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RESEARCH PAPER

Evidence for a differential opioidergic involvement in the analgesic effect of antidepressants: prediction for efficacy in animal models of neuropathic pain?

A-S Wattiez, F Libert, A-M Privat, S Loiodice, J Fialip, A Eschalier and C Courteix

Clermont Université, Université d'Auvergne, Pharmacologie Fondamentale et Clinique de la Douleur, BP 10448, F-63000 Clermont-Ferrand, Inserm, U 766, F-63001 Clermont-Ferrand, France

Correspondence
Christine Courteix, Clermont Université, Université d'Auvergne, Pharmacologie Fondamentale et Clinique de la Douleur, BP 10448, F-63000 Clermont-Ferrand, Inserm, U 766, F-63001 Clermont-Ferrand, France. E-mail: christine.courteix@u-clermont1.fr

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BACKGROUND AND PURPOSE
Antidepressants are one of the recommended treatments for neuropathic pain. However, their analgesic action remains unpredictable, and there are no selection criteria for clinical use. Better knowledge of their mechanism of action could help highlight differences underlying their unequal efficacy.

EXPERIMENTAL APPROACH
We compared the activity of a tricyclic antidepressant (clomipramine) with selective 5-HT and noradrenaline reuptake inhibitors (milnacipran and duloxetine) in streptozocin-induced diabetic and chronic constriction nerve injury-induced neuropathic rats, after repeated injections. We looked for an opioidergic mechanism in their action.

KEY RESULTS
Abolition of mechanical hyperalgesia was observed in mononeuropathic rats after five injections of clomipramine (5 mg·kg^{-1}, s.c.) and milnacipran (10 or 20 mg·kg^{-1}, i.p.) and in diabetic rats after clomipramine. An additional antinociceptive effect was obtained with five injections of duloxetine (3 mg·kg^{-1}, i.p.) in both models and milnacipran (10 mg·kg^{-1}, i.p.) in diabetic rats. These effects were observed with plasma antidepressant concentrations similar to those found in patients treated for neuropathic pain. Naloxone (1 mg·kg^{-1}, i.v.) only suppressed the anti-hyperalgesic effects of clomipramine in both models of pain and of milnacipran in the traumatic model.

CONCLUSIONS AND IMPLICATIONS
The opioid system appears to be involved in the mechanism of action of antidepressants that only have an anti-hyperalgesic effect but not in those that have a stronger (i.e. antinociceptive) effect. These differences between the antidepressants occurred whatever the aetiology of the neuropathy and, if confirmed in clinical trials, could be used to decide which antidepressant is administered to a patient with neuropathic pain.

Abbreviations
AU, arbitrary units; AUC, area under the time-course curve; CCI, chronic constriction injury; CMI, clomipramine; dulo, duloxetine; HPMC, hydroxy-propyl-methyl-cellulose; milna, milnacipran; MPE, maximal possible effect; SNL, spinal nerve ligation; SPC, summary of product characteristics; SSNRI, selective 5-HT (serotonin) and noradrenaline re-uptake inhibitors; STZ, streptozocin; TCA, tricyclic antidepressants; VT, vocalization threshold

Introduction

Neuropathic pain remains a significant clinical problem that is often refractory to conventional analgesics. Antidepressants, particularly tricyclic antidepressants (TCAs; nonspecific monoamine re-uptake inhibitors) have been widely used to treat neuropathic pain (Finnerup *et al.*, 2005) despite their undesirable side effects. The efficacy of selective 5-HT (serotonin) and noradrenaline re-uptake inhibitors (SSNRIs), which are better tolerated than TCAs, has been demonstrated in patients with neuropathic pain (Dworkin *et al.*, 2007). Nevertheless, data obtained from meta-analysis of randomized control trials show that a substantial percentage of patients do not respond favourably to antidepressants, with no more than 40–60% obtaining partial relief of pain (Dworkin *et al.*, 2007). Indeed, the estimated number needed to treat ranges from 2.7 to 3.7 for TCAs (Finnerup *et al.*, 2005; Sindrup *et al.*, 2005) and around 5 for duloxetine (Kajdasz *et al.*, 2007). Due to the limited number of head-to-head studies, there is little comparative data on their respective analgesic efficacy according to pain characteristics. Thus, there are no criteria other than tolerability for selecting between the various compounds available.

The mechanism generally proposed for the analgesic effect of antidepressants is the reinforcement of descending inhibitory pathways involving serotoninergic and noradrenergic projection neurones by inhibiting the re-uptake of 5-HT and noradrenaline and increasing their availability in the spinal cord. For some antidepressants, other mechanisms have been proposed, such as either direct or indirect involvement of the opioid system (Botney and Fields, 1983; Sacerdote *et al.*, 1987; Eschalier, 1990; Eschalier *et al.*, 2000). For example, in animal models of neuropathic pain, supra-spinal δ and spinal μ opioid receptors have been shown to be involved in the anti-hyperalgesic effect of clomipramine, a TCA (Marchand *et al.*, 2003b). Opioid receptors are also involved in the action of the SSNRI milnacipran (Bomholt *et al.*, 2005; Onal *et al.*, 2007), whereas the effect of another SSNRI, venlafaxine, does not involve these receptors (Marchand *et al.*, 2003a). We postulate that this differential involvement of the opioid system could account for differential efficacy between antidepressants.

The aim of the present study was to determine a link between antidepressant efficacy and the involvement of the opioid system in their analgesic effects. We thus decided to compare the analgesic efficacy of two SSNRIs, milnacipran and duloxetine, against the TCA clomipramine, using repeated administrations, on mechanical hyperalgesia in two animal models of neuropathic pain of metabolic and traumatic aetiologies. The first model, streptozocin (STZ)-induced diabetes in rats (Courteix *et al.*, 1993), would reproduce polyneuropathy with a systemic disease able to alter drug pharmacokinetics. The second model, due to a chronic constriction injury (CCI) of the sciatic nerve in rats (Bennett and Xie, 1988), results in a peripheral mononeuropathy but no systemic disorders. We assessed the relative contribution of opioid receptors by testing the effect of naloxone on antidepressant-induced anti-hyperalgesia. For validation purposes, the plasma levels of antidepressants were also determined to verify that the doses used in animals led to plasma levels that were similar in both models and close to those obtained in patients treated for neuropathic pain.

Methods

Animals

Male Sprague-Dawley rats (Charles Rivers, France) weighing 200–225 g and 250–275 g were housed in standard laboratory conditions under a 12 h light-dark schedule with free access to food and water, for 1 week before starting the experiments. The ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983) were respected, and the experimental protocol was approved by the Local Ethics Committee for Animal Experimentation (CEMEAA authorization No. CE-2-07).

