Neuropharmacology 70 (2013) 338–347

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

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

Levomilnacipran (F2695), a norepinephrine-preferring SNRI: Profile in vitro and in models of depression and anxiety

A.L. Auclair [a], J.C. Martel [a], M.B. Assié [a], L. Bardin [a], P. Heusler [b], D. Cussac [b], M. Marien [a], A. Newman-Tancredi [a,1], J.A. O'Connor [c], R. Depoortère [a,*]

[a] Neuropsychopharmacology Division, Centre de Recherche Pierre-Fabre, 17 Avenue Jean Moulin, 81106 Castres, France
[b] Molecular and Cellular Biology Division, Centre de Recherche Pierre-Fabre, 17 Avenue Jean Moulin, 81106 Castres, France
[c] Forest Research Institute, Harborside Financial Center, Plaza V, Jersey City, NJ 07311, USA

ARTICLE INFO

Article history:
Received 13 September 2012
Received in revised form
13 February 2013
Accepted 25 February 2013

Keywords:
5-HT
Depression
Levomilnacipran
Norepinephrine
Rat
SNRI

ABSTRACT

Levomilnacipran (LVM; F2695) is the more active enantiomer of the serotonin/norepinephrine (5-HT/NE) reuptake inhibitor (SNRI) milnacipran and is currently under development for the treatment of major depressive disorder. LVM was benchmarked against two other SNRIs, duloxetine and venlafaxine, in biochemical, neurochemical and pharmacological assays. LVM exhibited high affinity for human NE (K_i = 92.2 nM) and 5-HT (11.2 nM) transporters, and potently inhibited NE (IC_{50} = 10.5 nM) and 5-HT (19.0 nM) reuptake (human transporter) in vitro. LVM had 2-fold greater potency for norepinephrine relative to serotonin reuptake inhibition (i.e. NE/5-HT potency ratio: 0.6) and 17 and 27 times higher selectivity for NE reuptake inhibition compared with venlafaxine and duloxetine, respectively. LVM did not exhibit affinity for 23 off-target receptors. LVM (i.p.) increased cortical extracellular levels of 5-HT, and NE (minimal effective doses: MEDs = 20 and 10 mg/kg, respectively). In anti-depressive/anti-stress models, i.p. LVM diminished immobility time in the mouse forced swim (MED = 20 mg/kg) and tail suspension (MED = 2.5 mg/kg) tests, and reduced shock-induced ultrasonic vocalizations in rats (MED = 5 mg/kg). Duloxetine and venlafaxine were less potent (MEDs ≥ 10 mg/kg). At doses active in these three therapeutically-relevant models, LVM (i.p.) did not significantly affect spontaneous locomotor activity. In summary, LVM is a potent, selective inhibitor of NE and 5-HT transporters with preferential activity at the former. It is efficacious in models of anti-depressive/anti-stress activity, with minimal potential for locomotor side effects.

1. Introduction

About one in every 10 persons is expected to present at least one depressive episode during his/her lifetime (Andrade et al., 2003; Kessler et al., 2003). Recent projections for direct and indirect economic costs position depression near the top of the list for the year 2030 (Mathers and Loncar, 2006).

Pharmacological management of major depressive disorder (MDD) remains the cornerstone of treatment. Most effective anti-depressant agents increase synaptic concentrations of serotonin (5-HT) and/or norepinephrine (NE) by blocking reuptake of one or both of these neurotransmitters (Stahl et al., 2005). These treatments include mainly tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs), serotonin norepinephrine reuptake inhibitors (SNRIs) and norepinephrine selective reuptake inhibitors (NRIs). Selective reuptake inhibitors (SSRIs, SNRIs, and NRIs) have better tolerability than tricyclic antidepressants. In addition, there is some evidence suggesting the "dual action" of SNRIs may confer advantages over SSRIs or NRIs in treating MDD symptoms (Stahl et al., 2005; Papakostas et al., 2007).

Duloxetine, venlafaxine, desvenlafaxine and milnacipran (MLN) are all SNRIs used to treat MDD; however, only the first three are available in the US. MLN is only marketed as an antidepressant in Europe and Japan, but is available for the treatment of fibromyalgia in the US and Australia. SNRIs differ in their relative potency at NE and/or 5-HT transporters in vitro (and to some extent in vivo). In vitro, duloxetine, venlafaxine and desvenlafaxine preferentially inhibit 5-HT reuptake relative to NE reuptake (Bymaster et al., 2001; Vaishnavi et al., 2004; Deecher et al., 2006); meanwhile, MLN more potently inhibits NE reuptake relative to 5-HT reuptake by approximately 2-fold (Moret et al., 1985; Vaishnavi et al., 2004).

* Corresponding author. Tel.: +33 56371 4233; fax: +33 56371 4363.
E-mail address: ronan.depoortere@pierre-fabre.com (R. Depoortère).
[1] Present address: NeuroAct Communication, 25 rue des Généraux Ricard, 81100 Castres, France.

0028-3908
http://dx.doi.org/10.1016/j.neuropharm.2013.02.024

In vivo, in human subjects, venlafaxine inhibits 5-HT reuptake in platelets across the clinical dose range, but inhibits NE reuptake (as measured by the pressor response to intravenous tyramine) only at higher doses (≥225 mg/day) (Harvey et al., 2000; Debonnel et al., 2007). Similarly, duloxetine inhibits 5-HT reuptake at 20 mg/day (Turcotte et al., 2001) and 60 mg/day (Turcotte et al., 2001; Chalon et al., 2003) but does not inhibit NE reuptake at these doses (again using the pressor response to tyramine [Turcotte et al., 2001]). However, at 60 mg/day duloxetine does increase supine systolic blood pressure (Turcotte et al., 2001) suggesting it may have some effects on NE at this dose. Comparable studies with desvenlafaxine or MLN in human subjects have not been reported in the literature. Of interest, however, a recent positron emission tomography (PET) study reported similar occupancy at the norepinephrine (NET) and serotonin (SERT) transporters (~40% each) in the brain of MDD patients following 100 mg MLN (Nogami et al., 2012), suggesting it might block both transporters with equivalent potency at a clinically relevant dose. Unfortunately, PET studies investigating NET occupancy of venlafaxine, duloxetine, and desvenlafaxine have not yet been reported in the literature; therefore, comparisons of relative NET/SERT occupancy across this drug class can not be made at this time.

It has been proposed that the noted differences in the relative potency of SNRIs at blocking NET or SERT (in vitro and to some extent in vivo) may have clinical implications (Stahl et al., 2005; Shelton, 2009). MDD is a disorder with a broad range of symptoms; some of which are more associated with NE (attention, working memory, concentration, alertness, energy and social activity: Saha et al., 2004; Lapiz and Morilak, 2006; Briley and Moret, 2010; Kasper et al., 2011), while others are more related to 5-HT (agitation, appetite disturbance, and irritability: Nutt, 2008). Meanwhile, core symptoms/signs of MDD (depressed mood, sleep disturbance and anxiety) are attributed to changes in both 5-HT and NE neurotransmission. Therefore, while SNRIs in general seem to improve core symptoms of depression, differences in relative potency at NET and/or SERT between SNRIs could contribute to different levels of efficacy in treating the NE- or 5-HT-related symptoms of the pathology.

Milnacipran is the only marketed SNRI reported to have greater relative potency at NET than SERT in vitro and is the first SNRI to report equal occupancy of both transporters at clinical doses. MLN is a racemic mixture of the 1S, 2R (F2695/levomilnacipran [LVM], Fig. 1) and 1R, 2S (F2696) enantiomers. In general, enantiomers can present several advantages over racemates (i.e. the potential for a less complex, more selective pharmacodynamic profile, an improved therapeutic index, less complex pharmacokinetic profile, reduced potential for drug interactions, and less complicated relationship between plasma concentration and pharmacodynamic effect: Hutt and Vanetova, 2003). In addition, regulatory guidelines in the US and Europe recommend development of enantiomers over racemic drugs where appropriate (FDA guidance, 1992).

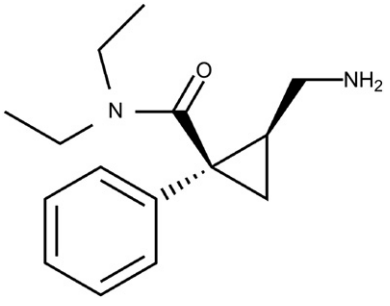

Fig. 1. Chemical structure of Levomilnacipran (LVM): (1S, 2R)-rel-2-(aminomethyl)-N,N-diethyl-1-phenyl-cyclopropane-1-carboxamide, a.k.a. F2695.

Historical (unpublished) data suggest that LVM is the more active enantiomer of MLN and LVM is currently in clinical development for the treatment of MDD.

The object of the present study was 1) to confirm that LVM is the more active enantiomer of MLN in relevant functional assays and 2) to characterize in more detail the in vitro and in vivo profile of LVM relative to two prototypical SNRIs commonly used to treat MDD in the US, duloxetine and venlafaxine. Thus, a first series of in vitro studies compared MLN and its two enantiomers for binding affinity at rat and human NET, SERT and DAT, functional potency for NE and 5-HT reuptake inhibition in rat transporters and potentiation of NE-induced seminal vesicle contraction. The activity of MLN and its two enantiomers was also compared in vivo in a mouse model of antidepressant-like activity (forced swim test, FST). Subsequent studies compared binding affinity of LVM with that of duloxetine and venlafaxine, at rat and human transporters, and reuptake inhibition of [^{3}H] monoamines at rat and human transporters. These latter in vitro studies were complemented by a microdialysis study in freely moving rats for measurement of cortical extracellular levels of monoamines, and behavioral models of depression and anxiety/stress in mice and rats.

2. Materials and methods

2.1. In vitro experiments

2.1.1. Binding affinity assays

All binding assays were performed in 96 well plates (DeepWell, Perkin Elmer Life Sciences, Courtaboeuf, France) in 50 mM TRIS buffer containing 120 mM NaCl and 5 mM KCl (human serotonin transporter [hSERT] and human norepinephrine transporter [hNET]) or $MgCl_2$ (human dopamine transporter [hDAT]), as described in the literature (Owens et al., 1997; Millan et al., 2001; Taber et al., 2005). Following incubation, assay media were rapidly filtered under vacuum through GF/B Unifilter® microplates (presoaked in 0.5% polyethyleneimine), and washed with 2 rinses of cold (4 °C) assay buffer using a unifilter apparatus. The radioactivity retained on the filters was measured by scintillation spectroscopy using a TopCount® radioactivity counter with 40 μl of scintillation fluid (Microscint 20, Perkin Elmer). See Supplementary Table 2 for overview of experimental conditions and corresponding references.

