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Non-Peptidergic Nociceptive Neurons Are Essential for Mechanical Inflammatory Hypersensitivity in Mice

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Abstract

Small nerve fibers that bind the isolectin B4 ($IB4^+$ C-fibers) are a subpopulation of primary afferent neurons that are involved in nociceptive sensory transduction and do not express the neuropeptides substance P and calcitonin-gene related peptide (CGRP). Several studies have attempted to elucidate the functional role of $IB4^+$-nociceptors in different models of pain. However, a functional characterization of the non-peptidergic nociceptors in mediating mechanical inflammatory hypersensitivity in mice is still lacking. To this end, in the present study, the neurotoxin IB4-Saporin (IB4-Sap) was employed to ablate non-peptidergic C-fibers. Firstly, we showed that intrathecal (i.t.) administration of IB4-Sap in mice depleted non-peptidergic C-fibers, since it decreased the expression of purinoceptor 3 ($P2X_3$) and transient receptor potential cation channel subfamily V member 1 (TRPV1) in the dorsal root ganglia (DRGs) as well as IB4 labelling in the spinal cord. Non-peptidergic C-fibers depletion did not alter the mechanical nociceptive threshold, but it inhibited the mechanical inflammatory hypersensitivity induced by glial cell-derived neurotrophic factor (GDNF), but not nerve growth factor (NGF). Depletion of non-peptidergic C-fibers abrogated mechanical inflammatory hypersensitivity induced by carrageenan. Finally, it was found that the inflammatory mediators PGE_2 and epinephrine produced a mechanical inflammatory hypersensitivity that was also blocked by depletion of non-peptidergic C-fibers. These data suggest that IB4-positive nociceptive nerve fibers are not involved in normal mechanical nociception but are sensitised by inflammatory stimuli and play a crucial role in mediating mechanical inflammatory hypersensitivity.

Keywords Non-peptidergic C-fibers · IB4-saporin · Mechanical hypersensitivity · Inflammatory pain · Nociceptors · Mice

Abbreviations

ATF-3	Activating transcription factor 3
AUC	Area under the curve
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CGRP	Calcitonin-gene related peptide
DRG	Dorsal root ganglion
EP	E prostanoid receptor
g	Grams
GDNF	Glial cell-derived neurotrophic factor
GFRα-1	GDNF family receptor alpha 1
GPCR	G protein-coupled receptor
HCN	Hyperpolarization-activated cyclic nucleotide-gated
HTMRs	High-threshold mechanoreceptors
IB4	Isolectin B4
IB4-Sap	IB4-saporin
i.pl.	Intraplantar
i.t.	Intrathecal
LTMRs	Low-threshold mechanoreceptors
MCP-1	Monocyte chemoattractant protein 1
Na_v	Voltage-gated sodium ion channel
NGF	Nerve growth factor
OCT	Optimum cutting temperature
PBS	Phosphate-buffered saline
$P2X_3$	Purinoceptor 3
PFA	Paraformaldehyde

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PGE_2	Prostaglandin E2
PKA	Protein kinase A
PKCε	Epsilon isozyme of protein kinase C
Sap	Unconjugated saporin
SEM	Standard error of the mean
T-PER	Tissue protein extraction reagent
TrkA	Tropomyosin receptor kinase A
TRPV1	Transient receptor potential cation channel subfamily V member 1
WT	Wild type

Introduction

The capacity to detect and transmit noxious stimuli protects the individual from damage and thus is an essential physiological function. Pain is initiated by activation of peripheral nociceptive nerve fibers, triggered by a range of harmful chemical, thermal, and mechanical stimuli [1]. Nociceptors are the first-order neurons in the pain sensory transduction pathway and are classified accordingly to axon size, degree of myelination, and speed of conduction into the subsets such as C-, Aδ-, and (to a lesser extent) Aβ-fibers [2]. Most of the C-fibers subpopulation, the small-diameter, unmyelinated, slow conducting fibers, express transient receptor potential cation channel subfamily V member 1 (TRPV1) and are classically subdivided into two main subsets: peptidergic and non-peptidergic [3]. These subgroups are heterogeneous, exhibiting distinct molecular and neuroanatomical characteristics. Peptidergic C-fibers produce and release neuropeptides such as substance P and calcitonin-gene related peptide (CGRP) and express the tropomyosin receptor kinase A (TrkA), which responds to nerve growth factor (NGF) [4–6]. In contrast, the non-peptidergic population of C-fibers is characterised by binding to isolectin B4 (IB4), a plant lectin from *Griffonia simplicifolia*, express the c-Ret neurotrophin and the GDNF family receptor alpha 1 (GFRα-1) that are targeted by glial cell-line-derived neurotrophic factor (GDNF), and also express the purinergic receptor $P2X_3$ [7–9]. These subpopulations also differ in their central terminations in the spinal cord. The peptidergic subset terminates more superficially, in lamina I and the outer part of lamina II, while the $IB4^+$-nociceptors (the non-peptidergic subset) project mainly to inner lamina II of the dorsal horn of the spinal cord [3, 10].

The differences between these two subsets of C-nociceptors suggest that they may have distinct functional properties in processing normal and/or pathological pain conditions. In recent years, the contribution of $IB4^+$- and $IB4^-$-nociceptors in different pain states has been investigated in animal models, mainly in rats, using the cytotoxin saporin conjugated to IB4 to deplete the subpopulation of non-peptidergic C-fibers [11–13]. In fact, the inflammatory pain induced by the chemokine monocyte chemoattractant protein-1 (MCP-1) and acute muscle pain were attenuated by IB4-Saporin (IB4-Sap) administration in rats [12, 14]. In addition, the treatment with IB4-Sap decreased chronic neuropathic pain induced by peripheral nerve injury [11] and chemotherapy [15], and also in a chronic muscle pain model [14].

Recently, the neurotoxin IB4-Sap has also been used to investigate the role of non-peptidergic neurons in mice [16, 17]. In a cancer pain model, the mechanical, but not thermal, allodynia was decreased by selective removal of non-peptidergic neurons in mice [16, 18]. However, it was shown that $IB4^+$-C-fibers in visceral sensory neurons are not involved in colorectal mechanical nociception in mice [17]. Despite the emerging evidence that non-peptidergic fibers are involved in different pain conditions, the impact of the $IB4^+$-nociceptors on mechanical inflammatory hypersensitivity in mice is less clear and has not been examined so far. In the present study, the effect of intrathecal (i.t.) injection of different doses of IB4-Sap in mice was characterised, through molecular biology tools as well as in animal models of acute nociception and mechanical inflammatory hypersensitivity.

Material and Methods

Animals

The experiments were performed on male C57BL/6 wild type (WT) mice weighing between 20 and 30 g. Mice were housed in temperature-controlled rooms (22–25 °C) and given water and food ad libitum at the animal facility in the Department of Pharmacology, Ribeirão Preto Medical School, University of São Paulo, Brazil. Mice were taken to the testing room at least 1 h before experiments. All behavioral tests were performed between 9:00 a.m. and 5:00 p.m., double-blinded and conducted in accordance with the prescribed guidelines on experimental animal welfare of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80-23) revised 1996 and were approved by the Ethics Committee of the Ribeirão Preto Medical School, University of São Paulo (Process no. 101/2010).