Induction of diabetes

Rats (250–275 g) were injected i.p. with STZ (Sigma Aldrich, St-Quentin-Fallavier, France; 72 $mg \cdot kg^{-1}$, 5 $mL \cdot kg^{-1}$ of body weight). Diabetes was confirmed 1 week after injection by measuring tail vein blood glucose levels (blood samples were obtain from the tail by pinprick) with a glucometer (ACCU-CHEK® ACTIVE, Roche Diagnostics, Meylan, France). Only animals with a blood glucose level >14 mM were considered diabetic and included in the study. At this point, in order to ensure good clinical status and minimize discomfort due to diabetes, hyperglycaemic rats were injected s.c. with insulin (LANTUS®, Sanofi-Aventis, Paris, France; 2 IU = per rat) every other day. Furthermore, we paid special attention to the general health status of the animals: weight loss greater than 10% of initial body weight, decrease of activity, or hair erection were criteria that prompted removal of the animals. This animal model of chronic pain with mechanical, thermal and chemical hyperalgesia has been described elsewhere (Courteix *et al.*, 1993). Some rats only received an i.p. injection of NaCl and were used as normal (healthy) rats.

Induction of mononeuropathy: chronic constriction nerve injury (CCI)

Unilateral peripheral mononeuropathy was induced according to the method described by Bennett and Xie (1988). Briefly, rats (200–225 g) were anaesthetized with sodium pentobarbital (6%, 1 $mL \cdot kg^{-1}$ of body weight i.p.). After skin incision, the left sciatic nerve was exposed and four polyester sutures (MERSUTURE® 3-0, Ethicon, Johnson & Johnson, France) were tied loosely around it at 1 mm intervals, so that the nerve was constricted but the circulation was not interrupted. The skin was then sutured (MONOCRYL® 5-0, Ethicon, Johnson & Johnson, Issy-les-Moulineaux, France). The CCI model as described by Bennett and Xie produces allodynia and hyperalgesia. The animals were allowed to recover and were monitored routinely to assure good health.

Assessment of mechanical hyperalgesia

The rats were submitted to the paw pressure test previously described by Randall and Selitto (Randall and Selitto, 1957). Mechanical hyperalgesia was assessed using a Ugo Basile

analgesimeter (Bioseb®, France) by applying a linearly increasing mechanical force to the dorsum of the left hind paw until a squeak was obtained. As this test involves animal handling, the experimenter got the rats used to being handled as follows: 3 days before the experiment, rats were held by the experimenter for 20 s without escaping, two or three times depending on their capacity to be quiet. On the day of the experiment, rats were again handled two or three times by the experimenter for 20 s and, simultaneously, the Ugo Basile apparatus was started to get the rat used to the noise of the apparatus. No rats showed aversive reactions during handling. Then, the paw of the rat was placed under the tip, and the pressure progressively applied until the rat vocalized. Three to four measures were performed at 10 min intervals in order to obtain two consecutive values that differed no more than 10%. The vocalization threshold (VT) was defined as the mean score of two measures, expressed in arbitrary units (AU). The maximal pressure (cut-off) applied was 75 AU.

Drugs and chemicals

Insulin (LANTUS®) was purchased from Sanofi-Aventis (Paris, France), STZ, naloxone and clomipramine from Sigma-Aldrich (Saint Quentin Fallavier, France), milnacipran (IXEL®) from Pierre Fabre Médicaments (Castres, France) and duloxetine (CYMBALTA®) from Eli Lilly (Belgium). Insulin, naloxone and clomipramine were dissolved in physiological saline (NaCl 0.9%), STZ in distilled water, duloxetine and milnacipran in hydroxy-propyl-methyl-cellulose (HPMC 0.25%, Colorcon, Orpington, UK). Drugs were prepared just before the injections.

Experimental design

All experiments were performed by the same experimenter blinded to the treatment. Treatments were randomized and administered according to the method of blocks in order to assess their effect in the same conditions. Different animals were used in each experiment.

Baseline mechanical sensitivity was determined before nerve injury or STZ injection, then 14 or 21 days after respectively. At that point, rats were considered as hyperalgesic and included in the study if their vocalization thresholds in response to paw-pressure were reduced above 15% of the value obtained before neuropathy. Then, for each experimental series corresponding to each antidepressant tested, hyperalgesic rats were randomly assigned to the following treatment groups:

- Antidepressant + naloxone: rats receiving five injections of antidepressant (i.p. or s.c.), then an i.v. injection of naloxone just after testing the effect of the fifth injection of antidepressant
- Antidepressant + NaCl: rats receiving five injections of antidepressant (i.p. or s.c.), then an i.v. injection of NaCl just after testing the effect of the fifth injection of antidepressant
- Vehicle (HPMC or NaCl) + naloxone: rats receiving five injections of HPMC (i.p.) or NaCl (s.c.), then an i.v. injection of naloxone just after testing the effect of the fifth injection of vehicle
- Vehicle (HPMC or NaCl) + NaCl: rats receiving five injections of HPMC (i.p.) or NaCl (s.c.), then an i.v. injection of NaCl just after testing the effect of the fifth injection of vehicle

As the results obtained with clomipramine and duloxetine in neuropathic rats suggest that the involvement of an opioid mechanism could be either related to the drug or to the drug in a pathological state, we performed two additional experimental series using healthy rats in order to test this hypothesis. Thus, normal (healthy) rats were randomly assigned to the same treatment groups as described above.

In order to perform experiments in conditions mirroring clinical use, (i) we chose doses of antidepressants to obtain plasma levels close to those of patients treated with the same antidepressants; and (ii) we administered the drugs repeatedly, every plasma half-life time ($T_{1/2}$) as in patients.

The chosen doses were 5, 3 and 10 mg·kg^{-1} per injection for clomipramine, duloxetine and milnacipran respectively. Five successive injections were performed every 155 min, 150 min and 420 min (Figure 1), that is, at intervals corresponding to the rat plasma $T_{1/2}$ of clomipramine, duloxetine and milnacipran respectively (Ardid and Guilbaud, 1992; Neliat *et al.*, 1996). Behavioural testing was performed at Tmax after the first injection and once after each of the five antidepressant injections in order to track time-course action, that is, at 88 min, 82 min and 240 min after each injection for clomipramine, duloxetine and milnacipran respectively (Figure 1). This delay was calculated using the formula (Tmax + $T_{1/2}$)/2 (Eschalier *et al.*, 1988) so that the experiments could be performed when plasma levels reached the steady-state mean value. Naloxone (1 mg·kg^{-1}) or NaCl (i.v.) was injected just after the effect of the fifth injection of antidepressants had been tested. VTs were then determined at 15, 30, 45, 60 and 75 min after the injection of naloxone or NaCl (Figure 1).