2.1.1.1. Human transporters. Radioligand binding was determined in HEK-293 cells permanently transfected with human SERT, NET, or DAT (65, 70, and 9.2 pmol/mg protein, respectively). Protein concentrations were determined by the method of Bradford (1976), using a commercially available kit (Bio-Rad, USA). Membranes were thawed and diluted in TRIS buffer to a final protein concentration of 3 μg per 400 μl for SERT and 5 μg per 400 μl for NET and DAT. 50 μl of [^{3}H] citalopram (2 nM, final concentration), [^{3}H]nisoxetine (2 nM), or [^{3}H]WIN35,428 (5 nM) and 50 μl drug/buffer were added to the membrane/buffer mixture for a final assay volume of 500 μl and incubated for 2 h at room temperature (25 °C) for SERT and at 4 °C for NET and DAT. Incubation was terminated by filtration and radioactivity quantified as stated above. Non-specific binding was defined with 10 μM fluoxetine, 10 μM desipramine, and 10 μM mazindol for SERT, NET, and DAT experiments, respectively. Saturation binding experiments for SERT and NET were performed with [^{3}H] citalopram (8 concentrations ranging from 0.125 to 16 nM) and [^{3}H]nisoxetine (12 concentrations ranging from 0.125 to 128 nM), respectively. For DAT, "cold" saturation binding experiments were performed with 5 nM [^{3}H]WIN35,428 and 11 concentrations of unlabeled WIN35,428 ranging from 0.01 to 10 nM. For competition binding experiments, the binding of 2 nM [^{3}H] citalopram, 2 nM [^{3}H]nisoxetine, or 5 nM [^{3}H] WIN35,428 was displaced with increasing concentrations of test compound.

2.1.1.2. Rat transporters. Binding assays on rat (r) SERT and rNET were performed on membrane preparations from rat brain cortex. Briefly, frozen brains were thawed, homogenized and centrifuged 3 times in ice cold TRIS buffer containing 120 mM NaCl and 5 mM KCl (rSERT) or 300 mM NaCl and 5 mM KCl(rNET). Final pellet was diluted in TRIS buffer to give a final concentration of 1.75 mg tissue (based on original weight) per well, and the binding of 1 nM [^{3}H] citalopram (rSERT) or 2 nM [^{3}H] N-methyl nisoxetine (rNET) was displaced with increasing concentrations of test compound. At the end of a 2 h incubation at room temperature (~23 °C for rSERT) or 4 °C (rNET), the plates were rapidly filtered and rinsed with ice-cold TRIS buffer. Non-specific binding was defined with 0.5 μM paroxetine (rSERT) or 10 μM desipramine (rNET).

2.1.2. Reuptake assays on human transporters

Experiments were performed on CHO cells stably expressing hSERT, hNET or hDAT (densities of 623 ± 19, 1272 ± 309 and 897 ± 112 fmol/mg protein,

respectively). Cell cultures were maintained in Ham's F12 buffer plus 10% FCS, 1.25 mg/ml geneticin and an antibiotic solution (10,000 units/ml penicillin, 10,000 units/ml streptomycin, 29.2 mg/ml L-glutamine; Invitrogen, Cergy Pontoise, France) at 37 °C in an air/CO_2 (95%/5%) water-saturated atmosphere and sub-cultured weekly. Cells were seeded in opaque 24 well-plates in cell culture medium and reuptake assays were run once cells reached confluence. The day of the experiment, cell culture medium was removed from the plate, cells were washed once with HBSS buffer and incubated at room temperature for 5 min in HBSS containing pargyline (100 μM) and ascorbate (100 μM). Cells were incubated with test compound or no drug for 15 min before adding [^{3}H]5-HT, [^{3}H]NE or [^{3}H]DA (final concentration 10 nM). Following addition of radiolabeled neurotransmitter, cells were allowed to equilibrate at room temperature for 5 min. Experiments were terminated by rapid aspiration of reuptake buffer. Cells were carefully and rapidly rinsed once with HBSS and the cell layer was incubated with Microscint 20 scintillation buffer (300 μl/well; Perkin Elmer) for at least 2 h under gentle shaking. Radioactivity incorporated in the cells was determined for each well by liquid scintillation counting using a TopCount microplate scintillation counter (Perkin Elmer Life Sciences, Courtaboeuf, France). The non-specific signal was defined as the radioactivity remaining in the presence of the selective reuptake blockers fluoxetine (for hSERT; 100 μM), desipramine (for hNET; 10 μM) or GBR12935 (for hDAT; 10 μM).

Experiments were performed in duplicate or triplicate. [^{3}H]Neurotransmitter reuptake values were corrected for non-specific radioactivity incorporation and expressed as percent of non-treated cells in wells of the same assay plate.

2.1.3. Reuptake assays in rat synaptosomes

Methods differed slightly for the reuptake assays in the initial set of experiments with MLN, LVM, and F2696 and the second set of experiments with LVM, duloxetine, and venlafaxine. Most notably, different brain regions were used for synaptosomal preparation (hypothalamus and cortex/striatum, respectively). The methods for reuptake assays with MLN, LVM, and F2696 are presented in the Supplementary File. Procedures for reuptake assays in rat synaptosomes with LVM, duloxetine, and venlafaxine were as follows.

Rats were killed by decapitation and the brain region (cortex for SERT and NET, striatum for DAT) was rapidly dissected, weighed and homogenized in 20 vol (cortex) or 200 vol (striatum) of a 0.32 M sucrose solution using a glass/glass homogenizer. The homogenate was then centrifuged 10 min at 1000 g at 4 °C; the supernatant was removed and used immediately for the reuptake assay. First, 50 μl of membrane preparation, 350 μl Krebs buffer solution and 50 μl test compound solution were incubated for 5 min at 37 °C. Then, either 50 nM [^{3}H]NE, 10 nM [^{3}H]5-HT, or 25 nM [^{3}H]DA was added to the membrane preparation and incubated at 37 °C for an additional 15, 5, or 4 min, respectively. The incubation was stopped by addition of 1 ml of ice-cold (4 °C) Krebs buffer and rapid filtration on 24-well plate filters (Unifilter GF/B). Each well was washed with ice-cold Krebs buffer. The radioactivity retained on the filters was measured by liquid scintillation (Topcount, Perkin Elmer Life Sciences, Courtaboeuf, France) after addition of scintillation fluid (MicroScint 20, Perkin Elmer, Life Sciences). Non-specific reuptake was defined by addition of 10 μM desipramine for [^{3}H]NE, 10 μM citalopram for [^{3}H]5-HT and 100 μM nomifensine for [^{3}H]DA reuptakes. Results are expressed as the mean of 3 or 4 EC_{50} determinations (using one brain homogenate for each determination).

2.1.4. Seminal vesicle assay

The experimental procedures are essentially the same as those described by Sharif and Gokhale (1986); details are provided in the Supplementary File.

2.2. In vivo experiments

2.2.1. Animals

Male Sprague-Dawley rats (Iffa-Credo, Les Oncins, France), 180–200 g (USV experiments) or 240–260 g (microdialysis experiment) at the start of the experiments, were quarantined for 4–5 days. They were then housed 5 to a cage and kept in temperature- and humidity-controlled rooms (21 ± 1 °C, relative humidity: 55 ± 5%) on a 12:12 h light:dark cycle (lights on at 07:00 h). Food (A04 rodent chow, SAFE, Epinay sur Orge, France) and water were available *ad libitum*.

Male Swiss mice weighing 18–24 g obtained from the Depre Breeding Center (St. Doulchard, France) were used in FST studies comparing MLN, LVM, and F2696. OF1 mice (FST), NMRI (TST) and C57Bl6J (locomotor activity), weighing between 20 and 22 g upon arrival, were obtained from Charles River Laboratories France (L'Arbresle, France) and were used for behavioral studies comparing LVM, duloxetine, and venlafaxine. All animals were housed in air-conditioned rooms (temperature 21 ± 1 °C; humidity 55 ± 5%), with lighting on from 7:00 to 19:00 h.

Animals were handled and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2010). Animals were housed and tested in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility in strict compliance with all applicable regulations, and the protocol was carried out in compliance with French regulations and with local Ethical Committee guidelines for animal research. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2.2. Microdialysis in freely moving rats

On the day before the microdialysis experiment, rats (270–340 g body weight at the time of surgery) were anaesthetized with isoflurane (induction with 4.5%, maintenance with 2.0%) and positioned in a stereotaxic apparatus (model 900, Kopf Instruments, USA) with the incisor bar fixed at −3.5 mm. Body temperature was maintained at 37.5 °C with a CMA/150 temperature controller (CMA-Microdialysis AB, Stockholm). The surface of the skull was exposed, a small hole was drilled through the bone and the dura was carefully perforated using a 28-gauge syringe needle. A commercially-available microdialysis probe (CMA/11, CMA-Microdialysis AB, Stockholm, 3 mm membrane length, 0.24 mm external diameter, in vitro recovery rate in the range of 17–20% at flow rate of 2 μl/min at room temperature) was slowly inserted to terminate in the left medial prefrontal cortex (mPFC), at the following coordinates: 3.0 mm anterior to bregma, 1.5 mm lateral to the midline suture, 4.5 mm ventral from the dura, at a medially-directed vertical angle of 14°. The probes were anchored to the skull with three stainless steel screws and dental acrylic cement. Following recovery from anesthesia, rats were transferred to bowl-shaped Perspex microdialysis cages (CMA-Microdialysis AB, Stockholm) with free access to food and water, and the probes were connected via a liquid swivel and counterbalance arm assembly to perfusion syringes containing filtered degassed Ringer's solution (composition in mM: NaCl, 147; KCl, 4.0; $CaCl_2$, 2.4). The probes were perfused at a rate of 0.2 μl/min overnight. The following morning, the perfusion syringes were refilled with fresh degassed Ringer's and the pump infusion rate was increased to 2.0 μl/min. After a 2-h equilibration period, microdialysate samples were collected in glass microtubes containing 15 μl of HCl (0.05 N) + EDTA (1 mM) using refrigerated microfraction collectors (Univentor model 820).

