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Spinal activation of the NPY Y1 receptor reduces mechanical and cold allodynia in rats with chronic constriction injury

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ABSTRACT

Neuropeptide tyrosine (NPY) and its associated receptors Y1R and Y2R have been previously implicated in the spinal modulation of neuropathic pain induced by total or partial sectioning of the sciatic nerve. However, their role in chronic constrictive injuries of the sciatic nerve has not yet been described. In the present study, we analyzed the consequences of pharmacological activation of spinal Y1R, by using the specific Y1R agonist $Leu^{31}Pro^{34}$-NPY, in rats with chronic constriction injury (CCI). CCI and sham-injury rats were implanted with a permanent intrathecal catheter (at day 7 after injury), and their response to the administration of different doses (2.5, 5, 7, 10 or 20 μg) of $Leu^{31}Pro^{34}$-NPY (at a volume of 10 μl) through the implanted catheter, recorded 14 days after injury. Mechanical allodynia was tested by means of the up-and-down method, using von Frey filaments. Cold allodynia was tested by application of an acetone drop to the affected hindpaw. Intrathecal $Leu^{31}Pro^{34}$-NPY induced an increase of mechanical thresholds in rats with CCI, starting at doses of 5 μg and becoming stronger with higher doses. Intrathecal $Leu^{31}Pro^{34}$ also resulted in reductions in the frequency of withdrawal to cold stimuli, although the effect was somewhat more moderate and mostly observed for doses of 7 μg and higher. We thus show that spinal activation of the Y1R is able to reduce neuropathic pain due to a chronic constrictive injury and, together with other studies, support the use of a spinal Y1R agonist as a therapeutic agent against chronic pain induced by peripheral neuropathy.

1. Introduction

Neuropathic pain induced by peripheral nerve injury is a serious public health concern [1,2]. Patients undergoing neuropathic pain typically suffer allodynia (pain induced by normally innocuous stimuli), hyperalgesia (an exaggerated response to painful stimuli), and paresthesia (tingling, tickling, pricking, numbness or burning sensations) [2–4]. In addition, neuropathic pain patients also manifest progressive alterations in quality of life, including severe depression, alterations of sleep, eating and memory, and functional limitations [5–7]. Unfortunately, and despite the existence of a number of analgesic drugs available against neuropathic pain, a large percentage remains refractory to treatment [2,8,9]. Moreover, most of these drugs also cause some type of adverse effect, limiting their use in high doses or for prolonged periods of time [8].

Neuropeptide tyrosine (NPY), a 36 aa peptide [10], is broadly distributed across the central [11] and peripheral nervous systems [12], and is strongly conserved through evolution, including in humans [13]. NPY acts through 5 different receptors known so far [14–16]. However, types 1 and 2 receptors (Y1R, Y2R) seem to be the most relevant in the mechanisms of pain [16–23]. Only very few dorsal root ganglion (DRG) neurons normally express NPY, but its expression is strongly upregulated by peripheral nerve injury. In contrast, both Y1R and Y2R are regularly expressed in DRG neurons where they exhibit a complementary expression, the former primarily in small neurons, and the latter in medium-sized and large ones. Finally, at the spinal cord level, an abundant NPY-expressing neuropil is normally detected, and produced primarily by local superficial dorsal horn interneurons, but also contributed by primary afferent neurons and descending inputs. Y1R and Y2R expression is also detected in the spinal cord, both in pre- (Y1R, Y2R) and postsynaptic (Y1R) locations (for a detailed description of the expression and distribution patterns of NPY, Y1R and Y2R in DRGs and the spinal cord, see [16,17] and references therein).

An increasing number of studies strongly supports the pain-modulating role of NPY [19,20,24–30], suggesting that along with its associated receptors, they could be attractive targets for the development of drugs against pain. In

Abbreviations: AUC, area under the curve; CCI, chronic constriction injury; CFA, complete freund's adjuvant; DRG, dorsal root ganglion; NPY, neuropeptide tyrosine; SLNC, single ligature nerve constriction; SNI, spared nerve injury; VGLUT2, vesicular glutamate transporter type 2; Y1R, NPY receptor type 1; Y2R, NPY receptor type 2.

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fact, the analgesic impact of the spinal activation of Y1- and Y2Rs in rats has been analyzed using a variety of pain models such as sciatic nerve axotomy [31] or spared nerve injury (SNI) [22], skin incision [32], and acute [22,33] or chronic [21,33,34] inflammation. However, no study has yet established the potential antiallodynic role of NPY or the selective activation of its associated receptors in rats with neuropathic pain induced by a compressive injury of the sciatic nerve.

In the present study, we explored the potential analgesic role of the pharmacological activation of spinal Y1Rs in rats with CCI [35]. Mechanical and thermal (cold) allodynia were used as parameters of change in rats with CCI treated either with vehicle or the specific Y1R agonist Leu^{31}, Pro^{34}-NPY.

