Pharmacology, Biochemistry and Behavior 150–151 (2016) 57–67

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Mechanical antihypersensitivity effect induced by repeated spinal administrations of a TRPA1 antagonist or a gap junction decoupler in peripheral neuropathy

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ARTICLE INFO

Article history:
Received 20 June 2016
Received in revised form 21 September 2016
Accepted 23 September 2016
Available online 24 September 2016

Keywords:
Astrocytes
D-Amino acid oxidase
Gabapentin
Hydrogen peroxide: Pain
Hypersensitivity
Spinal cord dorsal horn
TRPA1

ABSTRACT

Spinal transient receptor potential ankyrin 1 (TRPA1) channel is associated with various pain hypersensitivity conditions. Spinally, TRPA1 is expressed by central terminals of nociceptive nerve fibers and astrocytes. Among potential endogenous agonists of TRPA1 is H_2O_2 generated by D-amino acid oxidase (DAAO) in astrocytes. Here we studied whether prolonged block of the spinal TRPA1 or astrocytes starting at time of injury attenuates development and/or maintenance of neuropathic hypersensitivity. Additionally, TRPA1 and DAAO mRNA were determined in the dorsal root ganglion (DRG) and spinal dorsal horn (SDH). Experiments were performed in rats with spared nerve injury (SNI) and chronic intrathecal catheter. Drugs were administered twice daily for the first seven injury days or only once seven days after injury. Mechanical hypersensitivity was assessed with monofilaments. Acute and prolonged treatment with Chembridge-5861528 (a TRPA1 antagonist), carbenoxolone (an inhibitor of activated astrocytes), or gabapentin (a comparison drug) attenuated tactile allodynia-like responses evoked by low (2 g) stimulus. However, antihypersensitivity effect of these compounds was short of significance at a high (15 g) stimulus intensity. No preemptive effects were observed. In healthy controls, carbenoxolone failed to prevent hypersensitivity induced by spinal cinnamaldehyde, a TRPA1 agonist. TRPA1 and DAAO mRNA in the DRG but not SDH were slightly increased in SNI, independent of drug treatment. The results indicate that prolonged peri-injury block of spinal TRPA1 or inhibition of spinal astrocyte activation attenuates maintenance but not development of mechanical (tactile allodynia-like) hypersensitivity after nerve injury.

1. Introduction

Transient receptor potential ankyrin 1 (TRPA1) is a calcium-permeable nonselective cation channel. In the nervous system, it is expressed on a subpopulation of nociceptive primary afferent nerve fibers (Story et al., 2003). On their peripheral nerve endings, TRPA1 is involved in transduction of harmful signals into electric signals and on their central endings within the spinal dorsal horn TRPA1 is involved in amplification of glutamatergic transmission (see for review e.g., Andrade et al., 2012). Another cell type in the nervous system expressing TRPA1 is astrocyte on which TRPA1 contributes to regulation of the inhibitory synapse efficacy in adjacent neurons (Shigetomi et al., 2011). Moreover, oligodendrocytes in the nervous system were recently shown to express TRPA1 (Hamilton et al., 2016).

Nerve injuries may cause long-lasting pain accompanied by hypersensitivity particularly to mechanical stimulation and cooling. There is earlier evidence indicating that nerve injury-induced (neuropathic) pain is at least partly mediated by TRPA1. This is indicated by the findings that systemic block of TRPA1 genetically or pharmacologically suppressed pain hypersensitivity in animals with a traumatic, chemical or metabolic injury of peripheral nerves (e.g., del Camino et al., 2010; Eid et al., 2008; Obata et al., 2005; Wei et al., 2009, 2012). Suppression of neuropathic pain induced by systemically administered TRPA1

Abbreviations: DAAO, D-amino acid oxidase; DRG, dorsal root ganglion; ROS, reactive oxygen species; SDH, spinal dorsal horn; TRPA1, transient receptor potential ankyrin 1.

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http://dx.doi.org/10.1016/j.pbb.2016.09.007
0091-3057/

antagonists is at least partly due to spinal action, since blocking spinal TRPA1 has proved effective in attenuating hypersensitivity in experimental models of neuropathy (Wei et al., 2010a, 2011).