Immediately following the collection of four 30-min samples for measurement of pre-treatment "baseline" levels of monoamines, test compounds or saline (0.9% NaCl) was administered i.p. (10 ml/kg body weight), and microdialysate samples were collected continuously over the next 4 h. At the end of the experiment, rats were decapitated and the brains removed, cooled on ice and cut into coronal sections with the aid of a stainless steel brain matrix. Probe placement was verified by visual inspection of the probe tract in the tissue slices (which, although not as precise as verification following fixation of tissue, is deemed sufficient when large brain structures are targeted).

Each acidified microdialysate sample was divided into 2 aliquots for parallel analyses by 2 independent HPLC systems, one optimized for the quantification of NE and one optimized for 5-HT and DA levels.

NE in acidified microdialysate sample aliquots (30 μl) was derivatized and quantified by HPLC with fluorescence detection as described by Yoshitake et al. (2003). This method is based on precolumn derivatization of NE with benzylamine that results in a highly fluorescent and stable benzoxazole derivative. Briefly, 30 μl of a derivatizing reagent mixture containing benzylamine HCl (33 mM), CAPS buffer (3-cyclohexyl-aminopropanesulfonic acid, 25 mM) and potassium hexacyanoferrate(III) (0.55 mM) in 83% methanol were added to 30 μl of acidified microdialysate sample. Samples were capped, mixed and incubated for 20 min at 50 °C, and loaded into a HPLC system (WATERS 2690 Alliance Separation Module) equipped for programmed autoinjection (8 °C). Chromatographic conditions consisted of a C18 reverse-phase column (WATERS Symmetry Shield 2.1 × 150 mm, 5 μm particle size) maintained at 30 °C. A gradient mobile phase (final pH 4.5) consisted of sodium acetate (15 mM), EDTA (0.5 mM) and acetonitrile (30–90% vol/vol), and was delivered at a constant flow rate of 0.4 mL/min. Fluorescent derivatives were detected with a Waters Model 474 scanning fluorescence detector (lex = 345 nm, lem = 480 nm). Detector signal output was acquired and analyzed with chromatography application software (Empower Chromatography Manager, Waters).

DA and 5-HT in acidified sample aliquots (30 μl) were quantified directly by HPLC coupled with electrochemical detection. Samples were loaded into a HPLC system (WATERS 2690 Alliance Separation Module) equipped for programmed auto-injection (8 °C). Chromatographic conditions consisted of a cationic exchange column (Eicom CAX, 2.0 × 200 mm, 5 μm particle size) maintained at 40 °C. The mobile phase (final pH 6.2) consisted of sodium phosphate $C_2H_7NO_2$ (100 mM), Na_2SO_4 (50 mM), EDTA (0.125 mM), KCl (8 mM) and 25% methanol, and was delivered at a constant flow rate of 0.30 mL/min. Detection was performed with a WATERS 2465 electrochemical detector equipped with a glassy carbon working electrode set at an oxidation potential of +0.430 V. Detector signal output is acquired and analyzed with chromatography application software (Empower Chromatography Manager, Waters). Quantification of chromatographic peaks was based on the comparison with calibration curves derived from authentic standards of NA, 5-HT and DA prepared in acidified Ringer's solution and carried through the respective analytical procedure. Blanks consisted of Ringer's solution that were recovered from the infusion syringes at the end of the experiment, acidified and carried through the respective analytical procedure.

Monoamine levels in the microdialysate samples were calculated as absolute amount (fmoles) per 30 μl injected aliquot, and were expressed as a percentage of "baseline" values, defined here as the average amount of monoamine measured in the 4 microdialysate samples preceding the intraperitoneal administration of drug or saline solutions.

2.2.3. Forced swimming test (FST)

Both sets of FST experiments (those comparing MLN, LVM, and F2696 and those comparing LVM, duloxetine, and venlafaxine) were conducted using the same

overall procedure, with the exception that the cylinder was 23 × 11.5 cm, with 6 cm water, and the observation period was 5 min for the MLN, LVM, F2696 study. The rest was similar to the procedure described below for the study comparing LVM to duloxetine and venlafaxine.

Experimentally naïve mice were injected with vehicle or test compound i.p. (10 ml kg/kg) and placed individually 30 min later into a glass cylinder (height: 24 cm, diameter: 13 cm) containing 10 cm of water (22 ± 0.5 °C) for 6 min. The latency time to the first bout of immobility was recorded starting immediately after placing the mouse in the cylinders (precision 0.1 s). During the last 4 min, the duration of immobility was also measured (precision 0.1 s). A mouse was judged immobile when it ceased all active behaviors (i.e. struggling, swimming, jumping) and remained passively floating in the water or made minimal movements necessary to maintain its nostrils above water.

Note: Mice were utilized in the FST experiments as they are more sensitive than rats to the acute effects of SNRIs and SSRIs in this model (Castagne et al., 2009; in-house unpublished data). Also, the use of mice in this test presents the advantage of having the same species run in the two "depression" models (FST and tail suspension test [TST]). The same experimenter performed the FST and TST, and experiments were performed under blind conditions.

2.2.4. Tail suspension test (TST)

Movements of experimentally naïve mice, individually suspended by the tail (2 cm from the tip) to a vertical tail hanger (18 cm from the floor), were recorded by an automated animal activity monitor (Med Associates, Inc, Georgia, USA). The duration of immobility, defined as the time that mice no longer exhibit escape-oriented behaviors, was recorded during a 6 min test session. The animals were administered either with an injection of saline or test compound i.p. (10 ml/kg), and returned to their individual home cages for 30 min before being tested in the tail suspension chamber.

2.2.5. Stress-induced ultrasonic vocalization (USV)

The procedure was carried out as described in details by Bardin et al. (2010). Succinctly, rats were trained to associate unavoidable scrambled foot-shocks (sinusoidal, 0.4 mA, 1 s duration) to a conditioned light stimulus. During the test sessions, rats were injected i.p. (10 ml/kg) with drug or vehicle, 30 min before being placed into the cage for automatic recording of the time spent emitting ultrasonic vocalization calls (around 22 kHz) using the UltraVox system (Noldus, Wageningen, The Netherlands). During these test sessions, only the first foot shock was delivered with the conditional stimulus; subsequently, only the conditional stimulus was presented.

2.2.6. Spontaneous locomotor activity

Locomotor activity was recorded by an automated animal activity monitor (Multivarimex, Columbus Instruments, Columbus, Ohio, USA), by counting interruptions of at least 0.1 s of two parallel, horizontal infrared beams positioned 2 cm above the floor of the cage and spaced 7.5 cm apart. Experimentally naïve mice were injected with test compound (i.p. 10 ml/kg) and put back in their home cage during 30 min. Immediately thereafter, mice were placed in an acrylic (22 × 8.5 × 18 cm, $L \times W \times H$) cage without sawdust positioned in the activity monitor for recording of their spontaneous locomotor activity (number of interrupted light beams) during 60 min.

2.3. Statistical analysis

Binding studies were analyzed using built-in equations from the non-linear regression program Prism v4.03 (GraphPad Software, San Diego, CA, USA). Inhibition constants (K_i) were calculated from estimates of concentrations required to inhibit binding by 50% (IC_{50}) using the built-in "*Chen-Prusoff approximation*":

$$K_i = IC_{50}/(1 - ([\text{LIGAND}]/K_D\text{LIGAND})).$$

KD values for [^{3}H] citalopram: 3.2 (hSERT) and 0.48 nM (rSERT); [^{3}H] N-methyl nisoxetine: 8.5 (hNET) and 0.62 nM (rNET); and [^{3}H]WIN35,428: 43 nM (hDAT).

In vitro reuptake inhibition data (human) were analyzed by non-linear regression to determine an IC_{50}; concentration-response data (rat) were analyzed according to a 4-parameter sigmoid curve fit logistic model to determine an IC_{50}. (Prism v4.03 software).

In vitro seminal vesicle assay data were analyzed by a one-way ANOVA with the concentration as the between-subjects factor, followed by a Holm–Sidak post-hoc test for comparison with the appropriate control group.

Microdialysis data were analyzed using a one-way ANOVA with the treatment as the between-subjects factor, followed if appropriate by a Holm–Sidak post-hoc test for comparison with the appropriate vehicle-treated group (for NE and 5-HT measures collated across t_{0-240} post-injection). In addition, for the time-course analysis, NE 5-HT and DA measures were analyzed using a two-way ANOVA with the treatment as the between-subjects and time as the within-subject factors, followed if appropriate by a one-way ANOVA for post-hoc comparisons with the vehicle-injected group.

For behavioral studies, data were analyzed with a one-way ANOVA with the dose as the between-subjects factor, followed by a Holm–Sidak post-hoc test for comparison with the appropriate vehicle-treated group. ANOVAs were run with the Sigmastat 3.5 software (Systat software, Inc., Point Richmond, CA, USA).

2.4. Drugs

LVM and venlafaxine were synthesized in-house. Duloxetine was obtained from Sigma RBI or Bepharm (Shangai, China). [^{3}H] Citalopram (TRK1068; 70–90 Ci/mmol) and [^{3}H]nisoxetine (TRK942; 70–90 Ci/mmol) were purchased from GE-Healthcare (Orsay, France). [^{3}H]WIN35,428 (NET1033; 70–90 Ci/mmol) was obtained from Perkin-Elmer (Courtaboeuf, France). [^{3}H]DA (NET673, specific activity: 30–60 Ci/mmol) and [^{3}H]NE (NET377, specific activity: 10–30 Ci/mmol) were purchased from Perkin Elmer. [^{3}H]5-HT trifluoroacetate (TRK1006, specific activity: 80–130 Ci/mmol) was purchased from GEHealthcare. Desipramine hydrochloride was purchased from Sigma-RBI (St Quentin-Fallavier, France); citalopram was a gift from Lundbeck.

All drugs were dissolved in ultrapure water (binding assays, rat reuptake inhibition assays), or DMSO (human reuptake inhibition assays). In behavioral experiments, doses (expressed as the weight of the free base) were adjusted on the basis of body weight measured on the day of administration; control animals received vehicle (saline). The treatments were administered under blind conditions.

