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ORIGINAL INVESTIGATION

**Daisuke Mochizuki · Ryuichi Tsujita · Shinji Yamada
Koh Kawasaki · Yuji Otsuka · Shinji Hashimoto
Tsuyoshi Hattori · Yoshihisa Kitamura · Naomasa Miki**

Neurochemical and behavioural characterization of milnacipran, a serotonin and noradrenaline reuptake inhibitor in rats

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Abstract *Rationale:* The prefrontal cortex is implicated in the pathophysiology of depression, and hypoactivity of this brain area has been found in depressed patients. Reduced function of the serotonergic and noradrenergic systems is another feature of depression. *Objectives:* The present study was aimed at characterizing neurochemically and behaviorally the serotonin and noradrenaline reuptake inhibitor (SNRI), milnacipran, in the prefrontal cortex in comparison with tricyclic antidepressants and selective serotonin reuptake inhibitors. *Methods:* Sodium-dependent monoamine uptake measurement, radioligand binding assays, microdialysis procedure, forced swimming test and conditioned fear stress test were carried out in rats. *Results:* Milnacipran selectively inhibited sodium-dependent [^{3}H]serotonin (5-hydroxytryptamine, 5-HT) and [^{3}H]noradrenaline (NA) uptake into the synaptosomes from rat cerebral cortex (IC_{50}=28.0 and 29.6 nM, respectively) without any affinities for various neuroreceptors. In the medial prefrontal cortex, milnacipran (10 and 30 mg/kg, PO) caused a dose-related increase in the extracellular levels of 5-HT and NA with similar potency, whereas imipramine (10 and 30 mg/kg, PO) caused a dominant increase in the output of NA compared to 5-HT. Milnacipran (30 and 60 mg/kg, PO) significantly reduced the duration of both the immobility time in the forced swimming test and the freezing time in the conditioned fear stress test in rats, which are animal behavioral models for depression and anxiety, respectively. Imipramine and maprotiline were active in the former test, but not in the latter. Fluoxetine and fluvoxamine on the other hand were more active in the conditioned fear test. *Conclusion:* These findings show that milnacipran acts as a SNRI in vitro and in vivo and may be useful for the treatment of anxiety as well as depression.

Keywords Milnacipran · Serotonin and noradrenaline reuptake inhibitor (SNRI) · Sodium-dependent monoamine uptake · Microdialysis · Forced swimming test · Conditioned fear stress test · Rat

D. Mochizuki (✉) · R. Tsujita · S. Yamada · K. Kawasaki
Y. Otsuka · S. Hashimoto · T. Hattori · Y. Kitamura
Life Science Research Center for Pharmacology,
Asahi Kasei Corporation, 632-1 Mifuku, Ohito, Shizuoka, Japan
e-mail: mochizuki.db@om.asahi-kasei.co.jp
Tel.: +81-3-32595804, Fax: +81-3-32595818

D. Mochizuki
Asahi Kasei Corporation, 9-1 Kanda, Mitoshiro-cho, Chiyoda-ku,
Tokyo 101-8481, Japan

N. Miki
Department of Pharmacology, Osaka University Medical School,
2-2 Yamadaoka, Suita, Osaka, Japan

Introduction

The cerebral cortex is one of the most targeted areas of research on depression (Mann et al. 2000). In addition, the prefrontal cortex has been implicated in the mediation of emotional response to stress (Davidson et al. 2000; Drevets 2000; Rilling et al. 2001). Signs of hypoactivity, such as decreased cerebral blood flow and reduced rates of glucose metabolism, have been consistently found in the prefrontal cortex of depressed patients (Drevets et al. 1997; Soares and Mann 1997; Merriam et al. 1999; Videbech 2000). Neurochemically decreased activity of serotonergic and/or noradrenergic central neurotransmission is considered to be a key feature of depression (see reviews of Blier and de Montigny 1994; Briley and Moret 1997). Although the precise relationship between monoaminergic activity and the hypoactivity of the prefrontal cortex in the pathophysiology of depression is still unclear, the fact that most antidepressants act to increase the synaptic concentrations of 5-HT and/or NA in the brain suggests the importance of this dysfunction. In addition, it has also been reported that electrical stimulation of the rat medial prefrontal cortex enhances the extracellular levels of 5-HT in the forebrain (Juckel et al. 1999) and activates locus coeruleus noradrenergic neurons (Jodo et al. 1998). This observation may be relevant to the mechanism of electroconvulsive therapy in depression.

Milnacipran [(±)-cis-2-aminomethyl-N,N-diethyl-1-phenylcyclopropanecarboxamide monohydrochloride] is a clinically effective antidepressant (Kasper et al. 1996; Lopez-Ibor et al. 1996; Puech et al. 1997). Moret et al. (1985) showed that it inhibits 5-HT and NA uptake into rat hypothalamus slices with no affinity for adrenergic, muscarinic, histaminergic H_1, dopaminergic D_2 or serotonergic 5-HT_2 receptors. These authors also showed that milnacipran increases the extracellular levels of 5-HT and NA in the hypothalamus of freely moving guinea pigs (Moret and Briley 1997). Unlike many antidepressant drugs, milnacipran does not produce pharmacologically active metabolites (Puozzo and Leonard 1996) so that the behavioral, neurochemical and clinical effects of milnacipran are a result of the pharmacological profile of the drug itself. Although, as mentioned above, the cerebral cortex is one of the most important regions of research in depression to date, few experimental studies of milnacipran have used this tissue.

In the present study, we investigated the effects of milnacipran in rat cortical tissue on in vitro monoamine uptake and monoamine oxidase (MAO) activity, and the extracellular monoamine levels in vivo in the cortex in comparison with imipramine. We also studied the affinity of milnacipran for a wider range of receptors than those previously reported (Moret et al. 1985). To evaluate the behavioural effects of milnacipran, we chose two models, the forced swimming test and the conditioned fear stress test. The former is a useful rodent model that predicts the clinical efficacy of many types of antidepressants (Porsolt et al. 1977, 1978), including the selective serotonin reuptake inhibitors (SSRI) (Rénéric and Lucki 1998). The drugs that increase central noradrenergic activity tend to show a strong activity in this model (Porsolt et al. 1979). The conditioned fear stress test, on the other hand, is considered to be an animal model of anxiety or fear (Inoue et al. 1993), and it is particularly sensitive to the drugs that increase central serotonergic activity (Inoue et al. 1996). The effects of milnacipran in these behavioural models were compared to those of imipramine and maprotiline and the SSRIs, fluoxetine and fluvoxamine.

Materials and methods

Animals

These experiments were performed on male Sprague-Dawley or Wistar rats (Charles River, Yokohama, Japan). Rats were housed in a room at a 12-h dark/light cycle at 22–25°C with food and water ad libitum. All studies reported here were approved by the Animal Care and Use Committee of the Institute for Life Science Research, Asahi Kasei Corporation, and were carried out in compliance with the Guide for the Care and Use of Laboratory Animals of the Institute for Life Science Research, Asahi Kasei Corporation.

