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Research report

Indirect involvement of α_2-adrenoceptors in the mechanical antihypersensitivity effect induced by the spinally administered imidazoline I_1 receptor ligand LNP599 in a rat model of experimental neuropathy

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ABSTRACT

Here we assess whether neuropathic pain hypersensitivity is attenuated by spinal administration of the imidazoline I_1-receptor agonist LNP599 and whether the attenuation involves co-activation of α_2-adrenoceptors. Spared nerve injury (SNI) model of neuropathy was used to induce mechanical hypersensitivity in male and female rats with a chronic catheter for intrathecal drug administrations. Mechanical sensitivity and heat nociception were assessed behaviorally in the injured limb. Additionally, GTPγS radioligand binding assay, β-arrestin recruitment and intracellular cAMP levels were used for receptor profiling *in vitro*. LNP599 (imidazoline I_1 receptor agonist) and clonidine (α_2-adrenoceptor agonist) produced equal dose-related mechanical antihypersensitivity effects in both sexes. LNP599 attenuated heat nociception preferentially in males, while clonidine reduced heat nociception equally in males and females. Carbophenyline (another imidazoline I_1 receptor agonist) had no significant effect on mechanical hypersensitivity or heat nociception in males or females. Mechanical antihypersensitivity and heat antinociception induced by LNP599 in SNI males was prevented by pretreatments with yohimbine or atipamezole (two α_2-adrenoceptor antagonists) but not by efaroxan (a mixed imidazoline I_1 receptor/α_2-adrenoceptor antagonist). *In vitro* assays indicated that LNP599 does not activate α_{2A}- or other subtypes of α_2-adrenoceptors. However, LNP599 was a weak partial agonist for 5-HT_{2B} receptors and bound to sigma-1 and sigma-2 receptors that all are involved in modulation of spinal nociception. The results indicate that the suppression of neuropathic pain hypersensitivity by LNP599 is not due to action on spinal imidazoline I_1 receptors, but rather due to indirect activation of spinal α_2-adrenoceptors.

1. Introduction

Imidazoline receptors consist of a group of nonadrenergic binding sites that have affinity to endogenous ligands of the imidazoline receptors, such as agmatine (Bousquet et al., 2020). One major subclass of imidazoline receptors is the I_1 receptor, which is postulated to play a role in mediating the central hypotensive action of α_2-adrenoceptor agonists in the medulla (Regunathan and Reis, 1996). The potential role of imidazoline I_1 receptors in suppression of pain is less clear. From clinical point of view, attenuation of pain by a spinally administered imidazoline I_1 receptor agonist would be of considerable interest allowing a direct exposure of the drug to the spinal site of the ascending pain pathway and thereby, reducing side effects that are most commonly due to supraspinal actions.

Among earlier findings supporting a potential involvement of spinal imidazoline I_1 receptors in pain regulation are that imidazoline receptor-like protein (Ruggiero et al., 1998) and imidazoline I_1 receptor binding (De Vos et al., 1994) have been localized in the spinal dorsal horn. A number of studies have shown that spinal administration of compounds with affinity for imidazoline I_1 receptors attenuates pain-related

Abbreviations: IA, intrinsic activity; I.t., intrathecal; SNI, spared nerve injury.

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responses under physiological or various pathophysiological conditions. Spinal administration of agmatine, an endogenous agonist of imidazoline receptors (Horváth et al., 1999; Fairbanks et al., 2000; Hou et al., 2003; Peterson et al., 2023), or moxonidine, a mixed imidazoline I_1 receptor/α_2 adrenoceptor agonist (Stone et al., 2003), have induced antinociception or antihypersensitivity effect. However, mechanisms other than spinal imidazoline I_1 receptors may play a key role in the attenuation of pain-related responses by these compounds. For example, blocking of N-methyl-D-aspartate receptor and nitric oxide synthetase provide a plausible mechanism for the antihypersensitivity effect of spinal agmatine (Fairbanks et al., 2000), although this proposal is not supported by all studies (Courteix et al., 2007). Furthermore, spinal $\alpha_{2\,C}$-adrenoceptors may mediate the moxonidine-induced suppression of pain-related responses (Stone et al., 2003).

It may be argued that to exclude a potential contribution of spinal imidazoline I_1 receptors to pain regulation, one should still study more selective imidazoline I_1 receptor agonists with no significant action on α_2-adrenergic or other pain suppressive mechanisms activated by agmatine or moxonidine.

Here we studied whether the selective imidazoline I_1 receptor agonist LNP599 (Gasparik et al., 2015) suppresses pain-related behavior following intrathecal (i.t.) administration in the rat. For comparison, we administered i.t. another selective imidazoline I_1 receptor agonist carbophenyline (Del Bello et al., 2015). As an additional comparison drug was the prototype α_2-adrenoceptor agonist clonidine that has an affinity also for the imidazoline I_1 receptor (Szabo, 2002). Moreover, we attempted to reverse the LNP599-induced effects with i.t. administered α_2-adrenoceptor antagonists and a mixed imidazoline I_1 receptor/α_2-adrenoceptor antagonist.

For the sake of clinical relevance, experiments were performed in a rat model of chronic neuropathic pain using both male and female animals. Additionally, we assessed *in vitro* the affinity of the imidazoline receptor I_1 receptor agonist LNP599 for various subtypes of α_2-adrenoceptors and some other neurotransmitter receptors involved in spinal modulation of pain.

2. Materials and methods

2.1. Experimental animals

Adult, male and female Hannover-Wistar rats were used in behavioral experiments (weight: 180–230 g; Envigo, Horst, The Netherlands). The Ethical Committee on Animal Experiments of the regional government of Southern Finland accepted the experimental protocol (permission # ESAVI-41116–2019). The guidelines of European Communities Council Directive 2010/63/EU on the use of animals for scientific purposes were followed. To reduce animal suffering, only a minimal number of animals necessary to produce reliable scientific data were used. Polycarbonate cages, in which the animals were housed, had a deep layer of sawdust. Intrathecal catheter allowed only one animal in each cage, but animals had auditory, visual and olfactory contact with rats in the adjacent cages. The temperature of the animal room was kept at 24.0 ± 0.5ºC. Artificial illumination of the animal room was on from 8.30 a.m. to 8.30 p.m. The cages of the animals were environmentally enriched. Food consisted of commercial pelleted rat pellets (CRM-P pellets, Special Diets Services, Witham, Essex, England) and tap water was available *ad libitum*.

2.2. Techniques for producing neuropathy

To induce peripheral neuropathy, spared nerve injury (SNI) was induced as described by Decosterd and Woolf (2000). For the surgery, anesthesia was induced with sodium pentobarbital (60 mg/kg i.p.; OrionPharma, Espoo, Finland) and maintained at deep level (i.e.; no withdrawal response to noxious pinch) by administering 15–20 mg/kg of sodium pentobarbital as needed for the duration of surgery. Intrathecal (i.t.) catheter was implanted in the same session on Day 0 (D0) as the SNI operation. The SNI operation started with an incision of the skin on the lateral surface of the left thigh that was followed by a section of the biceps femoris muscle. This procedure exposed the sciatic nerve and its three terminal branches: the sural, common peroneal and tibial nerves. Following tight ligation of the common peroneal and tibial nerves with 4–0 silk, the nerve stump 3–4 mm distal to the ligation was removed. The operation left the sural nerve intact. Before moving rats to their individual home cages for recovery, the muscle and skin were sutured. Buprenorphine was given for postoperative pain treatment (0.01 mg/kg twice daily up to the third postoperative day).