Drugs

The following materials were obtained from the indicated sources: IB4-Sap and unconjugated saporin (Sap) (Advanced Targeting Systems, USA); capsaicin, epinephrine, and prostaglandin E_2 (PGE_2) (Sigma Aldrich, USA); carrageenan (FMC, USA); β-NGF (R&D systems, USA) and GDNF (Peprotech, USA). The drugs were diluted in sterile saline, except capsaicin that was diluted in a mixture of Tween-80 (10%), ethanol (10%), and saline, and the stock solution of PGE_2 (1 μg/μl) was prepared in 10% ethanol,

and dilutions were made in saline; the final concentration of ethanol was 1%.

Ablation of IB4$^+$ Afferents: i.t. Administration of IB4-Sap

Mice were anesthetised with 2% isoflurane and trichotomy was performed in the mice dorsal region. Using a 30-unit insulin syringe (BD Ultra Fine ™ II), IB4-Sap (0.16–3.2 μg/5 μl, i.t.), Sap (as control, 1.8 μg/5 μl, i.t.), or saline (vehicle, 5 μl/i.t.) were injected into the subarachnoid space on the midline between the L5 and L6 vertebrae. The animal's tail reflex findings were expected to confirm the correct performance of the procedure [19]. IB4-conjugated saporin (0.9 mg/mL) or unconjugated saporin (1 mg/mL) were diluted with saline, and the dose of 1.8 μg is the amount equivalent to that of saporin in 3.2 μg IB4-Sap once the vial had 56% saporin/mol IB4 of conjugate (approximately three molecules of saporin per molecule of IB4-Sap).

Mechanical Nociceptive Paw Tests

Electronic von Frey

Mechanical nociception was tested in mice as previously reported [20]. In a quiet room, mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors, at least 30 min before the start of testing. The test consisted of evoking a hind paw flexion reflex with a hand-held force transducer (electronic anesthesiometer; IITC Life Science, Woodland Hills, CA) adapted with a 0.5-mm^2 polypropylene tip. The investigator (blinded to group allocation) was trained to apply the tip perpendicularly to the central area of the plantar surface of the hind paw with a gradual increase in pressure. The gradual increase in pressure was manually performed in blinded experiments. The upper limit pressure was 15 g. The endpoint was characterised by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was automatically recorded, and the final value for response was obtained by averaging three measurements. The mechanical threshold was expressed in g.

von Frey Filaments

Mice were placed on an elevated wire grid and the plantar surfaces of both their hind paws were stimulated with a series of ascending force von Frey filaments (Ugo Basile, Italy) as previously reported [21]. Mice were first habituated to the experimental environment (room and apparatus) for a period of at least 30 min. Briefly, von Frey filaments of increasing stiffness (0.008–1.4 g) were applied on both hind paws plantar surface with enough pressure to bend the filament, to determine the stimulus intensity threshold stiffness required to elicit a paw withdrawal response. The absence of paw lifting after 3 s led to the use of the next filament with increasing stiffness, and paw lifting indicated a positive response and led to the use of the next weakest filament. This paradigm continued until a total of six measurements were taken or until four consecutive positive or four consecutive negative responses had occurred. The mechanical threshold was represented as withdrawal threshold (g). The animals' baseline for both von Frey tests, electronic and filaments, were measured at least twice before intraplantar (i.pl.) or i.t. injections. In addition, to avoid significant differences among the groups, mice were randomised in order to have small variability in the mechanical nociceptive threshold at baselines.

Chemical Nociception Test

Capsaicin Test

The capsaicin test was evaluated as previously described by [22]. Briefly, after an adaptation period, 20 μl of capsaicin (1.6 μg/paw) was injected i.pl. into the right hind paw of mice. Nociceptive response (paw licking) of each animal was assessed for 5 min following capsaicin administration. The amount of time (sec) spent licking the injected paw was considered indicative of nociception.

Induction of Paw Inflammation

Mice received an i.pl. administration of GDNF (0.1–10 ng/paw), NGF (1–100 ng/paw), carrageenan (100 μg/paw), epinephrine (300 ng/paw) or PGE_2 (100 ng/paw) injected subcutaneously into the plantar region of the hind paw of mice.

Percentage of Pain Inhibition

To determine the anti-hypersensitivity effect of IB4-Sap in inflammatory pain induced by i.pl. administration of different mediators (carrageenan, epinephrine, and PGE_2), the percentage of pain inhibition in mice pre-treated with different doses of IB4-Sap (0.16–3.2 μg) i.t. was calculated using the following formula:

$$\%\text{inhibition} = \frac{[(T-B)\text{control} - (T-B)\text{IB4-Sap}]}{(T-B)\text{control}} \times 100$$

where T means the threshold post inflammatory stimulus and B means the baseline threshold. Control is the animal group that received saline i.t., and IB4-Sap are the mice groups that were injected with different doses of IB4-Sap (0.16; 0.5; 1.5 or 3.2 μg/i.t.). It is important to mention that the % of pain inhibition take into account the baseline of each animal. Therefore, individual responses of experimental mice were normalised to their individual baseline responses.

The % of pain inhibition in capsaicin test was determined by the following formula: % inhibition = $C - T/C \times 100$, where C means the latency in seconds of control group (saline, i.t.) and T means the latency in seconds of IB4-Sap group (3.2 μg/i.t.).

Western Blotting Analysis

Mice were terminally anesthetised and the dorsal root ganglia (DRG) (segments L4, L5, and L6) were collected and homogenised in a lysis buffer T-PER (Tissue Protein Extraction Reagent, Thermo Scientific, USA) containing protease inhibitors (Protease inhibitor cocktail, Thermo Scientific, USA). The protein concentrations of the lysate were determined using the Coomassie colorimetric method (Bradford Reagent, Thermo Scientific, USA) and 30 μg of protein was loaded for each lane. The protein samples were separated by electrophoresis on a SDS-PAGE acrylamide gel and transferred to nitrocellulose membranes (Amersham Biosciences, UK). The membranes incubation consisted of 1 h at room temperature with blocking solution containing 5% dry milk (β-actin, TRPV1), 2% bovine serum albumin (BSA) plus 7% dry milk ($P2X_3$), or 2% BSA plus 5% dry milk (activating transcription factor 3, ATF-3), followed by incubation overnight at 4 °C with primary antibody against ATF-3 (1:500; Santa Cruz Biotechnology), $P2X_3$ (1:500; Sigma-Aldrich), and TRPV1 (1:400; Santa Cruz Biotechnology). Then, the membranes were washed and incubated for 1 h at room temperature with secondary antibodies conjugated with peroxidase (1: 3000; Jackson ImmunoResearch). The membranes were incubated with HRP Substrate Luminata™ Fort Western (Millipore, Brazil) and were then revealed, as indicated by the manufacturer. Image capture was performed with a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories) as directed by the manufacturer. A mouse monoclonal against β-actin (1: 4.000; Millipore) was used for loading controls. Images were used as representative blots. The densitometric data were measured following normalisation to the control (housekeeping gene) using Scientific Imaging-Systems (Image Lab™ 3.0 software, Bio-Rad Laboratories, USA).