The final behavioural measurements (i.e. after the last injection of the antidepressant and after naloxone injection) were scheduled according to the $T_{1/2}$ of the compounds. Thus, we were careful to start the first injection at different times (0500 h for clomipramine, 0800 h for milnacipran and 0524 h for duloxetine) so that the last behavioural testing (after naloxone injection) was done between 1600 h and 1800 h for clomipramine, 1715 h for milnacipran and 1800 h for duloxetine. In this way, the final behavioural testing was done at the same time of the cycle for each condition.

At the end of the experiment, the animals were anaesthetized with volatile isoflurane (3.5%) and 1.5 mL of blood was sampled by retro-orbital puncture using a heparin-coated Pasteur pipette (Heparin Choay). Samples were then centrifuged for 5 min at 4000× *g* and the plasma was stored at –20°C until the antidepressants were assayed in order to determine plasma levels and confirm that they were in the therapeutic range.

In additional animals, motor function was estimated by measuring spontaneous locomotor activity using an actimeter (Actisystem, Penlab, Apelex). The rats were placed in a rectangular cage (45 × 30 × 20 cm) and allowed to investigate the cage for 3 min, during which the total number of horizontal and vertical movements were recorded by the

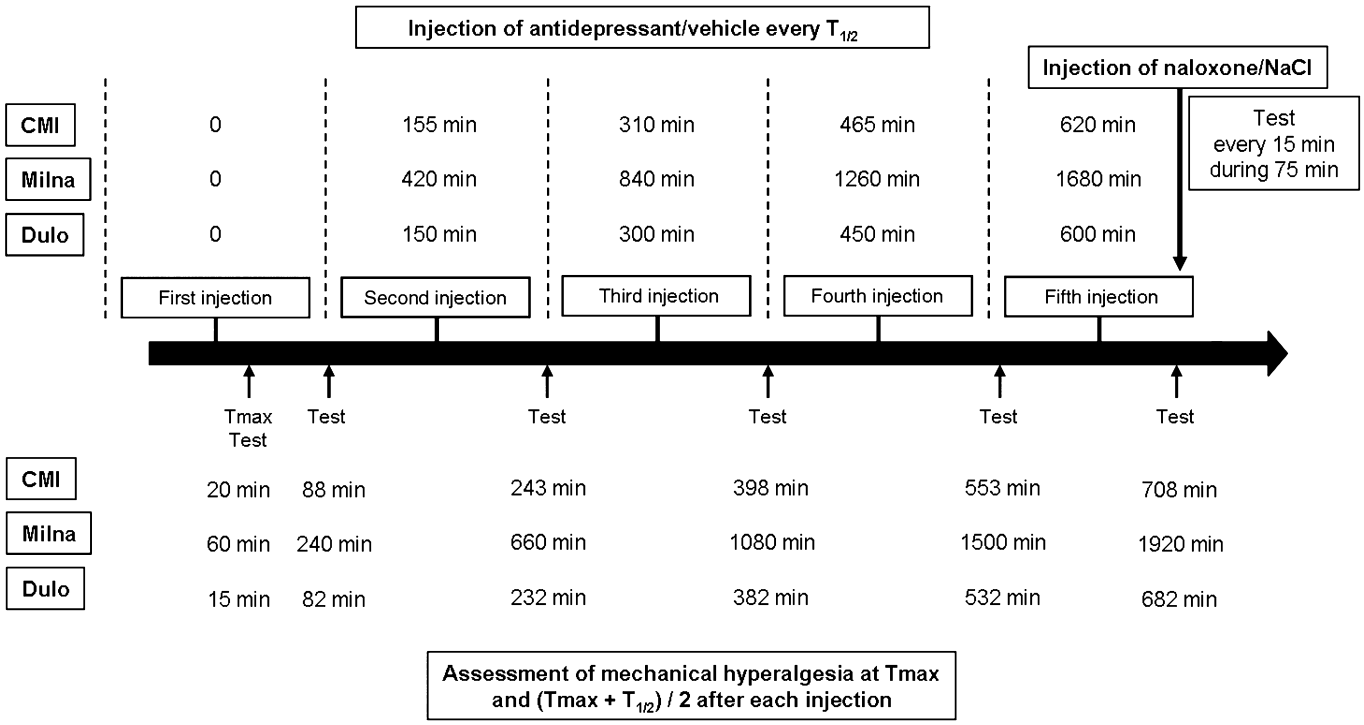

Figure 1

Experimental design for the administration of the antidepressants and naloxone. CMI, clomipramine; Dulo, Duloxetine; Milna, Milnacipran.

actimeter. The parameters were determined 88, 82 or 240 min after the last injection of either NaCl or clomipramine (5 mg·kg^{-1}), HMPC or duloxetine (3 mg·kg^{-1}), HPMC or milnacipran (10 mg·kg^{-1}), respectively, in STZ and CCI rats.

Determination of plasma antidepressant levels

Plasma levels of clomipramine (and its active desmethylated metabolite desmethyl-clomipramine), duloxetine and milnacipran were determined by HPLC coupled with diode array detection, using a two-step extraction protocol. Briefly, 400 µL of phosphate buffer (pH 10.5) and 200 µL of a 30 µg·mL^{-1} trazodone solution were added to 1.5 mL of plasma as internal standard. The sample was centrifuged (10 000× *g*) for 10 min with 7 mL of hexane/diethyl ether/isoamyl-alcohol (49/49/2). The organic layer was back-extracted with 300 µL of a H_2SO_4 solution (0.1 N). The acid solution was injected into a liquid chromatograph. The standard curve was calculated with spiked serum at the following concentrations: 0–5–20–100–200–1000 ng·mL^{-1}. All calibration samples were taken through the extraction protocol described above.

Separation was performed on a 250 mm × 4.6 mm Nucleodur® C8 Gravity column (Macherey-Nagel). The mobile phase consisted of a gradient of phosphate buffer 50 mM pH 3.6 – acetonitrile, increasing linearity from 15 to 35% acetonitrile over 8 min (flow rate: 1.5 mL·min^{-1}), and to 65% for the next 9 min (flow rate: 1.5 mL·min^{-1}). The system was then stabilized for an additional 2 min at initial conditions (flow rate: 1.5 mL·min^{-1}).

Detection was performed using a diode array detector (DIONEX PDA 100) set at 215 nm for duloxetine and milnacipran and at 240 nm for clomipramine and desmethyl-clomipramine, and spectral data were analysed in order to assess purity of peaks.

A linear relationship ($r > 0.999$) was found for all calibration curves, and the lower limit of quantification was 5 ng·mL^{-1} for each drug tested.

Data analysis

VTs are expressed as mean ± SE of raw data (in AU).

Behavioural data were examined using a two-way ANOVA (repeated measures). If significant, the ANOVA was followed by a Tukey's test in order to compare the different groups at the same time and to analyse the time-course of effect of antidepressants. Student's unpaired *t*-test was used to compare values after five injections versus values obtained before neuropathy.

The percentage of maximal possible effect (% MPE) was calculated using the equation % MPE = [Maximal post-drug threshold – Pre-drug (T0) threshold] × 100/[cut-off – Pre-drug (T0) threshold].

