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Action of mefloquine/amitriptyline THN101 combination on neuropathic mechanical hypersensitivity in mice

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Abstract

Tricyclic antidepressants that inhibit serotonin and noradrenaline reuptake, such as amitriptyline, are among the first-line treatments for neuropathic pain, which is caused by a lesion or disease affecting the somatosensory nervous system. These treatments are, however, partially efficient to alleviate neuropathic pain symptoms, and better treatments are still highly required. Interactions between neurons and glial cells participate in neuropathic pain processes, and importantly, connexins—transmembrane proteins involved in cell–cell communication—contribute to these interactions. In a neuropathic pain model in rats, mefloquine, a connexin inhibitor, has been shown to potentiate the antihyperalgesic effect of amitriptyline, a widely used antidepressant. In this study, we further investigated this improvement of amitriptyline action by mefloquine, using the cuff model of neuropathic pain in mice. We first observed that oral mefloquine co-treatment prolonged the effect of amitriptyline on mechanical hypersensitivity by 12 hours after administration. In addition, we showed that this potentiation was not due to pharmacokinetic interactions between the 2 drugs. Besides, lesional and pharmacological approaches showed that the prolonged effect was induced through noradrenergic descending pathways and the recruitment of α_2 adrenoceptors. Another connexin blocker, carbenoxolone, also improved amitriptyline action. Additional in vitro studies suggested that mefloquine may also directly act on serotonin transporters and on adenosine A_1 and A_{2A} receptors, but drugs acting on these other targets failed to amplify amitriptyline action. Together, our data indicate that pharmacological blockade of connexins potentiates the therapeutic effect of amitriptyline in neuropathic pain.

Keywords: Neuropathic pain, Amitriptyline, Mefloquine

1. Introduction

Neuropathic pain is defined as pain arising as a consequence of a lesion or disease affecting the somatosensory nervous system.[40,47] Like all forms of chronic pain, it is a major public health issue.[15] Although some antidepressant drugs are one of the best therapeutic options, their efficacy remains limited, with a number needed to treat around 3.6 to 6.4[13,37] and a pain relief that is usually partial. Enhancing the efficacy of antidepressants on neuropathic pain might thus be of therapeutic interest.

From the periphery to the central nervous system, interactions between neurons and nonneuronal cells, including glial cells, contribute to the encoding and modulation of sensory information.[4,10,17,21,27] Connexins may partly mediate some of these interactions, particularly through astrocyte networking activity.[4] These transmembrane proteins form hexa-protein complexes to form hemichannels or gap junctions, facilitating the passage of ions or small molecules such as adenosine triphosphate (ATP) or glutamate.[25] In nociceptive pathways, this interaction can occur in the dorsal root ganglia, the spinal cord, and the brain. In various models of neuropathic pain (including the chronic constriction injury [CCI] of the sciatic nerve, CCI of the infraorbital nerve, spinal nerve ligation, partial sciatic nerve ligation, spared nerve injury, spinal cord injury, and oxaliplatin-induced neuropathy), dynamic changes have been shown in the expression of connexins within these structures (for review: Refs. 31,43), particularly concerning Cx43, which is the most abundant isoform in the nervous system. With mostly intrathecal delivery, drugs or molecular tools used to modulate gap junctions and connexins have shown some efficacy to reduce mechanical hypersensitivity in animal models of neuropathic pain,[5,6,26,32,46,52] suggesting that it may be of interest to target neuroglial mechanisms through connexin manipulation. One of these drugs, mefloquine, displays inhibitory properties on gap junctions and connexins[19,35,36] at doses below its classical use in malaria.[42,45] With intracerebroventricular delivery in the spared nerve injury model and intracisternal delivery in the model of infraorbital nerve CCI, mefloquine

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has been shown to reduce nociceptive symptoms.[5,34] In addition, systemic injection at a dose much lower than those used for malaria potentiates the antihyperalgesic effect of the antidepressant amitriptyline in the CCI model in rats.[19]

In this work, we characterized and studied the potentiating action of systemic mefloquine/amitriptyline combination, designed as THN101, on mechanical hypersensitivity, using the cuff model of neuropathic pain in mice.[2,53] To advance mechanistic understanding, we aimed here to identify the targets of THN101 components that were critical to the potentiating effect. For the amitriptyline component of THN101, we thus tested noradrenergic contribution at anatomical and receptor level. Concerning mefloquine, it has been suggested that it may have multiple targets besides connexins, such as an action on serotonin-related and adenosine-related factors.[16,18,44,51] We thus tested the relevance of mefloquine action on a large set of targets, and we evaluated whether the confirmed ones may potentiate amitriptyline action.

2. Material and methods

2.1. Animals

C57BL/6 J male mice (Charles River, L'Arbresle, France), 8 to 16 weeks old, were used for this study. They were habituated to the animal facility and to the testing environment before procedures. Mice were group housed with food and water ad libitum, in facilities with controlled temperature and hygrometry, under a 12-hour light/dark cycle (lights on at 7 AM). Animal killing at the end of the protocols was performed using either controlled carbon dioxide exposure (automatic TEM SEGA system, 20% per min) or after an overdose of anesthetic (ketamine (Imalgene1000, 300 mg/kg)/xylazine (Rompun 2%, 20 mg/kg, Centravet, Tadden, France). The Chronobiotron (UMS3415) animal facility has an agreement for animal housing and experimentation, delivered by the French veterinary services (D-67-2018-38). Protocols were performed following the European ethical guidelines (EU 2010/63) and approved by the local ethical committee "Comité d'Ethique en Matière d'Expérimentation Animale de Strasbourg" (CREMEAS, CEEA35). Chronograms detailing the experiments are presented in the Supplemental Figure 1 (available at http://links.lww.com/PAIN/B343).

2.2. Cuff surgery

Surgeries were performed under either ketamine (Imalgene 1000, 100 mg/kg)/xylazine (Rompun 2%, 10 mg/kg, Centravet) anesthesia or under tiletamine–zolazepam (Zoletil 50, 80 mg/kg, Virbac Sante Animale, France)/xylazine (Rompun 2%, 20 mg/kg, Centravet) anesthesia, with atipamezole (Antisedan, 0.2 mg/kg, Vetoquinol, France) delivered for anesthesia reversal at least 1 hour after the induction. Anesthesia compounds were delivered intraperitoneally (i.p.; 5 mL/kg). After having exposed the common branch of the right sciatic nerve (sham group), a hemisectioned 2-mm long PE-20 polyethylene cuff (Harvard Apparatus, Les Ulis, France) was placed around the nerve to induce a compression (cuff group) according to previously published procedures.[2,53] The skin was closed with one or 2 stitches along the length of the incision.[2,53]

2.3. Treatments

To test the combination of amitriptyline and mefloquine, independent groups of mice were treated per os (oral gavage, p.o.) with either amitriptyline hydrochloride (Sigma Aldrich, St Quentin Fallavier, France, PubChem Substance ID 24278073) from 5 to 20 mg/kg or mefloquine hydrochloride (Sigma Aldrich, PubChem Substance ID 24724536), 0.05 or 0.1 mg/kg, or with their combination (designed, regardless of doses, as THN101 throughout the article). The drugs were dissolved in sterile 0.9% NaCl (B. Braun, Dublin, Ireland) with DMSO from 0.02% to 1% (Sigma Aldrich, PubChem Substance ID 329757302). For most experiments, mice received the treatments either (1) in the morning for tests at 1, 2, 4, 7, and 30 hours after administration or (2) at the end of the day for tests at 12, 16, and 20 hours after administration. Experimental design is presented in Supplemental Figure 1A (available at http://links.lww.com/PAIN/B343).

To test the central effect of mefloquine, alone or in combination with amitriptyline, mice were first treated with amitriptyline 10 mg/kg p.o. or received a vehicle solution (p.o., 10 mL/kg). Then, under 3% isoflurane anesthesia, they received a single dose of 3.6 μg of mefloquine dissolved in 10 μL of vehicle solution (DMSO, 1% in 0.9% NaCl) or 10 μL of vehicle solution, delivered intrathecally (i.t.) at the lumbar level through a 27-gauge needle connected to a 50-μL Hamilton syringe. Experimental design is presented in Supplemental Figure 1B (available at http://links.lww.com/PAIN/B343).

To study the adrenergic component of THN101 and of mefloquine action at a high dose, mice were treated with yohimbine hydrochloride (α_2-adrenoceptor antagonist, Tocris, Abingdon, United Kingdom, PubChem ID 6169), either i.p. (0.5 mg/kg) or p.o. dissolved in the drinking water (20 μg/mL), or with propranolol hydrochloride (β-adrenoceptor antagonist; Tocris, PubChem ID 66366), given p.o. dissolved in the drinking water (50 μg/mL).[22] Antagonist treatments in the drinking water started 5 days before THN101 administration. In another experiment, 5 μg of atipamezole (α_2-adrenoceptor antagonist, Antisedan; Vétoquinol, Lure, France, PubChem ID 71310) or 3 μg of sotalol hydrochloride (β-adrenoceptor antagonist, Sigma-Aldrich, PubChem ID 66245) were delivered i.t. (10 μL) at the lumbar level in 0.9% NaCl. Experimental designs are presented in Supplemental Figures 1B and 1C (available at http://links.lww.com/PAIN/B343).