2. Materials and methods

2.1. Animals

All experiments were performed in 109 male Sprague-Dawley rats (weight 180–280 g). Animals were maintained in 12 h day/night cycles (lights on from 7 a.m. to 7 p.m.), with water and food ad libitum. All experiments were performed according to the recommendations of the International Association for the Study of Pain (IASP) and the Society for Neuroscience (SFN) on the use of animals in research, and were approved by the IACUC of the IRTM (CICUAL-IIMT-12-04).

2.2. Chronic constriction injury (CCI)

CCI was induced in rats previously anaesthesized using Isoflurane (5% induction, 3.0% maintenance, 0.8 L/min O_2 flow rate; Piramal Healthcare, UK), according to the method by Bennett and Xie [35]. In brief, the right sciatic nerve was exposed at the midthigh level, and carefully dissected from the surrounding tissue. The nerve was ligated using 4 loose 5.0 silk ligatures, followed by suture in layers of the surgical wound using Vicryl 5.0 (muscle) and mononylon 5.0 (skin) (Ethicon, Livingston, Scotland). After a single subcutaneous dose of dexketoprofen trometamol (5 mg/kg; Lab Argentia, Bs As, Argentina) and the topical application of 2% lidocaine hydrochloride gel (AstraZeneca, Buenos Aires, Argentina) on the surgical wound, the animals were left to recover in a warm environment, before being returned to the animal house.

2.3. Intrathecal catheterization implantation and agonist administration

A chronic intrathecal (i.t.) catheter (PE 10, o.d. 0.61 mm; Intramedic, Clay Adams, Becton Dickinson and Company, New Jersey, USA) was implanted under anesthesia (as above), according to the method by Storkson and cols. [36], between the L5 and L6 vertebrae, with its tip at the lumbar enlargement, in sham rats and rats 7 days after induction of CCI. The proper location of the tip of the catheter was tested 24 h before the pharmacological experiments by assessing sensory and motor blockade after i.t. injection of 10 μl of lidocaine (50 mg/ml; Xylocaína, AstraZeneca, Buenos Aires, Argentina). All animals failing to show signs of sensory and motor blockade, or that manifested signs only in the left leg (contralateral to the CCI) were not included in the study. The pharmacological experiments were conducted on day 14 after CCI.

We used the Y1R agonist Leu^{31}, Pro^{34}-NPY (Tocris Bioscience, Bristol, UK). Leu^{31}, Pro^{34}-NPY, dissolved at a concentration of 1 mg/ml in 0.25% acetic acid and diluted to working concentrations in vehicle (sterile saline), was tested at doses of 2.5, 5, 7, 10 and 20 μg, in all cases to a final volume of 10 μl. The effect of the agonist was exposed by analysis of pain-like behavior in sham and injured rats (see below).

2.4. Control experiments

Two types of control experiments were performed: (1) Rats (n = 6) where the sciatic nerve was exposed but not ligated (sham rats), and with catheterization and injection of 10 μg of Leu^{31}, Pro^{34}-NPY; (2) Rats (n = 8) with CCI, and with catheterization and injection of vehicle (0.25% acetic acid).

2.5. Pain-like behavioral testing

2.5.1. Measurement of mechanical threshold

Mechanical threshold was evaluated using von Frey filaments (1.4, 2, 4, 6, 8, 10, 15 y 26 g; Stoelting, Inc., Wooddale, IL, USA). The medial aspect of the plantar surface of the ipsilateral hindpaw was mechanically stimulated, following the modified up-down method of Dixon, as described by Chaplan and cols. [37], to establish the 50% withdrawal threshold. Mechanical withdrawal threshold was tested previous to the injection of the agonist (0 min; basal response), and 5, 15, 30, 45, 60, 75 and 90 min after the application of the agonist. A paw withdrawal reflex obtained with 4.0 g force or less was considered an allodynic response.

2.5.2. Assessment of cold allodynia

Cold allodynia was assessed using a modified version of the method established by Choi and cols. [38]. After acclimatization in individual cubicles for 15 min, a drop of acetone was gently brought in contact with the plantar surface of the ipsilateral hindpaw. Applications were made four times every four minutes, for a total of 108 min from the beginning of the pharmacological experiment. Foot withdrawal was scored as positive (1) and lack of withdrawal as negative (0). The frequency of withdrawal was evaluated in 16 min bins (totaling 7 bins), each bin consisting of the average obtained from 4 consecutive stimulations. The first 16 min bin represents basal response, previous to agonist injection.

After all behavioral testing, animals were deeply anesthetized using an overdose of chloral hydrate (1.5 g/kg, intraperitoneal) followed by cervical dislocation.

2.6. Statistical analysis

All data is expressed as mean ± SEM, and presented as curve graphs and area under the curve (AUC) bar graphs. Statistical analysis was performed using Two-way repeated measures ANOVA followed by the Bonferroni posthoc test (curve graphs), or One-way ANOVA followed by the Tukey posthoc test (AUC graphs).

In all cases, levels of significance were established as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3. Results

All rats with CCI showed changes in the position of the injured leg, including retraction and protection (pain-like behavior). In contrast, injured or sham rats virtually never showed signs of altered pain-like behavior in the contralateral paw.