3. Results

3.1. Confirmation that LVM is the pharmacologically more active enantiomer of milnacipran

LVM exhibited high affinity for NET and SERT in native rat (r) tissue (K_i = 11.4 and 7.6 nM, respectively) and human (h) recombinant NET and SERT (K_i = 92.2 and 11.2 nM, respectively). All these affinities were slightly higher than those of MLN, and at least 10 times higher than those of F2696 (Table 1). None of the three compounds had detectable affinity for hDAT (K_i > 10 uM; rDAT not tested). In pharmacodynamic models, LVM, compared to F2696, was 50 and 13 times more potent in inhibiting NE and 5-HT reuptake in rat hypothalamic synaptosomes. All three compounds significantly (all F's > 3.2, all p's ≤ 0.01) potentiated NE-induced contraction of rat seminal vesicles in vitro, but LVM was 300 times more potent than F2696. Lastly, there was a significant effect in the forced swim test (FST) in mice ($F(6,42) = 56.9$, $p < 0.001$), but again, LVM was 33 times more potent than F2696 (Table 2). In the latter two models, LVM was also more potent than MLN, albeit less so than F2696. Collectively, these data confirm previous unpublished data suggesting that LVM is the more pharmacologically active enantiomer of MLN.

3.2. In vitro profile of LVM compared to duloxetine and venlafaxine

Having confirmed that LVM is the more active enantiomer of MLN, a second set of studies was performed to further characterize the pharmacological profile of LVM relative to two prototypical SNRIs currently used to treat MDD, duloxetine and venlafaxine.

Table 1
Comparative affinities of milnacipran and its enantiomers at human recombinant and rat native transporters.

	Milnacipran	LVM (F2695)	F2696
Human			
SERT	16.9 ± 1.3	11.2 ± 0.3	290 ± 40
NET	139 ± 24	92.2 ± 11.9	$>10^4$
DAT	$>10^4$	$>10^4$	$>10^4$
Rat			
SERT	16.0 ± 1.7	7.6 ± 1.5	1960 ± 218
NET	34.3 ± 1.7	11.4 ± 0.9	124 ± 20

Numbers represent the average ± S.E.M. K_i (nM); all experiments were performed in triplicate except hNET and hDAT experiments with F2696 (5 determinations), and rSERT with F2696 (4 determinations). Data for rat DAT not available.

Table 2
Comparison of activity of milnacipran (MLN), LVM and its 1R, 2S enantiomer F2696 in pharmacodynamic models related to the norepinephrine or serotonin systems.

		MLN	LVM	F2696
Inhibition of [^{3}H] monoamine reuptake, rat hypothalamus synaptosomes (IC_{50}, nM)	*NE*	30	15	750
	5-HT	150	46	600
Potentiation of NE-mediated contraction of rat seminal vesicle (MEC, μM)		0.1	0.1	30
Reduction of immobility time, FST in mice (MED, mg/kg i.p.)		10	3	100

5-HT = 5-hydroxytryptamine; ED_{50} = median effective dose; FST = forced swim test; IC_{50} = median inhibitory concentration; MEC = minimum effective concentration; NE = norepinephrine; PFC = prefrontal cortex.
$N = 4$ for the reuptake assay, 5–7 for the vesicle model, and 10 for the FST.

LVM has selective affinity for SERT and NET, with a potency in-between that of duloxetine and venlafaxine.

Duloxetine had higher affinity for r and h SERT and NET than LVM, while venlafaxine exhibited low affinity for both h and r NET. Duloxetine also showed modest affinity ($K_i = 446$ nM) for DAT, but LVM and venlafaxine had no appreciable affinity for this transporter (Table 3).

LVM did not exhibit affinity ($K_i \geq 10$ μM) for any of the 23 other targets tested; venlafaxine was similarly devoid of affinity for these targets. Duloxetine showed rather weak affinity (K_i ranging from 0.24 to 0.65 μM) for some targets (i.e. 5-HT, dopamine D_3 and histamine H_1 receptors; Supplementary Table 2).

3.3. LVM is a norepinephrine-preferring SNRI in functional assays

In CHO cells stably expressing transfected human transporters, LVM reduced the reuptake of [^{3}H]NE and [^{3}H]5-HT in a concentration-dependent manner (Fig. 2, top panel), with approximately two-fold higher potency at NET ($IC_{50} = 10.5$ and 19.0 nM, for [^{3}H]NE and [^{3}H]5-HT, respectively) (Table 4). Thus, LVM had an NE/5-HT potency ratio of 0.6. LVM did not reduce the reuptake of [^{3}H]DA ($IC_{50} > 100$ μM), consistent with its very weak affinity for the DAT. In native tissue (rat synaptosomes), LVM inhibited [^{3}H]NE and [^{3}H]5-HT reuptake with an IC_{50} of 62.2 and 72.6 nM, respectively. LVM was much less potent in inhibiting [^{3}H]DA reuptake (Table 4).

In contrast, both duloxetine and venlafaxine inhibited [^{3}H]5-HT reuptake more potently than that of [^{3}H]NE at human transporters (NE/5-HT reuptake selectivity ratios of 16 and 10, respectively) (Fig. 2, middle and bottom panels, and Table 4), and interfered with DA reuptake to some extent (IC_{50}: 2.6 and 22.7 μM, for duloxetine and venlafaxine, respectively). Similarly, in rat synaptosomes, duloxetine and venlafaxine blocked 5-HT reuptake with ~10 times greater potency than NE reuptake (duloxetine: IC_{50} = 10 and 90.7 nM, respectively; venlafaxine: IC_{50} = 75.8 and 726.3 nM, respectively) and had modest effects on DA reuptake ($IC_{50} = 402.4$ and 5270 nM for duloxetine and venlafaxine, respectively).

Amongst 6 other reference antidepressants tested under the same experimental conditions (SSRIs: escitalopram and citalopram; SNRIs: duloxetine, venlafaxine; NARI: reboxetine; TCA: desipramine; data on file), LVM is the only compound showing ~2-fold preferential NET over SERT blocking capacities (based on

Table 3
Affinities at human recombinant and rat native transporters.

	LVM	Duloxetine	Venlafaxine
Human			
SERT	11.2 ± 0.3	0.2 ± 0.0	17.9 ± 2.5
NET	92.2 ± 11.9	8.9 ± 0.8	$>10^4$
DAT	$>10^4$	446 ± 31.1	$>10^4$
Rat			
SERT	7.6 ± 1.5	0.3 ± 0.0	20.6 ± 2.4
NET	11.4 ± 0.9	2.0 ± 0.2	1187 ± 317

Numbers represent the average ± S.E.M. K_i (nM); all experiments were performed in triplicate except hSERT and hNET experiments with duloxetine (6 determinations), hNET experiments with venlafaxine (6 determinations), and hSERT with venlafaxine (5 determinations). Data for rat DAT not available.
Note: the LVM data presented in this table are the same as those presented in Table 1, but are provided here for the sake of facilitating comparison to duloxetine and venlafaxine.

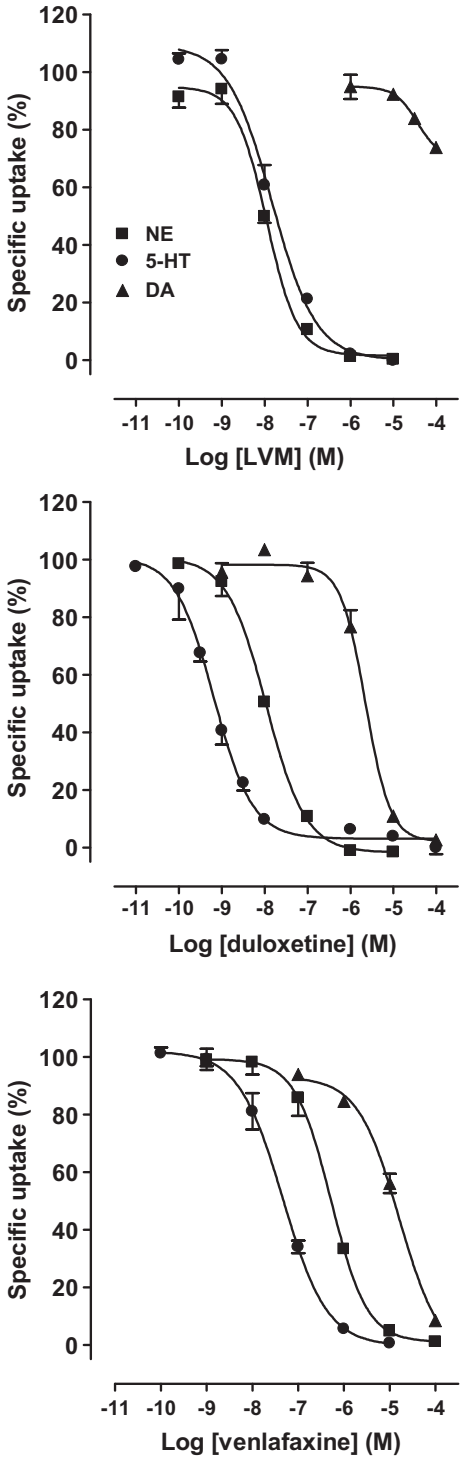

Fig. 2. Effects of LVM, duloxetine and venlafaxine on reuptake of [^{3}H]5-HT [^{3}H] norepinephrine (NE) and [^{3}H]dopamine (DA) in CHO cells stably expressing human SERT, NET or DAT. Symbols are mean ± SEM. $N = 2–3$ determinations per point.

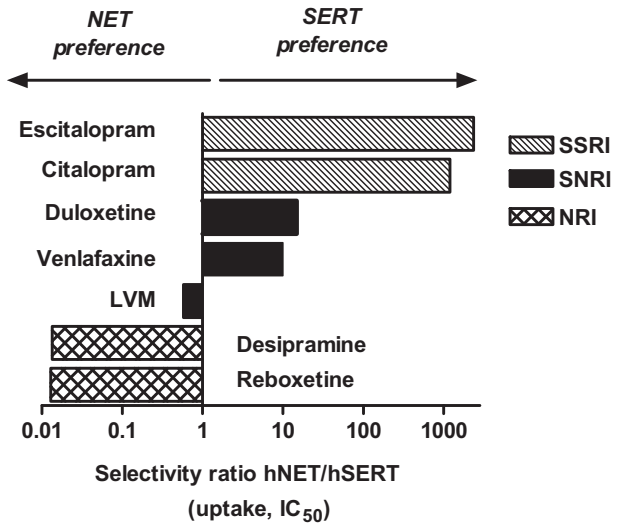

Fig. 3. Summary of selectivity ratios of reuptake potencies using human (h) transporters. Ratios were calculated by dividing IC_{50} (potency in reuptake assays) for NET by that for SERT, for LVM and a host of comparators.