To evaluate the relevance of potential mefloquine pharmacological targets, mice were treated with either (1) fluoxetine hydrochloride (serotonin reuptake inhibitor; Sigma Aldrich, PubChem Substance ID 24278096), 10 mg/kg p.o.; (2) DPCPX, 8-cyclopentyl-1,3-dipropylxanthine (A_1 adenosine receptor antagonist; Tocris, PubChem ID 1329), 1 mg/kg p.o.; (3) SCH58261, 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (A_{2A} adenosine receptor antagonist; Tocris, PubChem ID 176408), 0.1 mg/kg p.o.; or (4) carbenoxolone, 3β-hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate (blocker of gap junctions; Sigma Aldrich, PubChem Substance ID 57654021), 5 nmol injected i.t. (10 μL in 1% DMSO, 0.9% NaCl). These drugs were administered alone or in combination with amitriptyline, 10 mg/kg p.o. (10 mL/kg). Experimental design is presented in Supplemental Figure 1A (available at http://links.lww.com/PAIN/B343).

2.4. Behavioral testing

Mechanical sensitivity was assessed using the von Frey test. The investigators performing the test were blinded to the surgery and drug treatment. Animals were placed into clear Plexiglas boxes (7 × 9 × 7 cm) on an elevated metallic grid. After habituation, ascending calibers of von Frey filaments (Aesthesio; DanMic Global LLC, San Jose, CA) were applied to the plantar surface of

hind paws until they just bent, as previously described.[2,3,53] The paw withdrawal threshold (PWT), expressed in grams, corresponds to the first filament that resulted in at least 3 paw withdrawals of 5 applications.[2,3,53] For some experiments, areas under the curve (AUC) were also calculated over the duration of the considered test. For each animal, these AUC were calculated as the surface (g.h) above its PWT value at time 0.

2.5. Mass spectrometry

The concentrations of amitriptyline, its metabolite nortriptyline, and of mefloquine were measured by mass spectrometry in blood, plasma, brain samples, and spinal cord lumbar sections taken from mice treated orally with amitriptyline (10 mg/kg) or THN101 (amitriptyline 10 mg/kg and mefloquine 0.1 mg/kg). Experimental design is presented in Supplemental Figure 1A (available at http://links.lww.com/PAIN/B343).

2.5.1. Blood, plasma, and tissue collection

Blood was collected from the tip of the tail (sectioned at its soft nonbone level with scissors) using 20-μL heparinized capillaries (Minicap Capillary Pipette, Hirschmann Instruments, ref 11772533). Blood was recovered 1, 2, 4, and 7 hours after p.o. administration of amitriptyline or THN101. Immediately after collection, the blood was mixed with 10 μL of 5000 IU/mL sodium heparin (Sanofi, France) and stored at −80°C. After the last blood collection, mice were anesthetized with ketamine/xylazine (100/10 mg/kg) and killed by cardiac exsanguination. The blood collected in vacuumed lithium-heparinized tubes (BD Vacutainer LH PST II REF 367374) was centrifuged at 425*g* for 10 minutes at 4°C, and plasma was frozen at −80°C. In parallel, the brain and spinal cord were recovered in Eppendorf tubes on dry ice and stored at −80°C.

2.5.2. Plasma, blood, and tissue preparation

Plasma (50 μL) and blood (20 μL) were spiked with 20 pmol of D3-amitriptyline, D3-nortriptyline, and D10-mefloquine (Merck, Molsheim, France and Alsachim, Illkirch, France) to perform a quantification using the isotopic dilution method,[50] which allows absolute quantification of the compounds. In brief, a known amount of a stable isotope-containing target compound (ie, 20 pmol of D3-amitriptyline, D3-nortriptyline, and D10-mefloquine) is added to the sample. An MS analysis allows determination of the intensity ratio between the studied compound and its heavy counterpart. As the studied compound and its heavy counterpart behave the same way, such a method overcomes problems of degradation and loss due to extraction steps as well as matrix impact. Finally, it permits analysis of the presence of different compounds using a unique protocol even with low recovery yields. Recovery yields calculated from standard curves (n = 3) are reported in the Supplemental Table 1A (available at http://links.lww.com/PAIN/B343). Volumes were adjusted to 100 μL with ascorbic acid (50 μM final) and samples submitted to a solid-phase extraction (SPE). Samples were loaded on Phree SPE-cartridges (1 cc, Phenomenex, Le Pecq, France), and elution was performed with 300 μL of 99% acetonitrile (ACN)/1% formic acid (FA, vol/vol). Eluted fractions were dried under vacuum and suspended in 10 μL of 20% ACN/0.1% FA before MS analysis.

Brains and spinal cords were homogenized with a tissue mixer in 0.1 mM ascorbic acid. Homogenates were sonicated (2x5s, 100 W) and centrifuged (20,000*g*, 20 minutes, 4°C). One hundred microliter of the supernatant was spiked with 20 pmol of D3-amitriptyline, D3-nortriptyline, and D10-mefloquine and extracted on Phree SPE-cartridges as described above. Eluted fractions were dried under vacuum and suspended in 10 μL of 20% ACN/0.1% FA before MS analysis.

2.5.3. Liquid chromatography coupled to tandem mass spectrometry instrumentation and analytical conditions

Quantifications were performed using the multiple reaction monitoring (MRM) mode and the isotopic dilution method. Analyses were performed on a Dionex Ultimate 3000 HPLC system (Thermo Scientific, San Jose, CA) coupled with an Endura triple quadrupole mass spectrometer (Thermo Electron). Dried samples were suspended in 10 or 5 μL of 99.9% H_2O/0.1% FA (vol/vol), and 1 μL of the solution was loaded into a Luna Omega Polar reverse-phase capillary column (#00D-4760-AF; 100 x 0.5 mm 3 μm, Phenomenex). Elutions were performed by applying a linear gradient of buffers described in Supplemental Table 1B (available at http://links.lww.com/PAIN/B343). The MRM mode was completed at 3500 V (positive mode) of liquid junction voltage and 276°C capillary temperature. The selectivity for both Q1 and Q3 was set to 0.7 Da (FWHM). The collision gas pressure of Q2 was set at 2 mTorr of argon. The selection of the monitored transitions and the optimization of the collision energy were manually determined. The transitions and the corresponding collision energies used for MRM are reported in the Supplemental Table 1C (available at http://links.lww.com/PAIN/B343).

2.6. Noradrenergic lesions

The lesion of noradrenergic fibers from the descending pathways was performed with intrathecal (i.t.) administration of 6-hydroxydopamine (6-OHDA; 20 μg per mouse, in 5 μL of 0.9% NaCl solution containing 100 μg/mL ascorbic acid) on 8-week-old mice under 3% isoflurane anesthesia. For this i.t. injection, an incision was made at the T9 to T11 level so that a 27-gauge needle connected to a 50-μL Hamilton syringe could be inserted into the space between the thoracic T12 and lumbar L1 vertebrae.[24] For the peripheral pharmacological lesions affecting sympathetic peripheral sprouting,[24] 5 daily intraperitoneal injections of guanethidine monosulfate (30 mg/kg, Sigma Aldrich) were given in a volume of 5 mL/kg in 8-week-old mice.

Mice underwent sham or cuff surgery 2 weeks after the above procedures, and 2 weeks later, they were treated with THN101 (amitriptyline 10 mg/kg and mefloquine 0.1 mg/kg) or with mefloquine alone (0.4 mg/kg) for the central lesions, in a single p.o. administration. The control groups of nonlesioned mice (solvent injection) underwent the same nerve cuffing procedure as the lesioned groups. Experimental design is presented in Supplemental Figure 1D (available at http://links.lww.com/PAIN/B343).

2.7. Immunohistochemistry

Mice with and without 6-OHDA lesion were anesthetized under tiletamine–zolazepam (Zoletil50, 80 mg/kg, Virbac Sante Animale, France)/xylazine (Rompun 2%, 20 mg/kg, Centravet) and perfused with 30 mL of 0.1 M phosphate buffer (PB, pH 7.4), followed by 100 mL of 4% paraformaldehyde solution (PFA) in PB (10 mL/min). Brain and spinal cord were dissected. Brains were postfixed overnight in PFA, included in 2% agar, and cut into 40-μm thick sections (Vibratome VT1000S, Leica). The spinal cord was postfixed in PFA for 1 hour and cryoprotected (4 hours in

15% sucrose in phosphate buffered saline (PBS) followed by 24 hours in 30% sucrose in PBS), lumbar area was embedded in optimal cutting temperature compound (Sakura Finetek), frozen, and cut into 10-μm thick sections (Cryostat CM3050S, Leica) that were mounted on Superfrost Plus slides (Thermo Scientific).

Brain sections were processed for chromogenic immunostaining, as previously described.[12] All steps were performed on free-floating sections on a rotary shaker at room temperature. Sections were washed 3 × 5 minutes in PBS, incubated 15 minutes in a 1% H_2O_2/50% ethanol solution for peroxidase extinction, washed 3 × 5 minutes in PBS, incubated 45 minutes in PBS-Triton X100 (0.3%) with 5% goat serum, and then incubated overnight in PBS-Triton X100 (0.3%) with 1% goat serum and the primary chicken antibody against TH (1:2500, #ab76442; Abcam, Cambridge, United Kingdom). Sections were then washed 3 × 5 minutes in PBS, incubated for 2 hours with a biotinylated goat anti-chicken secondary antibody (1:400, #BA9010, Vector Laboratories, Burlingame, CA) in PBS, washed 3 × 5 minutes in PBS, incubated 90 minutes with the avidin–biotin–peroxidase complex (#PK4000, ABC Elite, 0.2% A and 0.2% B, Vector Laboratories) in PBS, washed 3 × 10 minutes in 0.05 M Tris-HCl buffer (TB; pH 7.5), and the chromogenic signal was revealed by incubation for 10 minutes in 0.025% 3.3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.0006% H_2O_2 (Sigma) in TB. After washes 2 × 10 minutes in TB and 3 × 5 minutes in PBS, sections were serially mounted on Superfrost Plus slides (Thermo Scientific), air-dried, dehydrated in graded alcohols baths (1 × 70%, 1 × 90%, and 2 × 100%), cleared in Roti-Histol (Carl Roth, Karlsruhe, Germany), and coverslipped with Eukitt. Evaluation of TH+ cells was performed using a Nikon Eclipse 80i microscope and pictures were taken with a digital camera (CX 9000, MBF biosciences). For each animal, all TH+ cells were bilaterally counted every other section over the whole anteroposterior extent of the considered cell group: A7 from −4.64 to −5.04 mm (distance from bregma), locus coeruleus from −5.36 to −5.84 mm, and A5 from −5.12 to −5.92 mm.