To compare the effect of naloxone on antidepressant activity, the area under the time-course (0–75 min) curve (AUC) of VT variations (thresholds at time T – thresholds obtained before drug treatments) after naloxone injection was calculated by the trapezoidal rule and expressed as mean ± SE (in AU × min).

A one-way ANOVA was used to compare MPE and AUC, and if significant, followed by a Bonferroni test.

Table 1

Plasma concentrations of the antidepressants

	Diabetic rats AD + NaCl	Diabetic rats AD + Naloxone	Mononeuropathic rats AD + NaCl	Mononeuropathic rats AD + Naloxone
[CMI + desmethyl-CMI] ($ng \cdot mL^{-1}$)	235 ± 10	233 ± 12	283 ± 24	328 ± 31
[Milnacipran] ($ng \cdot mL^{-1}$)	86 ± 18	93 ± 13	54 ± 13	55 ± 9
[Duloxetine] ($ng \cdot mL^{-1}$)	118 ± 49	132 ± 34	162 ± 40	155 ± 38

The plasma concentrations of clomipramine (CMI) and desmethyl-clomipramine (desmethyl-CMI), milnacipran and duloxetine in streptozocin and chronic constriction injury rats that had received five systemic injections of 5 $mg \cdot kg^{-1}$ clomipramine, 10 $mg \cdot kg^{-1}$ milnacipran or 3 $mg \cdot kg^{-1}$ duloxetine followed by naloxone (1 $mg \cdot kg^{-1}$) or NaCl. AD, antidepressant.

The correlation between the involvement of the opioid system [i.e. AUC of VT variations after naloxone injection (measured by the trapezoidal rule and expressed as mean ± SE in AU × min)] and the efficacy of the antidepressants (% MPE) was assessed by a linear regression.

The significance level was set at $P < 0.05$. Statistical analyses were run using SigmaStat 3.10/Systat Software, Inc.

Results

Clinical status of the animals

The mean body weight of STZ hyperglycaemic rats (n = 166 rats) was 250 ± 12 g 3 weeks after STZ injection, versus 258 ± 6 g before STZ injection. None of the 166 diabetic rats displayed weight loss greater than 10% of their initial weight, and none were excluded from this experiment.

Plasma levels of antidepressants in diabetic and mononeuropathic rats

Plasma levels of the three antidepressants were not statistically different between CCI and diabetic rats, and were in the range of levels commonly found in patients treated with the usual doses of these drugs (Table 1, second and fourth column). Naloxone failed to induce any change in these levels whatever the aetiology, which excludes any pharmacokinetic explanation for the effect of this antagonist on the anti-hyperalgesic action of antidepressants (Table 1, third and fifth column).

Compared efficacy of antidepressants on mechanical hyperalgesia

Two and 3 weeks after surgery and STZ injection, 99% of CCI rats and 81% of STZ rats displayed mechanical hyperalgesia corresponding to a significant reduction in paw pressure-induced VTs from 35.8 ± 0.9 and 36.7 ± 0.8 to 18 ± 0.5 and 16.9 ± 0.4 AU respectively.

Whereas the timing of the handling of the animals was different according to the drug (every 67 and 88 min, 180 and 240 min, 68 and 82 min for clomipramine, milnacipran and duloxetine respectively), no difference was observed in the pain scores of the different groups of vehicle-treated animals: five successive injections of either HMPC or NaCl did not induce any significant change in VTs in either CCI or STZ rats (Figures 2A,B,C and 3A,B,C), they had very constant thresholds throughout the experiments and whatever the time of the handling.

In both models, there was no modification of the motor function assessed with an actimeter (data not shown). The motor function was not affected by the treatments either.

Diabetic animals. In STZ rats, repeated administration of clomipramine (5 × 5 $mg \cdot kg^{-1}$, s.c.) induced a progressive and significant increase in VTs. At steady state (after the fifth injection), the thresholds were not different from those measured before STZ (Figure 2A). Five successive injections of milnacipran (5 × 10 $mg \cdot kg^{-1}$, i.p.) also induced a progressive and significant increase in VTs, which became higher after the fifth injection than those measured before STZ injection (Figure 2B). Duloxetine (5 × 3 $mg \cdot kg^{-1}$, i.p.) induced a strong increase in VTs, which were significantly higher after the fourth injection than before STZ administration (Figure 2C). Expressed as % MPE, the results show that duloxetine and milnacipran were the most effective antidepressants followed by clomipramine (Figure 2D).

Mononeuropathic animals. In CCI rats, clomipramine (5 × 5 $mg \cdot kg^{-1}$, s.c.) significantly increased the paw pressure-induced VTs leading to a total suppression of mechanical hyperalgesia after the fifth injection (Figure 3A). Repeated injection of milnacipran (5 × 10 $mg \cdot kg^{-1}$, i.p.) resulted in a progressive and significant increase in VTs. The VTs obtained after the fifth injection were not significantly different from those obtained before surgery (Figure 3B). Due to low plasma levels of milnacipran, we performed the same experiment with 20 $mg \cdot kg^{-1}$ milnacipran. Plasma levels were markedly increased whereas the analgesic effect plateaued (Figure 3B).

Chronic administration of duloxetine (5 × 3 $mg \cdot kg^{-1}$, i.p.) greatly increased the VTs of CCI rats. The thresholds obtained after the fifth injection were significantly higher than those measured before sciatic nerve ligation (Figure 3C). Expressed as % MPE, the results show that duloxetine was the most effective; all the other treatments showed only modest activity (Figure 3D).

Normal rats. In healthy rats, clomipramine (5 × 5 $mg \cdot kg^{-1}$, s.c.) had no effect on paw pressure-induced VTs (data not