Binding assay

Radioligand binding assays were performed using the crude synaptosomal membrane fraction of male Sprague-Dawley rat brains or the membranes expressing the cloned receptor. Whole brains, discrete brain regions (Table 1) or COS-7 cells expressing 5-HT receptor subtypes (Mochizuki et al. 1992) were homogenized in 10 volumes of ice-cold 0.32 M sucrose. The homogenate was centrifuged at 1000 *g* for 10 min. The supernatant was centrifuged at 48,000 *g* for 20 min. The resulting membrane pellet was resuspended in 50 mM TRIS-HCl buffer (pH 7.4) or appropriate buffer and incubated at 37°C for 30 min to degrade or dissociate any bound endogenous ligands. The membranes were then centrifuged at 48,000 *g* for 20 min and resuspended in appropriate buffer. The assay condition used for monoamine transporter binding and various receptor binding are indicated in Table 1. The incubations were stopped by rapid filtration through Whatman GF/C filters. The filters were rinsed three times with 4 ml ice-cold buffer and the retained radioactivity was measured by liquid scintillation (2300TR, Packard, USA). The K_i was calculated from the Chang and Prusoff equation $K_i=IC_{50}/(1+L/K_d)$, where L is the concentration of radiolabelled ligand.

Assays for MAO-A and MAO-B

Sprague-Dawley rats were decapitated, their brains removed and the cerebral cortex dissected out. The tissues were homogenized in 4 vol potassium phosphate buffer (50 mM; pH 7.4) and centrifuged at 1000 *g* for 3 min at 4°C. The supernatant was used as an enzyme source for assays. MAO activity was determined essentially as previously described (Wurtman and Axelrod 1963; Itzhak et al. 1991). In brief, the brain homogenate was preincubated with the drug to be tested in a final volume of 180 µl of the buffer at 37°C for 20 min. At the end of the preincubation period, the enzyme reaction was started by the addition of 20 µl of 1.5 µM [^{14}C]-5-HT (MAO-A substrate) including 100 µM non-radioactive 5-HT, or 20 µl of 2 µM [^{14}C]-β-phenylethylamine (β-PEA; MAO-B substrate) including 20 µM non-radioactive β-PEA. The incubation was continued for 10 min at 37°C. Then, the reaction was stopped by addition of 200 µl of 2 N HCl, and the deaminated metabolites were extracted by vigorous shaking with 4 ml diethylether (5-HT extraction) or *n*-heptane (β-PEA extraction). After centrifugation at 1000 *g* for 1 min, the water-phase was frozen in dry ice and the organic layer poured into vials. Reaction mixtures as described above but lacking the homogenate served as blanks. The radioactivity was measured by liquid scintillation (2300TR, Packard, USA). Under our experimental conditions, the enzyme reaction was linear with time and homogenate concentration. The percentage inhibition of drugs was calculated by comparing the blank value. IC_{50} values were determined from log dose-inhibition curves.

Assays for sodium-dependent monoamine uptake

Sprague-Dawley rats were killed by decapitation. Their brains were rapidly removed and the cerebral cortex and the striatum were immediately dissected out, and homogenized in 10 and 20 vol, respectively, of 0.32 M cold sucrose. The homogenates were centrifuged at 1000 *g* for 10 min and the resulting supernatants were centrifuged at 12,000 *g* for 20 min. The pellets obtained were resuspended in the same volume of sucrose solution, homogenized, and centrifuged at 12,000 *g* for 20 min. The final pellets were suspended in appropriate volumes of the same medium to obtain final protein concentrations that were on the linear portion of the protein curve. These synaptosomal solutions were immediately used for uptake measurements.

Synaptosomes from cerebral cortex (5-HT, NA) or striatum (dopamine, DA) were preincubated with the drug to be tested in a final volume of 450 µl of incubation buffer (119 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, 10 mM glucose, 0.1% ascorbic acid, 10 µM pargyline, 25 mM TRIS, pH 7.4) bubbled with 95% O_2 and 5% CO_2 at 37°C for 10 min. Thereafter, [^{3}H]5-HT, [^{3}H]NA or [^{3}H]DA was added at a final concentration of 4 nM, 2 nM or 5 nM, respectively. The uptake process was terminated after 5 min at 37°C by addition of 4 ml ice-cold buffer

Table 1 Methodologies for the binding assays used to determine the selectivity of milnacipran