Previous pharmacological studies demonstrating the antihypersensitivity effect of spinal TRPA1 antagonists in chronic neuropathic pain models were performed following the development of fulminant neuropathy and using acute single-dose treatments. This leaves open whether a TRPA1 antagonist administered spinally at the time of injury, e.g. when performing an elective operation in the clinic that potentially causes nerve damage, and continuation of the treatment for several days thereafter effectively attenuates the development and/or maintenance of pain hypersensitivity. Therefore, we assessed whether twice daily spinal treatment with a selective TRPA1 antagonist starting at the time of nerve injury and continuing for a week attenuates the development and maintenance of hypersensitivity. Moreover, activation of spinal astrocytes has been associated with pain hypersensitivity (Hansson, 2010). Astrocytes express TRPA1 (Shigetomi et al., 2011) and their activation leads to increased spinal level of astroglial D-amino-acid oxidase (DAAO) that generates H_2O_2 (Zhao et al., 2010), an established TRPA1 agonist (Andersson et al., 2008). To assess the potential role of spinal astrocytes in the TRPA1 antagonist-induced antihypersensitivity effect, we also assessed mechanical hypersensitivity following prolonged treatment of nerve-injured animals with spinally administered carbenoxolone, a gap junction blocker that inhibits activation of astrocytes.

It was hypothesized that if prolonged blocking of spinal TRPA1 or astrocytes during the first injury days prevents the development of neuropathic hypersensitivity, then the baseline hypersensitivity should be attenuated (i.e., attenuation of hypersensitivity at a time point when the pharmacological effect of the studied drug was over). On the other hand, by testing the acute antihypersensitivity effect of the studied compounds after a prolonged treatment we expected to reveal whether the studied drugs attenuate maintenance of neuropathic hypersensitivity as effectively after prolonged as acute single treatment.

For additional information about the potential interaction of astrocytes and TRPA1, we studied whether carbenoxolone attenuates hypersensitivity induced by spinal administration of cinnamaldehyde, a selective TRPA1 agonist, in healthy controls. Gabapentin that has been widely used in the treatment of neuropathic pain (Finnerup et al., 2015) was the comparison drug. We also determined TRPA1 and DAAO mRNA in the dorsal root ganglion (DRG) and the spinal dorsal horn (SDH) to find out whether nerve injury or the currently used drug treatments are associated with upregulations of TRPA1 or DAAO mRNA.

2. Materials and methods

2.1. Experimental animals

The experiments were performed in 55 adult, male Hannover-Wistar (HW) rats (weight: 150–200 g; CAS, Shanghai, China). None of the animals died before the completion of the behavioral experiments. Experiments were approved by the Science and Technology Commission of Shanghai Municipality (permission number SYXK 2012-0017) and all experimental procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

2.2. Techniques for intrathecal catheter installation and drug administration

For intrathecal (i.t.) drug injections a catheter (PE-10) was administered on day 0 (D0) into the lumbar level of the spinal cord under sodium pentobarbital anesthesia (60 mg/kg i.p.) as described in detail elsewhere (Størkson et al., 1996). For i.t. administration, the drugs were microinjected with a 50 μl syringe in a volume of 5 μl followed by a saline flush in a volume of 20 μl. I.t. administrations of drugs were performed in awake animals that were well-habituated to handling. During i.t. drug administration, the animal was gently restrained by one hand of the experimenter, while the other hand was used for drug injection. The animals expressed no obvious sign of discomfort due to restraint or i.t. injections.

2.3. Techniques for producing neuropathy

There are a number of surgically induced models of peripheral neuropathy (Honoré et al., 2011), of which the spared nerve injury model (SNI; Decosterd and Woolf, 2000) was adopted. Prior to surgery, the rat was anesthetized with sodium pentobarbital administered intraperitoneally at the dose of 60 mg/kg. Further doses of sodium pentobarbital were given at the dose of 15–20 mg/kg as needed to keep the depth of anesthesia deep enough so that the animal did not react to noxious stimulation. SNI surgery was performed on D0 in the same session as installation of i.t. catheter.