2.3. Techniques for microinjections

The surgical procedure for the catheter (PE-10) installation through the lumbar route is described in detail in an earlier publication (Størkson et al., 1996). The correct placing of the catheter was verified following recovery from anesthesia by administering lidocaine i.t. (4 %, 7–10 μl followed by a 15 μl of saline for flushing) using a 50 μl Hamilton syringe. Rats that were studied further had no motor impairment before lidocaine test but had a bilateral paralysis of hind limbs following lidocaine administration. The minimum interval between the lidocaine test and the start of the actual drug testing sessions was 3 days. In all experiments of the present study, the test compounds were microinjected i.t. with a 50 μl Hamilton microsyringe at a volume of 5–7 μl followed by a saline flush at a volume of 15 μl.

2.4. Assessment of mechanical sensitivity

In the clinic, peripheral nerve injury most commonly produces tactile allodynia as shown by cutaneous hypersensitivity to mechanical stimulation, whereas heat hyperalgesia is less frequent (Jensen and Finnerup, 2014). Therefore, here the focus of behavioral assessments was in the tactile allodynia-like symptoms, the index of which was the hind limb withdrawal evoked by stimulation of the intact sural nerve area with monofilaments in the operated side.

The animals were habituated to the experimental laboratory for 1–2 h daily during 2–3 days before actual testing sessions. When assessing tactile allodynia-like hypersensitivity in the rat standing on a metal grid, calibrated monofilaments were applied to the lateral part of the paw that is innervated by the intact sural nerve in the operated limb. The paw was stimulated five times at each stimulus intensity in an ascending series of stimulus forces (1–10 g; North Coast Medical, Inc., Morgan Hill, CA, USA). The index of mechanical sensitivity was the frequency of the limb withdrawal response. An increased withdrawal response rate was considered to represent mechanical hypersensitivity effect.

Previous studies (e.g., Decosterd and Woolf, 2000; Gonçalves et al., 2008; Wei et al., 2022b) have reported that SNI induces strong and long-lasting hypersensitivity to mechanical stimulation of the skin that mimicks tactile allodynia and that is caused by nerve injury rather than an injury of the skin and muscle, except for the first 3 postoperative days. In the present study, the redundancy in reporting is reduced by showing only results for a stimulus force producing a submaximal response that allows observing treatment-induced increases as well as decreases in hypersensitivity. In the present sample of male and female rats, a submaximal stimulus force that proved to produce equal withdrawal response was 6 g in males and 1 g in females. Therefore, these stimulus forces were chosen for assessing sex-related differences in the treatment effect on mechanical hypersensitivity.

In our preceding study, these stimulus forces (6 g in males, and 1 g in females) induced no limb withdrawals in naïve male (n = 11) or female (n = 8) animals of the same rat strain (Wei et al., 2022a). In animals of the present study, the 95 % confidence limits of the response rates evoked by these stimuli varied from 57 % to 72 % in SNI males and from 50 % to 64 % in SNI females indicating development of a significant

SNI-induced mechanical hypersensitivity in both sexes.

2.5. Assessment of heat nociception

Cutaneous heat nociception in the intact suralis nerve-innervated (lateral) area in the operated side was assessed by measuring the latency of the heat-induced limb withdrawal using a radiant heat device (Plantar test model 7370, Ugo Basile, Varese, Italy). Two measurements were made at each time point at one min intervals, and the mean latency of these two measurements was used in further calculations. The mean baseline latency was about 6 s and the cut-off latency was set at 15 s.

The strong mechanical hypersensitivity induced by SNI is not invariably accompanied by heat allodynia/hypersensitivity (Decosterd and Woolf, 2000). In line with this, the 95 % confidence limits of the baseline heat-evoked paw flick latency of naïve male rats of the same strain in our earlier study (Wei et al., 2022a) overlapped with that of SNI males of the present study (from 5.1 s to 6.5 s and from 4.6 s to 6.4 s, respectively). Therefore, the heat-evoked paw withdrawal latency in SNI animals of the current study represents an index of heat nociception rather than heat hypersensitivity or heat hyperalgesia.

2.6. Motor performance

To exclude a motor effect of LNP599, motor activity of the rats was assessed in the Rotarod test using a commercially available device (Ugo Basile) as done in nerve-injured animals earlier (Wei et al., 2016). The revolution speed was 12 revolutions per minute. The rats were put on the drum 30 min after i.t. administration of 10 μg of LNP599 or vehicle on separate days in a counterbalanced order. An additional control group of nerve-injured males was treated with a low sedative dose of sodium pentobarbital (20 mg/kg i.p.) to assess sensitivity of the Rotarod assay. The time animals were able to stay on the drum was calculated. Cut off time was set at 60 s.

2.7. Course of the behavioral study

The nerve injury and the i.t. catheter installation were performed in one operation, after which the animals recovered from the operation for one week. Habituation to the experimental conditions (1–2 h/day for 2–3 days) took place during the recovery period.

All drugs were administered i.t. When assessing the effect of LNP599 (3 μg or 10 μg), clonidine (3 μg or 10 μg), carbophenyline (1 μg or 10 μg) or vehicle on mechanical hypersensitivity or heat nociception, the response to monofilaments or to heat was determined before, 15 min, 30 min and 60 min after drug administration. With attempts to attenuate the antihypersensitivity effect induced by 10 μg of LNP599, the receptor antagonists (yohimbine, atipamezole or efaroxan) were administered 5 min before LNP599; the response to monofilaments or heat was administered before and 30 min after the drug administrations.

When assessing the effect of studied compounds on test stimulus-evoked pain behavior, there were the following treatment groups: i) LNP599 alone at the dose of 3 μg or 10 μg, ii) clonidine alone at the dose of 3 μg or 10 μg, iii) carbophenyline alone at the dose of 1 μg or 10 μg, iv) vehicle alone, v) receptor antagonist (3 μg of yohimbine or 2.5 μg of atipamezole or 1.5 μg of efaroxan) followed 5 min later by 10 μg of LNP599, vi) receptor antagonist alone (3 μg of yohimbine, 2.5 μg of atipamezole or 1.5 μg of efaroxan). In each treatment condition, mechanical hypersensitivity or heat nociception was determined before treatment and for one hour after treatment at 15 min intervals. Testing sessions were performed 7–21 days following SNI operation. Each drug condition was tested on a separate day, at varying order between animals and at an interval of at least 2 days. The number of i.t. injections per one animal varied from 3 to 4.

For comparison of different treatments on mechanical hypersensitivity, the response rates to repetitive stimulation at a force of 6 g (males) or 1 g (females) were standardized in the following way: [the drug-induced change in the response rate = response rate 30 min after drug treatments – response rate before drug treatments]. A change in the response rate < 0 % represents a reduction of hypersensitivity by the studied treatment. The effects of drug treatments on heat nociception (heat-evoked response latency) were standardized in the following way: [the drug-induced change in the latency = latency 30 min after drug treatment – latency before drug treatment]. A change in the latency > 0 s represents a reduction in heat nociception induced by the studied treatment.

Males and females, animals were randomly selected to various experimental groups. Drug treatments were given in a blinded fashion. At the completion of the experiments, the animals were given a lethal dose of sodium pentobarbital.