	Radioligand (concn)	Blank (concn)	Tissue	Buffer	Incubation conditions
Receptor					
5-HT_{1A}	[^{3}H]8-OH-DPAT (0.5 nM)	5-HT (10 µM)	Rat hippocampus	50 mM TRIS-HCl, pH 7.4, 2.5 mM $MgCl_2$, 10 µM pargyline	30°C, 30 min
5-HT_{1B}	[^{3}H]5-HT (4 nM)	5-HT (10 µM)	Human cloned	50 mM TRIS-HCl, pH 7.7. 4 mM $CaCl_2$, 0.1% ascorbic acid, 10 µM pargyline	25°C, 30 min
5-HT_{1D}	[^{3}H]5-HT (4 nM)	5-HT (10 µM)	Human cloned	50 mM TRIS-HCl, pH 7.7. 4 mM $CaCl_2$, 0.1% ascorbic acid, 10 µM pargyline	25°C, 30 min
5-HT_{1E}	[^{3}H]5-HT (4 nM)	5-HT (10 µM)	Human cloned	50 mM TRIS-HCl, pH 7.7. 4 mM $CaCl_2$, 0.1% ascorbic acid, 10 µM pargyline	25°C, 30 min
5-HT_2	[^{3}H]Ketanserin (1 nM)	Mianserin (10 µM)	Rat cerebral cortex	50 mM TRIS-HCl, pH 7.4, 0.1 µM prazosin	25°C, 30 min
5-HT_7	[^{3}H]5-HT (2 nM)	5-HT (10 µM)	Rat cloned	50 mM TRIS-HCl, pH 7.7, 4 mM $CaCl_2$, 0.1% ascorbic acid, 10 µM pargyline	25°C, 45 min
α_1	[^{3}H]Prazosin) (0.2 nM	Prazosin (0.1 µM)	Rat cerebral cortex	50 mM TRIS-HCl, pH 7.4, 0.005% ascorbic acid	25°C, 30 min
α_2	[^{3}H]Rauwolscine (2 nM)	Phentolamine (10 µM)	Rat cerebral cortex	50 mM TRIS-HCl, pH 7.4, 0.5 mM EDTA	25°C, 30 min
β	[^{3}H]DHA (2 nM)	(–)Alprenolol (0.1 µM)	Rat cerebral cortex	50 mM TRIS-HCl, pH 7.4, 0.005% ascorbic acid, 10 µM pargyline	23°C, 20 min
D_1	[^{3}H]SCH23390 (0.2 nM)	SKF38393 (10 µM)	Rat striatum	50 mM TRIS-HCl, pH 7.4, 1 mM $MgCl_2$	25°C, 60 min
D_2	[^{3}H]YM-09151-2 (0.1 nM)	(+)Butaclamol (1 µM)	Rat striatum	50 mM TRIS-HCl, pH 7.4, 120 mM NaCl	25°C, 60 min
D_4	[^{3}H]YM-09151-2 (0.15 nM)	Clozapine (10 µM)	Human cloned	50 mM TRIS-HCl, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, 120 mM NaCl, 5 mM EDTA, pH 7.4	25°C, 60 min
mACh[a]	[^{3}H]QNB (0.1 nM)	Atropine (10 µM)	Rat cerebral cortex	50 mM phosphate buffer, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$	25°C, 60 min
H_1	[^{3}H]Pyrilamine (3 nM)	Tryprolidine (2 µM)	Rat cerebral cortex	50 mM TRIS-HCl, pH 7.4	25°C, 30 min
NMDA	[^{3}H]MK-801) (3 nM	Phencyclidine (10 µM)	Rat cerebral cortex	10 mM HEPES/TRIS, pH 7.4, 10 µM glutamate, 10 µM glycine	25°C, 60 min
Sigma	[^{3}H]DTG (2 nM)	Haloperidol (10 µM)	Rat whole brain	50 mM TRIS-HCl, pH 8.0	25°C, 60 min
Opioid	[^{3}H]Naloxone (2 nM)	Naloxone (10 µM)	Rat whole brain	50 mM TRIS-HCl, pH 7.4	25°C, 30 min
$GABA_A$	[^{3}H]GABA (5 nM)	GABA (1 mM)	Rat cerebral cortex	50 mM TRIS-HCl, pH 7.4	4°C, 30 min
$GABA_B$	[^{3}H]Baclofen (10 nM)	GABA (1 mM)	Rat cerebral cortex	50 mM TRIS-HCl, pH 7.4, 2.5 mM $CaCl_2$	25°C, 30 min
BDZ[b]	[^{3}H]Ro15-4513 (6 nM)	Ro15-1788 (10 µM)	Rat cerebral cortex	50 mM TRIS-HCl, pH 7.4	4°C, 45 min
Transporter					
5-HT	[^{3}H]Paroxetine (0.2 nM)	Femoxetine (10 µM)	Rat cerebral cortex	50 mM TRIS-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl	20°C, 45 min
Noradrenaline	[^{3}H]Nisoxetine (1 nM)	Desipramine (1 µM)	Rat cerebral cortex	50 mM TRIS-HCl, pH 7.4, 300 mM NaCl, 5 mM KCl	20°C, 60 min
Dopamine	[^{3}H]BTCP (0.2 nM)	GBR12909 (10 µM)	Rat striatum	50 mM phosphate buffer, pH 7.4	4°C, 90 min

[a] Muscarinic acetylcholine [b] Benzodiazepine

and rapid filtration through Whatman GF/C filters. The filters were rinsed three times with 4 ml ice-cold buffer and the retained radioactivity was measured by liquid scintillation (2300TR, Packard, USA). To measure sodium-dependent monoamine uptake, non-specific uptake was determined by replacing NaCl by 119 mM LiCl. IC_{50} values were determined from log concentration-inhibition curves.

Microdialysis procedure

Wistar rats weighing 200–400 g were anesthetized with pentobarbital (40 mg/kg, IP) and mounted on a stereotaxic apparatus (Narishige, Tokyo, Japan). A guide cannula (G-1-4-03, Eicom, Japan) was implanted unilaterally to the surface of medial prefrontal cortex (A +3.2, ML +0.8, DV –1.0). The coordinates were with respect to bregma, and according to the atlas of Paxinos and Watson (1986). Microdialysis experiments were conducted in freely moving rats 20–24 h after the cannula implantation. A microdialysis probe (A-1-4-03, Eicom, Japan) was inserted into the guide cannula so that 3.0 mm of the probe was exposed to the medial prefrontal cortex. The probe was perfused with the artificial cerebrospinal fluid (145 mM NaCl, 3.0 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH 7.4) at a flow rate of 2 µl/min. Thirty-minute samples of the dialysates were collected in microtubes containing 10 µl of 0.2 M acetic acid with 0.02 mg/ml cysteine. 5-HT and NA in dia-

lysates were separated by a reverse-phase high performance liquid chromatography (column for 5-HT: Ultra-sphere IP, Beckman, USA; column for NA: MA5-ODS, Eicom, Japan) and detected with a graphite electrode (ECD-300; Eicom, Japan). The mobile phase consisted of 0.1 M acetate buffer (pH 4.5) containing 27 mg/l sodium octylsulfate, 60 mg/l sodium EDTA and 10% methanol for 5-HT, and of 0.1 M phosphate buffer (pH 6.0), containing 400 mg/l sodium 1-octanesulfonate, 50 mg/l sodium EDTA and 5% methanol for NA. Drugs were administered orally after the collection of three basal fractions containing similar concentrations of 5-HT or NA. Data are given as percentages of the basal values.

Forced swimming test

We essentially followed the method of Porsolt et al. (1978). Wistar rats, weighing 170–200 g, were placed individually in a plastic cylinder (height 37 cm, diameter 15.5 cm) containing water at 25°C to a depth of 20 cm. An initial 13 min pre-test swimming session was conducted on the day before the test. Twenty-four hours later, a 6-min measurement test was carried out. The total period of immobility was recorded during the 6-min test with an analysis program (TARGET series/7 M: Neuroscience Inc., Japan). All tested drugs were administered as a single oral dose 60 min before the test measurement. The doses of milnacipran, imipramine and maprotiline were 10, 30 and 60 mg/kg and the doses of fluoxetine and fluvoxamine were 30, 60 and 100 mg/kg. Sixteen animals were used for each dose.

Conditioned fear stress test

Conditioned fear stress test was performed as previously described (Hashimoto et al. 1999). Sprague-Dawley rats, weighing 230–270 g, were subjected to inescapable electric foot shock (2.5 mA of scrambled shock, 10 ms shock every 100 ms; shock duration of 30 s×5; and an intershock interval of 30 s) in a chamber with a grid floor. Electric shock was provided via an ENV-410 shock generator (Med Associates, USA). About 24 h after the electric foot shock, the rats were again placed and observed for 10 min in the shock chamber but no current was applied to the floor of the chamber. During the observation, the duration of freezing behavior was recorded using a modification time-sampling procedure (Fanselow 1980). Freezing was defined as the absence of all observable movement of the skeleton and the vibrissae, except those related to respiration. All other behaviors were scored as activity. The percentage score represented the number of 10-s periods during which the animal froze for the entire 10-s period. Behavior was recorded on videotape and scored by two independent observers who were blind to the treatment. Drugs were administered orally 60 min before the observation period. The doses of milnacipran, imipramine and maprotiline were 10, 30 and 60 mg/kg and the doses of fluoxetine and fluvoxamine were 30, 60 and 100 mg/kg. Sixteen animals were used for each dose.

