be implicated in the anxiolytic-like responses detected, with 5-HT_2 receptors in the peri-aqueductal gray area (PAG) involved in the anxiolytic response observed in the mouse EPM (unconditioned fear), whereas the same receptors in the amygdala may be involved in the response which was provoked in the FPT (conditioned fear), thus explaining different or opposing effects being observed with the same molecule, depending on the paradigm used.

In a more recent study (Nic Dhonnchadha et al., 2005), the anti-punishment action of venlafaxine, another SNRI, was eliminated by both SR 46349B and SB 206553 which when administered alone were without effect. The 5-HT_{2C} receptor antagonist RS 10-2221 failed to alter the effects of venlafaxine, implicating both the 5-HT_{2A} and 5-HT_{2B} receptor subtypes but not the 5-HT_{2C} receptors in the anxiolytic-like action of venlafaxine in the FPT. In addition, in the conditioned fear stress paradigm Miyamoto et al. (2002) suggested that since 5-HT_{2A} receptors are localized postsynaptically, both fluvoxamine and milnacipran may indirectly activate 5-HT_{2A} receptors by increasing the amount of endogenous 5-HT at nerve terminal.

In order to investigate the role of the noradrenergic system in the anxiolytic-like effect of milnacipran, a lesion was induced by systemic administration of the neurotoxin DSP-4, which destroys noradrenergic axon terminals from the locus coeruleus but not those from non-locus coeruleus neurones.

Following DSP-4 lesions, nearly all noradrenergic axons terminals are destroyed in the neocortex, hippocampus, olfactory bulb, thalamus, tectum, cerebellum and spinal cord horn. In contrast most noradrenergic axons are unaffected in the basal forebrain, hypothalamus reticular formation, brain stem motor nuclei and spinal cord ventral horn (Fritschy and Grzanna, 1989). Our present results agree with the above finding that the hypothalamus was not affected by the neurotoxin effect but that the NA concentration was significantly decreased in both hippocampus and cortex but left 5-HT and dopamine intact. The DSP-4 lesion reduced the noradrenaline content of the cortex and the hippocampus to 61.9% and 46.1% of control levels, respectively. These results are in accordance with those of Haddjari et al. (1997).

The resulting lesion completely prevented milnacipran-induced anxiolytic-like effects in the FPT suggesting a strong influence of the noradrenergic axon terminals from

the locus coeruleus. Furthermore, depletion of brain 5-HT content by administration of *p*-CPA also resulted in complete abolition of the anxiolytic-like effect of milnacipran. The depletion of 5-HT was about 50% in all structures studied. Although *p*-CPA administered alone was found to produce anxiolytic-like effects in a modified light–dark test in mice (Artaiz et al., 1998), the integrity of the 5-HT system seems to be necessary for the expression of milnacipran activity in the FPT.

We can conclude that the integrity of both systems NA and 5-HT is required for the effect of milnacipran in the FPT.

Our results have also to be discussed in terms of the antinociceptive effects seen in animals and man with various antidepressants and which can possibly participate in the anxiolytic-like effect observed in the FPT. In animal experiments, systemic administration of antidepressants has yielded confusing results in tests of nociception. Theoretically, a possible analgesic action could account for the effects observed in FPT. However, at doses active in alleviating pain in various tests, morphine did not increase the number of shocks received in the FPT (Boissier et al., 1968). Antinociceptive activity was not observed in the hot plate reaction test with citalopram (Hyttel, 1994) except at high doses (Fasmer et al., 1989), although citalopram was found to induce strong anxiolytic-like effects in the FPT (Hascoët et al., 2000). In addition the antinociceptive effect of paroxetine was found at higher doses than those producing anxiolytic-like effects and was affected by 5-HT_2 mechanism (Duman et al., 2004).

Although most SSRIs and SNRIs have both been found to have analgesic properties, they were not all found to be active in the FPT (Hascoët et al., 2000). For example, fluoxetine did not induce any anti-punishment effects in the FPT. Although milnacipran have been found to induce a dose-dependent antinociceptive effect using the formalin test in rat (Yokogawa et al., 1994), we did not find any effect in the hot plate test in mice at doses inducing anxiolytic-like effect in the FPT (Ripoll et al., in press) Thus, one may conclude that the effects found with antidepressants in the FPT were indeed anxiolytic-like and not analgesic effects.

In conclusion, our results strongly suggest that activation of 5-HT_{2A} receptors is critically involved in the anxiolytic activity of milnacipran but not the other 5-HT_2 receptor subtypes. These results also demonstrate the implication of this receptor in the anxiolytic-like activity on FPT even if benzodiazepines are strongly active on this test. There is evidence that increased level of extracellular 5-HT acting at hetero-receptor can modify noradrenaline release in the brain (Hughes and Stanford, 1996) perhaps via the 5-HT_{2A} receptor subtype. On the other hand, the NA system mediates presynaptic inhibition of the 5-HT system, probably via α_2-adrenoceptors, which may limit the efficacy of SNRI treatment. (Rénéric et al., 2002). The lack of action of milnacipran after NA depletion may result from the abolition of the activity of milnacipran on the locus coeruleus through which 5-HT release was increased (Mongeau, 1998) in addition to the 5-HT_{2A} receptor mediated enhancement of NA levels (Gobert and Millan, 1999) which would not be possible after depletion. The present data lead to the idea of a strong connection between 5-HT_{2A} receptors and NA. Studies using NA-deficient mice have already suggested interdependence between 5-HT and NA (Cryan et al., 2004), and that NA and 5-HT are both essential for the manifestation of the acute behavioural effects of milnacipran.

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