In the SNI operation, an incision was made into the skin on the lateral surface of the left thigh, followed by a section through the biceps femoris muscle to expose the sciatic nerve and its terminal branches: the sural, common peroneal and tibial nerves. The common peroneal and tibial nerves were then tightly ligated with 4–0 silk, sectioned distal to the ligation and 3–4 mm of the distal nerve stump removed. The sural nerve was left intact and care was exercised not to stretch it. For comparison, one group of sham-operated animals was studied. Sham operation was performed identically as the operation for inducing SNI, except that the common peroneal and tibial nerves were left intact. After the operations, the muscle and skin were sutured, and the rats were moved to their individual home cages for recovery. Since the studied compounds were presumed to have analgesic properties and the potential analgesic efficacy of the studied compounds given peri- vs only postoperatively was among the study questions, no additional analgesic treatment were in the study protocol, unless the animals showed signs of suffering or spontaneous pain. In the latter case, the animal was euthanized before completing the experiment, which, however, was not needed in the present sample of animals.

2.4. Behavioral testing

Since mechanical rather than heat hypersensitivity is a frequent problem in patients with peripheral neuropathy (Scadding and Koltzenburg, 2006), the focus was on mechanically evoked pain behavior. Furthermore, central mechanisms that were studied in the present experiments play an important role in hypersensitivity to mechanical rather than heat stimulation (Treede et al., 1992). Before the first assessment of pain behavior on D0, the animals were habituated to the experimental conditions at least for /day for two days. To assess mechanical hypersensitivity, the frequency of the withdrawal response to the application of monofilaments (von Frey hairs) to the sural nerve area in the hind paw was examined while the animal was standing on a metal grid that was covered by a plastic cage. A series of ten hairs with forces varying from 0.4 g to 60 g (North Coast Medical, Inc., Morgan Hill, CA) were applied five times each at a frequency of approximately of 0.5 Hz. Hairs were tested in ascending order of force. A visible lifting of the stimulated hind limb was considered a withdrawal response. If the rat failed to withdraw to any of the five presentations of a monofilament, the response rate for the studied force level was 0%. If the rat withdrew every time the monofilament was applied to the paw, the response rate for the studied force level was 100%. Thus, an increase in the response rate represents facilitation of mechanical stimulus-evoked pain behavior (hypersensitivity). In this study, results on testing mechanical sensitivity are reported at two different test stimulus intensities: a low test stimulus force (2 g) that activates only low-threshold

mechanoreceptors and a high test stimulus force (15 g) that produces co-activation of nociceptors (Leem et al., 1993).

Behavioral testing of mechanical hypersensitivity was performed on D0 (before induction of anesthesia for the installation of i.t. catheter and SNI surgery) and on D7, the last day of the experiment (Fig. 1). On D7, behavioral assessment of hypersensitivity was performed once before i.t. administration of the last drug/vehicle dose. This pre-drug assessment on D7, at a time point when the acute drug effect of the previous drug dose was over, allowed determining the potential effect of the prolonged twice daily drug treatment on the development of baseline hypersensitivity. The pre-drug assessment of hypersensitivity on D7 was followed by the assessment of the drug-induced acute antihypersensitivity effect 15, 30 and 60 min after the last drug/vehicle dose (Fig. 1).

2.5. Real-time quantitative polymerase chain reaction (RT qPCR)

Rat SDHs (L3-L5) and DRGs ipsilateral to injury were collected and homogenized using electronic microhomogenizer at 4000 rmp for 10 s in TRIzol (Invitrogen, Grand Island, NY, USA) on ice. Total RNA of the spinal lumbar enlargements was extracted and purified by use of TRIzol reagent. The cDNA was prepared from 1 μg of total RNA by using ReverTra Ace qPCR RT-Kit (Toyobo Co. Ltd., Osaka, Japan). Real-time quantitative PCR was performed on a Mastercycler ep realplex (Eppendorf, Hamburg, Germany) using Realmaster Mix (SYBR Green I) and synthetic primers. The sequences of primers were following: DAAO: 5′-CCC TTT CTG GAA AAG CAC AG-3′(forward), 5′-CTC CTC TCA CCA CCT CTT CG-3′(reverse) (Zhao et al., 2010); TRPA 1: 5′-CTC AGG TTC AAT GTG TCC GTTC-3′(forward), 5′-GTG CTG TGT TCC CTT CATC-3′(reverse) (Du et al., 2007); GAPDH: 5′-CCA AGG TCA TCCATG ACA AC-3′ (forward), 5′-TCC ACA GTC TTC TGA GTGGC-3′ (reverse) (Vahl et al., 2007). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression after normalization to the internal control gene GAPDH (Zhao et al., 2010).