2.8. In vitro methods

2.8.1. Membrane preparations and [^{35}S]GTPγS binding assays for α_2-adrenoceptor subtypes

Agonist-induced stimulation of [^{35}S]GTPγS binding was measured for the human α_2- adrenoceptor subtypes; α_{2A}, α_{2B} and $\alpha_{2\,C}$, as well as for rodent α_{2A}- adrenoceptor from cell membranes isolated from Chinese Hamster Ovary (CHO) cell lines stably expressing either of the human α_{2A}, human $\alpha_{2\,C}$ and rodent α_{2A}-adrenoceptors (Pohjanoksa et al., 1997; Sallinen et al., 2013). CHO-K1 cell line stably transfected with human α_{2B}-adrenoceptors (Euroscreen, Charleroi, Belgium) was used to isolate membranes containing α_{2B}- adrenoceptor. Before binding assays membrane preparations were prepared as described by Pohjanoksa and co-workers (1997). Briefly, cultured and frozen cell pellets were thawed and suspended in hypotonic lysis buffer (10 mM Tris–HCl, 0.1 mM EDTA, 0.32 mM sucrose, pH 7.4) and homogenised using an Ultra-Turrax homogeniser (3 × 10 s at 8000 rpm). The homogenate was centrifuged at 180 g for 15 min to remove cell nuclei, unbroken cells and aggregates. The supernatants were pooled and centrifuged at 50,227 g for 30 min. The pellet was washed with TE buffer (10 mM Tris, 0.1 mM EDTA) and re-centrifugated as above. The membranes were then suspended in TE buffer, aliquoted and stored at −70 °C until used. Protein concentrations were determined with the method of Bradford (Bradford, 1976) using bovine serum albumin as reference. In [^{35}S]GTPγS binding assays, frozen cell membranes were thawed and diluted with binding buffer (25 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, 20 mM NaCl, 1 μM GDP, 1 mM DTT, pH 7.4). Incubations were performed on 96-well Millipore MultiScreen MSFBN glass-fibre filter plates. Samples containing 10 μg of membrane protein were incubated with serial dilutions of the test compounds and 0.1 nM [^{35}S]GTPγS (NEGO30H, PerkinElmer). Reactions were terminated after 30 min incubation at RT by rapid vacuum filtration using a Millipore MultiScreen Vacuum Manifold. The filter plates were washed three times with ice-cold wash buffer (20 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, pH 7.4). Filters were dried and 50 μl SuperMix scintillation cocktail was added into each well. The incorporated radioactivity was measured using a MicroBeta2 microplate counter (PerkinElmer). All experiments were performed in duplicate. Results were analysed using GraphPad Prism software. All results were expressed as estimates of agonist potency (EC_{50}) and intrinsic activity (IA) in comparison to noradrenaline (10 μM), which was considered a full agonist on α_2-adrenoceptors.

2.8.2. β-arrestin recruitment and cAMP production for α_2-adrenoceptor subtypes

Investigation of agonist-induced β-arrestin-2 recruitment in cell lines stably overexpressing either human α_{2A}, α_{2B} or $\alpha_{2\,C}$- adrenoceptor was performed using PathHunter® β-Arrestin assay by Eurofins (CA, USA). DiscoverX HitHunter cAMP assay was used to study the effect of test compounds on cAMP production in presence of forskolin (20 μM) in cell lines stably overexpressing either human α_{2A}, α_{2B} or $\alpha_{2\,C}$ - adrenoceptor by Eurofins (CA, USA and Celle l'Evescault; France).

2.8.3. Selectivity profiling in binding and functional assays

The selectivity profile assessment of LNP599 was done at 10 μM concentration for 121 receptors, ion channels and enzymes by Eurofins CEREP SA (Celle-Lévescault, France; Table 1). LNP599 binding was calculated as a % inhibition of the binding of a ligand specific for each target. Compound enzyme inhibition effect was calculated as a % inhibition of control enzyme activity. Based on the hits in the binding profile, further dose–response assessment of LNP599 in binding assays for the $Sigma_{1/2}$ receptors was performed by Eurofins (Celle l'Evescault; France).

2.8.4. 5-HT_{2B} functional assay

Chinese hamster ovary cells stably expressing human 5-HT2B receptor (Euroscreen, Belgium) were cultured in Hamś F12 medium supplemented with 10 % dialysed foetal bovine serum (FBS), 25 mM HEPES, 100 IU/ml penicillin, 100 μg/ml streptomycin, 500 μg/ml G-418 and 250 μg/ml zeocin. Cells were maintained at 37°C in a 5 % CO_2/95 % air atmosphere and subcultured three times weekly with 0.25 % trypsin and 1 mM EDTA. The subculture ratios were 1:5–1:20.

The day before the experiment, the cells were plated into black-walled, clear bottom 96-well plates at a density of 30,000 cells/well in Hamś F12 medium supplemented with 1 % dialysed FBS. The medium was removed, and cells were incubated with the FLIPR Calcium 6 Assay reagent (Molecular Devices, CA, USA) for 1.5 hr at 37°C in dark followed by agonist stimulation. LNP599 (0.1–10 000 nM) was diluted in Probenecid–Ringer consisting of (in mM): 150 NaCl, 3 KCl, 1.2 MgCl2, 1 CaCl2, 5 glucose, 20 HEPES and 2.5 probenecid (pH 7.4 adjusted with 1.0 M NaOH). Osmolarity was adjusted to 322 mOsm. Changes in intracellular calcium were monitored using fluorescence plate reader FLIPR Tetra (Molecular Devices, CA, USA). The samples were excited at 485 nm, and emission was detected at 525 nm with a 515 nm cut-off filter. The minimum fluorescence value subtracted from the maximum for each well used in the calculations.

2.9. Drugs

LNP599 (imidazoline I_1 receptor agonist; Fellmann et al., 2013) and carbophenyline (another imidazoline I_1 receptor agonist; Del Bello et al., 2015) were synthetized by Orion Pharma (Turku, Finland). Clonidine HCl (the prototype α_2-adrenoceptor agonist with imidazoline structure and with affinity for imidazoline I_1 receptors; Szabo, 2002) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Yohimbine HCl (α_2-adrenoceptor antagonist with no affinity for imidazoline receptors; Savola and Savola, 1996) and efaroxan HCl (mixed imidazoline I_1 receptor/α_2-adrenoceptor antagonist; Haxhiu et al., 1994) were purchased from Tocris Bioscience (Bristol, UK). Atipamezole (α_2-adrenoceptor antagonist that has affinity also for imidazoline receptors; (Savola and Savola, 1996) was synthetized by OrionPharma. Physiological saline was used as control. Additionally, noradrenaline and the α_2-adrenoceptor agonist dexmedetomidine (OrionPharma) were used only for *in vitro* assays. The structure of LNP599 is illustrated in Fig. 1 by Bousquet et al. (2020), those of efaroxan, clonidine and yohimbine in Fig. 1 by Szabo (2002), that of carbophenyline in Fig. 1 by Micheli et al. (2020), and that of atipamezole in Fig. 1 by Pertovaara et al. (2005).

Table 1
LNP599 (10 μM) in binding, enzyme and uptake assays (CEREP, Eurofins).

Assay	1.0E-0.5 M
α_2-Adrenoceptor (non-selective)(antagonist radioligand)	59.5 %
Imidazoline I_2 receptor (antagonist radioligand)	56.8 %
5-HT_{2b} (h) receptor (agonist radioligand)	66.5 %
Sigma (non-selective) (h) receptor (agonist radioligand)	95.6

Compound binding was calculated as a % inhibition of the binding of a ligand specific for each target. Compound enzyme inhibition effect was calculated as a % inhibition of control enzyme activity. Results showing an inhibition or stimulation higher than 50 % are considered to represent significant effects of the test compounds. Profiling included 121 assays but not assay for imidazoline I_1 receptor binding site.

The doses of the currently used neurotransmitter receptor antagonists for the *in vivo* study were chosen based on preliminary experiments and our earlier studies showing that following i.t. administration they reverse effects of agonists but have no effects alone in neuropathic animals (Wei et al., 2014).

2.10. Statistical analyses

Statistical evaluation of the data was performed using one- or two-way ANOVA, with mixed design when appropriate, followed by t-test with Bonferroni correction for multiple comparisons, or with unpaired t-test when comparing two groups. However, Rotarod data was analyzed using non-parametric Kruskal-Wallis test followed by Dunn's test. $P < 0.05$ (two-tailed) was considered to represent a significant difference.