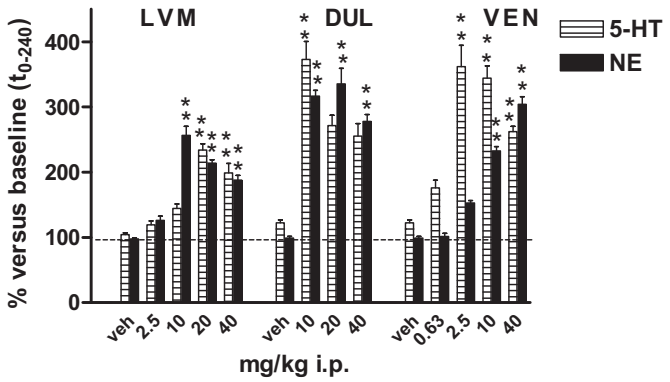

Fig. 4. Effects of LVM, duloxetine and venlafaxine on extracellular levels of 5-HT and NE in the medial prefrontal cortex of freely moving rats. Bars are mean + SEM of the % amount of neurotransmitter versus baseline (4 samples collected before test compound or vehicle injection) averaged from t_0 to t_{240} min post-injection. $N = 14–15$ for vehicle and 6–7 per dose for LVM; $n = 6–7$ rats for vehicle and per dose for duloxetine and venlafaxine. $^{**}p < 0.01$ compared to corresponding vehicle, Holm–Sidak post-hoc test following significant one-way ANOVA.

uptake assays on transfected human transporters: Fig. 3). Indeed, the NE/5-HT selectivity ratio of LVM is approximately 27 and 17 times that of duloxetine and venlafaxine, respectively.

3.4. LVM augments fronto-cortical extracellular levels of 5-HT, NE and DA

LVM significantly augmented extracellular levels of both 5-HT and NE ($F(4,38) = 8.90$, $p < 0.001$, and $F(4,40) = 14.54$, $p < 0.001$, respectively) in the frontal cortex of rats (Fig. 4). LVM increased NE levels more potently than those of 5-HT (MED = 10 and 20 mg/kg, respectively). At the lowest dose found to significantly increase NE or 5-HT levels in the prefrontal cortex (10 mg/kg), the ratio of NE/5-HT (percent increase) was approximately 4. At higher doses, this ratio was approximately 1 (Table 5).

Duloxetine also significantly increased NE and 5-HT in the rat mPFC ($F(3,26) = 4.44$, $p = 0.01$, and $F(3,26) = 11.54$, $p < 0.001$, for 5-HT and NE, respectively). Duloxetine significantly increased NE at all doses tested, and significantly increased 5-HT at 10 mg/kg with a (non-significant) tendency toward increase at 20 and 40 mg/kg (Fig. 4). The ratio of NE/5-HT (percent increase) was approximately 1 at all doses tested; except the 20 mg/kg dose (ratio = 1.6) (Table 5).

Venlafaxine significantly increased extracellular levels of both catecholamines as well ($F(4,33) = 4.33$, $p < 0.001$, and $F(4,32) = 24.3$, $p < 0.001$, for 5-HT and NE, respectively). Venlafaxine was 4 times more potent at increasing 5-HT levels versus NE (MED 2.5 and 10 mg/kg, respectively) (Fig. 4). The ratio of NE/5-HT (percent increase) was notably below 1 at doses lower than 10 mg/kg (Table 4).

Time-course data for NE and 5-HT extracellular levels are available in Supplementary Fig. 1, along with those for DA. All three SNRIs dose-dependently increased NE, 5-HT and DA in the rat prefrontal cortex. LVM augmented extracellular levels of DA starting from 10 mg/kg i.p. Venlafaxine showed an activity profile on the whole similar to that of LVM, although the MED was higher (40 mg/kg). There was a much more robust effect of duloxetine on DA, with levels reaching a peak of 10 times that of baseline 60 min post injection at 40 mg/kg.

3.5. LVM produces antidepressant-like effects in the forced swim and tail suspension tests in mice comparable to that of duloxetine and venlafaxine

Following injection of vehicle, mice spent time in immobility with average values that were homogenous across the different sets of forced swim experiments (from 165.7 ± 20.7 to 169.0 ± 18.7 s) (Fig. 5, white bars, left panels). LVM, duloxetine, and venlafaxine all dose-dependently and significantly decreased immobility relative to vehicle ($F(4,34) = 5.5$, $p < 0.01$; $F(3,24) = 21.0$, $p < 0.001$, and $F(3,27) = 3.7$, $p < 0.05$, respectively) with MEDs of 20 mg/kg i.p (Fig. 5). At the highest dose tested (40 mg/kg), the maximal decrease in immobility time was 78%, 69% and 52%, for duloxetine, LVM and venlafaxine respectively.

In the mouse tail suspension test, following injection of vehicle, mice spent from 124.0 ± 16.4 to 152.5 ± 12.8 s in immobility (Fig. 5, white bars, middle panels). LVM and duloxetine significantly

Table 4
Effects on uptake of norepinephrine, serotonin and dopamine using human transporters transfected in CHO cells, or rat synaptosomes.

	NE	5-HT	DA
Human			
LVM	10.5 ± 0.9	19.0 ± 3.9	>100,000
Duloxetine	11.2 ± 2.1	0.7 ± 0.0	2577 ± 205.6
Venlafaxine	449 ± 43.6	45.1 ± 1.3	22,663 ± 7374
Rat			
LVM	62.2 ± 8.7	72.6 ± 19.2	40,840 ± 5328
Duloxetine	90.7 ± 16.2	10.0 ± 1.1	402.4 ± 42.4
Venlafaxine	726.3 ± 150.9	75.8 ± 17.8	5270 ± 372

Numbers represent the average ± S.E.M. IC_{50} (nM), for blockade of norepinephrine (NE), serotonin (5-HT) and dopamine DA; all experiments were performed in triplicate except for LVM human DA (2 determinations), duloxetine rat NE and 5-HT, and venlafaxine rat 5-HT (4 determinations).

Table 5
Ratios of NE/5-HT efficacy based on microdialysis data.

	0.63 mg/kg	2.5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg
LVM	–	Nc	4.0	0.9	1.0
Duloxetine	–	–	0.9	1.6	1.3
Venlafaxine	Nc	0.2	0.6	–	1.5

Ratios were calculated as follows:

$$\frac{(\text{average \% increase for the given dose} - \text{average \% increase for vehicle}) \text{ for NE}}{(\text{average \% increase for the given dose} - \text{average \% increase for vehicle}) \text{ for 5-HT}}$$

for data collected at $t_{0–240}$ post-injection. –: not tested; Nc: not calculated, the increase being not significant at these doses.

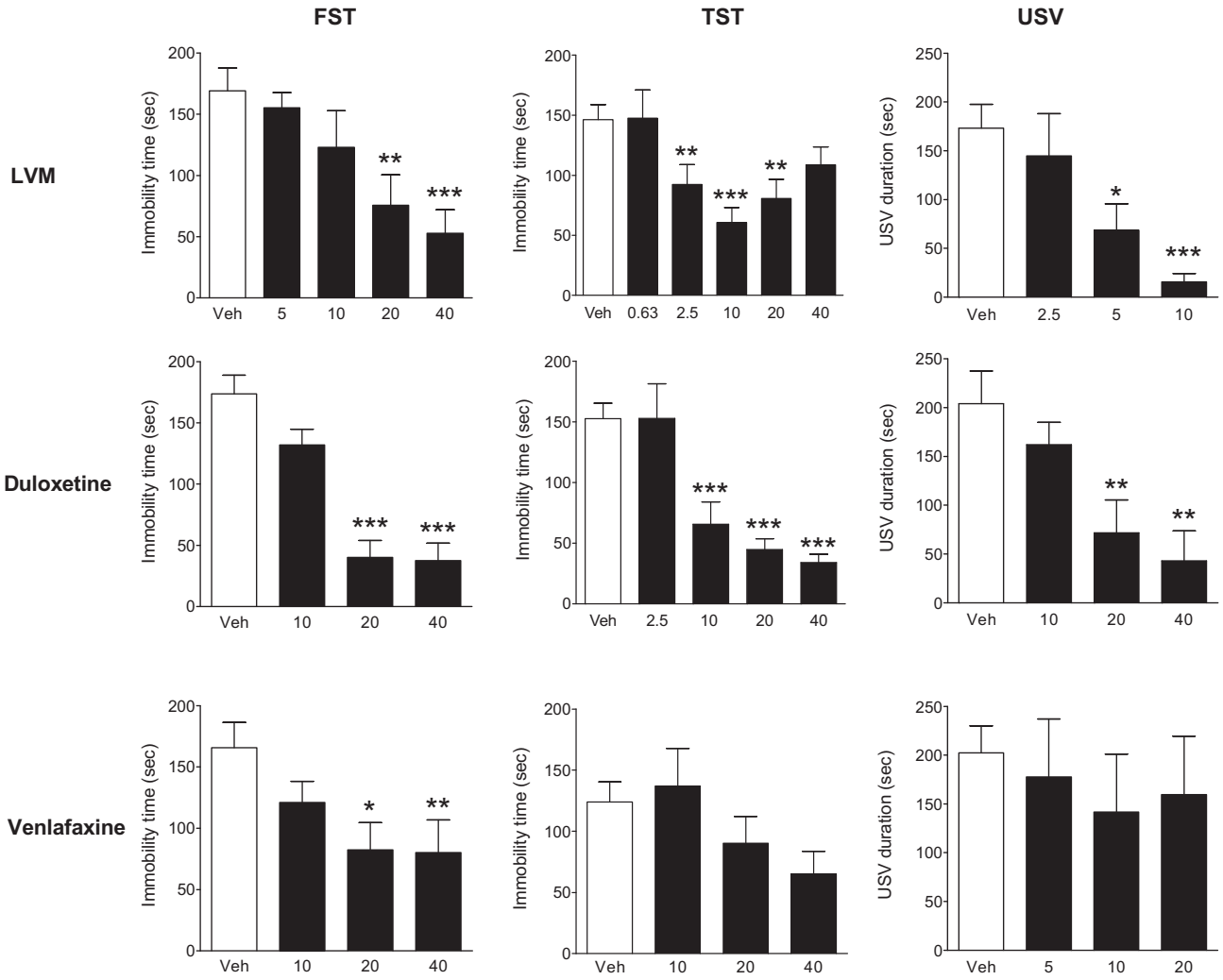

Fig. 5. Effects of LVM, duloxetine and venlafaxine in the forced swim test (FST) or tail suspension test (TST) in mice, and ultrasonic vocalization (USV) model in rats. Bars are mean ± SEM. Compound or vehicle (Veh) was administered i.p. 30 min before the test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, versus saline, Holm–Sidak post-hoc test following significant one-way ANOVA. For FST: $N = 8$–10 for vehicle and 7–8 per dose; for TST: $N = 11$–14 for vehicle and 7–9 per dose; for USV: $N = 7$–12 for vehicle and 9 per dose. Doses are in mg/kg i.p.