2.6. Drugs

Chembridge-5861528, TRPA1 antagonist (Koivisto and Pertovaara, 2015), was obtained from ChemBridge Corp. (San Diego, CA). Carbenoxolone (a gap junction decoupler), cinnamaldehyde (a TRPA1 agonist) and gabapentin (a widely used analgesic compound against neuropathic pain) were purchased from Sigma-Aldrich (St.Louis, MO). Vehicle consisted of polyethylene glycol (75%) and physiological saline (25%). All compounds were administered intrathecally (i.t.) to the lumbar level of the spinal cord. Drug doses were chosen based on earlier studies assessing the antihypersensitivity effect of spinally administered Chembridge-5861528 (e.g., Wei et al. 2010a & 2011) and carbenoxolone (Wei et al., 2010b), or hypersensitivity effect of cinnamaldehyde (Wei et al., 2011). When considering the dose of Chembridge-5861528, it should be noted that due to binding to brain proteins, only about 2.3% of Chembridge-5861528 is free in the brain (Wei et al., 2012). The dose of gabapentin, the comparison drug, was 50 μg that has been shown to be the ED_{50} value of intrathecally administered gabapentin against tactile-allodynia like symptoms in rodents (Cheng et al., 2000).

2.7. Course of the study

There were ten experimental groups, in all of which animals were equipped with a chronic catheter for i.t. drug deliveries (see Table 1 for the groups i–viii). The ten experimental groups were: i) CHEM D0–D7 group of SNI animals, in which Chembridge-5861528 was administered twice daily fourteen times at the dose of 10 μg (n = 6). ii) CHEM D7 group of SNI animals, in which vehicle was administered twice daily thirteen times, while the last (14th) i.t. treatment on D7 was Chembridge-5861528 at the dose of 10 μg (n = 6). iii) Carbenoxolone D0–D7 group of SNI animals, in which carbenoxolone was administered twice daily fourteen times at the dose of 10 μg (n = 6). iv) Carbenoxolone D7 group of SNI animals, in which vehicle was administered twice daily thirteen times, while the last i.t. treatment on D7 was 10 μg of carbenoxolone (n = 5). v) Vehicle group of SNI animals, in which vehicle was administered twice daily fourteen times (n = 5). vi) Sham-operated control group, in which vehicle was administered twice daily fourteen times (n = 5). vii) SNI animals treated twice daily, a total of 14 times, with 50 μg of gabapentin that served as a comparison drug that is widely used in the treatment of neuropathic pain in the clinic (n = 5), viii) SNI animals treated once with 50 μg of gabapentin (n = 5). ix) healthy control animals treated with a combination of vehicle followed 15 min later by 10 μg of cinnamaldehyde, a TRPA1 agonist (n = 6), x) healthy control animals treated with a combination of 10 μg of carbenoxolone followed 15 min later by 10 μg of cinnamaldehyde (n = 6).

Experiment started with a two-day habituation of the animals to the experimental conditions (handling, gentle restraint for i.t. drug delivery, and testing of the withdrawal threshold). On D0, first the preoperative sensitivity to mechanical stimulation was determined. Then, the animal was anesthetized for the installation of i.t. catheter and the SNI or sham operation in the same session. In groups i–viii, the first i.t. drug/vehicle delivery was performed on D0 when the animal was under anesthesia (immediately after installation of the i.t. catheter and 15 min before nerve injury or sham operation). The rationale for administering the first i.t. drug/vehicle dose about 15 min before nerve ligation in groups i–viii was to allow assessing potential pre-emptive effects of the studied compounds. Namely, earlier it was shown that intraperitoneal administration of an *N*-methyl-D-aspartate receptor antagonist 15 min before nerve ligation, in contrast to 15 min after nerve ligation, produced a significant pre-emptive effect as indicated by a reduction in hypersensitivity that lasted at least up to two weeks in spinal nerve-ligated animals (Wei and Pertovaara, 1999). After procedures performed on D0, animals in groups i–viii were allowed to recover from anesthesia and then, drug or vehicle was delivered twice daily on D1–D6 (Fig. 1). However, in healthy control groups ix–x, the first and only i.t. treatment was performed on D7.