Locomotor activity

Locomotor activity was determined in Wistar or Sprague-Dawley rats. The dose schedules were the same as those of the two behavioral models. In brief, in accord with the forced swimming test, the locomotor activity of rats was measured for 6 min beginning 60 min after the administration of drugs. For the conditioned fear stress test, it was measured for 10 min beginning 60 min after the administration of drugs. The SCANET system (Toyo Sangyo, Japan) was used to measure locomotor activity. Each cage (45×45 cm) was fitted with two sets of 72 infrared photo beams (two-dimensional measurements) placed 3 cm above the floor. Beam breaks were recorded automatically.

Drugs

Milnacipran hydrochloride (synthesized by Pierre Fabre Médicament, France), imipramine hydrochloride (Sigma, USA), maprotiline hydrochloride (Sigma), mianserin hydrochloride (Sigma), desipramine hydrochloride (Sigma), fluvoxamine maleate, fluoxetine hydrochloride and paroxetine hydrochloride (synthesized by Asahi Kasei Corporation) were used. [^{14}C]5-HT (1.8 GBq/mmol), [^{14}C]β-phenylethylamine (1.6 GBq/mmol), [^{3}H]8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT; 5320.6 GBq/mmol), [^{3}H]ketanserin (2852.7 GBq/mmol), [^{3}H]prazosin (2752.8 GBq/mmol), [^{3}H]rauwolscine (2978.5 GBq/mmol), [^{3}H]dihydroalprenolol (DHA; 2035.0 GBq/mmol), [^{3}H] SCH23390 (2638.1 GBq/mmol), [^{3}H]YM-09151-2 (3182.0 GBq/mmol), [^{3}H]quinuclidinyl benzilate (QNB; 1679.8 GBq/mmol), [^{3}H]pyrilamine (865.8 GBq/mmol), [^{3}H]MK-801 (751.1 GBq/mmol), [^{3}H]1,3-di(2-tolyl)-guanidine (DTG; 1417.1 GBq/mmol), [^{3}H]naloxone (1850.0 GBq/mmol), [^{3}H]γ-aminobutyric acid (GABA; 3688.9 GBq/mmol), [^{3}H]baclofen (1287.6 GBq/mmol), [^{3}H]Ro15-4513 (773.3 GBq/mmol), [^{3}H]paroxetine (854.7 GBq/mmol), [^{3}H]nisoxetine (2960.0 GBq/mmol), [^{3}H]N-[1-(2-benzo(b)thiophenyl)cyclophenyl]piperidine (BTCP, 1813.0 GBq/mmol), [^{3}H]5-HT (939.8 GBq/mmol), [^{3}H]noradrenaline (2597.4 GBq/mmol), [^{3}H]dopamine (751.1 GBq/mmol) were purchased from NEN DuPont. All other compounds used were analytical grade and were obtained from Wako Pure Chemical (Japan), Sigma and RBI (USA). Milnacipran and imipramine were dissolved in distilled water. Maprotiline, fluoxetine and fluvoxamine were dissolved in 0.5% methylcellulose. All drugs were administered PO in a volume of 0.2 ml/100 g body weight.

Statistical analysis

The effects of drugs in the microdialysis tests were analyzed by one-way analysis of variance (ANOVA) for repeated measures, and in the behavioral studies they were analyzed by one-way analysis of variance (ANOVA). When significant differences (P<0.05) were found, post hoc comparisons were made with Dunnett's test.

Results

In vitro radioligand binding studies

Milnacipran had high affinity for both 5-HT and NA transporters (K_i=8.5 and 31 nM, respectively), but no affinity for DA transporters (Table 2). Imipramine had high affinity for both 5-HT and NA transporters (K_i=6.0 and 18 nM, respectively).

Table 2 Affinity of milnacipran and imipramine for monoamine transporters in vitro. Drug inhibition of radioligand binding was studied as described in Table 1. Affinities are given as K_i values except where 10 μM of the displacing agent failed to displace 50% of the specific binding. Each value represents the mean±SEM of three separate experiments, which were performed in duplicate using nine concentrations ([^{3}H]paroxetine and [^{3}H]nisoxetine) and five concentrations ([^{3}H]BTCP)

Drug	K_i (nM)		
	[^{3}H]Paroxetine	[^{3}H]Nisoxetine	[^{3}H]BTCP
Milnacipran	8.5±0.63	31±1.1	>10,000
Imipramine	6.0±0.32	18±1.0	>10,000

Table 3 Binding profiles of milnacipran and reference compounds for various receptors in vitro. Drug inhibition of radioligand binding was studied as described in Table 1. Affinities are given as K_i values except where 10 µM of the displacing agent failed to displace 50% of the specific binding. Each value represents the mean of two separate experiments, which were performed in duplicate or triplicate using 5–11 concentrations. *n.d.* not determined

Receptor	Ki (nM)				
	Milnacipran	Imipramine	Maprotiline	Mianserin	Fluvoxamine
5-HT_{1A}	>10,000	>10,000	>10,000	390	>10,000
5-HT_{1B}	>10,000	n.d.	n.d.	n.d.	n.d.
5-HT_{1D}	>10,000	n.d.	n.d.	n.d.	n.d.
5-HT_{1E}	>10,000	n.d.	n.d.	n.d.	n.d.
5-HT_2	>10,000	110	80	2.4	4300
5-HT_7	>10,000	n.d.	n.d.	n.d.	n.d.
α_1	>10,000	85	68	88	1100
α_2	>10,000	920	3100	41	2900
β	>10,000	2000	4700	620	>10,000
D_1	>10,000	660	90	91	>10,000
D_2	>10,000	330	410	1000	3600
D_4	>10,000	n.d.	n.d.	n.d.	n.d.
mACh[a]	>10,000	52	350	2300	>10,000
H_1	>10,000	6.0	2.4	3.2	2800
NMDA	>10,000	>10,000	>10,000	>10,000	>10,000
Sigma	>10,000	260	610	2700	910
Opioid	>10,000	>10,000	n.d.	>10,000	n.d.
$GABA_A$	>10,000	>10,000	n.d.	>10,000	n.d.
$GABA_B$	>10,000	>10,000	n.d.	>10,000	n.d.
Benzodiazepine	>10,000	>10,000	n.d.	>10,000	n.d.