Immunofluorescence

The animals were deeply anesthetised with ketamine and xylazine (2:1) and perfused through the ascending aorta with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 (4 °C). After the perfusion, spinal cord sections (L4-L6) were removed and post-fixed in PFA 4% for 2 h and then replaced with 30% sucrose overnight. All of the spinal cords were embedded in optimum cutting temperature (OCT), and sections (30 μm) were cut in a cryostat and processed for immunofluorescence. All sections were blocked with 2% BSA in 0.3% Triton X-100 for 1 h at room temperature. Subsequently, the sections were incubated overnight at 4 °C with a polyclonal goat anti-TRPV1 (1:400, Santa Cruz Biotechnology, USA) antibody, followed of AlexaFluor-488 conjugated secondary antibody (Molecular Probes, USA) for 2 h at room temperature. For the examination of IB4-labeled non-peptidergic nociceptors, IB4-Alexa 594 conjugated (1:200, Invitrogen, USA) were incubated overnight at 4 °C. The sections were washed, mounted on glass slides with Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich, USA), and then covered with coverslips. Fluorescent images were captured in a Confocal Leica SP5 microscope (Leica, Germany).

Statistical Analysis

Data are reported as the means ± standard error of the mean (SEM) and are representative of two independent experiments. Two-way ANOVA was used to compare the groups and doses at all time points (curves) when the nociceptive responses were measured at different time points after the stimulus injection. The factors analysed were treatments, time, and time versus treatment interaction. If there was significant time versus treatment interaction, one-way ANOVA followed by Bonferroni's t test was performed for each time point. Alternatively, when the responses (mechanical inflammatory hypersensitivity, chemical nociception, and Western blot analyses) were measured only once after the stimulus injection, the differences between responses were evaluated by one-way ANOVA followed by Bonferroni's t test (for three or more groups). Pair-wise comparisons were made with the Student t test. P values less than 0.05 were considered significant. Statistical analysis was performed with GraphPad Prism (GraphPad Software, USA).

Results

IB4-Sap Treatment Selectively Depletes Non-Peptidergic Nociceptive Neurons in Mice

Initially, we characterised whether the intrathecal administration of neurotoxin IB4-Sap is able to eliminate non-peptidergic nociceptive neurons. For this purpose, firstly expression of the $P2X_3$ purinergic receptor (a marker for non-peptidergic nociceptors [23]) and ATF-3 (a marker of neuronal injury, [24]) was determined in DRGs after i.t. administration of neurotoxin IB4-Sap. I.t. treatment with neurotoxin IB4-Sap, at different doses, decreased the

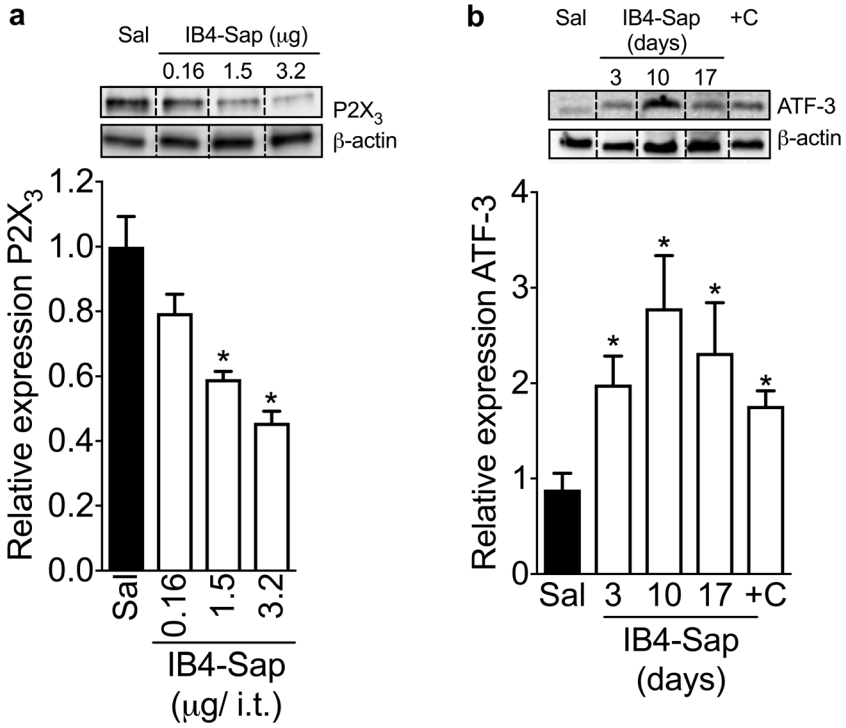

Fig. 1 Neurotoxin IB4-Sap depletes non-peptidergic C-fibers in mice. **a** At 24 days after i.t. administration of saline or IB4-Sap (0.16; 1.5; and 3.2 μg), bilateral L4, L5, and L6 DRGs were harvested and the $P2X_3$ (66 kDa) expression was evaluated by Western blot. **b** The DRGs (pooled of bilateral L4, L5, and L6) at 3, 10, or 17 days after the i.t. injection of neurotoxin IB4-Sap (IB4-Sap, 3.2 μg/5 μl) were collected to analyse the expression of neuronal marker of peripheral sensory neuron injury ATF-3 (21 kDa) by Western Blot. DRGs from mice with peripheral nerve injury were used as positive control (+C) group of ATF-3 expression. Representative blot and relative quantification. Beta actin (42 kDa) levels were used as an internal control. Data are expressed as the mean ± SEM ($n = 4$ per group). *$P < 0.05$ indicate statistically significant differences when compared with the control group (saline i.t)

expression of $P2X_3$ in DRGs samples at 24 days after treatment when compared to mice treated with saline (i.t.) (Fig. 1, panel a). Interestingly, i.t. injection of IB4-Sap produced a time-dependent increase in ATF-3 in DRG samples (Fig. 1, panel b). DRG samples from mice with peripheral nerve injury were used as a positive control (+C) group of ATF-3 expression (Fig. 1, panel b).

Subsequently, we investigated the effect of i.t. injection of IB4-Sap on the expression of TRPV1 and IB4 in the spinal cord 24 days after the neurotoxin administration. As observed in Fig. 2, panel a, treatment with neurotoxin IB4-Sap reduced significantly the expression of TRPV1 (green) and IB4 (red) binding sites in the dorsal horn of the spinal cord (L4-L6 level), compared to mice treated with saline (i.t.). In agreement, TRPV1 protein expression in the DRGs was decreased by treatment with IB4-Sap when compared to mice treated with saline (i.t.) or unconjugated Sap at 24 days after injections (Fig. 2, panel b). Corroborating these data, acute spontaneous nociception induced by capsaicin (licking behavior) was decreased in IB4-Sap pre-treated mice (Fig. 2, panel c). The percentage of pain inhibition was 60.32 ± 4.06. Notably, these data suggest that the i.t. injection of IB4-Sap decreases the population of non-peptidergic C-fibers in mice.

Non-Peptidergic C-Fibers Depletion Did Not Change Mechanical Nociceptive Threshold but Reduced GDNF-Induced Mechanical Inflammatory Hypersensitivity

We next determined whether the i.t. injection of IB4-Sap affects the mechanical nociceptive threshold during a period of 23 days following the treatment with the neurotoxin. Mice were treated intrathecally with different doses of IB4-Sap, unconjugated Saporin or saline (5 μl), and the mechanical nociceptive threshold was evaluated by von Frey (both filaments and electronic). In Fig. 3, a time-response curve is shown during the 23 days after i.t. injection of saline, saporin, or IB4-Sap in the mechanical nociceptive thresholds (electronic von Frey, Fig. 3, panel a; von Frey filaments, Fig. 3, panel b). The depletion of $IB4^+$-nociceptors induced by treatment with IB4-Sap did not modify the mechanical nociceptive threshold (Fig. 3, panels a and b) when compared to basal measure (before the i.t. injections) as well as compared with control groups (saline and saporin i.t.) for up to 23 days following treatments. The mechanical withdrawal thresholds were evaluated in both paws following i.t. administration.