Sham rats treated with 10 μg intrathecal Leu^{31}, Pro^{34}-NPY presented no significant changes in mechanical thresholds, as tested in the ipsilateral hindpaw (0 min: 13.41 ± 1.75; 5 min: 15.05 ± 0.12; 15 min: 15.0 ± 0.0; 30 min: 15.0 ± 0.0; 45 min: 15.0 ± 0.0; 60 min: 15.05 ± 0.12; 75 min: 15.0 ± 0.0; 90 min: 15:00 ± 0.0). In contrast, rats with CCI plus intrathecal injection of vehicle showed clear signs of mechanical (Figs. 1 and 3) and cold allodynia (Figs. 2 and 3). Conversely, as it will be described in more detail in the following sections, intrathecal injection of Leu^{31}, Pro^{34}-NPY in rats with CCI resulted in dose-dependent antiallodynic effects.

3.1. Dose-dependent effects of intrathecal Leu^{31}, Pro^{34}-NPY on mechanical allodynia in rats with CCI

Intrathecal injection of 2.5 μg Leu^{31}, Pro^{34}-NPY did not significantly alter the withdrawal threshold of CCI rats as compared to vehicle-treated CCI rats; all rats remained allodynic throughout the 90 min tested, even though a tendency towards increased withdrawal threshold could be appreciated (Fig. 1A).