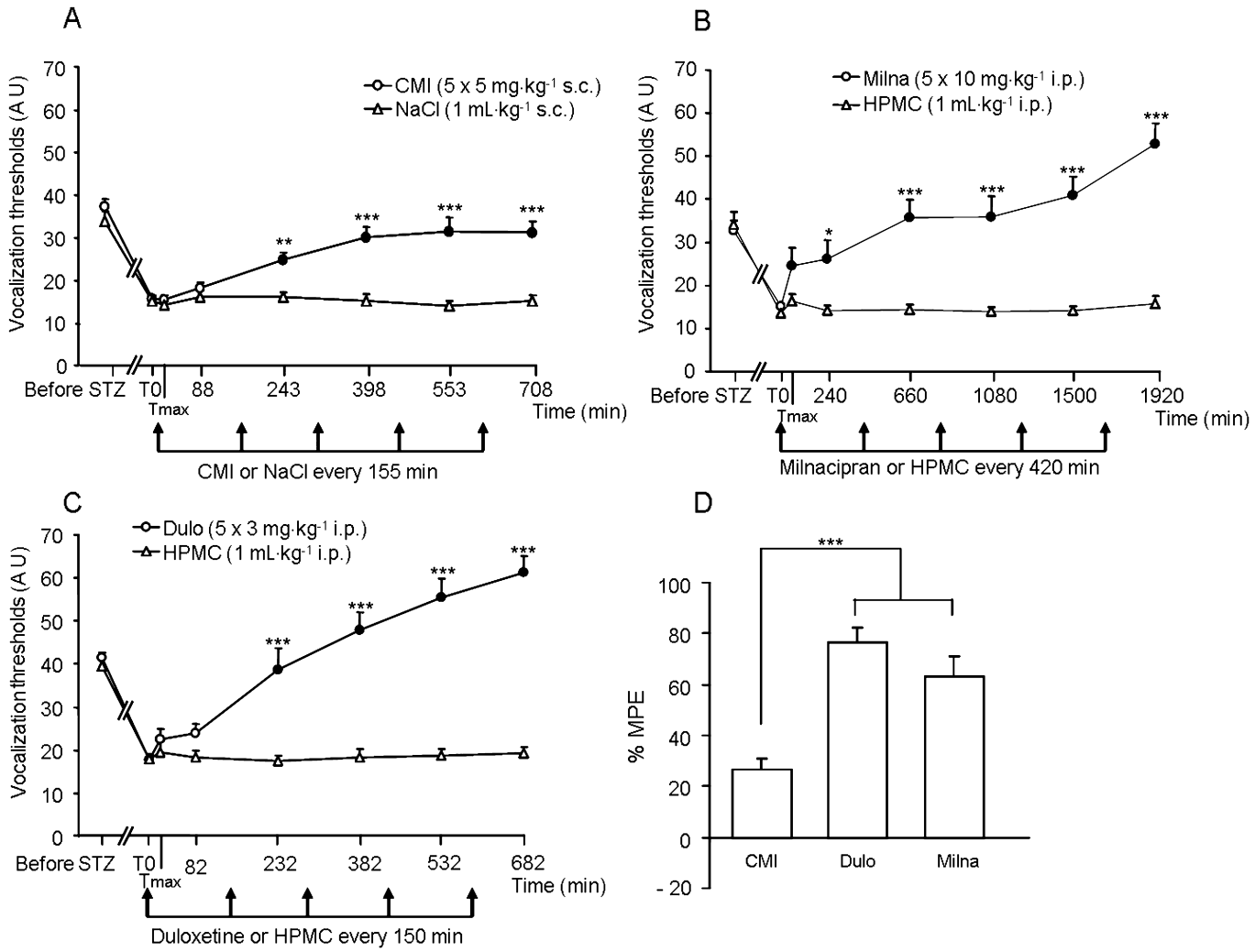

Figure 2

Time-course of the effect of repeated administration (five successive injections every $T_{1/2}$) of (A) NaCl (1 mL·kg^{-1}, s.c.) and clomipramine (CMI, 5 mg·kg^{-1}, s.c.) (B) Hydroxy-propyl-methyl-cellulose (HPMC, 0.25%, 1 mL·kg^{-1}, i.p.) and milnacipran (Milna, 10 mg·kg^{-1}, i.p.), and (C) HPMC (0.25%, 1 mL kg^{-1}, i.p.) and duloxetine (Dulo, 3 mg·kg^{-1}, i.p.) on paw pressure-induced vocalization thresholds in streptozocin (STZ) rats. Solid symbol: $P < 0.05$ compared with values measured before antidepressant or vehicle injection (T 0). $^{**}P < 0.01$; $^{***}P < 0.001$ versus vehicle group at the same time. (D) Percentage of maximal possible effect (% MPE) of the antidepressant obtained from seven to 16 rats. $^{***}P < 0.001$ versus corresponding group.

shown). Chronic administration of duloxetine (5 × 3 mg·kg^{-1}, i.p.) in healthy rats induced an increase in VTs. The values obtained after the fifth administration were significantly very different from the values obtained before injection (46.9 ± 17 AU and 38.9 ± 16 AU, respectively, data not shown).

Comparison of the involvement of the opioid system in the anti-hyperalgesic effect of the antidepressants

Clomipramine. In CCI and STZ rats, the anti-hyperalgesic effect of five injections of clomipramine is shown in Figure 4 expressed as the AUCs of VT variations (Figure 4A, clomipramine + NaCl treated groups). Naloxone (1 mg·kg^{-1}, i.v.) injected 88 min after the last injection of clomipramine abolished the clomipramine-induced anti-hyperalgesic effect in both STZ and CCI rats (Figure 4A).

Milnacipran. In STZ rats, the administration of naloxone 240 min after the last injection of milnacipran did not significantly change its antinociceptive effect, as shown by the AUCs of VT variations (Figure 4B). Conversely, in CCI rats, the anti-hyperalgesic effect of milnacipran was significantly reduced by naloxone injection at the doses tested (Figure 4B).

Duloxetine. In STZ and CCI rats, the antinociceptive effect of duloxetine was not changed by naloxone administered 82 min after the last injection of duloxetine (Figure 4C). This experiment was also done in normal rats where naloxone still had no significant effect (data not shown).