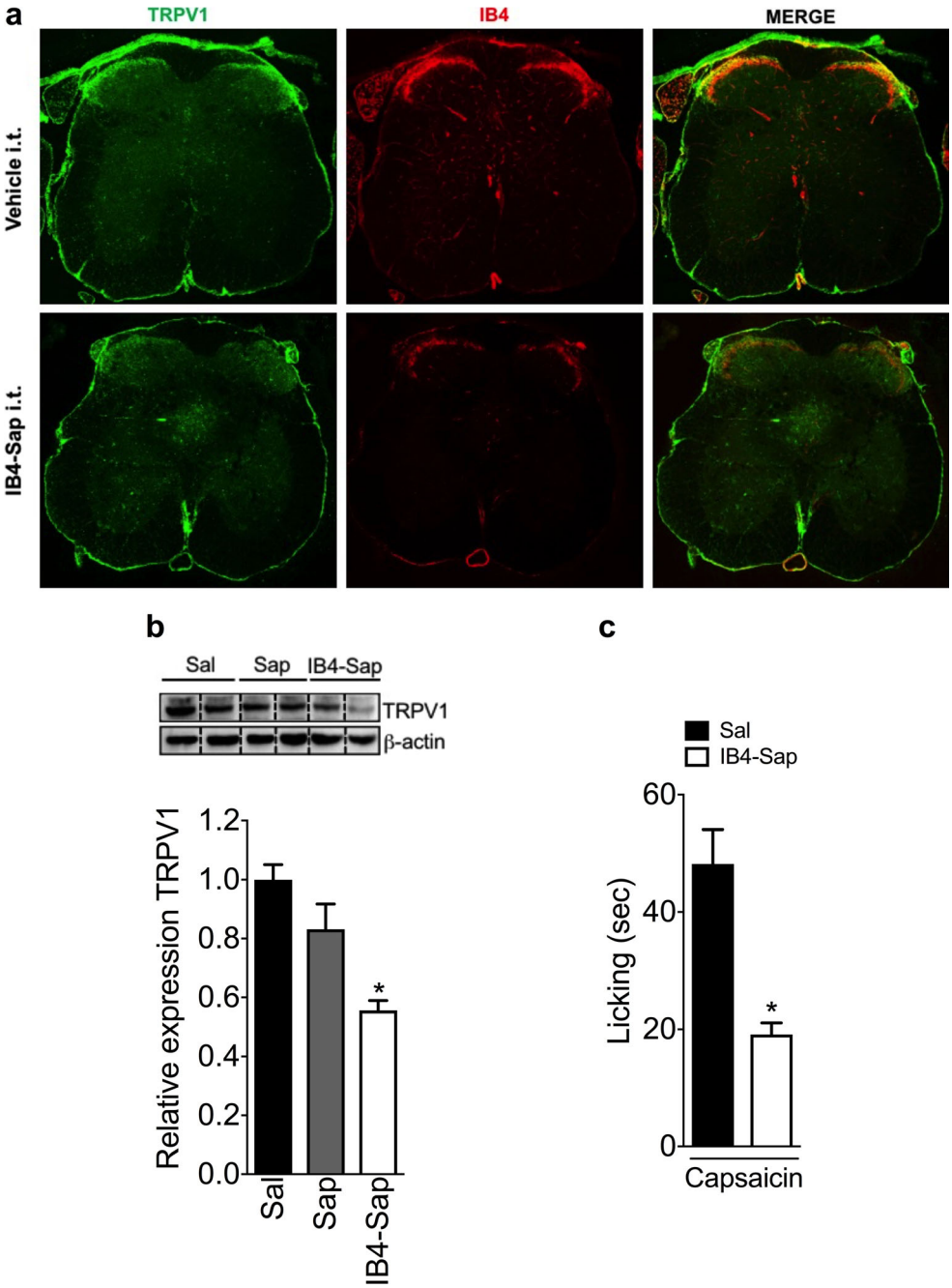

Fig. 2 Neurotoxin IB4-Sap decreases IB4 and TRPV1 expression and reduces the acute nociception induced by capsaicin. **a** At 24 days after i.t. administration of saline or IB4-Sap (3.2 μg), lumbar spinal cord samples were collected and the expression of TRPV1 (green) and IB4 (red) were evaluated by immunofluorescence (upper panel: saline i.t.; lower panel: IB4-Sap i.t.). Representative image. Scale bar = 100 μm. **b** The DRGs (pooled of bilateral L4, L5, and L6) were harvested 24 days after the i.t. injection of saline, Sap or neurotoxin IB4-Sap (3.2 μg/5 μl) to analyse the expression of TRPV1 (100 kDa) by Western Blot. Representative blot and relative quantification. Beta actin (42 kDa) levels were used as an internal control. **c** Capsaicin (1.6 μg/20 μl) was injected i.pl. 24 days after the administration i.t. of saline or IB4-Sap (3.2 μg). Nociceptive response characterised by licking was evaluated during 5 min period after capsaicin injection into hind paw. Data are expressed as the mean ± SEM ($n = 3$–4 mice per group in Western Blot experiment and $n = 6$–7 mice per group in behavior experiment). *$P < 0.05$ indicate statistically significant differences when compared with the control groups (saline and/or saporin i.t)

To confirm whether the IB4-Sap selectively depleted non-peptidergic C-nociceptors, we tested the effect of the neurotoxin on GDNF- and NGF-induced mechanical inflammatory hypersensitivity. Since there is evidence that GDNF and NGF promote pain hypersensitivity through non-peptidergic fibers and peptidergic nociceptors [7, 8], respectively, these mediators were used as a positive and negative control of neurotoxin treatment, respectively. First, we performed a dose- and time-response curve to determine the mechanical inflammatory hypersensitivity effect induced by i.pl. injection of these substances in mice. The i.pl. administration of different doses of NGF (Fig. 4, panel a, time- and dose-response curves and Fig. 4, panel b, area under curve; AUC) and GDNF (Fig. 4, panel e, time- and dose-response curves and Fig. 4, panel f, AUC) decreased the mechanical nociceptive threshold when compared to mice that received saline (i.pl.) and from the baseline. A dose of 100 ng and 10 ng per paw of NGF and GDNF, respectively, were used in subsequent experiments.