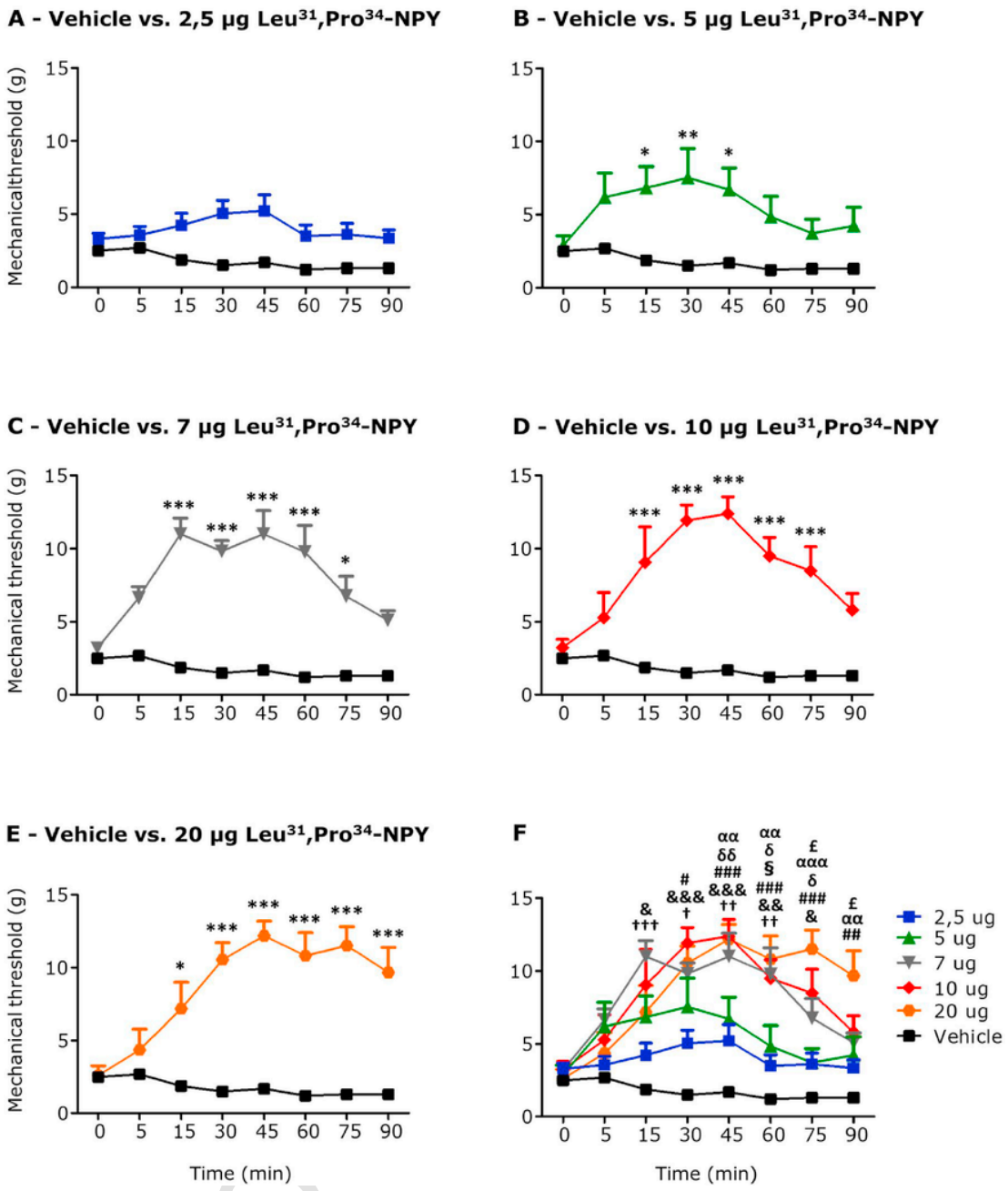

Fig. 1. Ipsilateral hindpaw mechanical withdrawal thresholds of CCI rats treated with 2.5 (A), 5 (B), 7 (C), 10 (D) or 20 µg (E) of Leu^{31}Pro^{34}-NPY (for clarity of the statistical description, A–E compare each dose of Leu^{31}Pro^{34}-NPY against the same vehicle-treated CCI rat control group; F shows the comparison among doses). Values correspond to the mean ± SEM and were analyzed by applying Two-Way repeated measures ANOVA, followed by Bonferronís Posthoc Test. P values are as follows: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. In F, the different tags are coded as follows: †2.5 vs. 7; $^{\&}$2.5 vs 10; $^{\#}$2.5 vs. 20; §5 vs. 7; $^{\delta}$5 vs. 10; $^{\alpha}$5 vs. 20; $^{£}$7 vs. 20.

In contrast, administration of 5 µg or more of Leu^{31}, Pro^{34}-NPY resulted in statistically significant increases across time in withdrawal thresholds in CCI rats, as compared to vehicle-treated injured rats (Two-way repeated measures ANOVA, $F = 12.35$, $P < 0.001$, for treatment as source of variation; $F = 19.78$, $P < 0.001$ for time as source of variation; Fig. 1B–E). The onset of antiallodynic effect, shown by statistically significant differences between Leu^{31}, Pro^{34}-NPY- and vehicle-treated CCI rats, begun at 15 min (5, 7, 10 and 20 µg) after injection of the agonist. Peak antiallodynic effects were observed between 15 and 45 min (5 µg), 15 and 60 min (7, 10 µg), and 30 and 90 min (20 µg) after intrathecal application of Leu^{31}, Pro^{34}-NPY (Fig. 1B–E). In addition, while rats treated with 5, 7 and 10 µg of Leu^{31}, Pro^{34}-NPY showed a progressive return to basal allodynic levels at 90 min, rats treated with 20 µg Leu^{31}, Pro^{34}-NPY maintained a significantly increased mechanical withdrawal threshold, even up to 90 min after intrathecal administration of the agonist (Bonferronís posthoc test, $P < 0.001$; Fig. 1E). Finally, statistically significant differences were found between doses at different time-points after injection of Leu^{31}, Pro^{34}-NPY, more often between 2.5 or 5 µg and 10 and 20 µg (Fig. 1F).

Area under the curve analysis of the mechanical thresholds after each dose confirmed the antiallodynic effect of Leu^{31}, Pro^{34}-NPY at doses of 5 µg and higher in CCI rats, as compared to vehicle-treated injured rats (One-way ANOVA, $P < 0.001$). It also showed presence of a stronger antiallodynic effect for Leu^{31}, Pro^{34}-NPY at 7, 10 and 20 µg, as compared to 2.5 µg (Tukey's post hoc test, $P < 0.05$ vs. 7 µg; $P < 0.01$ vs. 10 µg; $P < 0.001$ vs. 20 µg) or 5 µg (Tukey's post hoc test, $P < 0.01$ vs. 20 µg) (Fig. 3A).