decreased immobility relative to vehicle ($F(4,45) = 5.7$, $p < 0.001$ and $F(4,42) = 11.8$, $p < 0.001$, respectively) with a MED of 2.5 mg/kg i.p. and 10 mg/kg i.p, respectively. There was a trend toward reduced immobility with venlafaxine, but this effect was not statistically significant ($F(4,34) = 2.2$, n.s.). LVM reduced immobility in a U-shaped manner, not observed for duloxetine or venlafaxine.

3.6. LVM has anti-stress/anxiolytic activity in the USV model in rats

Under control (vehicle injection) conditions, rats emitted USV for an average of 173.2 ± 24.5 to 204.0 ± 33.5 s (Fig. 5, white bars, right panels). LVM and duloxetine significantly and dose-dependently reduced USV ($F(3,36) = 6.5$, $p < 0.001$ and $F(3,33) = 5.9$, $p < 0.01$, respectively). The MED for LVM was 5 mg/kg i.p and that for duloxetine was 20 mg/kg i.p. Venlafaxine (5–20 mg/kg) did not significantly modify the time spent emitting USV ($F(3,36) = 0.3$, ns).

3.7. LVM does not modify spontaneous locomotor activity of mice

LVM did not significantly modify spontaneous locomotion ($F(3,28) = 0.02$, n.s.), with values at all doses remarkably close to those of its control group (differing by less than 5%) (Table 6). By contrast, duloxetine significantly reduced locomotor counts ($F(3,29) = 8.15$, $p < 0.001$), with a marked reduction (82%) at 40 mg/kg. Although venlafaxine tended to increase spontaneous locomotor activity (62% at 40 mg/kg), the effect was not significant ($F(3,28) = 2.44$, n.s.).

On the whole, in rodent models of depression/stress, LVM was at least as potent as duloxetine and venlafaxine, albeit slightly less efficacious than the former in the TST model. Additionally, efficacy

Table 6
Effects on spontaneous locomotor activity.

	Veh	2.5 mg/kg	10 mg/kg	40 mg/kg
LVM	174.0 ± 23.3	169.0 ± 45.1	166.7 ± 22.8	174.0 ± 26.2
Duloxetine	244.4 ± 34.1	253.6 ± 40.2	182.1 ± 25.3	44.6 ± 9.3**
Venlafaxine	189.8 ± 32.5	229.0 ± 28.4	251.3 ± 29.6	308.7 ± 35.0

Numbers represent the average ± S.E.M. value (arbitrary units). Locomotor activity was recorded for 1 h, 30 min following i.p. administration of compounds or vehicle (Veh). $^{**}p < 0.01$, compared with vehicle, Holm–Sidak post-hoc test following significant one-way ANOVA. For LVM: $N = 11$ for vehicle and 7 per dose; for duloxetine: $N = 12$ for vehicle and 7 per dose; for venlafaxine: $N = 11$ for vehicle and 7 per dose.

Table 7
Summary of pharmacological activity of LVM and its comparators in behavioral models.

		LVM	Duloxetine	Venlafaxine
Therapeutically relevant models	FST	20	20	20
	TST	2.5[a]	10	>40
	USV	5	20	>40
Model related to unwanted side-effects	Spontaneous locomotion	>40	40	>40

Numbers indicate minimal effective dose, in mg/kg, i.p.
FST: forced swim test, TST: tail suspension test, USV: ultrasonic vocalization test.
[a] Partial effect at higher dose.

of LVM in the FST, TST, and USV assays was observed at doses that did not produce sedation (Table 7).

4. Discussion

This is the first paper to characterize the in vitro and in vivo pharmacological profile of the new SNRI LVM. Two sets of data are presented in this manuscript: 1) initial pharmacological studies comparing milnacipran and its two enantiomers LVM (F2695) and F2696 and 2) more detailed pharmacological studies characterizing the in vitro and in vivo pharmacological profile of LVM relative to two prototypical SNRIs, duloxetine and venlafaxine.

The initial pharmacological studies presented in this manuscript confirmed LVM as the more pharmacologically active enantiomer of milnacipran, and provide a rationale for why levomilnacipran was selected for development for the treatment of MDD in the US.

The focus of this manuscript was to characterize the in vitro and in vivo pharmacological profile of LVM relative to duloxetine and venlafaxine, two SNRIs currently marketed as antidepressants. In this manuscript, we show that LVM binds with high affinity to human and rat NET and SERT in vitro (but lacks affinity for DAT). Neither LVM, duloxetine, nor venlafaxine had significant affinity for any of the 23 off-target receptors tested (including those associated with side-effects: muscarinic M_1, M_3, 5-HT_{2B}, μ opioid, adrenergic α_1, α_2). However, duloxetine did show sub-micromolar affinity for 7 targets. Furthermore, LVM, duloxetine, and venlafaxine all potently inhibit NE and 5-HT reuptake in vitro (for both species).

Relative to the other SNRIs tested, LVM has less absolute affinity for NET and SERT than duloxetine (6–56 fold, depending on the transporter and the species [human or rat]), but quite similar affinity for h and r SERT (around 2 fold) and greater affinity for NET (at least 100 fold) than venlafaxine. However, in terms of functional potency in vitro (reuptake inhibition), LVM has similar potency to duloxetine for NE reuptake (both species), but lesser potency for 5-HT reuptake (7 and 27 fold less, for rat and human transporters, respectively). Interestingly though, in the mice FST assay, LVM was as efficacious and potent as duloxetine, and even more potent in the rat USV model, which would suggest the importance of the NE system in the activity of compounds in these two models.

In terms of relative affinity (NET/SERT affinity ratio), LVM presents a more balanced affinity for NET versus SERT (NET/SERT ratio around 1.5 and 8 for rat and human transporter) than either duloxetine (ratio around 7 and 45) or venlafaxine (ratio around 58 and above 500). Interestingly, in vitro LVM more potently inhibits NE than 5-HT reuptake (NE/5-HT ratio 0.9 and 0.6 for rat and human transporter, respectively) which is in contrast to duloxetine or venlafaxine which more potently inhibit 5-HT than NE reuptake (NE/5-HT ratio of 9 and 16 for duloxetine at rat and human transporters and 9.5 and 10 for venlafaxine at rat and human transporters). The NE/5-HT reuptake inhibition ratios reported here for duloxetine and venlafaxine are consistent with those of previous reports (Wong, 1998; Bymaster et al., 2001; Vaishnavi et al., 2004), and the NE/5-HT ratios reported here for LVM are similar to those previously reported for milnacipran (Vaishnavi et al., 2004).

Differences in NE/5-HT reuptake inhibition ratios amongst SNRIs may have clinical relevance (Stahl et al., 2005; Shelton, 2009; Blier and Briley, 2011; Kasper et al., 2011). For example, different potencies at NET and SERT in vivo could contribute to relative efficacy in treating different MDD symptoms associated with either NE (e.g. attention, working memory, concentration, alertness, energy and social activity) (Lapiz and Morilak, 2006; Briley and Moret, 2010; Kasper et al., 2011) or 5-HT (e.g. agitation, appetite disturbance, and irritability) (Nutt, 2008). In fact, some clinical data suggest MLN (which has a comparable in vitro profile to LVM) offers some benefit in terms of treating the noradrenergic symptom cluster (Blier and Briley, 2011; Kasper et al., 2011). Similar clinical data have been generated with LVM (publications in preparation).

Of note, LVM binds with higher affinity to SERT than NET (NET/SERT affinity ratio of 8 and 1.5 for human and rat respectively), but more potently inhibits NE relative to 5-HT reuptake (NE/5-HT potency ratio of 0.6 and 0.9 in human and rat, respectively). Reasons for this discrepancy between NET/SERT affinity ratios and NE/5-HT potency ratios are unclear, but similar disparities between binding and reuptake data have been reported for milnacipran as well as other SNRIs, SSRIs or TCAs (Vaishnavi et al., 2004; Stahl et al., 2005; in house data). This discrepancy may be due to the fact that [^{3}H] nisoxetine labels a site which may only partially overlap the endogenous ligand site, as suggested by Zhen et al. (2012). Thus, the potency to displace [^{3}H]nisoxetine binding may be an underestimate of the actual potency to block the reuptake of [^{3}H]NE.

Rat microdialysis studies were conducted to determine if the observed differences in potency at NET and SERT in vitro translated to differences in potency in vivo. In the present study, LVM, duloxetine, and venlafaxine significantly increased extracellular concentrations of NE and 5-HT in the rat prefrontal cortex. Observed effects of duloxetine and venlafaxine are consistent with previous reports (Kihara and Ikeda, 1995; Koch et al., 2003; Beyer et al., 2002). Furthermore, at the lowest effective dose of LVM (10 mg/kg), there was a significant increase in NE, but not 5-HT (NE/5-HT ratio of ~4). At subsequent doses (20 and 40 mg/kg), LVM increased NE and 5-HT concentrations with equal efficacy (NE/5-HT ratio of ~1 at both doses). Although not definitive, these data suggest that LVM may have a greater impact on extracellular NE levels than 5-HT levels at lower doses, but at higher doses, LVM may affect both catecholamines equally. It is unclear if similar effects would be observed in other brain regions, or if this greater potency at NET would be sustained following chronic administration; however, these data (in combination with the in vitro data above) suggest LVM may differ from other SNRIs in terms of relative potency at NE versus 5-HT in vitro and in vivo.