We evaluated the specificity of neurotoxin IB4-Sap treatment at 24 days after i.t. injection, on the mechanical inflammatory hypersensitivity induced by i.pl. administration of NGF and GDNF. Whereas the mechanical inflammatory

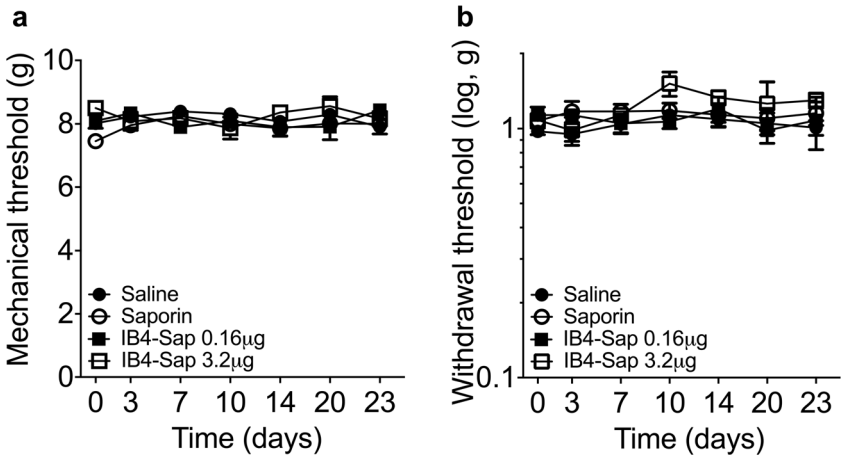

Fig. 3 IB4-Sap injection does not alter the mechanical nociceptive threshold in mice at several time points. The mechanical withdrawal threshold was evaluated using **a** electronic von Frey as well as **b** von Frey filaments before and 3, 7, 10, 14, 20, and 23 days after i.t. administration of saline, saporin, or IB4-Sap (0.16 or 3.2 μg/5 μl) in C57BL/6 mice. The data are presented as the mean ± SEM of 6–7 mice per group

hypersensitivity induced by i.pl. injection of NGF 100 ng was not altered in mice pre-treated with neurotoxin IB4-Sap compared to control groups (saline and saporin) (Fig. 4c, time-course response and Fig. 4, panel d, AUC), GDNF-induced mechanical inflammatory hypersensitivity was abolished by this treatment (Fig. 4, panel g, time-course response and Fig. 4, panel h, AUC). Thus, these data suggest the IB4-Sap depletes selectively the non-peptidergic subset of C-fibers in mice.

Non-Peptidergic Nociceptor Sensitisation Is Required for Mechanical Inflammatory Hypersensitivity

In order to identify whether the subpopulation of non-peptidergic nociceptors is involved in detecting and transmitting mechanical inflammatory hypersensitivity induced by peripheral inflammation, we treated animals with IB4-Sap and 24 days later, observed the mechanical inflammatory hypersensitivity produced by different noxious stimuli in mice. First, the mechanical inflammatory hypersensitivity induced by carrageenan (Fig. 5, panels a–d) was decreased by IB4-Sap 3 and 5 h (Fig. 5, panels a and c, panels b and d respectively) after injection, compared to control groups (saline or saporin, i.t.) in both von Frey tests (filaments and electronic). Interestingly, the higher dose of IB4-Sap (3.2 μg), but only at 5 h in the von Frey filament test, had more effect in the reduction of mechanical inflammatory hypersensitivity when compared with the lower doses (Fig. 5, panel d). Furthermore, as can be seen in Table 1, in von Frey filaments but not in the electronic version, the different doses of neurotoxin IB4-Sap promote a significant dose-dependent inhibition of mechanical inflammatory hypersensitivity (which takes into account the baseline of each group) at both evaluated times (3 and 5 h). Thus, a dose of 3.2 μg/i.t. of IB4-Sap was used in subsequent experiments. Afterwards, we investigated whether peripheral injection of the inflammatory mediator carrageenan could induce an elevated expression of the $P2X_3$ purinergic receptor in the DRGs. The i.pl. injection of carrageenan was found to promote an increase in $P2X_3$ expression in the DRGs at 1 and 3 h after i.pl. injection of the inflammatory stimulus compared to control mice (Fig. 5, panel e).

Mechanical inflammatory hypersensitivity is triggered by inflammatory mediators that sensitise primary nociceptive fibers through direct interaction with specific receptors present in the nociceptors [25]. PGE_2 and sympathomimetic amines (e.g., epinephrine) are well-known mediators that act to directly sensitise primary nociceptive neurons [26, 27]. In this context, we injected PGE_2 and epinephrine i.pl. in mice pre-treated with neurotoxin IB4-Sap to evaluate whether the mechanical inflammatory hypersensitivity induced by these inflammatory mediators is mediated by non-peptidergic nociceptors in mice. The non-peptidergic C-fibers depletion significantly attenuated the mechanical inflammatory hypersensitivity caused by PGE_2 and epinephrine injected into the mouse paw compared to control group (saline, i.t.) (Fig. 6, panels a and c, electronic von Frey; b and d, von Frey filaments). Table 1 shows a summary of the percentage of pain inhibition produced by different doses of neurotoxin IB4-Sap, in the diverse inflammatory pain models in mice analysed in this study. Altogether, our results suggest the non-peptidergic nociceptors are sensitised by inflammatory mediators and mediate mechanical inflammatory hypersensitivity in mice.

Discussion

Primary sensory neurons detect peripheral stimuli in order to discern touch, temperature, and nociception. Nociceptors are the subset of peripheral nerve fibers that selectively respond to noxious or potentially tissue-damaging stimulus and are sensory end organs that innervate skin, muscle, joints, and viscera [28, 29]. The C-fibers is the most common subpopulation of nociceptors in the skin, characterised as polymodal neurons, which react to multiple modalities of stimuli, like mechanical, thermal, and chemical [30]. Thus, understanding the functional properties of each subset of sensory neurons in pain