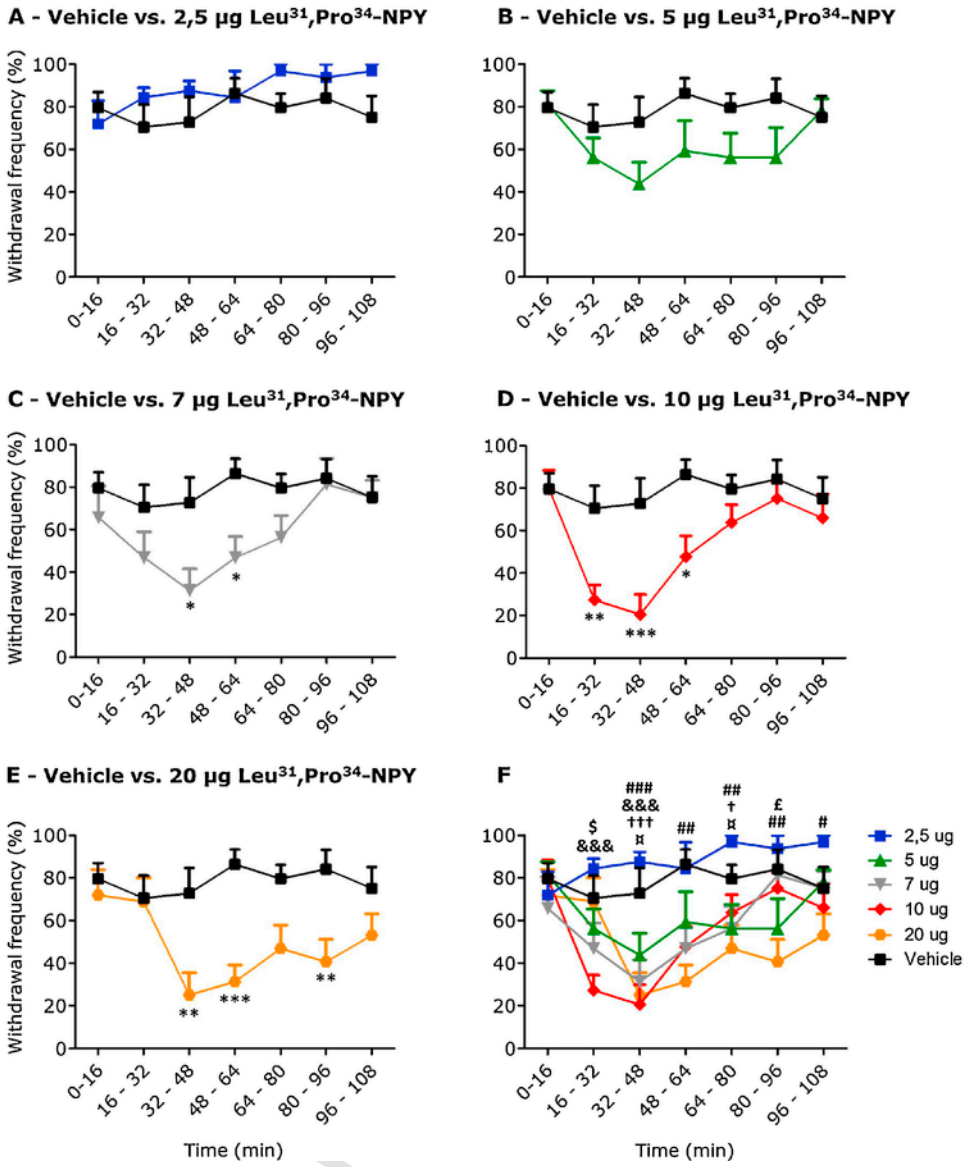

Fig. 2. Ipsilateral hindpaw withdrawal frequency to cold stimulation of CCI rats treated with 2.5 (A), 5 (B), 7 (C), 10 (D) or 20 µg (E) of Leu^{31}Pro^{34}-NPY (for clarity of the statistical description, A-E compare each dose of Leu^{31}Pro^{34}-NPY against the same vehicle-treated CCI rat control group; F shows the comparison among doses). Values correspond to the mean ± SEM and were analyzed by applying Two-Way repeated measures ANOVA, followed by Bonferroni's Posthoc Test. P values are as follows: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. In F, the different tags are coded as follows: $^{\text{¤}}$2.5 vs. 5; †2.5 vs. 7; $^{\&}$2.5 vs 10; $^{\#}$2.5 vs. 20; $^{£}$7 vs. 20; $^{\$}$10 vs. 20.

3.2. Dose-dependent effects of intrathecal Leu^{31}, Pro^{34}-NPY on cold allodynia in rats with CCI

Intrathecal injection of 2.5 or 5 µg Leu^{31}, Pro^{34}-NPY did not induce significant changes in the withdrawal frequency to cold stimuli in rats with CCI (Fig. 2A–B); animals presented with cold allodynia throughout the 108 min evaluated, even though a tendency towards a lesser withdrawal frequency could be observed for rats treated with 5 µg of the Y1R agonist (Fig. 2B). In contrast, statistically significant reductions across time in cold allodynia were observed in CCI rats injected with 7, 10 or 20 µg of Leu^{31}, Pro^{34}-NPY, as compared to vehicle-treated injured rats (Two-way repeated measures ANOVA, $F = 5.104$, $P < 0.001$, for treatment as source of variation; $F = 10.52$, $P < 0.001$ for time as a source of variation; Fig. 2C–E). The onset of antiallodynic effect begun at bins 16–32 (10 µg; Fig. 2D) and 32–48 min (7 and 20 µg; Fig. 2C, E) after intrathecal application of the agonist. Peak antiallodynic effects were of short duration for all three doses (Fig. 2C–E). However, while rats treated with 10 µg showed a return to basal allodynic levels 90 min after Leu^{31}, Pro^{34}-NPY administration, rats treated with 20 µg showed a slower return to basal levels (Fig. 2D, E). Finally, statistically significant differences were found between doses at different time-points after injection of Leu^{31}, Pro^{34}-NPY, more often between 2.5 µg and the other doses (Fig. 2F).

Area under the curve analysis of the withdrawal frequency to cold stimulation after each dose confirmed the antiallodynic effect of Leu^{31}, Pro^{34}-NPY at doses of 10 µg and higher in CCI rats, as compared to vehicle-treated injured rats (One-way ANOVA, $P < 0.001$). It also showed presence of a stronger antiallodynic effect for Leu^{31}, Pro^{34}-NPY at 7, 10 and 20 µg, as compared to 2.5 µg (Tukey's post hoc test, $P < 0.05$ vs. 7 µg; $P < 0.01$ vs. 10 or 20 µg) (Fig. 3B).