Spinal cord sections were processed for fluorescence immunostaining. All steps were performed on sections mounted on Superfrost slides. Sections were washed 3 × 5 minutes in PBS, incubated 45 minutes in PBS-Triton X100 (0.5%) with 10% goat serum, and incubated overnight (4°C) in PBS-Triton X100 (0.5%) with 1% goat serum and the primary chicken antibody against TH (1:100, #ab76442, Abcam). Sections were then washed 3 × 5 minutes in PBS-Tween20 (0.1%), incubated for 1 hour with a secondary Alexa Fluor 488-conjugated goat antibody (1:400, #A11039; Invitrogen, Carlsbad, CA) in PBS, washed 3 × 5 minutes in PBS-Tween20 (0.1%), and mounted with ProLong Gold (#P36930, Invitrogen). Images were acquired with a Nikon E80i microscope with the 20x objective and a digital camera (CX 9000, MBF biosciences, Williston, VT) using Neurolucida acquisition software. Evaluation of TH-positive fiber length was performed using ImageJ software. The total TH+ fiber length was measured within given zones (mean zone surface per section: 230066 ± 3171 μm^2) in the dorsal horn of 3 sections per animal at the lumbar L4-L5 level.

The analysis of the sections was performed by an investigator blinded to the lesion status of the animals.

2.8. In vitro pharmacology

To determine possible pharmacological targets for mefloquine, an in vitro pharmacological study was performed through the Contract Research Organization Eurofins CEREP (Le Bois l'Evêque, France), which consisted in identifying mefloquine activity on various transmembrane and soluble receptors. Radioligand binding and functional assays were performed with 1 μM of mefloquine, diluted in DMSO (10^{-2} M). EC_{50} and IC_{50} of mefloquine on A_1 and A_{2A} adenosine receptors, and on serotonin uptake transporters, were also measured using mefloquine at several concentrations. Detailed method information, as provided by the contractor, are given in the supplemental section (Supplemental Table 2, available at http://links.lww.com/PAIN/B343).

2.9. Statistics

Statistical analyses were performed with Statistica (Dell software, France). Student *t* tests (plasma, spinal cord, or brain drug levels; immunohistochemistry experiment) and analysis of variance (other figures) were performed. The surgery procedure (sham or cuff), peripheral lesion procedure (guanethidine or saline), central lesion procedure (6-OHDA or saline), and the various treatments when conducted on independent groups of mice were considered as between-group factors. When needed, the time of measurement, or the treatments when conducted at a week delay on same animals (drug tests in 6-OHDA-lesioned animals and their controls), was considered as within-subject factor. The Duncan test was used for post hoc analyses.

3. Results

3.1. Amitriptyline/mefloquine THN101 effect on mechanical hypersensitivity

To evaluate the potential synergy between amitriptyline (AMI) and mefloquine (MEF), we first performed dose–response studies. Mice from the vehicle-treated (VEH) cuff group displayed mechanical hypersensitivity (sham > cuff, $F1,137 = 223.0$, $P < 10^{-5}$). Oral amitriptyline (**Fig. 1A**) dose-dependently relieved mechanical hypersensitivity (group effect, $F4,47 = 21.19$, $P < 10^{-5}$; post hoc: sham>all other groups at $P < 0.043$), with no significant effect at 5 mg/kg, a partial and transient recovery at 10 mg/kg ($F4,188 = 5.51$, $P < 0.001$; post hoc AMI10>cuff VEH, at 2 hours $P = 0.013$), and a more complete and transient recovery at 20 mg/kg ($F4,188 = 5.51$, $P < 0.001$; post hoc: AMI20>cuff VEH, at 2 hours, $P < 0.001$). The 2 doses tested for oral mefloquine (**Fig. 1B**) had no significant action per se on the hypersensitivity (group effect, $F3,38 = 64.02$, $P < 10^{-5}$; post hoc: sham>all other groups at $P < 4*10^{-6}$ and cuff VEH-treated group similar to cuff MEF-treated groups at $P > 0.274$). The 2 lowest doses of amitriptyline (5 and 10 mg/kg) were then combined with mefloquine. When combined with mefloquine, amitriptyline 5 mg/kg led to a partial relief of hypersensitivity, limited within 4 hours when combined with mefloquine 0.05 mg/kg (Group*Time, $F4,64 = 3.47$, $P = 0.013$; post hoc: AMI5 + MEF0.05 > VEH, at 4 hours $P = 0.047$ post hoc) (**Fig. 1C**) and present at 7 hours when combined with mefloquine 0.1 mg/kg (Group*Time, $F4,68 = 6.99$, $P = 9*10^{-5}$; post hoc: AMI5+ MEF0.1>VEH, at 7 hours $P = 9*10^{-5}$) (**Fig. 1D**). Mefloquine further enhanced the action of amitriptyline 10 mg/kg, with a partial and transitory improvement with mefloquine 0.05 mg/kg (Group*Time, $F4,64 = 2.95$, $P = 0.026$; post hoc: AMI10+ MEF0.05>VEH, at 2 hours $P = 0.032$, at 7 hours $P = 0.040$ post hoc) (**Fig. 1E**) and a better and sustained effect with mefloquine 0.1 mg/kg (**Fig. 1F**, left), for which the relief of mechanical hypersensitivity was stably maintained for at least 7 hours (Group*Time, $F4,118 = 4.30$, $P = 1.6*10^{-4}$; post hoc: AMI10 + MEF0.1 > VEH, at 2 hours $P = 0.004$, at 4 hours $P = 1.8*10^{-5}$, at 7 hours $P = 1.1*10^{-5}$). We also observed that THN101 at this dose combination had no effect per se on PWTs in

the sham surgery group (**Fig. 1F**, left) (post hoc: $P > 0.51$ for SHAM-THN101 at 1-7 hours against value at 0 hours) or on the contralateral PWTs in the cuff group (**Fig. 1F**, right) (Group*Time, $F4,118 = 0.46$, $P = 0.88$). This prolonged benefit of the THN101 combination compared with individual treatments is further highlighted by the AUC presentation of data (**Fig. 1G**) (Group effect, $F4,51 = 6.02$, $P = 4.79*10^{-4}$; post hoc: THN101 > VEH, MEF0.1 and AMI10-treated groups at $P < 0.0396$). All the following experiments were thus conducted using amitriptyline 10 mg/kg + mefloquine 0.1 mg/kg as THN101 combination.

The duration of THN101 effect was then evaluated beyond 7 hours (**Fig. 2A**). This experiment allowed showing that the action of a single THN101 administration partially lasted at least 16 hours and was no more present after 20 hours (Group*Time, $F6,82 = 9.07$, $P < 10^{-6}$; post hoc: THN101>all others groups at 12 hours $P < 7*10^{-5}$ and at 16 hours $P < 4*10^{-4}$). Repeating the evening injection procedure in a separate set of mice allowed showing that relief of mechanical hypersensitivity can be repeatedly observed over 5 days, 12 hours after each THN101 administration (Group*Time, $F1,14 = 15.55$; $P = 0.0015$; post hoc: THN101>VEH at day 1 $P = 0.0011$, day 2 $P = 4*10^{-4}$, day 3 $P = 0.011$, day 4 $P = 0.028$, and day 5 $P = 0.029$) (Supplemental Figure 1E and **Fig. 2B**, available at http://links.lww.com/PAIN/B343).

3.2. Pharmacokinetic profile of THN101

We then tested whether mefloquine may affect the antidepressant pharmacokinetic profile. Blood levels of mefloquine, amitriptyline, and its metabolite nortriptyline were monitored 1, 2, 4, and 7 hours after administration of amitriptyline alone or THN101. Amitriptyline, nortriptyline, and mefloquine levels were also measured in plasma, brain, and spinal cord 7 hours after administration.