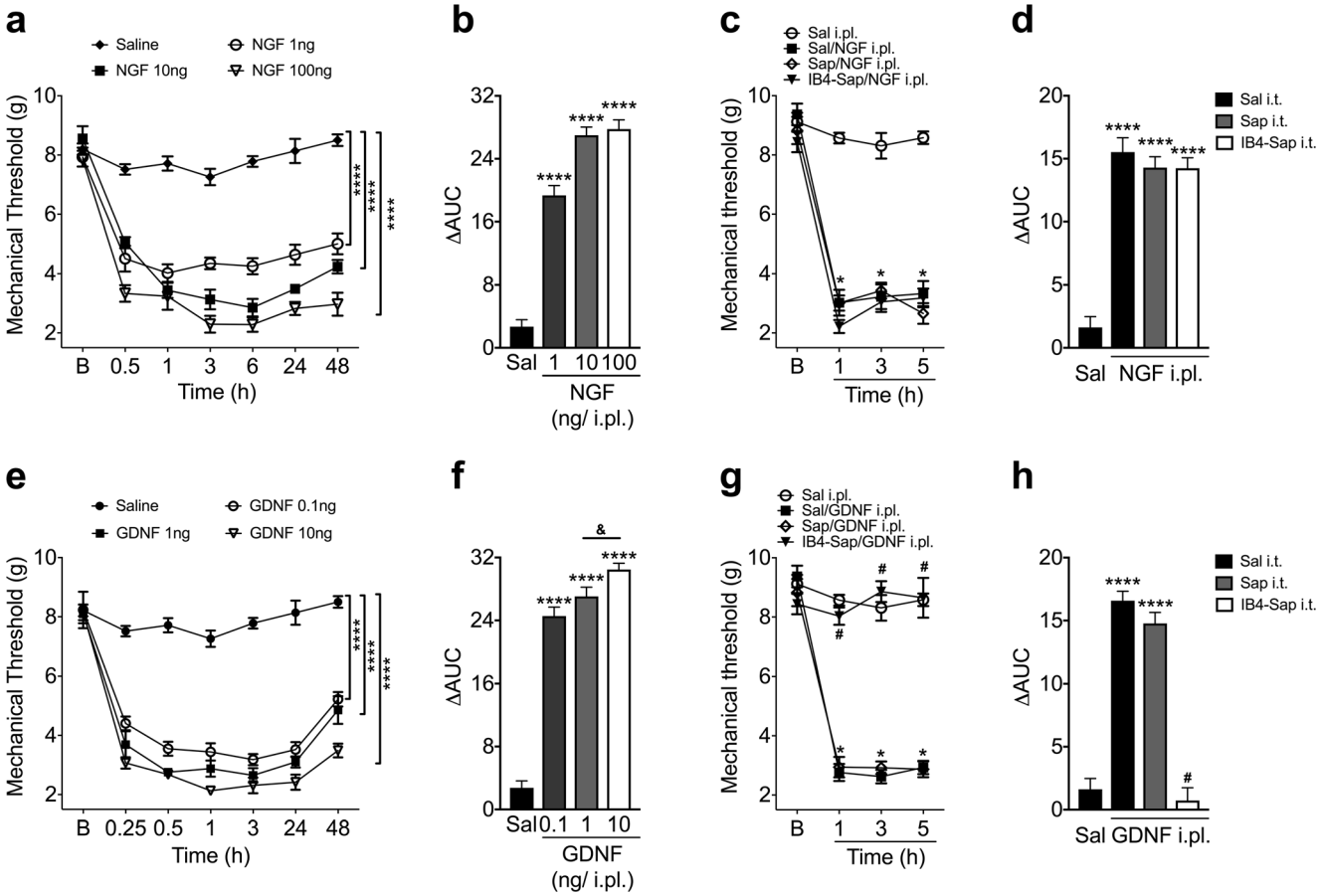

Fig. 4 Neurotoxin IB4-Sap depletes selectively non-peptidergic C-fibers. Time- and dose-response curves of NGF (**a** and **b**) and GDNF (**e** and **f**) in mechanical threshold in mice. NGF was injected i.pl. at different doses (1–100 ng/20 μl) and the mechanical withdrawal threshold was evaluated before (B, baseline threshold) and 0.5 to 48 h after inflammatory stimulus injection using electronic von Frey. Time- and dose-response curves (**a**) and ΔAUC for difference between the groups in relation to the baseline (integral between 0 and 48 h) (**b**) after the NGF i.pl. injection. **e** and **f** GDNF (0.1–10 ng/20 μl) was administered i.pl. and the mechanical withdrawal threshold was assessed before or 0.25, 0.5, 1, 3, 24, and 48 h after injection. Time- and dose-response curves (**e**) and ΔAUC for difference between the groups in relation to the baseline (integral between 0 and 48 h) (**f**) after the GDNF i.pl. administration. **c**, **d**, **g**, and **h** Mice were injected i.t. with saline, saporin (toxin non-conjugated, negative control), or IB4-Sap (3.2 μg) and 24 days after the treatments, mechanical withdrawal threshold was evaluated using electronic von Frey before (B-baseline) and after inflammatory stimuli injection (i.pl./ 20 μl). **c** and **d** NGF (100 ng/20 μl) was injected i.pl. and after 1, 3, and 5 h (**c**, time-response curve) the mechanical nociceptive threshold was evaluated. **d** ΔAUC for difference between the groups in relation to the baseline (integral between 0 and 5 h) (**g** and **h**) GDNF (10 ng/20 μl) was injected i.pl. and after 1, 3, and 5 h (**g**, time-response curve) the mechanical nociceptive threshold was evaluated. **h** ΔAUC for difference between the groups in relation to the baseline (integral between 0 and 5 h). Saline (20 μl) i.pl. was the control group. Data are expressed as mean ± SEM ($n = 5$–7 mice per group). $^{****}P < 0.0001$ indicate statistically significant differences when compared with the saline i.pl. group and from the baseline; $^{\&}P < 0.05$, compared with GDNF 1 ng/i.pl. and $^{\#}P < 0.001$, compared with the control groups (saline and saporin, i.t.) injected i.pl. with GDNF

processing can be important to the development of new therapies that target nociceptors, to treat pathological pain (clinical) without affecting the protective pain sensation (nociceptive). In this context, this study provides evidence of the essential role of non-peptidergic C-nociceptors in mechanical inflammatory hypersensitivity, but not in acute nociceptive pain, in mice.

Previous studies have used a cytotoxin to eliminate the $IB4^+$-neurons subpopulation in rats [31] and more recently in mice [16]. The cytotoxin saporin is a protein from *Saponaria officinalis*, which when conjugated to a specific marker for a particular type of neuron (like IB4 in non-peptidergic fibers) is internalised, inhibiting the protein synthesis and thus result in cell death [32, 33]. In fact, a single injection of saporin conjugated to IB4 induced a selective loss of $IB4^+$-neurons, which can be visualised in DRG, spinal cord, and skin of rats [34]. Moreover, mice treated intrathecally with IB4-Sap showed a decrease in IB4 staining in the spinal cord 2 weeks after injection [16]. Here we confirm that i.t. injection of IB4-Sap depletes permanently and selectively the non-peptidergic nociceptor population in mice. Expression of $P2X_3$, which is a characteristic of the non-peptidergic population [23, 35], was decreased in the DRGs after IB4-Sap treatment. Furthermore, ATF-3 which is a transcription factor

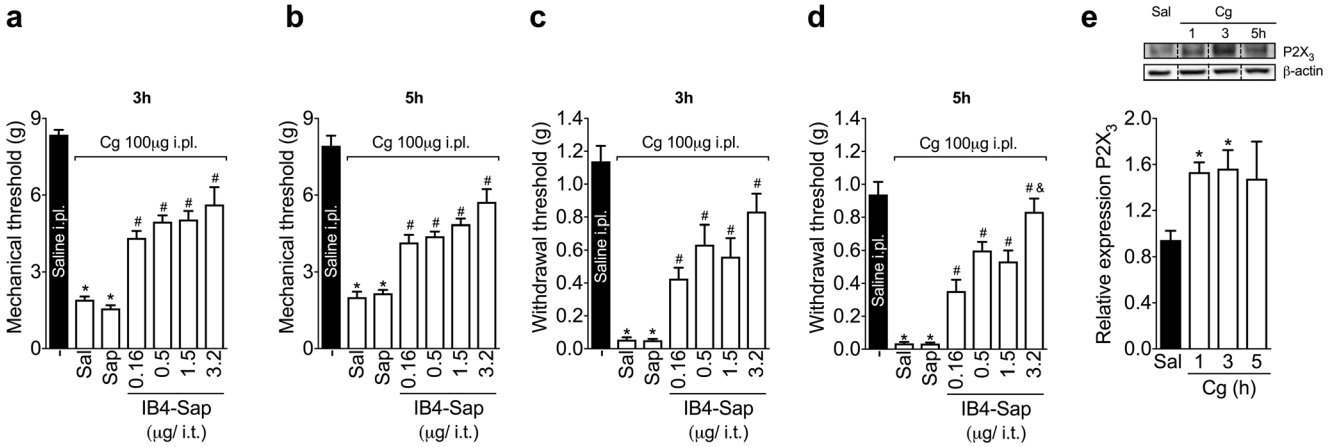

Fig. 5 Mechanical inflammatory hypersensitivity induced by carrageenan is dependent of IB4$^+$ nociceptors activation. **a–d** Mechanical withdrawal thresholds were assessed using electronic von Frey (**a and b**) and von Frey filaments (**c** and **d**), 3 (**a** and **c**), and 5 (**b** and **d**) hours after the i.pl. administration of carrageenan (100 µg/20 µl) or saline (20 µl/i.pl.) 24 days after i.t. injection of saline (5 µl), saporin control (5 µl) or IB4-Sap (0.16–3.2 µg/5 µl) in mice. **e** Saline or carrageenan (100 µg) were injected into the paw and at 1, 3, and 5 h after i.pl. injections, the L4, L5, and L6 DRGs (ipsilateral) were collected to analyse the $P2X_3$ (66 kDa) expression by Western blot. Representative blot and relative quantification. Beta actin (42 kDa) levels were used as an internal control. Data are expressed as the mean ± SEM ($n = 4$ mice per group in Western Blot experiment and $n = 5–7$ mice per group in behavior experiment). *$P < 0.05$ indicate statistically significant differences when compared with the saline i.pl. group; $^{\#}P < 0.05$, compared with the control groups (saline and saporin, i.t.) injected i.pl. with carrageenan; and $^{\&}P < 0.05$, compared with the IB4-Sap 0.16, 0.5, and 1.5 µg/i.t. groups