4. Discussion

In the present study, we show that the intrathecal administration of a selective Y1R agonist reduces mechanical and cold allodynia in a dose-dependent manner in rats with chronic constriction injury of the sciatic nerve. We

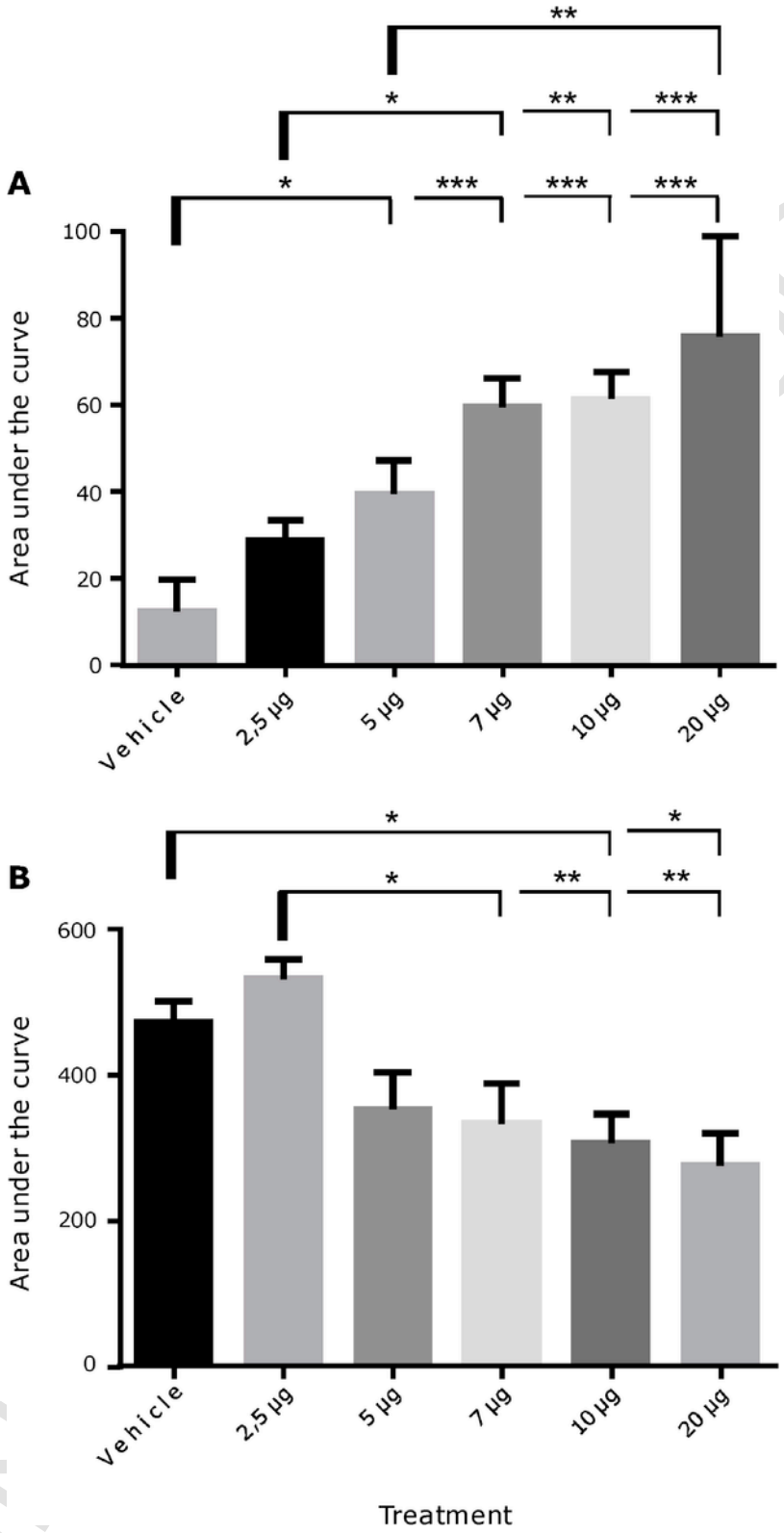

Fig. 3. Area under the curve analysis of ipsilateral mechanical withdrawal threshold (A) or withdrawal frequency to cold stimulation (B) of CCI rats treated with 2.5, 5, 7, 10 or 20 µg and compared with vehicle-treated injured rats. Values correspond to the mean ± SEM and were analyzed by applying One-Way ANOVA, followed by Tukey's Posthoc Test. P values are as follows: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

thus confirm previous observations in rats with other types of peripheral neuropathy or inflammation (see below), supporting the potential value of spinal Y1R activation for the induction of analgesia.