3. Results

3.1. Mechanical hypersensitivity

Mechanical hypersensitivity induced by SNI was significantly stronger in males than females (main effect of gender: $F_{1,\ 13} = 90.1$, $P < 0.0001$; Fig. 1 A). Stimulus forces of 6 g in SNI males and 1 g in SNI females produced submaximal response rates that were of equal magnitude ($t_{13} = 1.8$; Fig. 1 B). These submaximal stimulus forces producing equivalent responses in males and females were chosen for the assessment of the treatment-induced effect on mechanical hypersensitivity and its potential variation with sex.

LNP599 produced a mechanical antihypersensitivity effect that was significant 15–30 min after treatment and that disappeared by 60 min after treatment (Fig. 2 A). The mechanical antihypersensitivity effect induced by i.t. treatment with LNP599 was dose-related (3–10 μg; main effect of dose: $F_{2,\ 36} = 24.3$, $P < 0.0001$), and the antihypersensitivity effect did not vary with sex (interaction between force and sex: $F_{2,\ 36} = 0.6$; Fig. 2 B). *Post hoc* testing indicated that the mechanical antihypersensitivity effect induced by LNP599 was significant in both males and females at the dose of 10 μg but not yet at the dose of 3 μg (Fig. 2 B).

Clonidine, the prototype α_2-adrenoceptor agonist, induced a dose-related mechanical antihypersensitivity effect (main effect of dose: $F_{2,\ 33} = 28.8$, $P < 0.0001$) that did not vary with sex (interaction dose x sex: $F_{2,\ 33} = 0.03$; Fig. 2 C). According to *post hoc* tests, the mechanical antihypersensitivity effect of clonidine was significant in both sexes at the dose of 10 μg but not at the dose of 3 μg (Fig. 2 C).

Carbophenyline, a selective imidazoline I_1 receptor agonist, failed to induce a significant change in mechanical hypersensitivity when administered i.t. at doses of 1 μg or 10 μg (main effect of dose: $F_{2,\ 22} = 0.5$), independent of sex (interaction dose x sex: $F_{2,\ 22} = 1.1$; Fig. 2 D).

Three different i.t. administered receptor antagonists were used in attempts to prevent the development of the mechanical antihypersensitivity effect induced by 10 μg of LNP599 in male SNI animals. In attempts to attenuate the LNP599-induced mechanical antihypersensitivity effect with various receptor antagonists, the main effect of drug treatments was significant ($F_{4,\ 29} = 9.6$, $P < 0.0001$; Fig. 2 E). *Post hoc* testing indicated that pretreatments with α_2-adrenoceptor antagonist yohimbine (3 μg) or atipamezole (2.5 μg) reduced the LNP599-induced mechanical antihypersensitivity effect (Fig. 2 E). Pretreatment with the mixed imidazoline I_1 receptor/α_2-adrenoceptor antagonist efaroxan (1.5 μg) failed to reverse the LNP599-induced mechanical antihypersensitivity effect (Fig. 2 E). Yohimbine (3 μg), atipamezole (2.5 μg) or efaroxan (1.5 μg) alone had no significant effect on mechanical hypersensitivity in SNI males (main effect drugs: $F_{4,\ 25} = 1.2$; Fig. 2 F). Nor did yohimbine alone (3 μg) influence mechanical hypersensitivity in SNI females (Fig. 2 F).

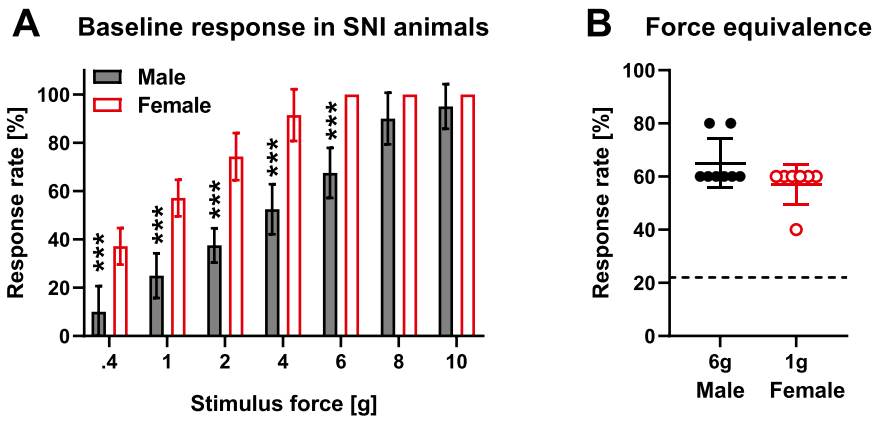

Fig. 1. Baseline mechanical hypersensitivity following spared nerve injury (SNI) varies with sex. (A). Hind limb withdrawal rate to repeated mechanical stimulation at various stimulus forces. (B) Stimulus forces chosen for assessing drug effects evoked equal responses in male and female SNI animals. The graphs show mean response rates in each stimulus condition and the error bars represent S.D. ($n_{Male} = 8$, $n_{Female} = 7$). The higher the response rate in Y-axis, the stronger the mechanical hypersensitivity. ***$P < 0.005$ (Bonferroni-corrected t-test; reference: the corresponding value in females).