[a] Muscarinic acetylcholine

Milnacipran was devoid of affinity (K_i>10,000 nM) for all of the neurotransmitter receptors tested, which attested its selectivity (Table 3). Imipramine had high affinity for histamine H_1 receptors, and relatively moderate affinities for muscarinic acetylcholine, α_1-adrenergic and 5-HT_2 receptors. Maprotiline had high affinity for histamine H_1 receptors, relatively moderate affinities for α_1-adrenergic, 5-HT_2 and dopamine D_1 receptors, and slightly lower affinity for muscarinic acetylcholine receptors. Mianserin had high affinities for H_1 and 5-HT_2 receptors, and relatively moderate affinities for α_2-adrenergic, α_1-adrenergic and dopamine D_1 receptors. Fluvoxamine had little or no affinity for receptors tested, except a weak affinity for sigma receptors.

MAO-A and MAO-B activity

In this study, as expected, clorgyline, a selective MAO-A inhibitor, potently inhibited MAO-A activity (IC_{50}=3.0 nM ±0.5) but was a weak inhibitor of MAO-B activity (IC_{50}=1600 nM±340). (–)Deprenyl, a selective MAO-B inhibitor, potently blocked MAO-B activity (IC_{50}=4.9 nM ±0.5) but was a weak inhibitor of MAO-A activity (IC_{50}=1100 nM±68). Milnacipran up to 100 µM had no effect on the activity of either MAO-A or MAO-B in rat brain homogenates (Table 4). Imipramine was only a very weak inhibitor of MAO-B (IC_{50}=16.1 µM±7.9), and inhibited about 40% of MAO-A activity at 100 µM. Mianserin inhibited about 40% of MAO-B activity at 100 µM, and had little effect on MAO-A activity.

Sodium-dependent monoamine uptake

The potency and selectivity of milnacipran in comparison to some classical antidepressants and SSRIs are shown in Table 5 and Fig. 1. Milnacipran inhibited sodium-dependent [^{3}H]5-HT and [^{3}H]NA uptake into the synaptosomes from rat cerebral cortex with equal potency (IC_{50}= 28.0±1.7 and 29.6±1.5 nM, respectively; ratio of IC_{50} of 5-HT uptake/IC_{50} of NA uptake=0.95). Imipramine also blocked [^{3}H]5-HT and [^{3}H]NA uptake with similar potency (IC_{50}=18.5 and 23.0 nM, respectively). Desipramine and maprotiline were 300- and 640-fold, respectively, more active at inhibiting NA uptake than 5-HT uptake while fluoxetine, fluvoxamine and paroxetine had approximately 20-, 100- and 200-fold, respectively, greater affinity for 5-HT uptake than for NA uptake.

Table 4 Effects of milnacipran, imipramine and mianserin on MAO-A and B activity in rat brain homogenate. Homogenate from rat cerebral cortex was used for MAO activity. The homogenate was incubated with drug and substrate ([^{3}H]5-HT for MAO-A or [^{3}H]β-PHA for MAO-B) at 37°C for 10 min. MAO activity (% of control) is given as the mean±SEM of three separate experiments

Drug	Percentage of control activity	
	MAO-A	MAO-B
Milnacipran		
10^{-6} M	95.9±2.7	98.4±1.0
10^{-5} M	97.9±1.3	94.8±2.0
10^{-4} M	95.2±1.5	92.9±2.5
Imipramine		
10^{-6} M	91.9±0.7	81.9±3.1
10^{-5} M	77.2±6.9	55.8±7.5
10^{-4} M	57.0±3.4	22.0±3.9
Mianserin		
10^{-6} M	91.6±4.4	92.7±3.8
10^{-5} M	90.3±1.6	80.0±4.6
10^{-4} M	86.2±0.3	56.8±4.0

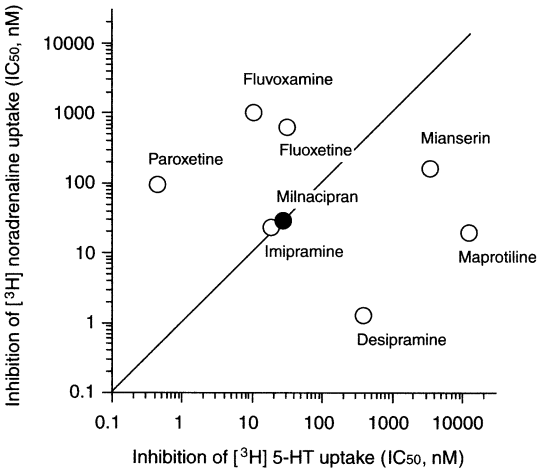

Fig. 1 Comparison of the inhibitory potency for sodium-dependent [^{3}H]5-HT and [^{3}H]NA uptake into the cerebral cortical synaptosomes. The values are taken from Table 5

Table 5 Effects of milnacipran and reference compounds on sodium-dependent monoamine uptake into rat cerebral cortical synaptosomes. Synaptosomes from rat cerebral cortex (for 5-HT and NA) or striatum (for DA) were used for the uptake of the [^{3}H]ligands (4 nM [^{3}H]5-HT, 2 nM [^{3}H]NA or 5 nM [^{3}H]DA at 37°C for 10 min). To determine sodium-dependent monoamine uptake, NaCl (in incubation buffer) was replaced by LiCl. Each value represents the mean±SEM of three separate experiments, which were performed in duplicate

Drug	Uptake inhibition (IC_{50}, nM)			5-HT/NA ratio
	[^{3}H]5-HT	[^{3}H]NA	[^{3}H]DA	
Milnacipran	28.0±1.7	29.6±1.5	>10,000	0.95
Imipramine	18.5±1.1	23.0±1.2	>10,000	0.80
Desipramine	382±52	1.26±0.16	3580±86	300
Maprotiline	12,700±890	19.8±0.74	6920±1100	640
Mianserin	3450±330	159±13	5350±760	22
Fluoxetine	32.1±3.8	632±53	5170±580	0.051
Fluvoxamine	10.6±1.0	1030±28	>10,000	0.010
Paroxetine	0.45±0.028	97.1±1.0	1170±140	0.0046

Microdialysis

The mean baseline levels of 5-HT and NA (pg/30 min dialysate sample) were 0.427 and 0.372, respectively. Baseline values for the different groups of treatment did not vary significantly. The administration of milnacipran at 10 and 30 mg/kg PO produced a dose-related increase in extracellular 5-HT concentrations [$F_{treatment}(2,105)=141.414$, $P<0.01$; $F_{time}(6,105)=8.819$, $P<0.01$; $F_{interaction}(12,105)=2.749$, $P<0.01$], with maximal increases of 256% and 317%, respectively, 1 h after administration (Fig. 2A). Imipramine also produced a significantly increase in extracellular 5-HT levels at 30 mg/kg PO [$F_{treatment}(2,103)=24.746$, $P<0.01$; $F_{time}(6,103)=0.693$, $P=0.655$; $F_{interaction}(12,103)=0.593$, $P=0.843$]; however, the magnitude of the increase (about 165% from 1 h until 3 h 30 after administration) was much less than that caused by milnacipran (Fig. 2B).