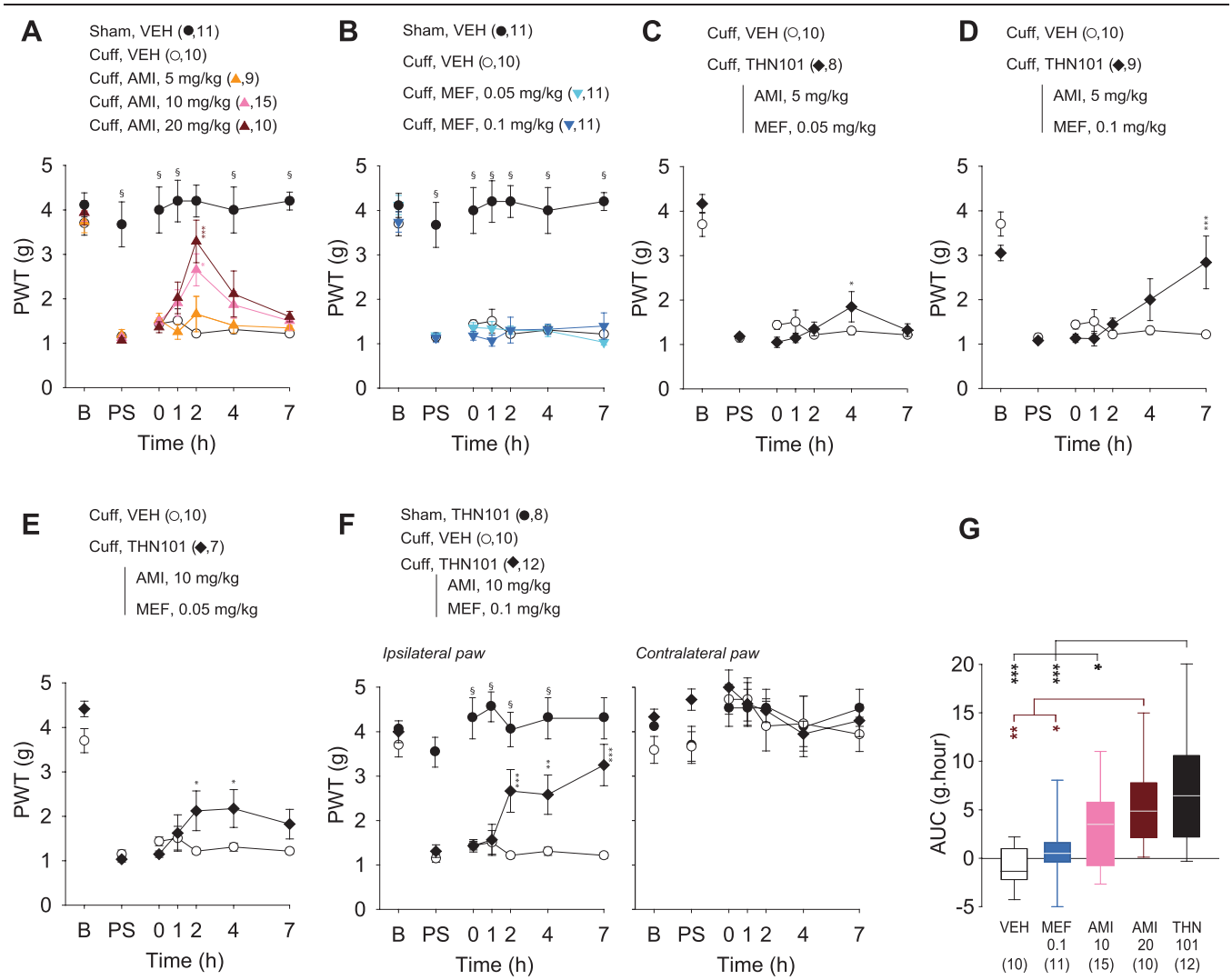


Figure 1. Effect of amitriptyline and mefloquine combination on mechanical hypersensitivity in mice with neuropathic pain. The mechanical right hind paw withdrawal threshold (PWT) was assessed with von Frey filaments before surgery (B: baseline), the mean of postsurgical values before treatment is displayed as PS (postsurgery baseline), and the oral drug treatment was performed 2 to 3 weeks after surgery. On the drug treatment day, sham (n=8-11) and cuff (n=7-15 per group) mice were tested before and from 1 to 7 hours after drug administration. Mice PWT was assessed after amitriptyline (A) or mefloquine (B) treatment at different doses and after treatment with amitriptyline and mefloquine combination (C-F). Comparison of areas under the curve (AUC) between cuff VEH, mefloquine, amitriptyline, and THN101 (AMI 10 mg/kg/MEF 0.1 mg/kg)-treated groups are shown with whisker boxes (G). Vehicle solution (NaCl 0.9%, DMSO 0.02% 10 mL/kg p.o., once after time 0 test) was used as negative control (A to G). Results are expressed as mean ± SEM. Symbols for some of the post hoc comparisons: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs cuff VEH (or vs the designated group in (G)); §$P < 0.05$ vs cuff AMI 20 mg/kg (A) or vs cuff MEF 0.1 mg/kg (B) or vs cuff THN101 (F).

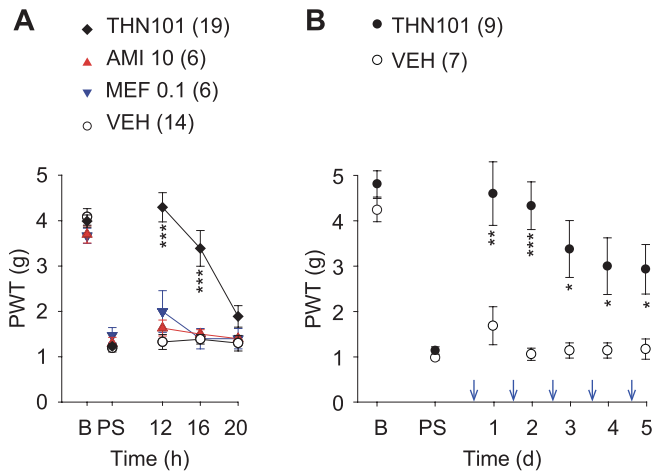


Figure 2. THN101 action on mechanical hypersensitivity. Experiments were conducted in cuff mice. The mechanical right hind paw withdrawal threshold (PWT) was assessed with von Frey filaments before surgery (B: baseline), the mean of postsurgical values before treatment is displayed as PS (postsurgery baseline), and the drug treatment was performed or started 2 to 3 weeks after surgery. Treatments were given orally in the evening, and mice tested on the following day. In a first experiment, time course of THN101 (amitriptyline 10 mg/kg/mefloquine 0.1 mg/kg) action was compared with control, amitriptyline, and mefloquine treatments (A). In a second experiment, THN101 was orally given each evening over 5 consecutive days, and mice tested each time 12 hours postadministration (B). Results are expressed as mean ± SEM. Symbols for some of the post hoc comparisons: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs cuff VEH.

Blood levels of mefloquine were found to gradually increase up to 7 hours after oral administration (**Fig. 3A**) (Time effect, F3,42 = 21.52, $P < 10^{-6}$), reaching 292.1 ± 18.9 μM. At 7 hours postadministration, plasma levels were at 92.6 ± 8.4 μM (**Fig. 3B**), and central concentrations were of 44.2 ± 9.1 pmol/mg of protein in the spinal cord (**Fig. 3C**) and 1.0 ± 0.1 pmol/mg of protein in the brain (**Fig. 3D**).

Conversely, blood amitriptyline and nortriptyline concentrations (**Fig. 3A**) were highest 1 hour after oral drug delivery and decreased similarly in the amitriptyline-treated and THN101-treated groups (Group*Time, amitriptyline: F3,42 = 0.55, P = 0,65, nortriptyline: F1,14 = 4.13, P = 0.061) (**Fig. 3A**). Similarly, no significant difference was found between amitriptyline alone and THN101 concerning the plasma (**Fig. 3B**), spinal cord (**Fig. 3C**), and brain (**Fig. 3D**) levels of amitriptyline (P = 0.41, 0.24 and 0.30, respectively) and nortriptyline (P = 0.32, 0.67 and 0.11, respectively). It should be noted that one of the mice displayed much higher plasma amitriptyline concentrations than the others; however, statistical conclusions remained the same whether this animal was included (see above) or no (without the potential outlier: plasma: P = 0.83; spinal cord: P = 0.37; brain: P = 0.66) (Supplemental Fig. 2, available at http://links.lww.com/PAIN/B343).

3.3. Contribution of descending aminergic pathways in THN101 and mefloquine action

We next investigated the mechanism of THN101 by studying the role of aminergic pathways.[24] To test the involvement of the sympathetic system, we injected guanethidine i.p. for 5 days.[3] This peripheral lesion did not impact on the THN101-sustained relief of mechanical hypersensitivity, as tested 12 hours after oral administration (treatment effect, F1,16 = 36.73, $P < 2^{*}10^{-5}$; Lesion*Treatment, F1,16 = 0.002, P = 0.96; post hoc: THN101 action similar between both groups at P = 0.80) (**Fig. 4A**).

To test the involvement of the noradrenergic-descending controls of pain, i.t. injections of 6-OHDA into the T12-L1 area were performed 2 weeks before the cuff surgery. Using immunohistochemistry, we completed our previous characterization[24] of this lesion procedure by studying its impact on 3 noradrenergic nuclei and on lumbar spinal cord TH+ fibers (Supplemental Fig. 3, available at http://links.lww.com/PAIN/B343). The i.t. 6-OHDA did not significantly change the total number of TH+ cells in the A7 cell group (P = 0.29), in the locus coeruleus (P = 0.56), and in the A5 cell group (P = 0.11). However, a nonsignificant trend toward some loss (A7: −17.1%; locus coeruleus: −11.5%; A5: −15.7%) was present in each group of cells, which may suggest that only a small subset of cells might have been affected. In the dorsal horn of the lumbar spinal cord, a significant 55.7% decrease in TH+ fibers was observed ($P < 7^{*}10^{-6}$), in line with a previous qualitative report.[24] In agreement with a previous report,[24] this lesion procedure did not change the mechanical PWTs of the mice and did not change either the development of the mechanical hypersensitivity after the cuff surgery (Lesion*Treatment*Time, F4,96 = 0.26, P = 0.90) (Supplemental Fig. 4, available at http://links.lww.com/PAIN/B343).

Oral VEH solution did not affect the time course of the PWTs (Group*Time effect, F4,56 = 1.22, P = 0.315) (**Fig. 4B**, left). A week later, we tested THN101 and observed that its action was significantly more potent in the nonlesioned mice (saline i.t.) than in the lesioned ones (6-OHDA i.t.) from 4 to 7 hours after administration (Group*Time effect, F4,56 = 4.69, P = 0.0024; post hoc: saline>6-OHDA at 4 hours P = 0.050 and at 7 hours P = 0.0001) (**Fig. 4B**, right). As observed above, neither vehicle, THN101, nor 6-OHDA changed the PWTs in mice from the sham group (Lesion*Time, F4,252 = 0.19, P = 0.94; Treatment*Time, F8,252 = 1.29, P = 0.25; Lesion*Treatment*Time, F8,252 = 0.77, P = 0.62) (**Fig. 4C**, left and center).