4.1. Methodological considerations

In the present study, we used the Y1R agonist $Leu^{31}Pro^{34}$, reportedly a high affinity, selective agonist (relative to the Y2R), but also presenting affin-

ity for the Y4R and Y5Rs (see [39]). This could imply that the effects of the Y1R agonist presented here may reflect actions other than through the Y1R, especially when testing the highest doses. However, to this date, there are no reports of the presence of Y4R and Y5R in the rodent spinal cord. Moreover, Y4 and Y5Rs are restricted to particular upper brain regions (see [40]). This would only leave potential for actions through the Y2R. However, early in vitro studies have shown that $Leu^{31}Pro^{34}$ has a high selectivity for the Y1R over Y2R in binding assays [41,42], and that it is devoid of agonistic potency at Y2Rs [42]. Nevertheless, future in vivo experiments, with co-application of $Leu^{31}Pro^{34}$ and a selective Y1R antagonist, will be needed to confirm the assumption of Y1R-only antiallodynic effects reported here and elsewhere. And, if high doses of $Leu^{31}Pro^{34}$ do have non-specific behavioral effects, it remains to be established.

Another issue to be addressed is the use of different concentrations of $Leu^{31}Pro^{34}$. In a previous study by Xu and cols. [31], the effects of $Leu^{31}Pro^{34}$ on the flexor reflex in decerebrated normal and axotomized rats was reported. The authors found that low doses of $Leu^{31}Pro^{34}$ result in facilitation of the reflex, whereas high doses induce its depression (and hence suggesting its analgesic potential). In our present behavioral study, we did not detect such dual effects; $Leu^{31}Pro^{34}$ application virtually always induced a dose-dependent decrease in mechanical and cold allodynia.

Finally, it should be mentioned that in the spinal cord of rat, axotomy or strong SLNC of the sciatic nerve induces a moderate decrease in the immunohistochemical expression of the Y1R in lamina II [43]. Two possible causes have been advanced: (1) a decrease in central primary afferents (see [44] and references therein), due to a concurrent decrease after peripheral nerve injury in the expression of Y1R, normally present in DRG neurons [43]; and (2) a decrease of Y1R expression in local spinal cord neurons ([43]); none of these hypotheses has yet been fully confirmed. Medium SLNC, comparable to the CCI model used here, has also been shown to induce moderate Y1R expression decrease in DRGs, but without altering its spinal expression [43]. Altogether, what would the extent of a potential decrease in pre- and postsynaptic Y1R expression after CCI is currently unknown. However, based on the data presented here, it appears as if it would not be enough to alter the capacity of an Y1R agonist to reduce the allodynic responses observed in CCI rats.

4.2. Spinal activation of the Y1R reduces mechanical and cold allodynia in rats with CCI

The observed reductions reported here in mechanical and cold allodynia in rats with CCI after intrathecal application of a Y1R agonist are in line with previous studies, where it was observed that intrathecal application of NPY [22,34,45] or Leu^{31}, Pro^{34}-NPY [31] in intact rats [31,34,45], rats with sciatic nerve axotomy [31,45] or rats with SNI [22] results in depression of the flexor reflex [31,45], increases in the nociceptive threshold in the hot plate and paw pressure tests [34], and reductions in mechanical and cold allodynia, and mechanical hyperalgesia [22].

Our results also correlate with studies using a different approach to show the effects of selective NPY-receptor activation, namely the co-administration of NPY and Y1R antagonists. Thus, co-application of NPY and an Y1R antagonist attenuates the analgesic effect of the peptide in rats with SNI [22] or plantar incision [32], as shown by increases in mechanical allodynia [22] and thermal hyperalgesia [32] after treatment. Similarly, it was observed that the analgesic effect of intrathecal NPY in rats with chronic [21] or acute [22,33] hindpaw inflammation induced by intraplantar injection of carrageenan-, CFA- [21] or formalin [22,33] is blocked by co-administration of a Y1R selective antagonist; animals showed an increase in thermal hypersensitivity [21] or increased aversive responses [22,33]. More recently, the analgesic effect of Y1R activation during chronic inflammation has been confirmed in rats with CFA-induced inflammation, where the intrathecal administration of Leu^{31}, Pro^{34}-NPY resulted in reduced thermal hyperalgesia [20].

More indirect evidence comes from studies in rats where death of Y1R-expressing spinal neurons was induced by receptor-mediated internalization of a conjugate consisting of the toxin saporin and NPY the rat the Y1R seems to be expressed in large quantities in spinal neurons [46], while the Y2R protein seems to be absent (see [17] but also next section for recent data from RT-PCR studies)), neuronal death putatively occurs only for Y1R-expressing neurons. In normal rats thus treated, the deletion of Y1R-expressing spinal neurons resulted in reductions in the nocifensive responses to heat and chemical stimuli of the skin [29,30]. Moreover, it also resulted in reduced thermal hyperalgesia in rats with CFA-induced hindpaw inflammation [29]. Altogether, these studies highlight the preponderant role of Y1R-expressing spinal neurons in pain modulation, and further support the value of Y1R activation for antinociception.