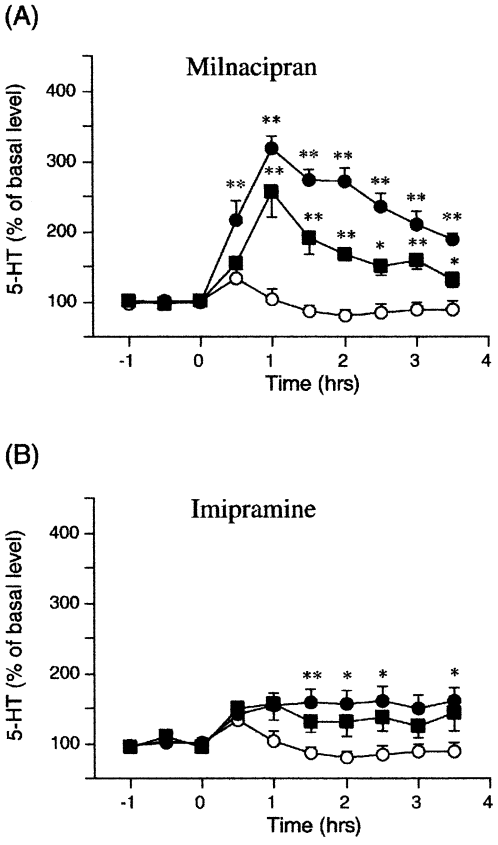

Fig. 2 Effects of milnacipran (**A**) and imipramine (**B**) on the extracellular 5-HT concentrations in the medial prefrontal cortex. The animals were given vehicle (*open circles*), 10 (*black squares*) or 30 (*black circles*) mg/kg of drugs PO at time 0. The results are expressed as percentages of the mean of three measurements taken before drug administration. Each point represents the mean±SEM from six rats. *$P<0.05$, **$P<0.01$ (ANOVA for repeated measures followed by Dunnett's test) when compared to the vehicle treated group

Milnacipran increased extracellular NA concentrations in a dose-related manner [$F_{treatment}(2,101)=77.345$, $P<0.01$; $F_{time}(6,101)=4.722$, $P<0.01$; $F_{interaction}(12,101)=3.393$, $P<0.01$]. The maximal value of the extracellular levels of NA with the administration of doses of 10 and 30 mg/kg, PO reached 223% and 352%, respectively, of basal level. The peak level at 30 mg/kg was reached 3 h after the administration (Fig. 3A). Imipramine also produced dose-dependently increases in extracellular noradrenaline levels [$F_{treatment}(2,102)=92.409$, $P<0.01$; $F_{time}(6,102)=11.686$, $P<0.01$; $F_{interaction}(12,102)=5.320$, $P<0.01$] (Fig. 3B). The maximal value of the extracellular levels of NA with the administration of doses of 10 and 30 mg/kg, PO reached 340% and 370%, respectively, of basal level. This magnitude of increase was approximately equal to that observed with milnacipran. Imipramine caused the maximal increase in extracellular levels of NA at 2.5–3 h.

Forced swimming

There was a significant effect of milnacipran treatment on the immobility time in the forced swimming test

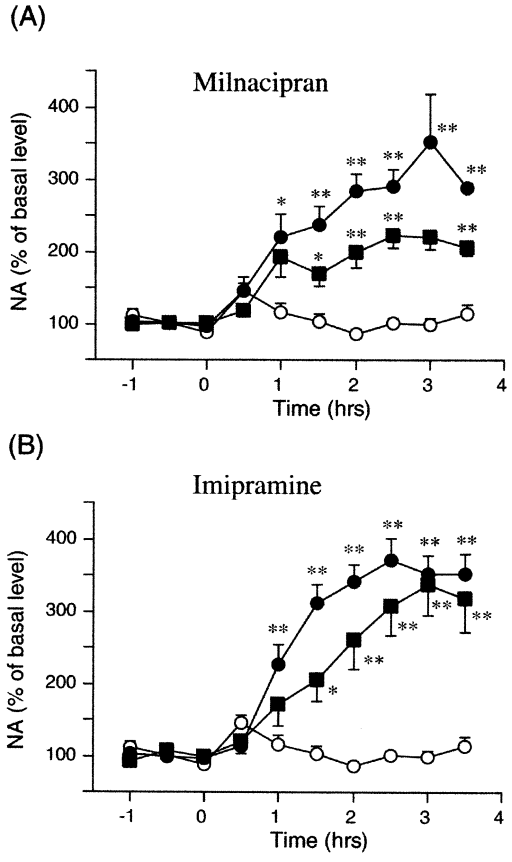

Fig. 3 Effects of milnacipran (**A**) and imipramine (**B**) on the extracellular noradrenaline concentrations in the medial prefrontal cortex. The animals were given vehicle (*open circles*), 10 (*black squares*) or 30 (*black circles*) mg/kg of drugs PO at time 0. The results are expressed as percentages of the mean of three measurements taken before drug administration. Each point represents the mean±SEM from six rats. **P*<0.05, ***P*<0.01 (ANOVA for repeated measures followed by Dunnett's test) when compared to the vehicle treated group

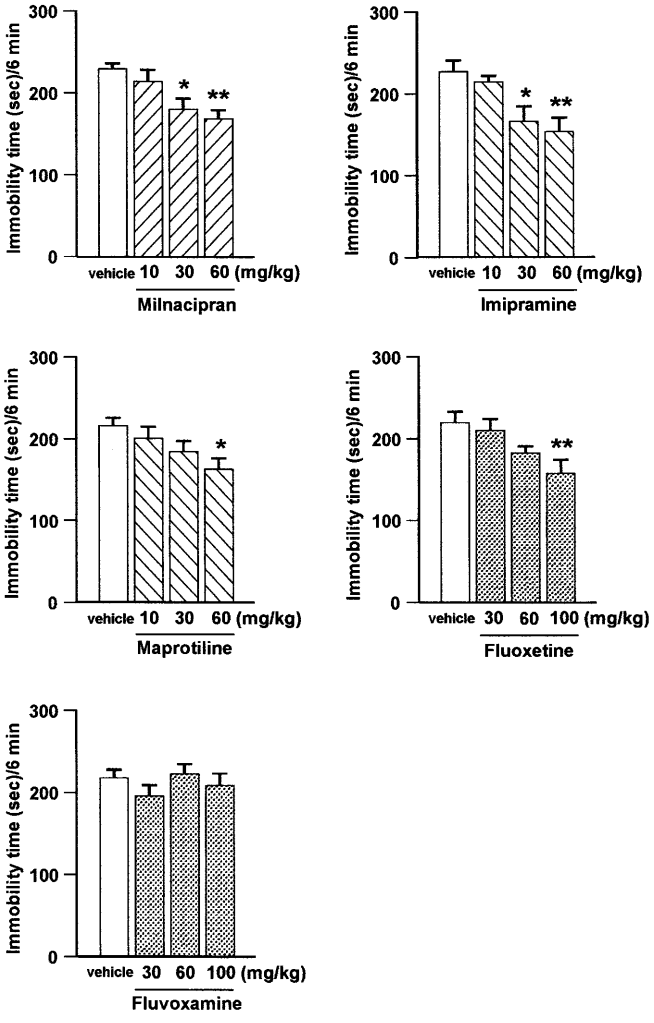

Fig. 4 Effects of milnacipran, imipramine, maprotiline, fluoxetine and fluvoxamine on the duration of immobility in the forced swimming test in rats. Each drug was given orally 60 min before the test. Means±SEM (n=16 per group) are represented. Data were analyzed by one-way ANOVA, followed by Dunnett's test. **P*<0.05, ***P*<0.01 when compared to the respective vehicle treated group