The above data were supportive of a critical role of descending noradrenergic pathways in the action of THN101. We then tested whether mefloquine alone at a higher dose may affect mechanical sensitivity and, if so, whether noradrenergic descending pathway functionality contributed to the effect observed. We thus administered 0.4 mg/kg mefloquine per os to cuff animals whose descending noradrenergic pathways were or not lesioned. We observed that mefloquine relief of hypersensitivity was lost after lesion (Lesion*Treatment*Time effect, F4,92 = 17.53, $P <$ 10-6, post hoc: saline/MEF>6-OHDA/MEF, at 1 hour P = 0.010, at 2 hours $P < 10^{-5}$) (**Fig. 4D**). Importantly, although it relieved the mechanical hypersensitivity in cuff mice, 0.4 mg/kg mefloquine had no effect on PWTs in the sham group (**Fig. 4C**, right).

To further support a potential spinal action of mefloquine in THN101 combination, we administered mefloquine i.t. after an oral administration of 10 mg/kg amitriptyline. Two hours after this procedure, the combination provided a significant improvement in PWTs compared with mice that received one only of the treatments (Group*Time, F6,62 = 3.26, P = 0.007; post hoc: MEF/AMI > all other groups at 2 hours $P < 0.002$) (**Fig. 5**).

3.4. Adrenergic mechanism of action of THN101 combination

Beside the lesion experiments, we also used a pharmacological approach to study the role of the noradrenergic system in an independent set of experiments. As downstream actors after noradrenaline recruitment, the respective contribution of both α and β adrenoceptors[24] in THN101 response was tested.

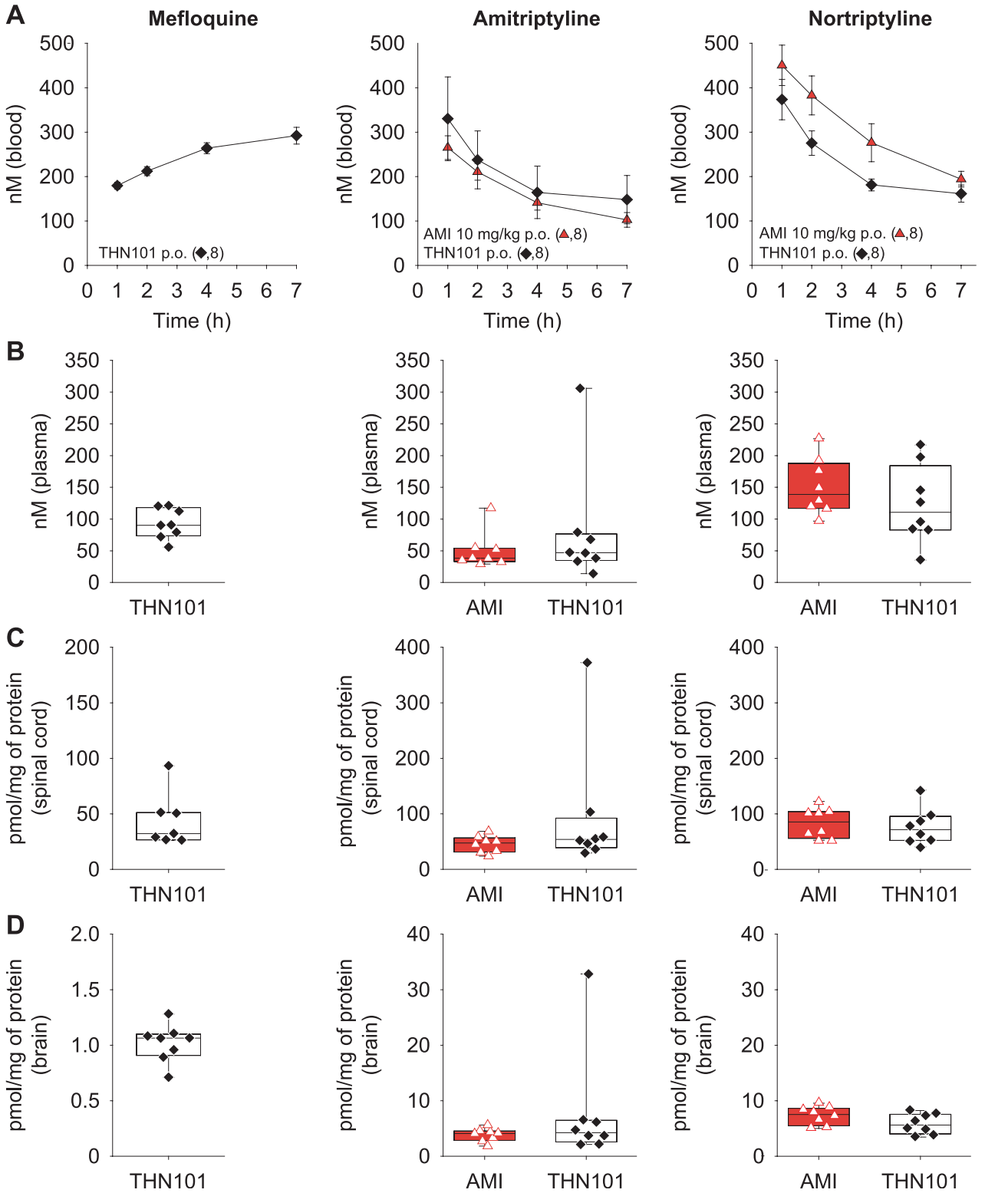


Figure 3. Evaluation of mefloquine, amitriptyline, and nortriptyline concentrations by mass spectrometry after amitriptyline or THN101 treatment. Measures were performed only in cuff mice. Mefloquine, amitriptyline, and nortriptyline concentrations (nM) were measured in blood from 1 to 7 hours after amitriptyline (10 mg/kg) or THN101 (AMI 10 mg/kg/MEF 0.1 mg/kg) per os treatment. Results are expressed as mean ± SEM (A). Mefloquine, amitriptyline, and nortriptyline plasma concentrations (nM) (B), and spinal cord (C) and brain (D) concentrations (pmol/mg of protein) were also assessed 7 hours after amitriptyline or THN101 per os treatment (whisker boxes).

Yohimbine and propranolol were delivered through drinking water, which had no effect per se on mechanical sensitivity thresholds (PS, for postsurgery baseline, vs time point "0" on **Fig. 6A**), as previously reported.[24] We thus tested the impact of these antagonists on THN101 action and observed that its lasting activity on mechanical hypersensitivity remained present under propranolol treatment but was suppressed under yohimbine treatment (**Fig. 6A**) (Group*Time, $F_{12,104} = 3.90$, $P < 6*10^{-5}$;

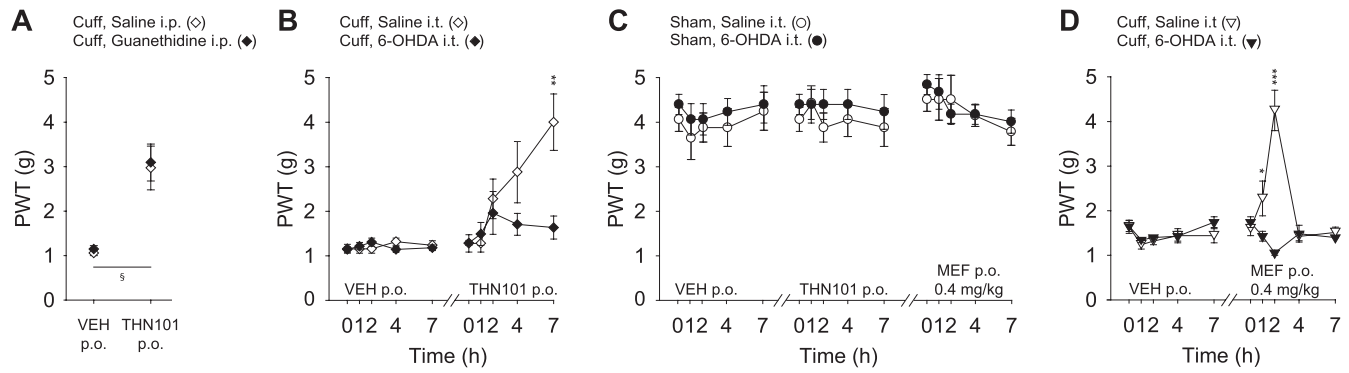


Figure 4. Central descending aminergic pathways are necessary to the action of THN101 and mefloquine. The mechanical hind paw withdrawal thresholds (PWTs) were assessed using von Frey filaments in mice 2 to 4 weeks after cuff or sham surgery. Peripheral (A) and central (B–D) lesions were performed before surgery. In separate sets of experiments, cohorts of saline-treated and guanethidine-treated cuff mice were tested for response to vehicle and THN101 a week apart (A), cohorts of saline-treated and 6-OHDA-treated cuff mice were tested for response to vehicle and THN101 a week apart (B), cohorts of saline-treated and 6-OHDA-treated sham mice were tested for response to vehicle, THN101, and mefloquine (0.4 mg/kg) a week apart (C), and cohorts of saline-treated and 6-OHDA-treated cuff mice were tested for response to vehicle and mefloquine a week apart (D). Mice were tested before (0) and from 1 to 7 hours after drug administration. Results are expressed as mean ± SEM. Symbols for some of the post hoc comparisons: §$P < 0.001$ for cuff THN101 vs cuff VEH (A); *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs cuff VEH and vs cuff 6-OHDA (B to D).

post hoc: (THN101 = SHAM THN101)>all others groups at 12 hours $P < 0.05$, at 16 hours THN101 < SHAM THN101 at $P < 0.005$ and THN101 > all others groups except PRO-THN101 at $P < 0.05$). This finding was further illustrated by the area under the curve (calculated for each mouse above its time point "0" threshold) (**Fig. 6A**, right) (Group effect, $F3,35 = 8.31$, $P = 2*10^{-4}$; post hoc: THN101>YOH-THN101 and VEH at $P < 5*10^{-4}$ and PRO-THN101>YOH-THN101 and VEH at $P < 0.02$). Finally, the THN101/yohimbine combination had no effect on PWTs in mice from the sham group (**Fig. 6A**).