synthesised de novo in sensory neurons in response to injury of peripheral axons [36] was increased in DRGs, at least, 17 days after neurotoxin administration, suggesting neuronal damage [24]. Several studies have reported that the maximal loss of IB4-binding occurs 21 days after IB4-Sap injection, which then persists indefinitely as a result of cell death [11, 34]. In the present work, we also demonstrated a loss in IB4-binding in the inner lamina II of the spinal cord 24 days after neurotoxin injection. Importantly, GDNF induces hypersensitivity via sensitisation of non-peptidergic neurons [37, 38], while the hypersensitivity effect induced by NGF is dependent on TrkA activation, which is mainly expressed in peptidergic neurons C-fibers [39, 40]. We used these facts to confirm the specificity of IB4-Sap in depleting non-peptidergic C-fibers, since the mechanical inflammatory hypersensitivity induced by GDNF, but not by NGF, was completely abolished in mice treated with the neurotoxin. Our results are in agreement with a previous study in rats, in which it was observed that GDNF hypersensitivity was blocked by IB4-Sap administration, while hypersensitivity induced by NGF was attenuated by

Table 1 Percentage of pain inhibition in mice treated with different doses of IB4-Saporin i.t. (relative to control group, saline i.t)

Inflammatory stimulus (i.pl.)	Carrageenan (100 µg)		Epinephrine (300 ng)	PGE_2 (100 ng)
Treatment (i.t.)	3 h	5 h		
Electronic von Frey				
IB4-Saporin 0.16 µg	47.91 ± 8.52	42.55 ± 8.33	–	–
IB4-Saporin 0.5 µg	54.24 ± 6.42	42.5 ± 8.43	–	–
IB4-Saporin 1.5 µg	51.01 ± 6.14	44.25 ± 6.53	–	–
IB4-Saporin 3.2 µg	69.62 ± 9.59	68.74 ± 7.39	66.24 ± 6.99	58.27 ± 9.57
von Frey filaments				
IB4-Saporin 0.16 µg	34.1 ± 2.2$^{*, \#, \&}$	35.5 ± 1.33$^{*, \#, \&}$	–	–
IB4-Saporin 0.5 µg	50.4 ± 2.42$^{@, \&}$	63.5 ± 2.43$^{@, \&}$	–	–
IB4-Saporin 1.5 µg	47.1 ± 1.14$^{@, \&}$	55.5 ± 2.53$^{@, \&}$	–	–
IB4-Saporin 3.2 µg	72.02 ± 2.59$^{@, *, \#}$	88.4 ± 1.39$^{@, *, \#}$	74.0 ± 1.5	48.9 ± 1.57

Values represent means ± SEM ($n = 5–7$ mice per group). $^{@}P < 0.05$ indicate statistically significant differences when compared with the IB4-Sap 0.16 µg/i.t. group; *$P < 0.05$ indicate statistically significant differences when compared with the IB4-Sap 0.5 µg/i.t. group; $^{\#}P < 0.05$ indicate statistically significant differences when compared with the IB4-Sap 1.5 µg/i.t., and $^{\&}P < 0.05$ indicate statistically significant differences when compared with the IB4-Sap 3.2 µg/i.t. group

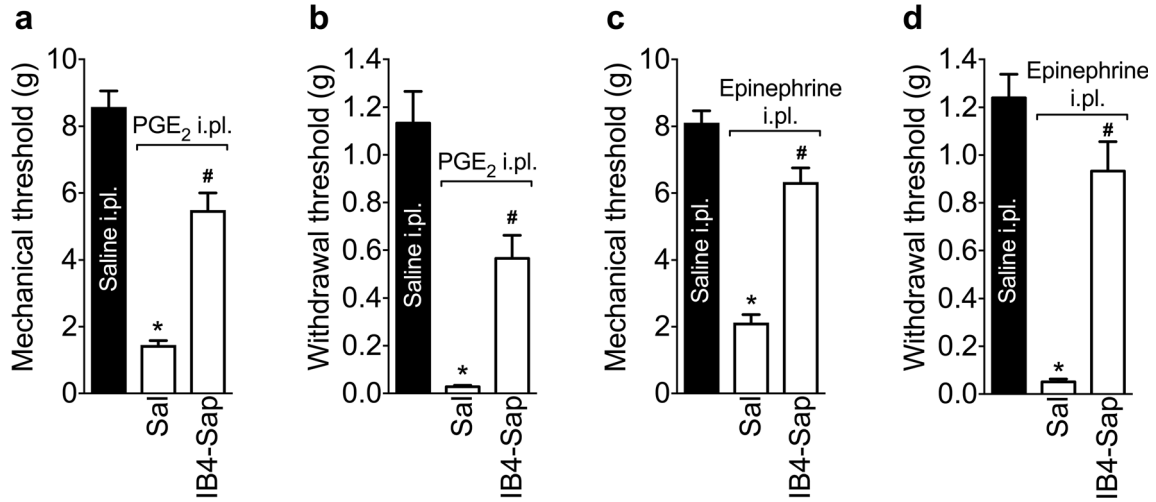

Fig. 6 PGE_2 and epinephrine sensitise non-peptidergic C-fibers. **a**–**d** At 24 days after i.t. administration of saline or IB4-Sap (3.2 μg), C57BL/6 mice were injected i.pl. with different inflammatory mediators and mechanical withdrawal threshold were evaluated using electronic von Frey (**a** and **c**) and von Frey filaments (**b** and **d**). **a** and **b** PGE_2 (100 ng/20 μl) or saline was injected i.pl. and the mechanical threshold was measured 30 min after inflammatory stimulus injection. **c** and **d** Mechanical withdrawal threshold was assessed 60 min after the i.pl. administration of saline or 300 ng of epinephrine into the hind paw of mice. Data are expressed as the mean ± SEM ($n = 5$–7 mice per group). $^{*}P < 0.05$ indicate statistically significant differences when compared with the saline i.pl. group; and $^{\#}P < 0.05$, compared with the control group (saline i.t.) injected i.pl. with inflammatory mediators (PGE_2 or epinephrine)

TrkA antisense injection [38]. Taken together, this evidence suggests that IB4-Sap injection selectively eliminates $IB4^{+}$-nociceptors in mice and rats.