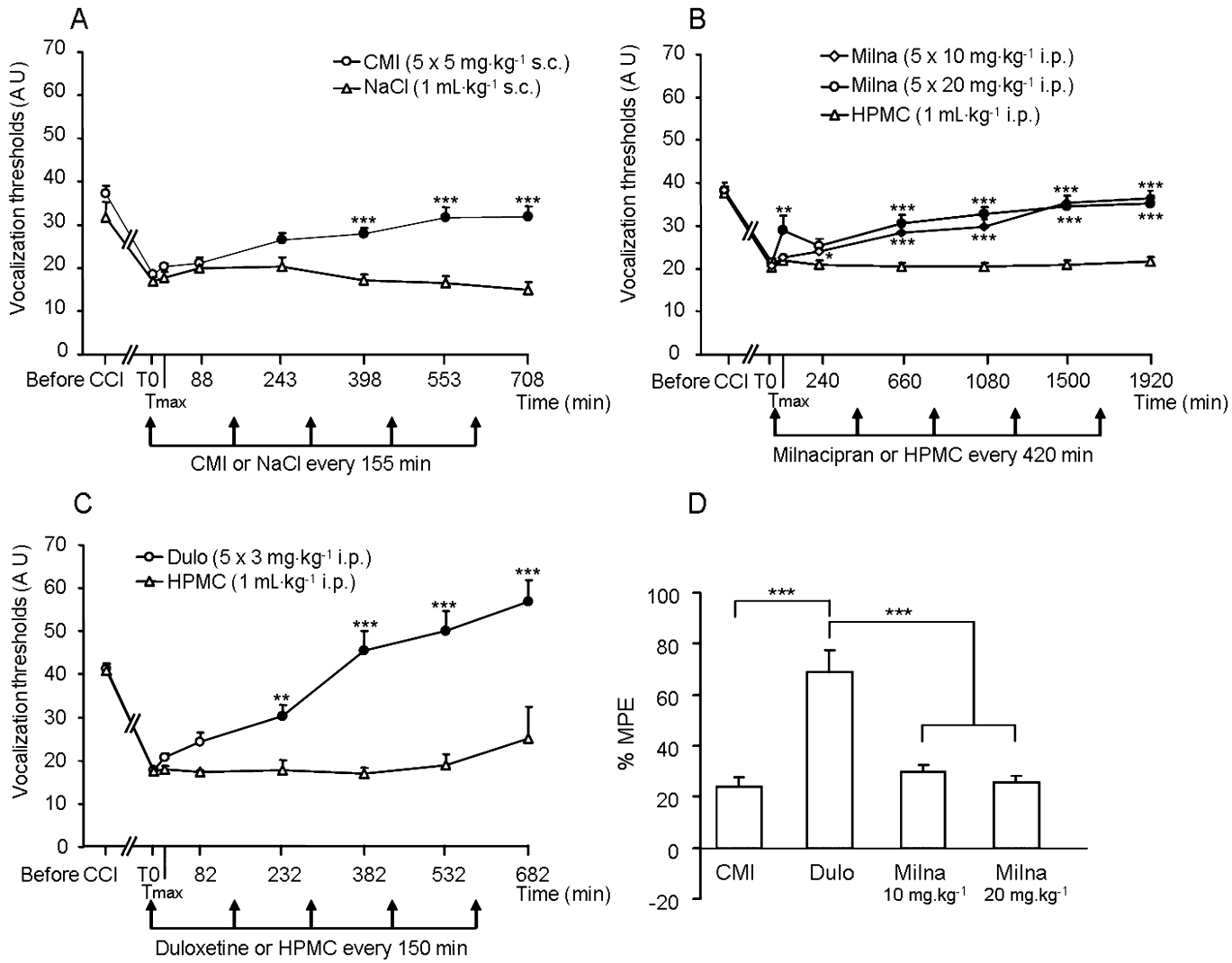

Figure 3

Time-course of the effect of repeated administration (five successive injections every $T_{1/2}$) of (A) NaCl (1 mL·kg^{-1}, s.c.) and clomipramine (CMI, 5 mg·kg^{-1} s.c.) (B) Hydroxy-propyl-methyl-cellulose (HPMC, 0.25%, 1 mL·kg^{-1}, i.p.; $n = 2 \times 7$ rats by pooling the results obtained for this group in two experiments) and milnacipran (Milna, 10 mg·kg^{-1}, i.p and 20 mg·kg^{-1}, i.p.), and (C) HPMC (0.25%, 1 mL·kg^{-1}, i.p.) and duloxetine (Dulo, 3 mg·kg^{-1}, i.p.), on paw pressure-induced vocalization thresholds in chronic constriction injury (CCI) rats. Solid symbol: $P < 0.05$ compared with values measured before antidepressant or vehicle injection (T 0). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus vehicle group at the same time. (D) Percentage of maximal possible effect (% MPE) of the antidepressant obtained from seven to 15 rats. ***$P < 0.001$ versus corresponding group.

Correlation of the inhibitory effect of naloxone with antidepressant-induced anti-hyperalgesia/antinociception

Figure 5 shows a significant inverse correlation ($r^2 = 0.74$, $P < 0.001$) between the opioidergic involvement in the effect of antidepressants and their anti-hyperalgesic efficacy in STZ and CCI rats. An opioidergic mechanism did not appear to be involved in the effect of the most active drugs.

Discussion

These results indicate that, with the focus on one symptom of neuropathic pain (mechanical hyperalgesia) in two models of different aetiologies, clomipramine (a TCA) reverses hyperalgesia while duloxetine (a SSNRI) increased scores compared with the pre-injury values indicating not only an anti-hyperalgesic but also an antinociceptive effect. In the same conditions, the effect of milnacipran depended on the aetiology; it induced a marked effect in diabetic rats and a limited effect in the CCI model. These effects were produced by patterns of administration (repeated injections every $T_{1/2}$) close to those used clinically, which led to plasma concentrations within the therapeutic range obtained in humans for each antidepressant. Moreover, the antidepressants less efficient at treating neuropathic pain in rats (i.e. those inducing only an anti-hyperalgesic but not an antinociceptive effect) are those in which an opioidergic