This experiment suggested a preferential role of α_2 adrenoceptors in THN101 action. As tests were conducted on the day following THN101 administration while adrenergic receptor blockade was continuous, we then tested whether α_2 adrenoceptors were more specifically involved in the induction or the maintenance of THN101 action. Yohimbine was thus delivered i.p. either at the same time as THN101 administration (induction) or 30 minutes before testing time (expression). When yohimbine was given at the same time as THN101, THN101 action was no longer observed 12 hours later (**Fig. 6B**, left), whereas its effect was still present when yohimbine was administered 30 minutes before the final test (**Fig. 6B**, right) (YOH0: Group*Time effect, $F4,38 = 5.94$, $P < 8*10^{-4}$; post hoc: THN101>YOH0-THN101 at $P < 0.005$ for 12hours and 13hours; YOH12: Group*Time effect, $F4,38 = 7.13$, $P < 2*10^{-4}$; post hoc: THN101=YOH12-THN101 > VEH at $P < 0.05$ for 12hours and 13hours). These data supported a role of α_2 adrenoceptors in the induction of THN101 long-lasting action.

To more specifically test the role of spinal adrenergic receptors in this induction process, we used i.t. delivery of antagonists just after the saline or THN101 per os administration (**Fig. 6C**). As the i.t. procedure may lead to shorter effect of the antagonist than oral or i.p. delivery, PWTs were tested at 4 hours. The α_2-adrenoceptor antagonist atipamezole fully suppressed THN101 action, whereas the β-adrenoceptor antagonist sotalol partially suppressed it (Antagonist*Treatment, $F1,51 = 15.61$, $P < 0.001$; post hoc: (cuff saline THN101 = all sham groups) > all others groups at $P < 0.05$, cuff sotalol THN101 > (all cuff saline groups and cuff atipamezole THN101) at $P < 0.05$). This suppression was present while these antagonists had no effect per se in mice from the cuff group and had no effect in THN101-treated mice from the sham group (**Fig. 6C**). Interestingly, this partial suppression observed with local (i.t.) sotalol at 4 hours is in line with the partial effect observed with chronic oral propranolol at the time point 16 hours (**Fig. 6A**). Together it suggests that spinal α_2 adrenoceptors are essential to THN101 action and that β adrenoceptor may also contribute (although perhaps to a lesser extent) to this action.

3.5. Contribution of mefloquine in the mechanisms of action of THN101

We then further explored the mefloquine component of THN101 action, by analyzing potential targets (**Fig. 7A and B**) besides its

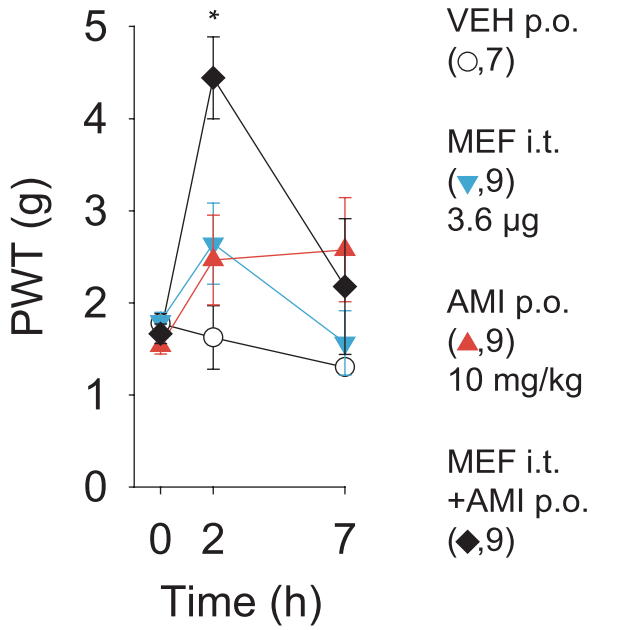


Figure 5. Spinal action of mefloquine in THN101 combination. The experiment was conducted in cuff mice. The mechanical hind paw withdrawal thresholds (PWTs) were assessed using von Frey filaments in mice 2 to 3 weeks after cuff surgery. Central (i.t.) administration of mefloquine combined or not with oral administration of amitriptyline (10 mg/kg) was tested and monitored since 7hours after drug administration. Results are expressed as mean ± SEM. Symbols for some of the post hoc comparisons: *$P < 0.05$ vs cuff VEH.

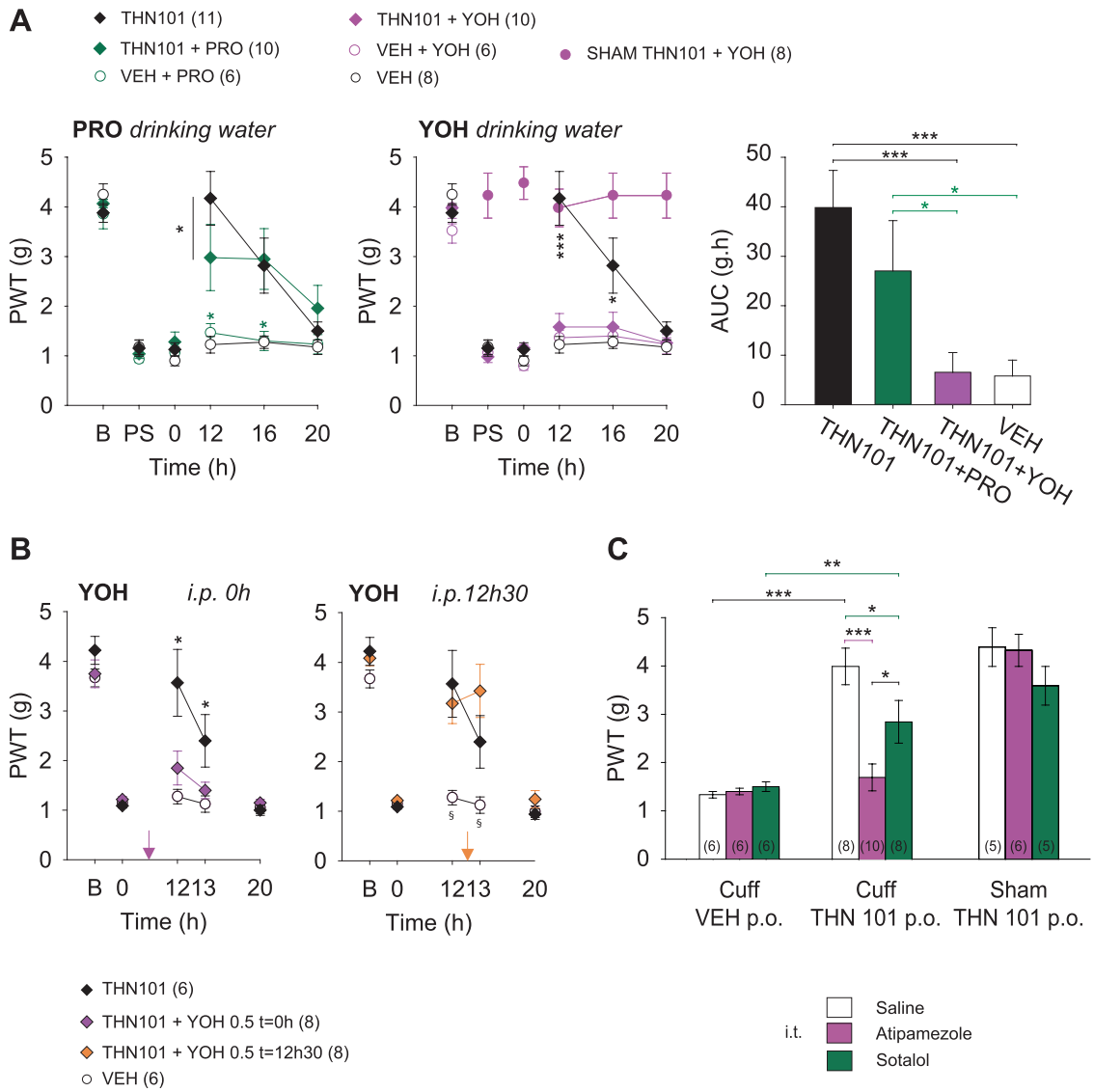


Figure 6. THN101 antiallodynic action and adrenoceptors. The mechanical hind paw withdrawal thresholds (PWTs) were assessed using von Frey filaments in mice 2 to 3 weeks after cuff or sham surgery. In a first set of experiments (A), the β_2 (propranolol, PRO)-adrenoceptor and the α_2 (yohimbine, YOH)-adrenoceptor antagonists were delivered through the drinking water. The treatment with these antagonists started 5 days before the THN101 acute administration (time course: $^*P < 0.05$ and $^{***}P < 0.001$ indicate post hoc significance for cuff THN101 vs (cuff THN101 + PRO or YOH); bar graph: $^*P < 0.05$ for (cuff THN101 + PRO) vs (cuff THN101 + YOH) and vs cuff VEH and $^{***}P < 0.001$ for cuff THN101 vs (cuff THN101 + YOH) and vs cuff VEH). In the second set of experiments (B), yohimbine was injected i.p. either at the same time as THN101 (left graph) or 30 minutes before the final test (right graph) ($^*P < 0.05$ for cuff THN101 vs (cuff THN101 + YOH); §$P <$ 0.001 for cuff VEH vs cuff THN101 and vs cuff THN101 + YOH). (C) Central (i.t.) administration of saline, α_2-adrenoceptor antagonist atipamezole or β-adrenoceptor antagonist sotalol combined with oral administration of vehicle or THN101 treatment was tested 4hours after drug administration ($^*P < 0.05$, $^{**}P <$ 0.01, and $^{***}P < 0.001$ for the indicated comparisons). Results are expressed as mean ± SEM. B, baseline before surgery; PS, postsurgery baseline before antagonist oral delivery in drinking water.