[$F(3,60)=6.000$, $P<0.01$]. A post hoc Dunnett's test revealed that milnacipran at 30 mg/kg ($P<0.05$) and 60 mg/kg ($P<0.01$) produced a dose-related decrease in immobility time (Fig. 4). Imipramine (30 and 60 mg/kg), maprotiline (60 mg/kg) and fluoxetine (100 mg/kg) also decreased the duration of immobility [imipramine, $F(3,60)=5.849$, $P<0.01$; maprotiline, $F(3,60)=3.133$, $P<0.05$; fluoxetine, $F(3,60)=4.383$, $P<0.01$]. Fluvoxamine had no significant effect on the forced swimming-induced immobility at any of the doses tested (30, 60 and 100 mg/kg) [fluvoxamine, $F(3,60)=0.857$, $P=0.488$].

Conditioned fear stress-induced freezing

Figure 5 shows the effects of milnacipran, imipramine, maprotiline, fluvoxamine and fluoxetine on the duration of the conditioned fear stress-induced freezing behavior. Milnacipran at 30 mg/kg ($P<0.05$) and 60 mg/kg ($P<0.05$) significantly reduced the freezing behavior [$F(3,60)=3.518$, $P<0.05$]. Fluvoxamine (60 and 100 mg/kg) and fluoxetine (60 mg/kg) also reduced the freezing behavior [fluvoxamine, $F(3,60)=4.393$, $P<0.01$; fluoxetine, $F(3,60)=3.195$, $P<0.05$]. Imipramine and maprotiline had no significant effect on the freezing behavior at any of the doses tested (10, 30 and 60 mg/kg) in the present experiments [imipramine, $F(3,59)=1.604$, $P=0.198$; maprotiline, $F(3,60)=0.451$, $P=0.718$].

Locomotor activity

Milnacipran at the highest dose (60 mg/kg) used in the behavioral tests did not modify the locomotor activity in either strain of rats (data not shown). Only fluoxetine among the tested drugs significantly decreased the locomotor activity at the highest dose (100 mg/kg) (fluoxe-

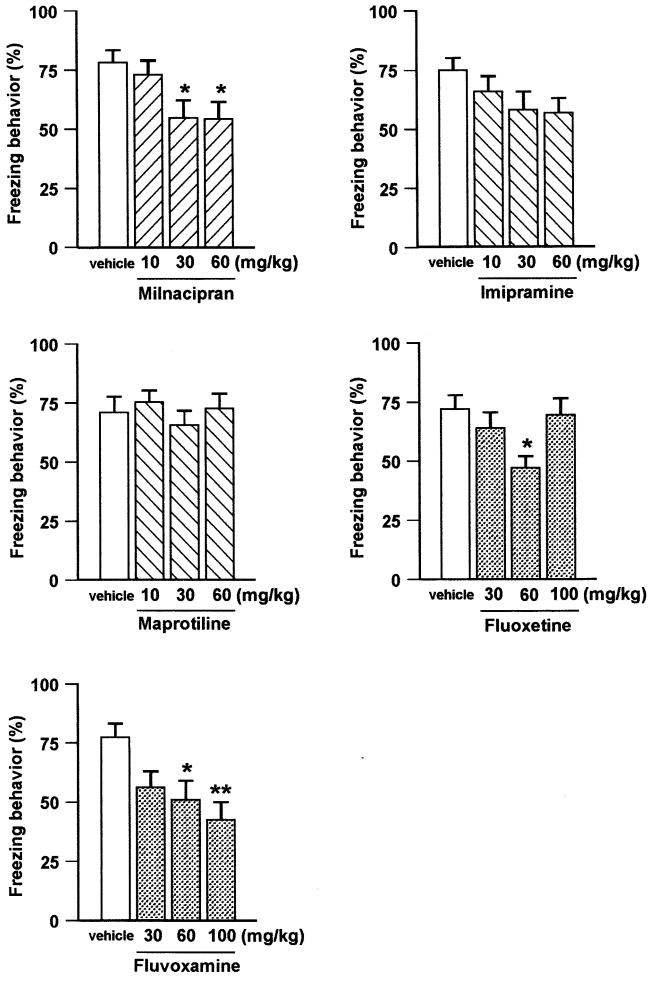

Fig. 5 Effects of milnacipran, imipramine, maprotiline, fluoxetine and fluvoxamine on the expression of freezing in the conditioned fear stress test in rats. Each drug was given orally 60 min before the test. Means±SEM (n=16 per group) are represented. Data were analyzed by one-way ANOVA, followed by Dunnett's test. *P<0.05, **P<0.01 when compared to the respective vehicle treated group

tine versus control; 1798±296 versus 4377±559 (–59%), P<0.05) when the test was performed in Sprague-Dawley rats under the conditions of the conditioned fear stress experiment, but not in Wistar rats under the conditions of the forced swimming test.

Discussion

Termination of the synaptic effects of monoamine neurotransmitters occurs through rapid reuptake across the presynaptic membranes. 5-HT, NA and DA transporters have been cloned and shown to act sodium-dependently on monoamine reuptake (Giros et al. 1992; Ramamoorthy et al. 1993; Lingen et al. 1994). It has previously been demonstrated that milnacipran inhibits 5-HT and NA uptake in slices of the rat hypothalamus with a slightly greater potency for NA uptake (Moret et al. 1985). The present study, however, is the first to show that milnacipran inhibits sodium-dependent [^{3}H]5-HT and [^{3}H]NA uptake into the synaptosomes from rat cerebral cortex. The ratio of IC_{50} of 5-HT uptake/IC_{50} of NA uptake (5-HT/NA) of milnacipran was 0.95, showing that milnacipran inhibits both 5-HT and NA uptake with similar potency. Milnacipran had no affinity for the DA transporter or any of the receptors examined. At 10 µM milnacipran inhibited less than 50% of the specific binding to any of the 20 receptors studied. Moreover milnacipran did not inhibit the activity of MAO-A and B in the rat brain tissue. These findings show that milnacipran may be classified as a selective serotonin and noradrenaline reuptake inhibitor (SNRI) in vitro.