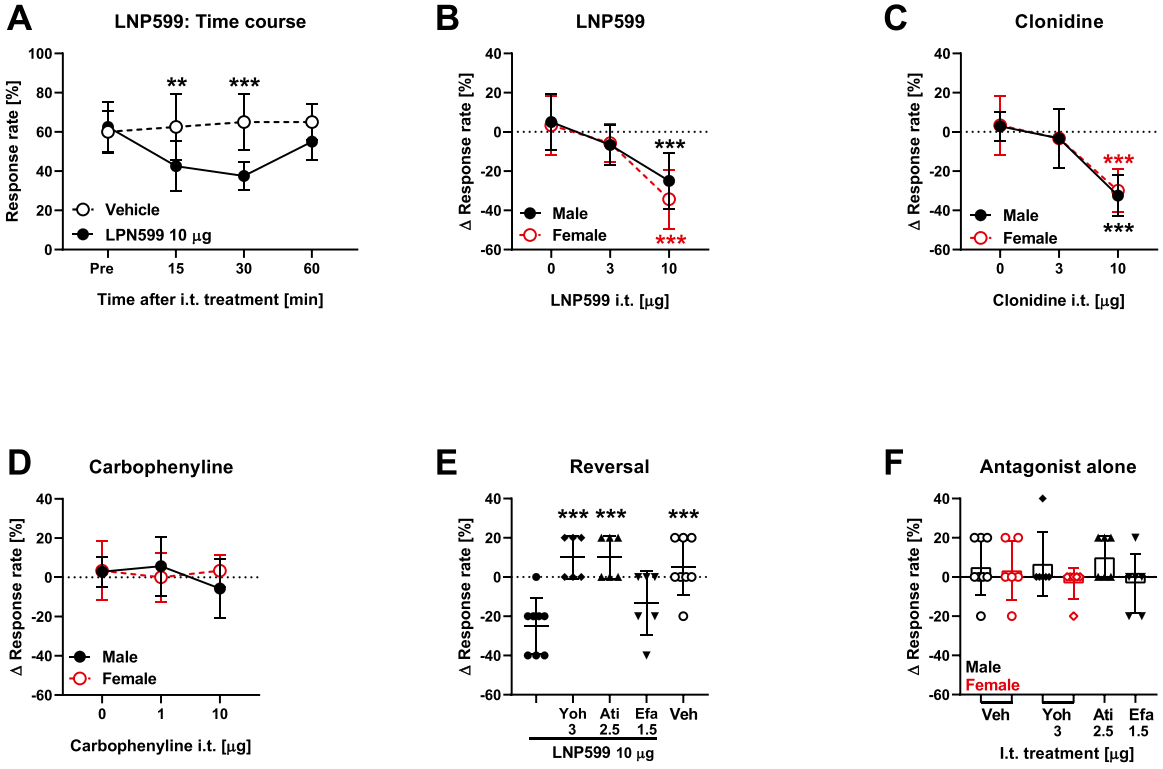

Fig. 2. Mechanical antihypersensitivity effect induced by LNP599 or clonidine in animals with spared nerve injury (SNI). (A) Time course of the anti-hypersensitivity effect induced by LNP599 (LNP) in male SNI animals. (B) Dose-related antihypersensitivity effects induced by LNP599. (C) Dose-related anti-hypersensitivity effect induced by clonidine. (D) Lack of effect by carbophenyline (CBP). (E) The antihypersensitivity effect of LNP599 (10 µg) was prevented by pretreatment with yohimbine (Yoh+; 3 µg) or atipamezole (Ati; 2.5 µg), but not by pretreatment with efaroxan (EFA; 1.5 µg) or by yohimbine administered after LNP599 (+Yoh; 3 µg). (F) Lack of effect by receptor antagonists alone. All drugs were administered intrathecally (i.t.). Veh, vehicle (= 0 µg in B-D). Testing was performed at the force of 6 g in males and 1 g in females that produce equal responses in pre-drug conditions. The graphs show mean values, and error bars represent S.D. (n = 6–8). In A, the Y-axis represents the absolute response rate to repeated mechanical stimulation; the higher the response rate, the stronger the mechanical hypersensitivity. In B-F, the Y-axis represents the drug-induced change in the response rate at the time point of maximal drug effect; values < 0 % represent drug treatment-induced reduction of hypersensitivity. **$P < 0.01$, ***$P < 0.005$ (Bonferroni-corrected t-test; Reference in B-C, the corresponding vehicle value. Reference in E, LNP599 alone at the dose of 10 µg).

3.2. Heat nociception

Baseline latencies of the noxious heat-evoked withdrawals of the injured hind limb were not significantly different between SNI males and SNI females ($t_{12} = 0.6$; Fig. 3 A). LNP599 had a significant main effect on heat nociception ($F_{2, 37} = 6.2$, $P = 0.0048$), and the effect of LNP599 was significantly different between SNI males and SNI females ($F_{1, 37} = 5.4$, $P = 0.0251$; Fig. 3 B). *Post hoc* testing indicated that LNP599 induced significant heat antinociception only in males and only at the dose of 10 µg (Fig. 3 B).

Clonidine had a significant effect on heat nociception (main effect of drug: $F_{2, 33} = 12.8$, $P < 0.0001$) that did not vary with sex (interaction

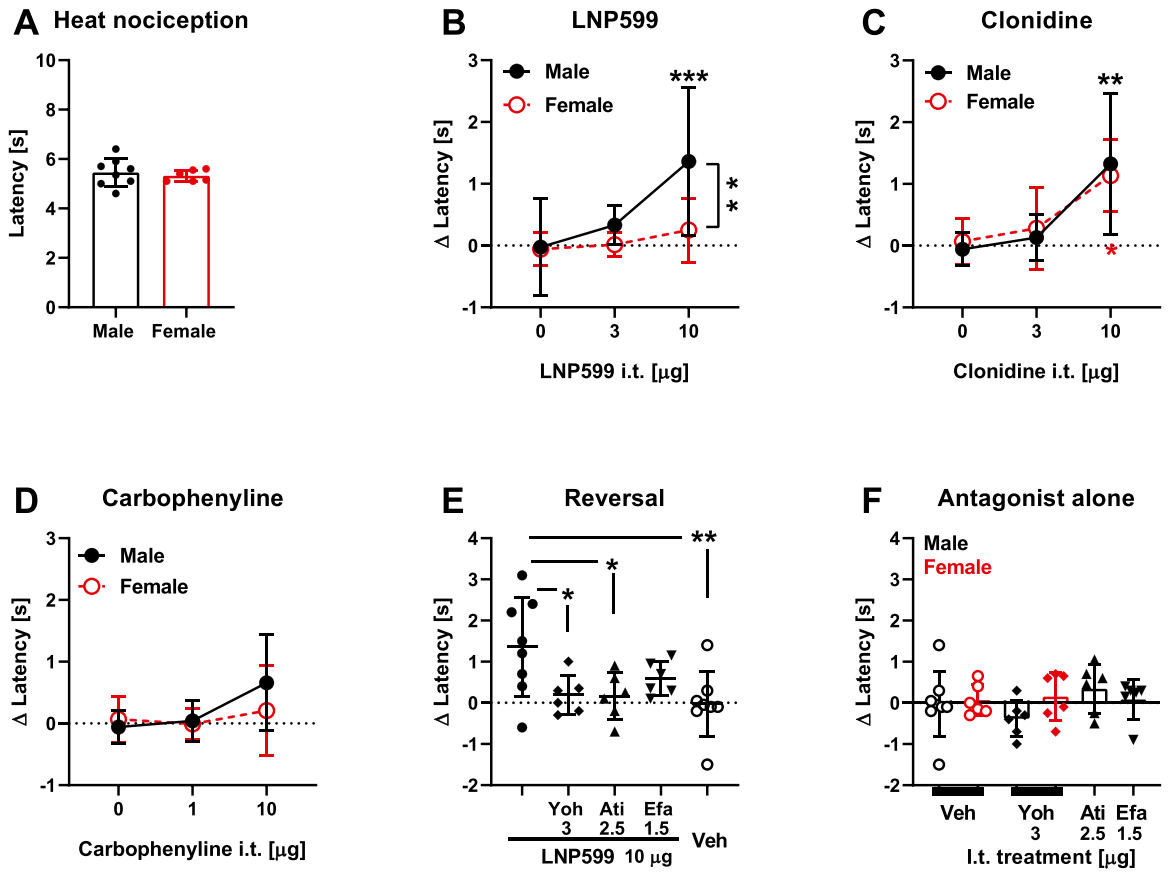

Fig. 3. Attenuation of heat nociception by LNP599 or clonidine in animals with spared nerve injury (SNI). (A) Baseline heat nociception in the injured limb. (B) Sexually dimorphic attenuation of heat nociception by LNP599 (LNP). (C) Attenuation of heat nociception by clonidine. (D) Lack of significant effect on heat nociception by carbophenyline. All drugs were administered intrathecally (i.t.). Veh, vehicle (= 0 µg in B-D). The graphs show mean values, and error bars represent S.D. (n = 6–8). In B-D, the Y-axis represents the drug-induced change in the latency of the heat-evoked limb withdrawal at the time point of maximal drug effect; values > 0 % represent drug treatment-induced heat antinociception. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ (Bonferroni-corrected t-test; Reference in B-C, the corresponding vehicle value.).

drug x sex: $F_{2, 33} = 0.3$; Fig. 3 C). *Post hoc* testing indicated that clonidine induced significant heat antinociception in both males and females at the dose of 10 µg but not yet at the dose of 3 µg (Fig. 3 C). Carbophenyline failed to induce a significant change in heat nociception (main effect of drug: $F_{2, 33} = 2.9$), independent of sex (interaction drug x sex: $F_{2, 33} = 1.1$; Fig. 3 D).

In attempts to attenuate the LNP599-induced heat antinociception with various receptor antagonists in SNI males, the main effect of drug treatments was significant ($F_{4, 29} = 3.8$, $P < 0.01$; Fig. 3 E). Heat antinociception induced by 10 µg of LNP599 was reversed by pretreatment with yohimbine (3 µg) or atipamezole (2.5 µg), but not with efaroxan (1.5 µg) in SNI males (Fig. 3 E). Yohimbine (3 µg), atipamezole (2.5 µg) or efaroxan (1.5 µg) alone had no significant effect on heat nociception in SNI males (Fig. 3 F). Nor did yohimbine alone (3 µg) influence heat nociception in SNI females (Fig. 3 F).