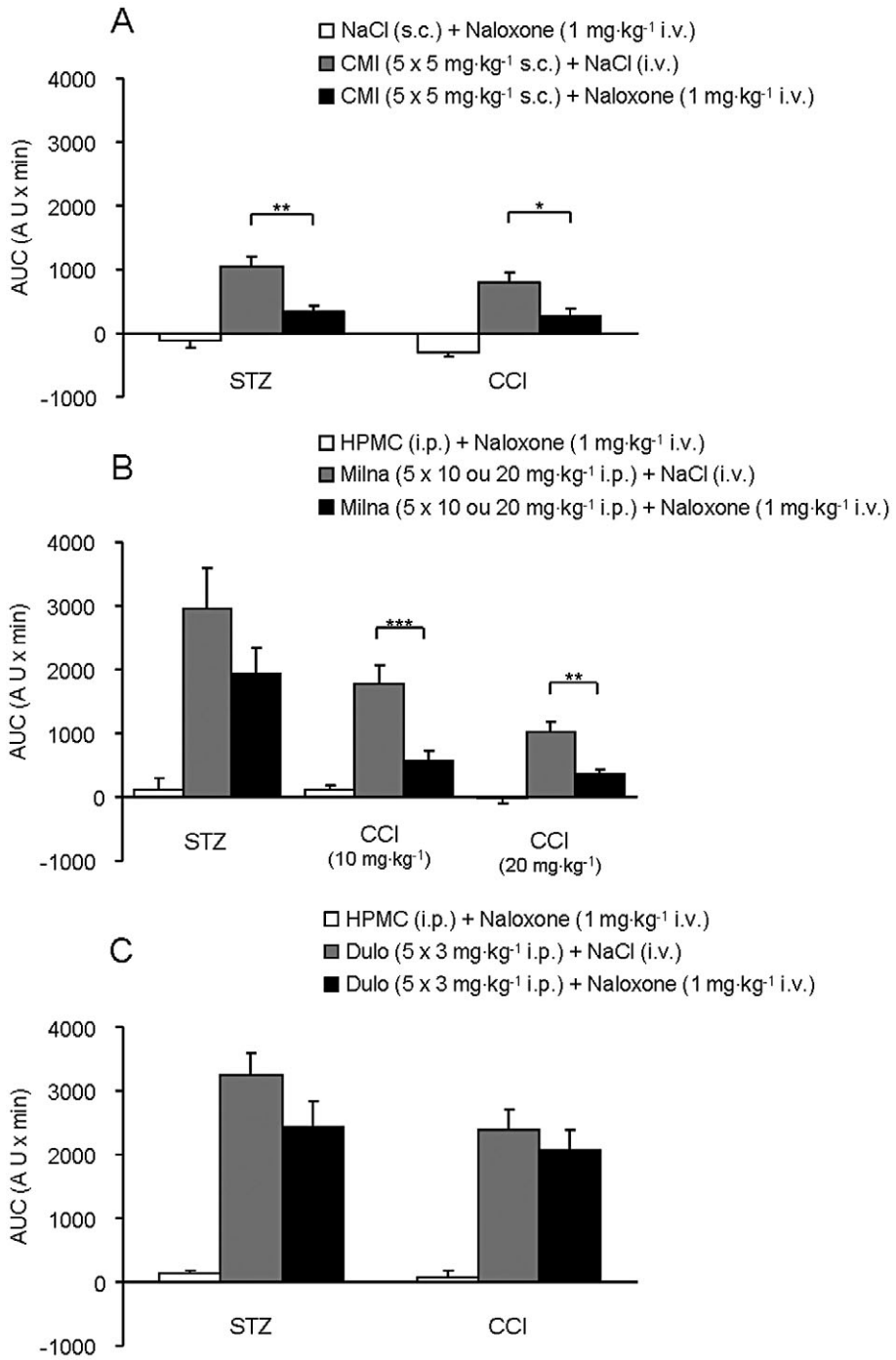

Figure 4

Influence of naloxone (1 mg·kg^{-1} i.v.) or NaCl (1 mL·kg^{-1} i.v.) on the effect of repeated administrations of (A) clomipramine (CMI) or NaCl (B) milnacipran (Milna) or HPMC, and (C) duloxetine (Dulo) or HPMC in streptozocin (STZ) rats and chronic constriction injury (CCI) rats. Area under the time-course (0–75 min) curve (AUC) of vocalization threshold variations (AU × min). Vocalization threshold variations correspond for each animal to the differences between post-naloxone or NaCl scores and scores obtained before their injection. These variations are used in order to eliminate changes in pre-injection differences between the naloxone and NaCl treated groups. Values obtained from seven to 16 rats. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus corresponding group.

mechanism is involved. Finally, a key result is that the aetiology of the neuropathy had no influence on the antidepressant effectiveness, at least for clomipramine and duloxetine.

Clinically, antidepressants are administered in cases of neuropathic pain and require a chronic treatment to show efficacy (Finnerup *et al.*, 2005; Mico *et al.*, 2006). Unfortunately, there are only a few animal studies that have investi-

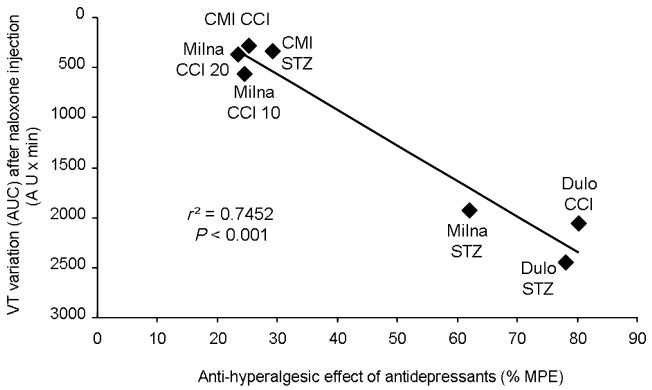

Figure 5

Correlation between the opioid involvement and the anti-hyperalgesic effect of antidepressants obtained after five systemic injections of 5 mg·kg^{-1} clomipramine (CMI), 10 or 20 mg·kg^{-1} milnacipran (Milna) or 3 mg·kg^{-1} duloxetine (Dulo) in streptozocin (STZ) and chronic constriction injury (CCI) rats. Ordinates correspond to the vocalization threshold (VT) variations [time-course curve (AUC)] obtained after naloxone injections (expressed in AU × min) and abscissae correspond to the anti-hyperalgesic effect of antidepressants expressed as percentage of maximal possible effect (% MPE). The higher the AUC are, the less the opioid system is involved.

gated the consequences of such chronic treatment with antidepressants and compared the efficacy of the different antidepressants. Our study confirms the necessity of chronic administration for the three antidepressants tested to have an effect. VTs assessed after the first injection were never different from pre-drug values, except for milnacipran in diabetic rats, demonstrating lack of effect of acute administration of either clomipramine, milnacipran and duloxetine and suggesting that antidepressants act through molecular and neuronal events (plasticity) which could affect gene expression, protein synthesis, transporter and receptor regulation that need time to occur. Similarly, other animal studies on antidepressants revealed that repeated administration of clomipramine suppresses mechanical hyperalgesia in CCI (Marchand *et al.*, 2003b) and oxaliplatin-induced (Ling *et al.*, 2007) neuropathic rats while a single i.v. injection either did not modify (3 mg·kg^{-1}) (Coudore-Civiale *et al.*, 2000) or slightly reduced it (8 mg·kg^{-1}) (Courteix *et al.*, 1994) in STZ-induced diabetic rats. In the same way, repeated administration of amitriptyline was shown to suppress pain behaviour in the Herpes Simplex virus type 1-induced neuropathic pain in mice (Kuraishi *et al.*, 2004) whereas acute administration (2 mg·kg^{-1}) only had a weak effect in diabetic rats (Courteix *et al.*, 1994). Even though acute systemically, intrathecally or intracerebroventricularly administered milnacipran was reported to suppress tactile allodynia or thermal hyperalgesia in nerve-ligated mice (Suzuki *et al.*, 2008) or rats (Obata *et al.*, 2005), another study comparing the effects of chronic versus acute systemic administration of milnacipran concluded that only chronic treatment alleviates the spinal nerve ligation (SNL)-induced shift in weight bearing in rats (King *et al.*, 2006). In our study, the effect of milnacipran was significant after one administration but only in diabetic rats. However, as Figure 2B shows, this acute effect is weak compared with the effect obtained after chronic administration, once again demonstrating the importance of repeated injections. The necessity of chronic administration of milnacipran was recently confirmed (Takeda *et al.*, 2009): CCI rats displayed analgesia 7 days after the initiation of the treatment. Venlafaxine showed the same pattern, with a single dose having no effect while repeated administrations had an anti-hyperalgesic effect (Marchand *et al.*, 2003a,b). Whereas duloxetine reduced SNL-induced tactile allodynia after a single injection (Iyengar *et al.*, 2004), we found here that the antidepressant was only effective after repeated injection in both models of neuropathic pain. Taken together, our results clearly show that using patterns of administration close to those used clinically is important to evaluate the effect of antidepressants in experimental neuropathic pain models. By determining plasma levels obtained after five injections of antidepressants (i.e. at the steady state), we verified that the 'every half-life time' administration procedure allows the antidepressant to reach plasma levels close to those obtained in clinical use. The plasma concentrations reached after five clomipramine injections (235 to 283 ng·mL^{-1} according to the model) were slightly higher than the therapeutic effective level (200 ng·mL^{-1}) (Langohr *et al.*, 1982; Sindrup *et al.*, 1990). For duloxetine, plasma levels (118 to 162 ng·mL^{-1}) were within the range considered as therapeutic [40 to 200 ng·mL^{-1}, data from summary of product characteristics (SPC)] even if to our knowledge, specific pharmacokinetics/pharmacodynamic studies have not yet been performed. Regarding milnacipran, plasma levels reached after 5 × 10 mg·kg^{-1} (54 to 86 ng·kg^{-1}) were lower than the Cmax obtained in humans (216 ng·mL^{-1}, data from SPC). Considering that the plasma level is not predictive of an analgesic action if the biological events underlying this action require time to develop, the possibility that a longer treatment could result in a stronger effect cannot be excluded. Indeed, a 7 day treatment with milnacipran was reported to be necessary to desensitize somatodendritic 5-HT$_{1A}$ autoreceptors (Mochizuki *et al.*, 2002a). Unfortunately, in this study, like many others, the plasma level of this antidepressant was not measured.