known action on connexins.[7] The in vitro binding assays showed that 1 μM mefloquine does not directly target noradrenaline and dopamine uptake sites or α and β adrenoceptors (**Fig. 7A**), although yohimbine was able to antagonize the action of mefloquine alone at a high dose (Group*Time, F4,40 = 11.16, $P < 5^*10^{-6}$; post hoc: MEF/saccharine>MEF/yohimbine at 2 hours $P < 2^*10^{-5}$, 4 hours $P < 2^*10^{-4}$ and 7 hours $P = 0.019$) (**Fig. 8A**). The analyses also reported a lack of direct binding to a wide range of other tested targets (**Fig. 7A** and Supplemental Fig. 5, available at http://links.lww.com/PAIN/B343). This analysis, however, showed a potential antagonistic action (ie, at least 50% binding displacement at 1 μM) for (1) the adenosine receptors A1 and A2A, (**Figs. 7A and B**) and (2) the serotonin transporter pathway (**Figs. 7A and B**).[18]

In agreement with lesion data showing that descending noradrenergic pathways are important to mefloquine action at a high dose (**Fig. 4C**), we observed that yohimbine blocked this action (Group*Time, F4,40 = 11.16, $P < 5^*10$-6; post hoc: MEF > MEF/yohimbine at 2 hours $P < 2^*10$-5, 4 hours $P < 2^*10$-4 and 7 hours $P = 0.019$) (**Fig. 8A**). However, in vitro analysis (**Fig. 7**) showed that mefloquine does not directly target these receptors. We then tested in vivo whether the selective manipulation of the mefloquine targets that were identified in vitro may also modulate amitriptyline action. Interestingly, we observed no potentiating effect of the serotonin uptake inhibitor fluoxetine (Group*Time, F8,72 = 6.18, $P = 0.52$) (**Fig. 8B**), the adenosine A1 antagonist DPCPX (Group*Time, F8,60 = 1.32, $P = 0.24$) (**Fig. 8C**), or the A2A antagonist SCH58261 (Group*Time, F16,52 = 0.59, $P =$

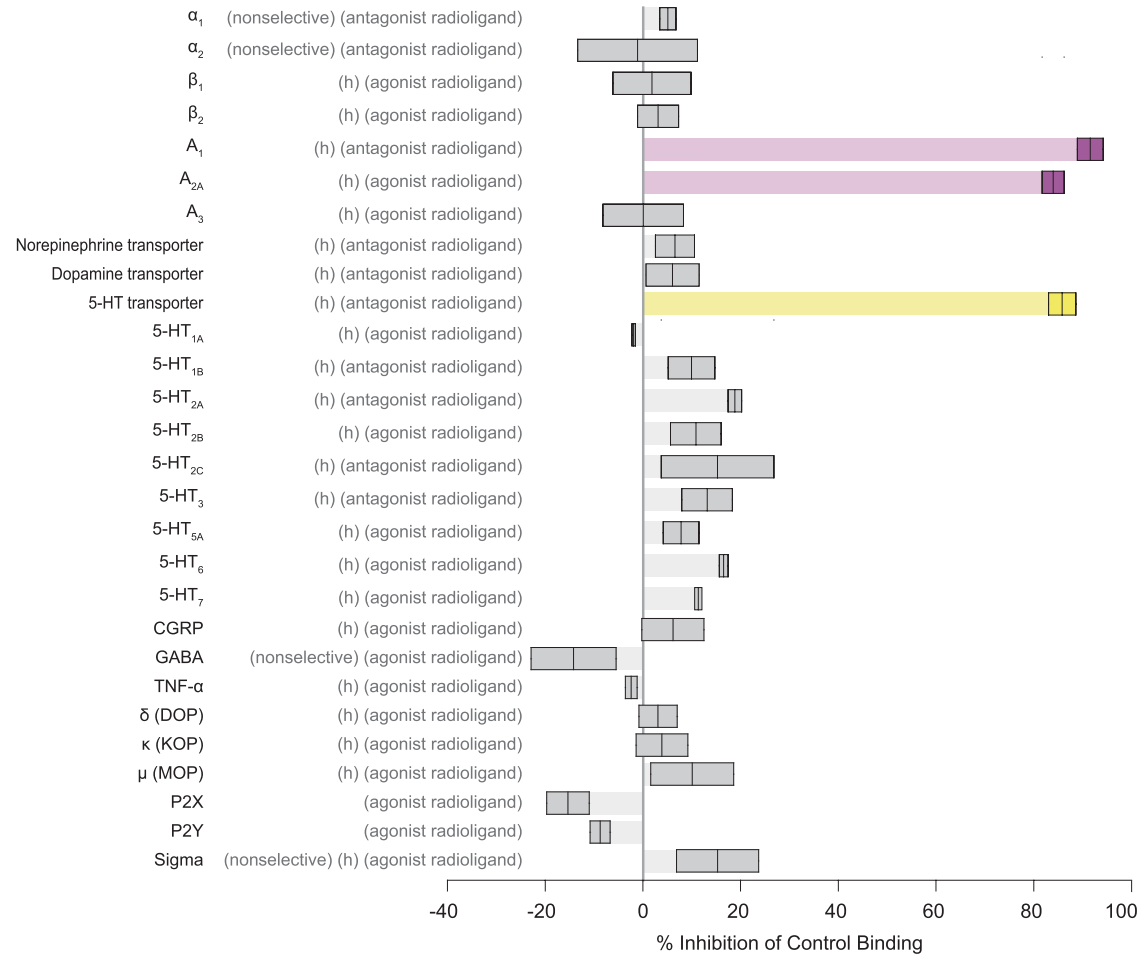
0.88) (**Fig. 8D**) on amitriptyline action. However, the concomitant i.t. blockade of gap junctions by carbenoxolone led to potentiate the action of oral amitriptyline (Group*Time, F6,40 = 5.65, P = 2.52*10-4; post hoc: AMI + CBX>all the other groups at 2 hours $P > 0.0048$) (**Fig. 8E**). These data suggested that mefloquine may preferentially improve amitriptyline effect through targeting of gap junctions.

4. Discussion

Modulators of connexins have been suggested to modulate the therapeutic action of drugs acting on the central nervous system, both in sleep disorders[9,38,49] and in neuropathic pain.[19,20] In this study, we showed that mefloquine enhanced the effect of the tricyclic antidepressant amitriptyline on mechanical hypersensitivity in a mouse model of neuropathic pain. Pharmacokinetic and pharmacological approaches allowed us to highlight some mechanistic features of this potentiation, pointing towards the role of descending noradrenergic pathways, α_2 adrenoceptors, and gap junctions.

A single THN101 (amitriptyline/mefloquine combination) administration led to a long-lasting (around 16 hours) effect on the mechanical hypersensitivity of mice with sciatic nerve compression. This action was likely synergistic and not a simple additive effect. Indeed, it was present for a dose of mefloquine that was ineffective, and it lasted for hours after the acute action of

A **Mefloquine 1 µM - duplicates**



B

Binding assay	Radioligand	IC50	Ki	nh
A_1(h) (antagonist radioligand)	[3H]DPCPX	2.0E-07 M	1.3E-07 M	1.1
A_{2A}(h) (agonist radioligand)	[3H]CGS21680	1.1E-07 M	9.0E-08 M	0.9
5-HT transporter (h) (antagonist radioligand)	[3H]imipramine	1.9E-07 M	8.6E-08 M	1.1

Functionnal assay	Referent	EC50	IC50	KB
A1(h) (agonist effect)	CPA	n.c.		
A2A(h) (agonist effect)	NECA	n.c.		
A1 (h) (antagonist effect)	DPCPX		1.1E-06 M	2.6E-07 M
A2A(h) (antagonist effect)	ZM 241385		1.6E-06 M	1.7E-07 M
5-HT uptake	imipramine		7.7E-07 M	

Figure 7. In vitro evaluation of mefloquine pharmacological potential targets. In vitro determination of pharmacological targets of mefloquine at 1 µM (A). Binding and functional properties of mefloquine on relevant adenosine and serotonin targets (B). α1, alpha 1 adrenoceptor; α2, alpha 2 adrenoceptor; β1, beta 1 adrenoceptor; β2, beta 2 adrenoceptor; A1, adenosine receptor A1; A2A, adenosine receptor A2A; A3, adenosine receptor A3; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; CPA, N6-cyclopentyladenosine; NECA, 59-N-ethylcarboxamidoadenosine.