There are many functionally distinct subsets of cutaneous somatosensory neurons with specific threshold sensitivities, each of which can detect a large range of sensory stimuli, including light touch, mechanical pressure, temperature, itch, and pain [41, 42]. In particular, the mechanosensory neurons are classified into distinct subtypes, for example, the low-threshold mechanoreceptors (LTMRs), which respond preferentially to innocuous mechanical forces, and the high-threshold mechanoreceptors (HTMRs) like mechanonociceptors, which exclusively detect noxious or potentially harmful mechanical forces [41, 43, 44]. However, it has been observed that the LTMRs are essential not only for touch sensation and innocuous mechanical stimulation but also play an important role in acute mechanical pain and mechanical hypersensitivity after nerve injury [45, 46]. In addition, the mechanically activated cation channel Piezo-2, which is expressed in LTMRs, plays a role in both innocuous touch and mechanical pain sensation [47, 48]. Although in our study, the ablation of non-peptidergic C-fibers did not affect acute mechanical nociceptive thresholds in mice, which is in agreement with a study in rats [49, 50], we cannot exclude the involvement of these fibers in detecting this type of sensation. It could be due to the nociceptive methods we have used, electronic and filaments of von Frey. In fact, the ablation of C-fibers ($Na_v1.8$ positive cells) did not change the mechanical threshold of mice detected by von Frey filaments but strongly impact mechanical threshold determined by Randall Selitto test [51]. Alternatively, distinct sensory neurons subsets (like $IB4^{+}$, $IB4^{-}$ as well LTMRs) might have overlap functions to encode mechanical nociception.

We also investigated the participation of non-peptidergic neurons in acute chemical nociception induced by capsaicin, and we observed a reduction in the nociceptive effect induced by capsaicin when $IB4^{+}$-neurons were ablated. Capsaicin, the main pungent component of hot-chili peppers, induces nociception through TRPV1 activation [52, 53]. The TRPV1 is a non-selective cation channel expressed by peptidergic and non-peptidergic primary sensory neurons [54, 55]. We also found that IB4-Sap decreases TRPV1 in both spinal cord and DRG, in agreement with a study in rats [34] and also reduces bladder nociceptive responses induced by intravesicular capsaicin injection [31]. Therefore, we propose that $IB4^{+}$-neurons are involved, at least in part, in acute nociception induced by TRPV1 activation.

In the last part of our study, we examined whether non-peptidergic C-fibers are the subpopulation of nociceptors involved in sensing and transducing mechanical inflammatory hypersensitivity in mice. Previous reports show that IB4-Sap decreases mechanical inflammatory pain induced by MCP-1 [12] as well as attenuating acute and chronic muscle pain induced by inflammatory and ergonomic intervention insults in rats [14]. Interestingly, the inflammatory model of muscle pain induced by intramuscular carrageenan was only slightly decreased by IB4-Sap injection, suggesting that $IB4^{+}$-neurons do not play a major role in this model of muscle

hyperalgesia [14]. On the other hand, in the present study, it was found that the acute mechanical inflammatory hypersensitivity induced by i.pl. injection of carrageenan was substantially reduced by non-peptidergic C-fibers depletion in mice. It was interesting that the participation of non-peptidergic C-fibers in mechanical inflammatory hypersensitivity was detected by both electronic and von Frey filaments. Several studies have discussed advantages, limitations, reproducibility among others features for each test in human and rodents [20, 56]. In this context, it has been demonstrated that different skin layers can be stimulated by each of them and consequently, distinct subsets of fibers might be activated. In fact, it is more likely that von Frey filaments assess tactile sensitivity, responses to low-threshold mechanical stimuli, which are characteristics of Aβ- and C-LTMRs fibers, localised in glabrous and hairy skin [57]. On the other hand, electronic von Frey is more sensitive to measure inflammatory hypersensitivity, high-threshold mechanical stimuli, that are detected by Aδ and C-fibers, which are found in intra-epidermal free nerve endings [41]. It is important to mention that in the present study, the inflammatory stimulus was injected subcutaneously, which means the inflammation was in a deeper skin layer that is different from a more superficial intradermal injection. Nevertheless, since von Frey filament was able to detect reversal of mechanical inflammatory hyperalgesia by IB4-Sap treatment, this result indicates that during inflammation, even von Frey filament is able to stimulate sensitised C-fibers. It is in line with the observation that mechanical inflammatory hyperalgesia, determined by von Frey filaments, was reduced in mice with depletion of $Na_v1.8^+$ nociceptors [51]. Further supporting the importance of non-peptidergic C-fibers for mechanical inflammatory hypersensitivity, we found that inflammation causes up-regulation in $P2X_3$ expression at the DRGs, which has been previously described as important for inflammatory pain [58–63]. The importance of non-peptidergic C-fibers has been also demonstrated in chronic inflammatory pain conditions [11, 14, 15], as well as in a phenomenon of transition from acute to chronic pain, the hyperalgesic priming [64]. Hyperalgesic priming is a long-lasting neuroplastic change in nociceptors function triggered by a previous pro-inflammatory insult, inducing now a marked and prolonged mechanical hyperalgesia, which is associated with chronic pain states [65]. Interestingly, Joseph and Levine [13] demonstrated that only $IB4^+$-neurons are involved in hyperalgesic priming. These evidences suggest that besides targeting non-peptidergic nociceptors to control acute inflammatory pain, they might be also an interesting target for the control of chronic inflammatory pain states.

Our group has previously shown that carrageenan induces mechanical inflammatory pain in mice through a mechanism dependent on release of prostaglandins and sympathomimetic amines such as epinephrine, which sensitise directly primary sensory neurons [66]. Therefore, we investigated whether non-peptidergic C-fibers are the nociceptor subpopulation sensitised by i.pl. injection of PGE_2 and epinephrine. We found that IB4-Sap significantly decreased the mechanical inflammatory hypersensitivity induced by PGE_2 and by epinephrine in mice. It is well known that inflammatory mediators sensitise nociceptors via binding to specific G protein-coupled receptors (GPCRs) expressed in primary sensory neurons [27, 67, 68]. For example, PGE_2 binds to prostanoid (EP4) receptors, leading to activation of adenylate cyclase and elevated cyclic adenosine monophosphate (cAMP) [69], which can activate protein kinase A (PKA) signaling pathway with subsequent sensitisation of ion channels (e.g., TRPV1, $P2X_3$, $Na_v1.8$, and $Na_v1.9$) in primary afferent neurons [70, 71]. In addition, cAMP can also bind directly to HCN2, an ion channel belonging to the hyperpolarization-activated cyclic nucleotide-gated (HCN) family, leading to an enhancement of inward current and activation of neuronal firing [72]. Furthermore, epsilon isozyme of protein kinase C (PKCε), a signaling pathway involved in epinephrine-induced sensory neurons sensitisation and pain hypersensitivity, can also be activated by cAMP [27]. Importantly, epinephrine via β2-adrenergic receptor activates cAMP/PKCε specifically in $IB4^+$-neurons [73]. Thus, we can suggest that these directly acting mediators might be involved in mechanical inflammatory hypersensitivity through the sensitisation of non-peptidergic nociceptors.

In summary, our results suggest that the non-peptidergic C-fibers have a critical role in mechanical inflammatory hypersensitivity. In addition, ablation of $IB4^+$-nociceptors did not affect acute nociceptive pain sensation. Thus, understanding the involvement of each nociceptive neuron subpopulation in physiological as well as pathological conditions could lead to a new strategy to treat pain disorders without affecting protective pain. Silencing a subpopulation of primary sensory neurons specifically might be particularly advantageous as it can reduce systemic side effects.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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