Before any conclusions can be drawn on the relationship between the pharmacology of milnacipran and its clinical effects it is necessary to confirm that this pharmacology exists under in vivo conditions similar to those used clinically i.e. by the oral route. Although species differences in bioavailability can sometimes make the interpretation more difficult, it is widely accepted (by regulatory authorities amongst others) that studies using the clinical route of administration (usually oral) are important for concluding any relationship between pharmacological effect and clinical efficacy.

The oral administration of milnacipran resulted in a dose-related increase in the extracellular levels of 5-HT and NA in the medial prefrontal cortex. Since milnacipran has a high bioavailability and no pharmacologically active metabolites (Puozzo and Leonard 1996), the increase of the extracellular levels of both 5-HT and NA is probably due to the principal pharmacological activity of the parent compound, namely the inhibition of the sodium-dependent 5-HT and NA uptake.

Acute treatment with SSRIs such as citalopram and fluvoxamine has been shown to increase markedly the extracellular concentrations of 5-HT in the dorsal raphe nucleus, but to have less effect on 5-HT levels in the frontal cortex (Bel and Artigas 1992; Invernizzi et al. 1992). This situation is thought to result from a negative control on cell firing exerted by somatodendritic 5-HT_{1A} autoreceptors in the raphe nuclei (Blier and de Montigny 1994). Acute co-administration of the 5-HT_{1A} receptor antagonist, (–)pindolol (10 mg/kg, IP), with milnacipran (10 mg/kg, IP) increased the extracellular levels of 5-HT in guinea pig hypothalamus compared to milnacipran alone (Moret and Briley 1997). This suggests that the action of milnacipran is probably also attenuated by the stimulation of somatodendritic 5-HT_{1A} autoreceptors in the raphe. In spite of this inhibition, however, a single administration of milnacipran increased both 5-HT and NA levels with similar potency, though the levels of 5-HT, but not NA, gradually reduced after they reached the maximal level. It is interesting to note that the maximal effect on the extracellular levels of 5-HT is observed 1 h after administration of milnacipran whereas NA levels are maximal after 2.5–3 h. The delay in the maximal effect on the NA levels may be explained by a difference in the mechanism of action

namely the implication of an indirect effect in the neuronal circuits.

Another SNRI, venlafaxine, was originally shown not to increase the concentration of extracellular 5-HT in the rat frontal cortex (Dawson et al. 1999). A more complete recent study by Millan et al. (2001) has, however, demonstrated that venlafaxine elicits a dose-dependent increase in extracellular levels of both 5-HT and NA.

Imipramine, like milnacipran, had high affinity for both [^{3}H]paroxetine binding site and [^{3}H]nisoxetine binding site, and inhibited both 5-HT and NA uptake with similar potency (5-HT/NA ratio=0.80). However, imipramine, in contrast to milnacipran, had a weak effect on the extracellular levels of 5-HT compared with that of noradrenaline in vivo. The principal metabolite of imipramine, desipramine, is, however, a potent NA uptake inhibitor. In the present study, desipramine is about 300-fold more potent at inhibiting the uptake of NA than that of 5-HT. Thus in vivo imipramine acts as a relatively selective inhibitor of the uptake of NA especially when administered orally.

The effects of milnacipran, imipramine, maprotiline, a selective noradrenaline reuptake inhibitor, fluoxetine and fluvoxamine, two selective 5-HT reuptake inhibitors, were studied on two behavioural models, the forced swimming test and the freezing behaviour induced by a conditioned fear.

The rat forced swimming is a popular behavioural test for antidepressants (Porsolt et al. 1978), which is sensitive to the effects of many types of antidepressant drugs, including tricyclics and tetracyclics, monoamine oxidase inhibitors and atypical antidepressants. Although the SSRIs have been shown to be only weakly active in the forced swimming test (Porsolt et al. 1979; Borsini 1995), several researchers have consistently observed antidepressant-like activity in this test (Rénéric and Lucki 1998). It would appear that the noradrenergic system plays an important role in this test.

The conditioned fear stress test is a useful behavioural model for detecting anxiolytics (Fanselow and Helmstetter 1988; Kalin et al. 1988). The conditioned fear stress selectively increases 5-HT metabolism in the medial prefrontal cortex (Inoue et al. 1993), and the freezing behavior induced by conditioned fear stress is reduced by serotonergic activation (Inoue et al. 1996).

As expected, milnacipran inhibited in a dose-related manner both the duration of the immobility time on the forced swimming test and of the freezing time on the conditioned fear stress test. Imipramine and maprotiline showed positive results in the forced swimming test, but not the conditioned fear stress. Fluoxetine had a positive effect only at the highest dose in the forced swimming test whereas fluvoxamine was inactive in this test. Fluoxetine and fluvoxamine showed positive results in the conditioned fear stress test, although the former did not reduce the duration of the freezing at the highest dose (100 mg/kg, PO). This might be attributable to the reduction of the locomotor activity at the same dose. This same high dose did not induce a change in the locomotor activity before the forced swim test however. This difference might be due to the fact that Wistar rats were used in the forced swimming test while Sprague-Dawley rats were used in the conditioned fear stress test. Recently, several studies using a modified forced swimming test with a novel behavioural scoring system have indicated that this model can reveal the effect of SSRIs (Detke et al. 1995). However, in the forced swimming test as studied here, the SSRIs, fluoxetine and fluvoxamine, were inactive or weakly active. Thus, the combination of the forced swimming test and the conditioned fear stress test can distinguish the selectivity of antidepressants for 5-HT or noradrenaline neurotransmitter systems in vivo. Milnacipran is effective in the conditioned fear stress test, which is a model of anxiety, as well as in the forced swimming test, which is a model of depression. Although there have been few clinical studies on anxiety except the report that milnacipran is effective in the treatment of panic disorders in a clinical pilot study (Ansseau et al. 1991), the present study supports a probable effectiveness in the treatment of anxiety.

In summary, the present findings show that milnacipran selectively inhibits both the sodium-dependent 5-HT and NA uptake into synaptosomes of rat cerebral cortex to the same extent with no affinity for a variety of neuroreceptors. The oral administration of milnacipran causes an increase in the extracellular levels of both 5-HT and NA with similar potency in the medial prefrontal cortex. Behaviorally, milnacipran reduced the duration of both the immobility time in the forced swimming test and the freezing time in the conditioned fear test with no effect on locomotor activity suggestive of clinical efficacy in the treatment of depression and anxiety, respectively. In conclusion, these results show that milnacipran acts as a SNRI in vivo as well as in vitro and may be useful for the treatment of anxiety as well as depression.

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