Transposing preclinical results to clinical implications is complex and often, preclinical results cannot be reproduced in patients. Working at clinically relevant plasma levels following chronic drug administration means the conditions are one step closer to those occurring clinically, and therefore we suggest that particular caution should be brought to the design of preclinical experimental procedures. Moreover, with regard to antidepressants, it is well known that even if they act faster on nociception than on depression (Mico *et al.*, 2006), they require a therapeutic lag between the beginning of their administration and the onset of their effect.

Our study, using clinically relevant experimental conditions, showed a differential effect of these three antidepressants on a similar symptom whatever the aetiology of the neuropathic pain model. In both models tested, clomipramine only reverses mechanical hyperalgesia, whereas after the fifth duloxetine injection, VTs were noticeably higher than pre-injury values. This difference justifies describing clomipramine as anti-hyperalgesic and duloxetine as anti-nociceptive. In the same conditions, milnacipran was anti-hyperalgesic in the CCI model after both 5 × 10 and 5 × 20 mg·kg^{-1} and antinociceptive in diabetic rats (after 5 ×

10 mg·kg^{-1}). We also observed that in normal healthy rats, clomipramine (5 × 5 mg·kg^{-1}) did not increase VTs, further supporting that it does not have an antinociceptive effect. Duloxetine (5 × 3 mg·kg^{-1}), still in healthy rats, had a significant antinociceptive effect, just as in diabetic or CCI rats. This leads us to conclude that the antidepressants can be classified in rank order of activity as follows: duloxetine = milnacipran > clomipramine in experimental painful diabetic neuropathy, and duloxetine > milnacipran = clomipramine in the CCI model. The SSNRI, venlafaxine, was shown to induce an antinociceptive effect in the same diabetic conditions (Marchand *et al.*, 2003a,b). Another study shows that duloxetine has a stronger anti-allodynic effect than milnacipran or venlafaxine in the rat SNL model (Iyengar *et al.*, 2004). These findings lead us to reconsider the use of TCAs that are still today reference compounds for the management of diabetic and traumatic neuropathic pain. However, this differential efficacy of antidepressants should be assessed using other testing procedures to evaluate antidepressant effects on other evoked pain symptoms and to ultimately determine clusters of symptoms sensitive to a particular antidepressant or group of antidepressants.

To understand the mechanism underlying the antinociceptive effect of antidepressants, the involvement of the opioid system was tested. The data suggest that there is no preferential implication of this opioid system with regard to the aetiology of the neuropathy. The opioid system appears to be involved in the mechanism of action of antidepressants that only have an anti-hyperalgesic action (clomipramine in both models and milnacipran in CCI rats) but not in those that have a stronger (i.e. antinociceptive) effect (duloxetine in both models and in normal rats and milnacipran in STZ rats), indicating that this opioid system is not essential for this effect.

A lack of involvement of the opioid system was observed previously with venlafaxine, another SSNRI (Marchand *et al.* 2003a,b). The mechanistic explanation of these differences needs to be clarified. First, TCAs are known not to bind to opioid receptors (Isenberg and Cicero, 1984) just as duloxetine (Carter and McCormack, 2009) and milnacipran (Mochizuki *et al.*, 2002b); therefore, the opioid system is involved indirectly probably through endogenous opioid release induced by the antidepressant. However, a physiological decrease in brain β-endorphin content has been reported in both STZ (Forman *et al.*, 1986) and CCI (Panerai *et al.*, 1987) rats which supports the finding that the differential involvement of the opioid system is not due to the aetiology but to the antidepressant used (consistent with the results obtained with clomipramine and duloxetine). The interaction of antidepressants with the opioid system needs to be further assessed. Among other putative reasons, one may evoke the fact that antidepressants are 'dirty' drugs exerting their effect, besides increasing the availability of 5-HT and noradrenaline, through sodium channel blockade or N-methyl-D-aspartate receptor inhibition (Gilron, 2010). Whatever the reason, this result re-asserts the reduced effectiveness of opioid therapy in neuropathic pain that requires higher doses to respond (Przewlocki and Przewlocka, 2001). Thus, in neuropathic pain, a pathophysiological mechanism-based treatment would appear to be more pertinent than an aetiology-based treatment (Woolf *et al.*, 1998). Conversely, results obtained with milnacipran suggest that its efficacy depends on aetiology, and subsequently that the profile of action of milnacipran is different depending on aetiology, which was not the case for the other antidepressants tested. To resolve this issue, further studies are needed to: (i) compare the metabolism of milnacipran in diabetic rats and in rats without any metabolic changes (healthy rats or CCI model); and (ii) study the involvement of the opioid system in the effect of milnacipran determined in another model of polyneuropathy (i.e. oxaliplatin-induced neuropathic pain).

In conclusion, the present results demonstrate that dosing to steady state is a valuable preclinical strategy to study the analgesic action of antidepressants and suggest that rather than the aetiology of the neuropathic pain, it is the mechanism of action of antidepressants that should be more predictive for optimal efficacy; the involvement of an opioidergic mechanism is not associated with strong analgesic activity. Further research is required to determine whether these criteria of mechanism-based choice could be applied to other pain symptoms (such as mechanical allodynia and cold allodynia, which are frequently observed in neuropathic pain) in neuropathic rats, or to other neuropathic pain models of various aetiologies (toxic or infectious). Thereafter, clinical studies could be performed to determine whether a cluster of symptoms can be identified in neuropathic patients as sensitive to some antidepressants depending on their mechanism of action.

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Conflict of interest

None.

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