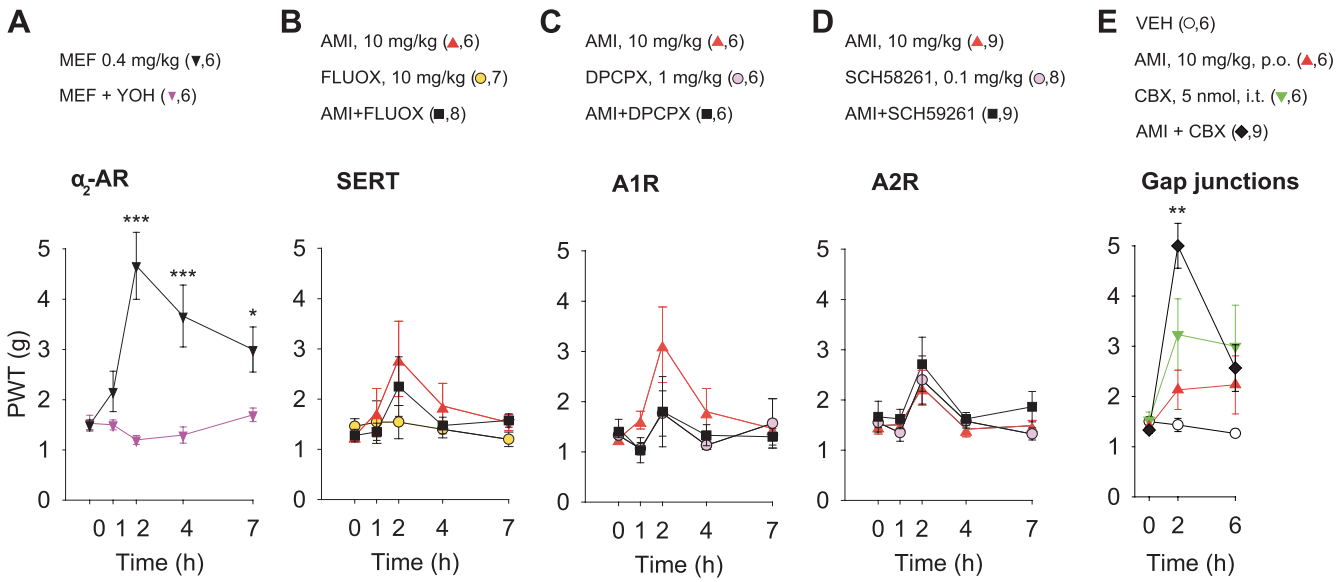


Figure 8. In vivo evaluation of mefloquine pharmacological potential targets. All experiments were conducted in cuff mice. The mechanical right hind paw withdrawal threshold (PWT) was assessed with von Frey filaments. We tested the role of α_2 adrenoceptors in mefloquine action using yohimbine (YOH) (A) (*$P <$ 0.05 ***$P < 0.001$ for MEF vs MEF + YOH). We also tested whether the serotonin uptake (SERT) inhibitor fluoxetine (B), the adenosine A1 receptor antagonist DPCPX (C), the adenosine receptor A2 antagonist SCH59261 (D), and the gap junction blocker carbenoxolone (CBX) (E) may potentiate oral amitriptyline action (**$P < 0.01$ for AMI + CBX vs VEH). Results are expressed as mean ± SEM.

amitriptyline alone stopped. This long-lasting effect with a single injection had not been previously detected when, at another dose combination and with another route of administration, amitriptyline/mefloquine had been first tested in rats with CCI.[19] In this previous study, the benefit of the combination was anyway present after a few days of co-administration. The interest of amitriptyline/mefloquine association is thus illustrated in 2 species and 2 different neuropathic pain models.

A prolonged action of the amitriptyline/mefloquine combination could be the basis of the faster and enhanced recovery observed in this previous rat study, but the detailed time course of hyperalgesia relief after each mefloquine administration had not then been tested. The present time course study highlighted that THN101 kinetic on mechanical hypersensitivity differs from the kinetic of its individual component. Although, besides the spinal noradrenergic and gap junction components, we cannot yet provide mechanistic insights explaining such prolonged effect, it should be noted that the kinetic of mefloquine blood levels strikingly differs from the one of amitriptyline. Although the antidepressant levels peak rapidly in the organism and decrease regularly, mefloquine bioavailability is slower and levels are high 7 hours after administration. This feature may contribute to the lasting potentiation observed in THN101.

An interaction of mefloquine with amitriptyline pharmacokinetics could also have explained the potentiating effect of THN101. The previous study conducted in rat with chronic delivery of amitriptyline and a higher dose of mefloquine showed that brain and serum levels of amitriptyline were overall not affected by mefloquine co-treatment.[19] Moreover, using mass spectrometry, we showed that under our procedures in mice the THN101 combination did not alter the time course of blood levels of mefloquine, of amitriptyline, and importantly, of its active metabolite nortriptyline, and we further showed that it did not alter either the plasma, brain, or spinal cord concentrations of these drugs. This finding is consistent with the fact that amitriptyline and mefloquine are mostly metabolized by different enzymes. Indeed, amitriptyline is mainly metabolized by the cytochrome P450 CYP2C19, leading to nortriptyline by demethylation.[33] By contrast, mefloquine is metabolized by CYP3A4, leading to the formation of a major nonactive metabolite in malaria, carboxymefloquine, and to a lesser extent to hydroxymefloquine.[14] The benefit of THN101 combination is thus not metabolism based.

To explore the action of THN101, we then focused on the target mechanisms of its drug components, amitriptyline and mefloquine. We first tested 2 mechanisms known to contribute to amitriptyline-mediated relief of mechanical hypersensitivity, a peripheral mechanism and a central one.[23,24] The peripheral component of antidepressant drugs relies on the recruitment of noradrenergic fibers in the dorsal root ganglia.[3,24] The downstream mechanism is then mediated by the recruitment of β_2 adrenoceptors present on nonneuronal cells and leading to a relief of the neuroimmune activation induced by the nerve lesion.[3,24] The guanethidine experiment, leading to a sympathectomy, showed that this peripheral component was not critical to THN101 prolonged action. This finding is in agreement with data from the rat study showing that amitriptyline/mefloquine combination had no synergistic impact on markers associated with neuroinflammation.[19] However, the pharmacological study, particularly the i.t. delivery of sotalol that partially blocked THN101 action, suggested that β adrenoceptors can nevertheless contribute to THN101 action with a potential central component.

A central mechanism for antidepressant drug action on neuropathic pain has also been described.[1,24] It is mostly based on the recruitment of noradrenergic descending inhibitory controls of pain[30] and on spinal α_2 adrenoceptors. We thus tested the role of these descending pathways by using i.t. 6-OHDA and observed that it suppressed THN101 action. 6-OHDA is a toxin targeting both noradrenergic and dopaminergic systems, and it is for example classically used to model Parkinson disease loss of midbrain dopamine cells and forebrain dopamine fibers.[11,48] Because of its homology with dopamine and noradrenaline, 6-OHDA enters cells through the transporters of dopamine (DAT) and noradrenaline (NET). However, dopamine projections to the spinal cord arise from hypothalamic A11 and

A13 cell groups, which have the peculiarity of not (or poorly) expressing the DAT.[22,41] High doses of i.t. 6-OHDA can still affect spinal dopamine,[28] but the dose that we used was shown (using mass spectrometry) to induce a 69% decrease in the spinal noradrenaline content without changing the spinal dopamine content[24] and was shown (using immunohistochemistry) to decrease the presence of TH-positive fibers in the lumbar dorsal horn of the spinal cord.[24] Completing the lesion characterization, we showed here that it had no significant impact on the total number of noradrenergic cells in A5, locus coeruleus, and A7, although a trend toward 11% to 17% loss was observed in each group of cells. It may be hypothetized that this trend could reflect the loss of a subset of noradrenergic cells corresponding to cells projecting to the spinal cord but masked by the overall population of the other noradrenegic neurons. The impact of the lesion in the spinal cord was anyway supported by the significant decrease (−55.7%) in TH-positive fibers at the lumbar level. This 6-OHDA experiment together with the use of α_2-adrenoceptor antagonists, particularly atipamezole with intrathecal delivery, highlighted that a spinal noradrenergic component was critical to THN101 mechanism. Interestingly, beyond amitriptyline, this central component seemed to be also present for mefloquine itself at a high dose, which supports a convergence of anatomical targets between the 2 drug components of THN101. Of course, this spinal action does not exclude the contribution of important supraspinal actors in THN101 action.

Mefloquine is known to target connexins, both in vitro[20,35,36] and in vivo,[8] but this drug could also act on other targets. Contract Research Organizations, such as CEREP/Eurofins, are mostly working for pharmaceutical companies and provide professional assessment of binding or activity of drugs on potential targets and off-targets. However, these profiles are rarely communicated to the scientific community, which constitutes a major loss of important information. The literature[16,18,44,51] and the present screening of 80 putative targets suggested that mefloquine may act below μM range on serotonin reuptake and around μM range on adenosine receptors A_1 and A_{2A}, in each case with blocker or antagonist properties. We thus tested the potential implication of these targets by using selective drugs in combination with amitriptyline. The selective serotonin reuptake inhibitor fluoxetine did not improve amitriptyline action (which already has a serotonergic component[23,29]). It suggests that although mefloquine may act on serotonin reuptake, this action alone could not explain the potentiation observed with THN101 combination. The manipulation of adenosine receptors is also known to significantly modulate nociceptive symptoms.[39] However, blocking adenosine A_1 and A_{2A} receptors did not improve amitriptyline action, which suggested that these targets may not be critical either in THN101-potentiating effect. Finally, results showing that the intrathecal delivery of another gap junction blocker, carbenoxolone, can amplify amitriptyline effect support the hypothesis that gap junctions are a relevant target for potentiating antidepressant drug action in neuropathic pain.

Together, our data indicate that amitriptyline action can be potentiated by the antimalaria drug mefloquine at a low dose. This potentiation seems to be mediated by blocking gap junctions and we show that it requires descending noradrenergic controls and α_2 adrenoceptors. The present data now support the interest to assess at the clinical level the effect of the TNH101 drug combination in patients with neuropathic pain, as well as support further exploration of the detailed mechanism that would link gap junctions and the noradrenergic system and lead to potentiate the action on neuropathic pain.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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