3.3. Motor performance

The effect of 10 µg of LNP599 versus vehicle on motor performance in the Rotarod test was assessed only in male SNI animals. A group of SNI males treated with a sedative dose of sodium pentobarbital (20 mg/kg i.p.) served as a control for testing assay sensitivity. The main effect of drug treatments was significant among the three test groups ($KW = 16.15$, $P = 0.0002$; Fig. 4). *Post hoc* testing indicated that all SNI males treated with LNP599 as well as all those treated with vehicle reached the cut-off latency and thereby showed no reduction in motor performance, whereas SNI males treated with a sedative dose of sodium pentobarbital had significantly reduced drop latencies (Fig. 4).

3.4. In vitro assays for α_2-adrenoceptors

α_2-Adrenoceptor pharmacology was studied using functional [^{35}S] GTPγS binding assays on membranes isolated from CHO cells stably expressing human α_2-adrenoceptor subtypes or rodent α_{2A}-adrenoceptor (Fig. 5). LNP599 induced only a minor if any activation of α_2-adrenoceptors that contrasts with the strong activation of α_2-adrenoceptors by established α_2-adrenoceptor agonists noradrenaline, clonidine or dexmedetomidine. Agonistic activity of LNP599 on human α_{2A}-, α_{2B}- and α_{2C}- adrenoceptors was also studied using HitHunter cAMP and PathHunter β-arrestin assays (Fig. 6). In these *in vitro* assays, LNP599 did not present any functional activity on α_2 adrenoceptor subtypes.

3.5. In vitro assay for 5-HT$_{2B}$ receptors

LNP599 was a weak, partial agonist for 5-HT$_{2B}$ receptors (EC_{50} = 6490 nM, IA = 0.42), as revealed by intracellular Ca^{2+} changes in CHO cells stably overexpressing 5-HT$_{2B}$ receptor (Fig. 7). LNP599 did not inhibit 5-HT induced Ca^{2+} responses in CHO cells stably overexpressing 5-HT$_{2B}$ receptor (data not shown).

3.6. In vitro assays for Sigma-1 and Sigma-2 receptors

Sigma-1 agonist binding assay was performed using ^{3}H-pentazocine, whereas Sigma-2 binding was performed using ^{3}H-di-o-tolylguanidine

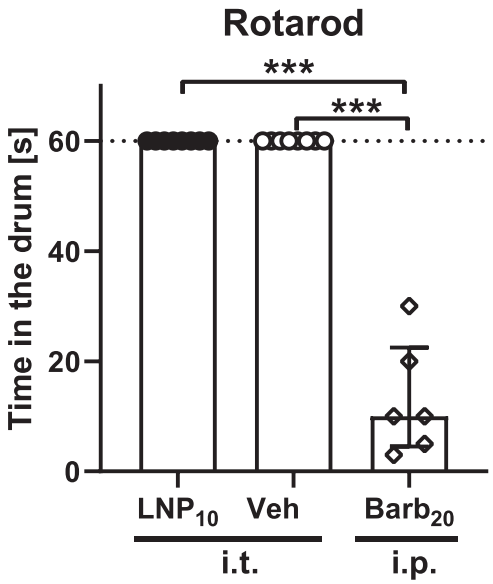

Fig. 4. Lack of effect by LNP599 on motor behavior in male animals with spared nerve injury. Lack of effect by i.t. treatment with 10 μg of LNP599 (LNP) on Rotarod performance, whereas a sedative dose of sodium pentobarbital (Barb; 20 mg/kg i.p.) significantly decreased the drop latency. Veh, vehicle. The cut-off latency of 60 s is shown by a dotted horizontal line. The bars show median values, and the error bars represent interquartile ranges. *** $P < 0.0002$ (Dunn's test) $n_{LNP/Veh} = 8$, $n_{Barb} = 6$.

(DTG) in presence of 1 μM pentazocine (Eurofins Discovery).LNP599 bound to Sigma-1 (IC_{50} = 710 nM, Ki = 360 nM) and Sigma-2 (IC_{50} = 400 nM, Ki= 300 nM) in Jurkat cells (Fig. 8).

4. Discussion

4.1. Attenuation of stimulus-evoked pain behavior in neuropathy

In the present study, spinally administered imidazoline I_1 receptor agonist LNP599 and the prototype α_2-adrenoceptor agonist clonidine induced equal dose-related mechanical antihypersensitivity effects in neuropathic animals. The LNP599-induced antihypersensitivity effect was prevented by α_2-adrenoceptor antagonists yohimbine or atipamezole. In contrast, a mixed imidazoline I_1 receptor/α_2-adrenoceptor antagonist efaroxan failed to prevent the LNP599-induced mechanical antihypersensitivity effect. These behavioral findings suggest that spinal α_2-adrenoceptors rather than imidazoline I_1 receptors play a role in the antihypersensitivity effect induced by LNP599 in neuropathy, although the role of α_2-adrenoceptors needs to be indirect (see below section on receptor binding *in vitro*). Further evidence against the involvement of spinal imidazoline I_1 receptors in pain regulation is that spinal administration of carbophenyline, another imidazoline I_1 receptor agonist, had no significant effect on mechanical hypersensitivity or heat nociception in neuropathic animals.

4.2. Receptor binding in vitro

Earlier *in vitro* results indicate that LNP599 has no affinity for α_{2A}-adrenoceptors (Gasparik et al., 2015). The present *in vitro* study confirms and extends this earlier finding by showing that LNP599 does not bind to α_{2A}-, α_{2B}- or α_{2C}-adrenoceptors. This finding indicates that the antihypersensitivity effect was not due to a direct action of LNP599 on spinal α_2-adrenoceptors. Furthermore, the present *in vitro* study showed that LNP599 is a weak partial agonist for 5-HT_{2B} receptors and binds to sigma-1 and sigma-2 receptors. All of these three receptors are involved in spinal regulation of neuropathic hypersensitivity. The 5-HT_{2B} receptor (Heijmans et al., 2021) and the sigma-1 receptor (Bravo-Caparrós