4.3. Possible mechanisms of antiallodynic actions of Y1R activation

A crucial aspect to take into consideration to understand the antiallodynic actions of spinal Y1R activation is where exactly this agonist is acting. For a number of years, the exact localization of the Y1R and Y2Rs in DRGs and the spinal cord was the subject of intense debate. On one hand, Y1R-like-immunoreactivity seemed to be exclusively associated to the cell body of a subpopulation of small DRG neurons (no evidence for its axonal transport was found at that time) [47–49], and in a number of interneurons localized in lamina II of the dorsal horn of the spinal cord [43,48,50], supporting its postsynaptic localization. On the other hand, the Y2R appeared to be not only expressed in a subpopulation of small and medium-sized DRG neurons, but also to undergo axonal transport, meaning that it could be present at spinal presynaptic sites [49,51]. Today, it is known that both Y1R and Y2Rs expressed in DRG neurons undergo axonal transportation and exhibit presynaptic localization in the central endings of primary afferents [52,53], and that the Y1R is also postsynaptic at the spinal cord level, expressed in a wide variety of neuronal types, as shown in rat [46] and mouse [25]. So far, presence of the Y2R in spinal cord neurons has been elusive (see [17]), although we have recently reported presence of Y2R mRNA in rat dorsal horn by means of RT-PCR analysis [54]. Therefore, when addressing the effects of NPY and its analogues, the antiallodynic effects could depend on mechanisms involving both pre- and postsynaptic locations.

From a presynaptic point of view, it has been proposed that Y1R activation would have a role in the release of excitatory neurotransmitters [55]. This has been recently confirmed in rats with chronic hindpaw inflammation [20], where it was observed that the spinal NPY-related analgesic effect depends on the Y1R-dependent inhibition of substance P release from primary afferent nerve endings. Moreover, the authors demonstrated in mouse that the inflammatory condition resulted in increased affinity of Y1R G-protein coupling [20]. Altogether, the antiallodynic effect of intrathecal Leu^{31}, Pro^{34}-NPY observed here in CCI rats may at least in part be dependent on the activation of presynaptic Y1Rs, leading to reductions in excitatory neurotransmitters release, which in turn would result in reduced excitability of spinal interneurons and projection neurons (see [17,18]). If CCI or other neuropathic conditions are also capable of altering Y1R G-protein coupling, it remains to be established.

In addition to its presynaptic action, intrathecal Leu^{31}, Pro^{34}-NPY most certainly acts upon postsynaptic Y1Rs. Between 70–80% of all neurons present in laminae I–III are excitatory interneurons [56–58]. These interneurons are considered glutamatergic, based on their content of the vesicular glutamatergic transporter type 2 (VGLUT2) [59,60], and they also express the neuropeptide somatostatin [48]. Conversely, several Y1R-expressing interneurons in laminae I–II colocalize with somatostatin [48], and 97.5% of axonal nerve endings produced by somatostatinergic interneurons in laminae I–II also coexpress VGLUT2 [61], supporting their excitatory nature. Finally, it has been demonstrated that the application of NPY onto rat spinal cord slices selectively inhibits the majority of laminae I–II excitatory interneurons [55,62,63] through induction of an Y1R-dependent hyperpolarizing potassium conductance [62,63].

The Y1R-dependent antiallodynic effect could also be based, even if seemingly contradictory, on the inhibition of inhibitory interneurons (see [18]). It has been demonstrated in the rat that peripheral neuropathy induces alter-

ations in the anionic homeostasis of lamina I interneurons due to a decrease in the expression of the potassium-chloride transporter (KCC2). This has been shown to lead to changes in the transmembrane anionic gradient, where activation of GABAergic receptors (typically expressed in inhibitory interneurons [64,65]) would result in excitatory instead of inhibitory effects [66]. As a consequence, the normally inhibitory GABAergic neurotransmission would become excitatory during neuropathic pain conditions. While co-expression of NPY and GABA has been demonstrated in inhibitory spinal interneurons [67,68], the presence of NPY receptors remains to be demonstrated. However, the potential expression and activation of Y1R in GABAergic inhibitory interneurons could, during neuropathic conditions, act blocking the above described aberrant GABAergic-dependent excitation.

Finally, it could be speculated that agonists targeting the Y1R could also act upon projection neurons. In fact, a number of projection neurons expressing the Y1R have been identified in laminae I, IV–VI and X [46]. Moreover, it is possible that these neurons also expressed the receptor for substance P, NK1 [69]. If the already described postsynaptic actions of NPY onto spinal laminae I–II Y1R-expressing interneurons [55,62,63] also apply to nociceptive projection neurons remains to be established. However, if true, it could imply that Y1R activation in a number of spinal projection neurons expressing this receptor was capable of directly modulating the transmission of pain-related information to the CNS during CCI.

In conclusion, and in agreement with previous studies using models of partial or complete transection of peripheral nerves, skin injury and acute and chronic inflammation, we here show that spinal pharmacological modulation of the Y1R significantly reduces mechanical and cold allodynia in rats subjected to constrictive injury of the sciatic nerve. It is likely that such modulation took place both at pre- and postsynaptic sites, inhibiting the transmission of pain signals to upper levels of the nervous system, although the exact mechanisms require further elucidation. Nevertheless, together with other studies, our data supports the concept of using spinal Y1R agonists as a therapeutic strategy against chronic pain induced by peripheral neuropathy.

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