The effects of LVM on extracellular levels of NE and 5-HT are in contrast to those of venlafaxine, which more potently increased concentrations of 5-HT relative to NE (MEDs of 2.5 and 10 mg/kg for NE and 5-HT, respectively; NE/5-HT ratio of 0.2 and 0.6, respectively). Ratios for venlafaxine reported in the present microdialysis study are contrary to previously reported data showing significant increases in NE and 5-HT in the rat prefrontal cortex following 5, 15, and 40 mg/kg venlafaxine ip (Koch et al., 2003) or equal increases in NE and 5-HT following 10 mg/kg ip (Weikop et al., 2004). However, the observed ratios in the present study are consistent with data in human subjects suggesting that venlafaxine primarily inhibits 5-HT reuptake at lower clinical doses and only inhibits both NE and 5-HT reuptake at the highest clinical doses (Harvey et al., 2000; Debonnel et al., 2007).

Duloxetine significantly increased NE and 5-HT at all the doses tested with an NE/5-HT ratio of approximately 1 at all doses. This is

consistent with the literature reporting significant and comparable increases in NE and 5-HT in the prefrontal cortex of rats following acute administration of duloxetine (5 and 15 mg/kg ip; Koch et al., 2003). Another study reported significant increases in NE and 5-HT in the prefrontal cortex following acute oral administration of duloxetine (3–12 mg/kg and 6–12 mg/kg, respectively; Kihara and Ikeda, 1995). Doses of duloxetine below 10 mg/kg were not tested in the present study, making true comparison of relative potency on NE and 5-HT (as measured by MED) impractical.

In the present microdialysis studies, there was a plateauing effect for the increase in NE and 5-HT observed with LVM and duloxetine. The reason(s) for this plateau for NE is not entirely clear, but may be due in part to an increased activation of adrenergic alpha2 autoreceptor-induced feedback inhibition of NE release, which counterbalances the high levels of extracellular NE induced by these drugs. Microdialysis studies in rats have shown that the combined systemic treatment of desipramine (a selective NRI) with idazoxan (an alpha2-adrenoceptor antagonist) results in a dramatic potentiation of cortical NE output that is orders of magnitude higher than the levels of NE attainable by either of these drug treatments alone (Dennis et al., 1987). Similar autofeedback inhibition effects have been described for the 5-HT system in the prefrontal cortex via activation of 5-HT_{1A} receptors (Gartside et al., 1995). It is unclear why a plateau effect was observed with duloxetine and LVM, but not venlafaxine, and additional experiments (out of the scope of the present report) would be needed to resolve/explore this matter.

All three SNRIs increased levels of DA; for duloxetine, this may reflect a direct effect of blockade of DAT (K_i: 446 nM). However, neither LVM nor venlafaxine have high affinity for DAT; therefore, increases in DA following LVM and venlafaxine administration is most likely due to an indirect effect on DA via blockade of NET. In the prefrontal cortex, where DA and NE terminals co-exist but NET sites greatly outnumber DAT sites, NET is responsible for most extracellular DA uptake (reviewed in El Mansari et al., 2010).

LVM, duloxetine, and venlafaxine were studied in two models considered to be predictive of antidepressant-like activity: the forced swim test (FST) and the tail suspension test (TST) (Porsolt et al., 2001). Mice were selected for the FST because this species is comparatively more sensitive than rats to the effects of SNRIs and other classes of antidepressants (Castagne et al., 2009; in-house unpublished data). In both models, LVM significantly decreased the time of immobility. Whereas LVM had a dose-dependent effect in the FST, it produced a U-shaped dose–response curve in the TST. Reasons for the U-shaped curve are unknown; however, they are unlikely to be related to motor-impairing/sedative effects, as LVM had no impact on spontaneous locomotor activity at similar doses in mice. The difference of sensitivity of LVM between the FST and TST tests could be related to the use of different strains (OF1 versus NMRI), as suggested previously for other SNRIs (Kulkarni and Dhir, 2007).

Duloxetine and LVM had similar efficacy and potency in the FST, but LVM was more potent than duloxetine in the TST. The duloxetine effects observed in the TST and FST in the present study are in agreement with those reported previously (Li et al., 2003; Andreasen et al., 2009; Berrocoso and Mico, 2009a,b; Castagne et al., 2009). Venlafaxine was less efficacious and potent than LVM and duloxetine in the FST, and only showed a moderate tendency to reduce immobility in the TST. However, previous studies have reported efficacy of venlafaxine in each of these assays across a similar dose range (Castagne et al., 2009). Lack of, or poor efficacy of venlafaxine in the present study could also be due to the use of different strains.

LVM was also studied in the FST in comparison to MLN and F2696. LVM differs in potency in these two sets of experiments. The potency for LVM in the FST study in comparison with duloxetine and venlafaxine is lower than that in the MLN/LVM/F2696 comparative study. However, one should be cautious in directly comparing these data, as the two sets of data were generated at different times (two decades apart), in different strains obtained from different breeders, and under slightly different conditions.

In the USV model in rats, “distress” calls in the 22 kHz range are emitted in response to various stressful/aversive stimuli (such as presentation of a conditioned stimulus previously associated with mild foot shocks), and are considered to reflect an underlying “anxiety-like/stress-like” affective state (Jelen et al., 2003). LVM reduced USV, suggesting “anxiolytic-like” and/or “anti-stress” effects, and was approximately four times more potent than duloxetine. Venlafaxine was inactive in this assay, consistent with previous reports (Sanchez, 2003). Previous studies have shown that the SNRIs duloxetine and milnacipran are effective in the USV model (Bardin et al., 2010); however, milnacipran (Bardin et al., 2010) and LVM (present study) appear to have greater potency than duloxetine in this assay. Perhaps the near 2-fold preference for NE versus 5-HT reuptake inhibition properties of LVM and milnacipran confer an advantage of efficacy in this model.

Taken together, the results from the FST, TST and USV models clearly suggest a potential “antidepressant-like” and/or “anxiety/anti-stress-like” activity of LVM, on the whole similar to that displayed by duloxetine, but apparently superior to that of venlafaxine. However, additional studies in these models following long-term administration are warranted, as antidepressants are prescribed on a chronic basis in the clinic; extrapolations from these acute pre-clinical experiments to the clinic should be made with caution. In addition, future studies should be conducted to evaluate the efficacy of LVM in models associated with NE-related depressive symptoms (e.g. fatigue, anhedonia).

In summary, LVM is the pharmacologically more active enantiomer of milnacipran. Like duloxetine and venlafaxine, it potently inhibited NE and 5-HT reuptake in vitro, increased NE and 5-HT in the rat prefrontal cortex, and like duloxetine, decreased immobility in the FST and TST in mice (venlafaxine being, under our experimental conditions, less or not active). However, LVM, unlike duloxetine or venlafaxine, exhibited a 2-fold preference for NE over 5-HT reuptake inhibition in vitro, and to some extent in vivo (at the lowest effective dose in the microdialysis study). At higher doses, LVM, like duloxetine and venlafaxine, increased extracellular levels of NE and 5-HT equally. Additional studies are needed to further explore the relative potency of LVM on NE and 5-HT reuptake in vivo and to determine the clinical relevance of this finding. However, overall, these data demonstrate that LVM is a selective and potent SNRI that may have potential therapeutic value in the treatment of MDD.

Acknowledgments

We wish to express our gratitude to Christelle Benas, Joël Besnard, Nathalie Consul-Denjean, Nathalie Danty, Alexandra Galinier, Jérôme Floutard, Jean Laval, Nathalie Leduc, Nathalie Malfètes, Anne-Marie Ormière, Gaëlle Pulou, Veronique Ravailhe, for their expert technical assistance.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2013.02.024.

References

Andrade, L., Caraveo-Anduaga, J.J., Berglund, P., Bijl, R.V., De Graaf, R., Vollebergh, W., et al., 2003. The epidemiology of major depressive episodes:

results from the International Consortium of Psychiatric Epidemiology (ICPE) surveys. Int. J. Methods Psychiatry Res. 12, 3–21.
Andreasen, J.T., Nielsen, E.O., Redrobe, J.P., 2009. Chronic oral nicotine increases brain [^{3}H]epibatidine binding and responsiveness to antidepressant drugs, but not nicotine, in the mouse forced swim test. Psychopharmacology 205, 517–528.
Bardin, L., Gregoire, S., Aliaga, M., Malfetes, N., Vitton, O., Ladure, P., et al., 2010. Comparison of milnacipran, duloxetine and pregabalin in the formalin pain test and in a model of stress-induced ultrasonic vocalizations in rats. Neurosci. Res. 66, 135–140.
Berrocoso, E., Mico, J.A., 2009a. Cooperative opioid and serotonergic mechanisms generate superior antidepressant-like effects in a mice model of depression. Int. J. Neuropsychopharmacol. 12, 1033–1044.
Berrocoso, E., Mico, J.A., 2009b. Role of serotonin 5-HT1A receptors in the antidepressant-like effect and the antinociceptive effect of venlafaxine in mice. Int. J. Neuropsychopharmacol. 12, 61–71.
Beyer, C.E., Boikess, S., Luo, B., Dawson, L.A., 2002. Comparison of the effects of antidepressants on norepinephrine and serotonin concentrations in the rat frontal cortex: an in-vivo microdialysis study. J. Psychopharmacol. 16, 297–304.
Blier, P., Briley, M., 2011. The noradrenergic symptom cluster: clinical expression and neuropharmacology. Neuropsychiatr. Dis. Treat. 7 (Suppl. 1), 15–20.
Bradford, M.M., 1976. An improved colorimetric technique for protein measurement. Anal. Biochem. 72, 348–354.
Briley, M., Moret, C., 2010. Improvement of social adaptation in depression with serotonin and norepinephrine reuptake inhibitors. Neuropsychiatr. Dis. Treat. 6, 647–655.
Bymaster, F.P., Dreshfield-Ahmad, L.J., Threlkeld, P.G., Shaw, J.L., Thompson, L., Nelson, D.L., et al., 2001. Comparative affinity of duloxetine and venlafaxine for serotonin and norepinephrine transporters in vitro and in vivo, human serotonin receptor subtypes, and other neuronal receptors. Neuropsychopharmacology 25, 871–880.
Castagne, V., Porsolt, R.D., Moser, P., 2009. Behavioral assessment of antidepressant activity in rodents. In: Buccafusco, J.J. (Ed.), Methods of Behaviour Analysis in Neuroscience. CRC Press, Boca Raton, pp. 103–117.
Chalon, S.A., Granier, L.A., Vandenhende, F.R., Bieck, P.R., Bymaster, F.P., Joliat, M.J., Hirth, C., Potter, W.Z., 2003. Duloxetine increases serotonin and norepinephrine availability in healthy subjects: a double-blind, controlled study. Neuropsychopharmacology 28, 1685–1693.
Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2010. Guide for the Care and Use of Laboratory Animals. National Academies Press, Washington, D.C., USA.
Debonnel, G., Saint-André, E., Hébert, C., de Montigny, C., Lavoie, N., Blier, P., 2007. Differential physiological effects of a low dose and high doses of venlafaxine in major depression. Int. J. Neuropsychopharmacol. 10, 51–61.
Deecher, D.C., Beyer, C.E., Johnston, G., Bray, J., Shah, S., Abou-Gharbia, M., et al., 2006. Desvenlafaxine succinate: a new serotonin and norepinephrine reuptake inhibitor. J. Pharmacol. Exp. Ther. 318, 657–665.
Dennis, T., L'Heureux, R., Carter, C., Scatton, B., 1987. Presynaptic alpha-2 adrenoceptors play a major role in the effects of idazoxan on cortical noradrenaline release (as measured by in vivo dialysis) in the rat. J. Pharmacol. Exp. Ther. 241, 642–649.
El Mansari, M., Guiard, B.P., Chernoloz, O., Ghanbari, R., Katz, N., Blier, P., 2010. Relevance of norepinephrine-dopamine interactions in the treatment of major depressive disorder. CNS Neurosci. Ther. 16, 1–17.
FDA Guidance on the Development of New Stereoisomeric Drugs, 1992. http://www.fda.gov/drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm122883.htm#.UKHEWm4ZyYE.email.
Gartside, S.E., Umbers, V., Hajos, M., Sharp, T., 1995. Interaction between a selective 5-HT$_{1A}$ receptor antagonist and an SSRI in vivo: effects on 5-HT cell firing and extracellular 5-HT. Br. J. Pharmacol. 115, 1064–1070.
Harvey, A.T., Rudolph, R.L., Preskorn, S.H., 2000. Evidence of the dual mechanisms of action of venlafaxine. Arch. Gen. Psychiatry 57, 503–509.
Hutt, A.J., Vanetova, J., 2003. The chiral switch: the development of single enantiomer drugs from racemates. Acta Facult. Pharm. Univ. Comenianae 50, 7–23.
Jelen, P., Soltysik, S., Zagrodzka, J., 2003. 22-kHz ultrasonic vocalization in rats as an index of anxiety but not fear: behavioral and pharmacological modulation of affective state. Behav. Brain Res. 141, 63–72.
Kasper, S., Meshkat, D., Kutzelnigg, A., 2011. Improvement of the noradrenergic symptom cluster following treatment with milnacipran. Neuropsychiatr. Dis. Treat. 7 (Suppl. 1), 21–27.
Kessler, R.C., Berglund, P., Demler, O., Jin, R., Koretz, D., Merikangas, K.R., et al., 2003. The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). J. Am. Med. Assoc. 289, 3095–3105.
Kihara, T., Ikeda, M., 1995. Effects of duloxetine, a new serotonin and norepinephrine uptake inhibitor, on extracellular monoamine levels in rat frontal cortex. J. Pharmacol. Exp. Ther. 272, 177–183.
Koch, S., Hemrick-Luecke, S.K., Thompson, L.K., Evans, D.C., Threlkeld, P.G., Nelson, D.L., Perry, K.W., et al., 2003. Comparison of effects of dual transporter inhibitors on monoamine transporters and extracellular levels in rats. Neuropharmacology 45, 935–944.
Kulkarni, S.K., Dhir, A., 2007. Effect of various classes of antidepressants in behavioral paradigms of despair. Prog. Neuropsychopharmacol. Biol. Psychiatry 31, 1248–1254.
Lapiz, M.D., Morilak, D.A., 2006. Noradrenergic modulation of cognitive function in rat medial prefrontal cortex as measured by attentional set shifting capability. Neuroscience 137, 1039–1049.
Li, X., Witkin, J.M., Need, A.B., Skolnick, P., 2003. Enhancement of antidepressant potency by a potentiator of AMPA receptors. Cell. Mol. Neurobiol. 23, 419–430.
Mathers, C.D., Loncar, D., 2006. Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med. 3, e442.
Millan, M.J., Gobert, A., Lejeune, F., Newman-Tancredi, A., Rivet, J.M., Auclair, A., Peglion, J.L., 2001. S33005, a novel ligand at both serotonin and norepinephrine transporters: I. Receptor binding, electrophysiological, and neurochemical profile in comparison with venlafaxine, reboxetine, citalopram, and clomipramine. J. Pharmacol. Exp. Ther. 298, 565–580.
Moret, C., Charveron, M., Finberg, J.P., Couzinier, J.P., Briley, M., 1985. Biochemical profile of midalcipran (F 2207), 1-phenyl-1-diethyl-aminocarbonyl-2-aminomethyl-cyclopropane (Z) hydrochloride, a potential fourth generation antidepressant drug. Neuropharmacology 24, 1211–1219.
Nogami, T., Takano, H., Arakawa, R., Ichimiya, T., Fujiwara, H., Kimura, Y., et al., 2012. Occupancy of serotonin and norepinephrine transporter by milnacipran in patients with major depressive disorder: a positron emission tomography study with [11C]DASB and (S, S)-[18F]FMeNER-D2. Int. J. Neuropsychopharmacol., 1–7. Oct 16 (Epub ahead of print).
Nutt, D.J., 2008. Relationship of neurotransmitters to the symptoms of major depressive disorder. Clin. Psychiatry 69 (Suppl. E1), 4–7.
Owens, M.J., Morgan, W.N., Plott, S.J., Nemeroff, C.B., 1997. Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites. J. Pharmacol. Exp. Ther. 283, 1305–1322.
Papakostas, G.I., Thase, M.E., Fava, M., Nelson, J.C., Shelton, R.C., 2007. Are antidepressant drugs that combine serotonergic and noradrenergic mechanisms of action more effective than the selective serotonin reuptake inhibitors in treating major depressive disorder? A meta-analysis of studies of newer agents. Biol. Psychiatry 62, 1217–1227.
Porsolt, R.D., Brossard, G., Hautbois, C., Roux, S., 2001. Rodent models of depression: forced swimming and tail suspension behavioral despair tests in rats and mice. Curr. Protoc. Neurosci. 55 (8.10A), 1–14.
Saha, S.N., Bhatia, S.C., Nayar, U., 2004. Adrenergic involvement in the locus ceruleus and adjoining regions in the facilitation of predatory attack behavior as induced by hypothalamic stimulation in cats. Indian J. Physiol. Pharmacol. 48, 51–58.
Sanchez, C., 2003. Stress-induced vocalisation in adult animals. A valid model of anxiety? Eur. J. Pharmacol. 463, 133–143.
Sharif, S.I., Gokhale, S.D., 1986. Pharmacological evaluation of the isolated rat seminal vesicle preparation. J. Pharmacol. Methods 15, 65–75.
Shelton, R.C., 2009. Serotonin norepinephrine reuptake inhibitors: similarities and differences. Prim. Psychiatry 16 (Suppl. 4), 25–35.
Stahl, S.M., Grady, M.M., Moret, C., Briley, M., 2005. SNRIs: their pharmacology, clinical efficacy, and tolerability in comparison with other classes of antidepressants. CNS Spectr. 10, 732–747.
Taber, M.T., Wright, R.N., Molski, T.F., Clarke, W.J., Brassil, P.J., Denhart, D.J., Mattson, R.J., Lodge, N.J., 2005. Neurochemical, pharmacokinetic, and behavioral effects of the novel selective serotonin reuptake inhibitor BMS-505130. Pharmacol. Biochem. Behav. 80, 521–528.
Turcotte, J.E., Debonnel, G., de Montigny, C., Hébert, C., Blier, P., 2001. Assessment of the serotonin and norepinephrine reuptake blocking properties of duloxetine in healthy subjects. Neuropsychopharmacology 24, 511–521.
Vaishnavi, S.N., Nemeroff, C.B., Plott, S.J., Rao, S.G., Kranzler, J., Owens, M.J., 2004. Milnacipran: a comparative analysis of human monoamine uptake and transporter binding affinity. Biol. Psychiatry 55, 320–322.
Weikop, P., Kehr, J., Scheel-Krüger, J., 2004. The role of alpha1- and alpha2-adrenoreceptors on venlafaxine-induced elevation of extracellular serotonin, noradrenaline and dopamine levels in the rat prefrontal cortex and hippocampus. J. Psychopharmacol. 18, 395–403.
Wong, D.T., 1998. Duloxetine (LY 248686): an inhibitor of serotonin and noradrenaline uptake and an antidepressant drug candidate. Expert Opin. Investig. Drugs 7, 1691–1699.
Yoshitake, T., Fujino, K., Kehr, J., Ishida, J., Nohta, H., Yamaguchi, M., 2003. Simultaneous determination of norepinephrine, serotonin, and 5-hydroxyindole-3-acetic acid in microdialysis samples from rat brain by microbore column liquid chromatography with fluorescence detection following derivatization with benzylamine. Anal. Biochem. 312, 125–133.
Zhen, J., Ali, S., Dutta, A.K., Reith, M.E., 2012. Characterization of [^{3}H]CFT binding to the norepinephrine transporter suggests that binding of CFT and nisoxetine is not mutually exclusive. J. Neurosci. Methods 203, 19–27.