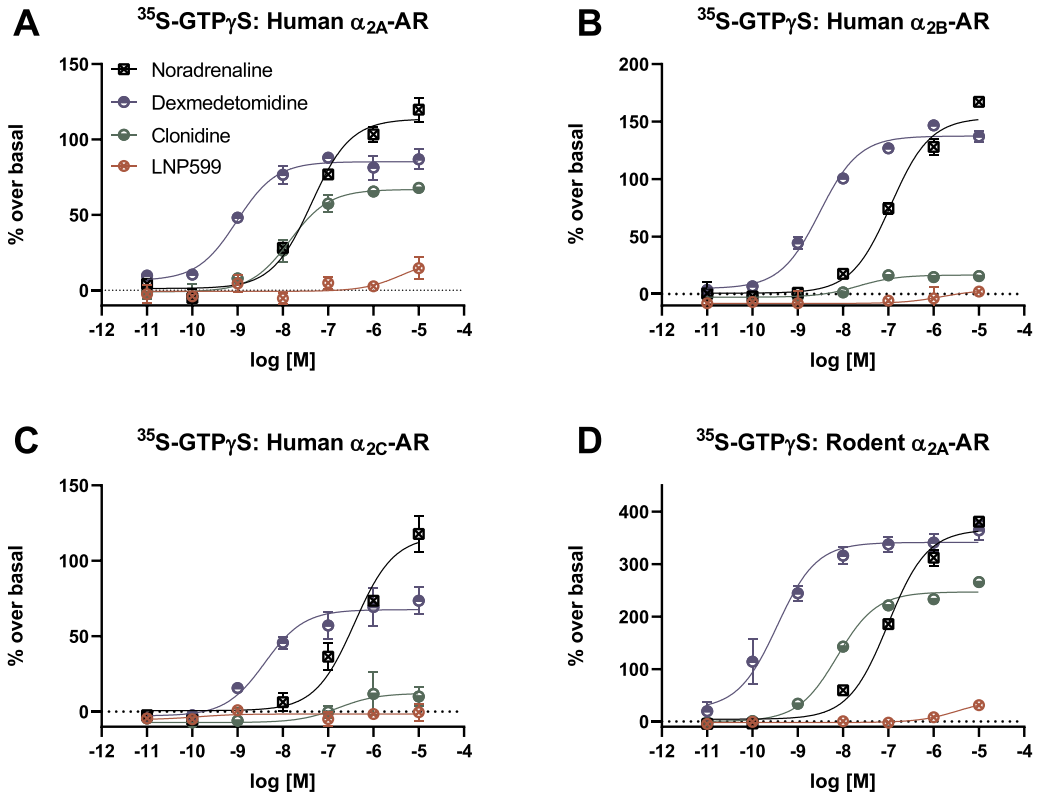

Fig. 5. LNP599 does not activate α_2-adrenoceptors in [^{35}S] GTPγS binding assays. LNP599 (unlike α_2-adrenoceptor agonists noradrenaline, clonidine and dexmedetomidine) did not induce [^{35}S]GTPγS binding on membranes isolated from CHO cells stably expressing human α_2-adrenoceptor subtypes α_{2A} (A), α_{2B} (B), α_{2C} (C), or rodent α_{2A}-adrenoceptors (D). Error bars represent S.D. AR, adrenoceptor.

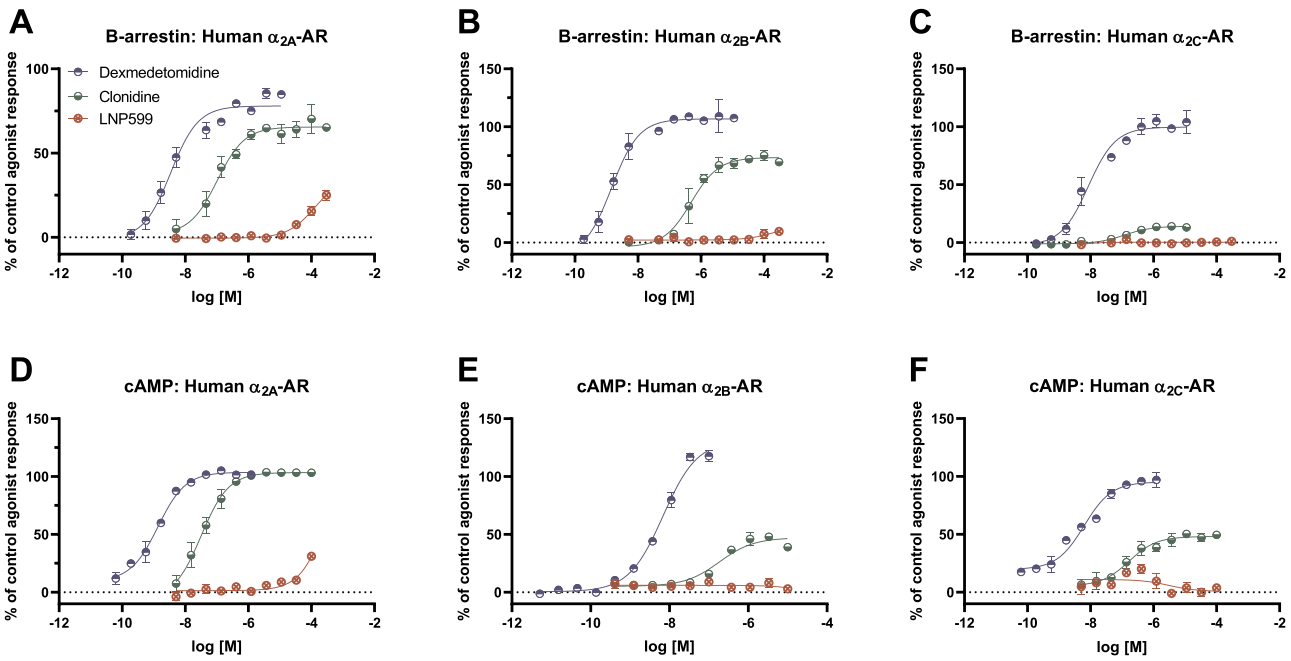

Fig. 6. LNP599 does not activate α_2-adrenoceptors in HitHunter cAMP or PathHunter β-arrestin assays. LNP599 did not present any functional activity on human α_{2A}-adrenoceptors (A, D), α_{2B}-adrenoceptors (B, E), or α_{2C}-adrenoceptors (C, F) either in the β-arrestin (A-C) or HitHunter cAMP assays (D-F), unlike α_2-adrenoceptor agonists dexmedetomidine or clonidine. Error bars represent S.D. AR, adrenoceptor.

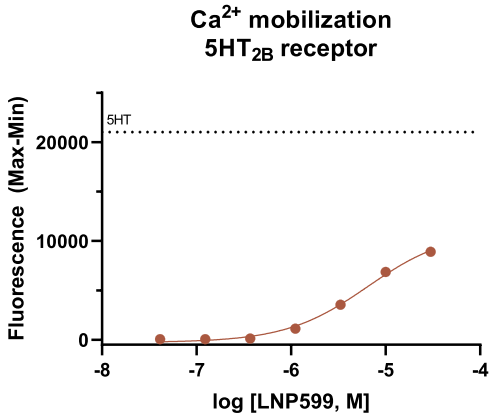

Fig. 7. LNP599 is a weak partial agonist for 5-HT_{2B} receptors. LNP599 was a weak, partial agonist for 5-HT_{2B} receptors (EC_{50} = 6490 nM, IA = 0.42). The dotted horizontal line represents the result induced by 1 μM of the endogenous ligand (5-HT).

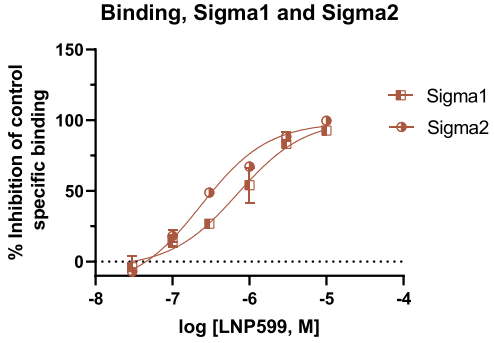

Fig. 8. LNP599 binds to sigma-1 and sigma-2 receptors. LNP599 bound to Sigma-1 (IC_{50}=710 nM, Ki = 360 nM) and Sigma-2 (IC_{50}=400 nM, Ki= 300 nM) receptors. Error bars represent S.D.

et al., 2019; Choi et al., 2022) promote neuropathic hypersensitivity, whereas the sigma-2 receptor attenuates neuropathic hypersensitivity (Sahn et al., 2017). Thus, direct action of LNP599 on the sigma-2 receptor may have contributed to the attenuation of neuropathic hypersensitivity. However, the onset and duration of the antihypersensitivity effect induced by spinally administered sigma-2 receptor agonists (Sahn et al., 2017) is an order of magnitude longer than following LNP599 in the present study. Moreover, the proposed involvement of the sigma-2 receptor as a mediator of the LNP599-induced effect does not explain why α_2-adrenoceptor antagonists prevented the antihypersensitivity effect of LNP599.

Interestingly, earlier results indicate that sigma-1 receptor activation potentiates release of noradrenaline in the nervous system (Monnet et al., 1995). Based on this earlier finding, it may be speculated that among upstream mechanisms contributing to the LNP599-induced antihypersensitivity effect is sigma-1 receptor-mediated potentiation of noradrenaline release leading to α_2-adrenoceptor-mediated attenuation of pain hypersensitivity.

4.3. Sexual dimorphism

Pain and its underlying mechanisms may vary with sex (Mogil, 2020). Earlier studies assessing sexual dimorphism of pain hypersensitivity in experimental models of neuropathy other than SNI have produced variable results (Mogil et al., 2024). Previous studies using SNI model in rats have, however, have reported significantly stronger mechanical hypersensitivity in female than male rats (Ahlström et al., 2021; Wei et al., 2022b, 2023), which is in line with current results in SNI rats. In contrast to findings in SNI rats, some earlier studies in the mouse SNI model have failed to demonstrate a sex difference in mechanical hypersensitivity (see for references and potential explanations Discussion in Wei et al., 2023). The sex-difference of neuropathic hypersensitivity in SNI rats can reflect sexual dimorphism in pain processing and modulating pathways at multiple levels of the nervous system (Mogil, 2020).

In the present study, the mechanical antihypersensitivity effect of LNP599 or that of the prototype α_2-adrenoceptor agonist clonidine did

not vary with sex. However, LNP599 induced significant heat antinociception only in males, whereas clonidine induced equal heat antinociception in both males and females. These findings further emphasize that spinal mechanisms underlying the modulation of pain-related behavior by LNP599 at least partly differ from those of clonidine.

Yohimbine alone had no effect on mechanical hypersensitivity or heat nociception in males or females suggesting that independent of sex, there was no tonic noradrenergic feedback inhibition controlling pain-related behavior in neuropathic animals. Thereby, sexual dimorphism in descending noradrenergic inhibition is not likely to explain the observed sex difference in neuropathic hypersensitivity. In line with the present behavioral results obtained more than week after SNI operation, recent results in SNI males indicate that tonic noradrenergic inhibition is observed only for the first 1–3 days after the SNI operation (Llorca-Torralba et al., 2022).

4.4. Caveats

Earlier studies have shown attenuation of pain-related responses following spinal administration of a presumed endogenous imidazoline I_1 receptor agonist agmatine or the mixed imidazoline I_1/α_2-adrenocceptor agonist moxonidine (see Introduction). These earlier results need not be in discrepancy with the present findings, since there is evidence indicating that the attenuation of pain-related behavior by spinally administered agmatine is mediated by non-imidazoline and non-α_2-adrenergic mechanisms ((Fairbanks et al., 2000); however, (Courteix et al., 2007)). Moreover, spinal $\alpha_{2\,C}$-adrenoceptors mediate the pain suppression induced by moxonidine (Stone et al., 2003).

Interestingly, α_2-adrenoceptor antagonist yohimbine has affinity for 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1A} receptors (Maroteaux et al., 1992; Hamblin and Metcalf, 1991; Millan et al., 2000). However, the α_2-adrenoceptor antagonist atipamezole that has only negligible affinity for these subtypes of 5-HT_1 receptors (Pertovaara et al., 2005) was as effective as yohimbine in reversing the LNP599-induced antihypersensitivity effect. This finding supports the interpretation that α_2-adrenoceptors rather than 5-$HT_{1B/1D/1A}$ receptors were involved in the LNP599-induced antihypersensitivity effect.

The failure to reverse the LNP599-induced antihypersensitivity effect with the mixed imidazoline I_1/α_2-adrenoceptor antagonist efaroxan needs to be interpreted cautiously. While only one dose of efaroxan was tested, the dose used was higher than that reversing the antinociceptive actions of moxonidine or the α_2-adrenoceptor agonist dexmedetomidine in the mouse spinal cord (Fairbanks and Wilcox, 1999). Importantly, the failure to recapitulate the LNP599-induced antihypersensitivity effect with a selective imidazoline I_1 receptor agonist carbophenyline is in line with the efaroxan result. Still, it remains a puzzling finding why two established α_2-adrenoceptor antagonists (yohimbine and atipamezole) reversed the antihypersensitivity effect induced by LNP599, unlike efaroxan, a mixed imidazoline I_1 receptor/α_2-adrenoceptor antagonist.

A recent study reported attenuation of neuropathic hypersensitivity by systemic administration of a selective imidazoline I_1 receptor agonist carbophenyline (Micheli et al., 2020). This recent finding with systemic drug administration together with the lack of pain suppressive effect by spinally administered imidazoline I_1 receptor agonists in the present study suggests that activation of supraspinal rather than spinal imidazoline I_1 receptors attenuates neuropathic pain. It should also pointed out that imidazoline I_2 receptors, which play a significant role in pain modulation (see references in Bousquet et al., 2020), were outside the focus of the present study.

Rotarod assay for excluding treatment-induced motor effects was performed only in males. It should be noted, however, that in females LNP599 attenuated only mechanically but not heat-evoked limb flexion reflex responses, although the motor part of the flexion reflex response evoked by heat and mechanical stimulation did not vary with the modality of test stimulation. Therefore, the LNP599-induced antihypersensitivity effect in females is due to suppression of spinal sensory rather than motor neurons.

Current *in vitro* assays indicate that LNP599 binds to sigma-1 and sigma-2 receptors. However, our *in vitro* results do not allow concluding whether LNP599 activates or inhibits sigma-1 or sigma-2 receptors. Nor did we assess noradrenaline release in the present study. Therefore, further experiments are still needed to test the proposal that activation of sigma-1 receptors by LNP599 causes noradrenaline release providing the link for α_2-adrenoceptors in the LNP-induced antihypersensitivity effect. Moreover, an additional caveat concerning this hypothesis is that while activation of sigma-1 receptors is expected to potentiate noradrenaline release (Monnet et al., 1995) and thereby enhance noradrenergic inhibition of pain, sigma-1 receptor activation is expected to have in parallel an opposite effect, promotion of neuropathic hypersensitivity (Bravo-Caparrós et al., 2019; Choi et al., 2022).

5. Conclusions

Spinal administration of LNP599, an imidazoline I_1 receptor agonist, attenuated mechanical hypersensitivity and heat nociception in a rat model of peripheral neuropathy. The mechanical antihypersensitivity of effect of LNP599 did not vary with sex, while heat antinociception was induced only in males. Since the mechanical antihypersensitivity effect was not recapitulated by carbophenyline, another imidazoline I_1 receptor agonist, nor prevented by efaroxan, a mixed imidazoline I_1/α_2-adrenoceptor antagonist, spinal imidazoline I_1 receptors were not likely to mediate the effect of LNP599. The mechanical antihypersensitivity effect of LNP599 was prevented by yohimbine or atipamezole, two α_2-adrenoceptor antagonists. The role of spinal α_2-adrenoceptors was indirect, since *in vitro* assays showed that LNP599 does not bind to α_2-adrenoceptors. Interestingly, *in vitro* assays showed that LNP599 has affinity for 5-HT_{2B} and sigma1/2 receptors, the contributions of which to the LNP599-induced pain modulation remain to be studied.

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CRediT authorship contribution statement

Antti Pertovaara: Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Ari Koivisto:** Writing – original draft, Supervision, Conceptualization. **Andrii Domanskyi:** Validation, Methodology, Investigation. **Hong Wei:** Visualization, Validation, Methodology, Investigation. **Jonne Laurila:** Validation, Methodology, Investigation. **Anne Vuorenpää:** Visualization, Validation, Methodology, Investigation, Formal analysis.

Declaration of Competing Interest

Three of the authors (A.V., A.D., and A.K.) are employees of a pharmaceutical company (Orion Pharma). One of the authors (J.L.) has received funding from a pharmaceutical company (Orion Pharma). Other authors (H.W. and A.P.) declare no competing interests.

Data availability

Data will be made available on request.

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