On D7, the monofilament test was performed in all animals once before the last injection of the studied compound (for assessment of baseline hypersensitivity) and then 15, 30, and 60 min after i.t. administration of the compound (for assessment of acute antihypersensitivity effect of the studied drugs). Each animal participated only in one of the experimental conditions.

The animals were euthanized about 30 min after performing the last monofilament test on D7, and the L_5–L_6 DRGs and the SDH ipsilateral to

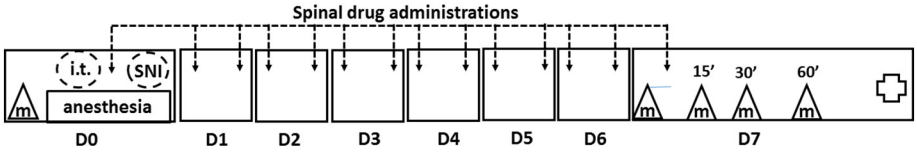

Fig. 1. Course of the study. D0–D7 represent Day 0–Day 7. Triangles with letter m represent time points for monofilament testing; 15′-60′ on D7 indicate time of testing in minutes after the last spinal drug/vehicle administration. The circle with letters i.t. represents time point for installation of intrathecal catheter, and the circle with letters SNI represents time point for nerve ligation or sham operation. Broken arrows represent time points for repeated spinal drug/vehicle applications. The cross on D7 represents the time point (30 min after the last monofilament test) when animals were euthanized.

Table 1
Drug treatment procedures in experimental groups i–viii.

Group #	Twice daily i.t. treatment on D0–D6	Last i.t. treatment on D7
i CHEM D0–D7	Chembridge-5861528 10 μg	Chembridge-5861528 10 μg
ii CHEM D7	Vehicle	Chembridge-5861528 10 μg
iii Carbenoxolone D0–D7	Carbenoxolone 10 μg	Carbenoxolone 10 μg
iv Carbenoxolone D7	Vehicle	Carbenoxolone 10 μg
v Vehicle D0–D7	Vehicle	Vehicle
vi Sham	Vehicle	Vehicle
vii Gabapentin D0–D7	Gabapentin 50 μg	Gabapentin 50 μg
viii Gabapentin D7	Vehicle	Gabapentin 50 μg

D0–D7 refer to postinjury days 0–day 7.

nerve injury were harvested for qPCR analyses that were performed as described in Section 2.5. Due to various technical errors, DRG or SDH sampling was not successful in all cases and therefore, in some groups the number of DRG or SDH samples is smaller than the number of behaviorally tested animals in the same experimental condition.

2.8. Statistical analysis of data

Since the response rate to monofilament stimulation in some test conditions reached the floor (0%) or the ceiling (100%), statistical analyses of the data on mechanical sensitivity were performed using non-parametric Kruskal-Wallis or Friedman's test followed by Dunn's test (comparisons of three or more groups), or by using Mann-Whitney *U* test (comparison of two groups). mRNA data was normally distributed in all mRNA data groups in which normality could be tested using Kolmogorov-Smirnov test. However, in some mRNA data groups the number of samples was too low ($n < 5$) to allow normality test. Therefore, statistical analysis of all mRNA data was performed using non-parametric Kruskal-Wallis test followed by Dunn's test. $P < 0.05$ was considered to represent a significant difference.

3. Results

3.1. Induction of mechanical pain hypersensitivity by SNI

Installation of i.t. catheter, sham operation and the seven-day vehicle treatment had no long-term influence on pain behavior as indicated by comparison of monofilament-evoked responses at an innocuous stimulus force of 2 g or at a noxious stimulus force of 15 g in vehicle-treated sham controls preoperatively on day 0 versus the seventh vehicle treatment day (Fig. 2A). SNI produced a significant mechanical hypersensitivity effect as revealed by comparison of monofilament-evoked responses in sham controls versus SNI animals at an innocuous force of 2 g or a noxious force of 15 g (Fig. 2B).

3.2. Antihypersensitivity effect induced by i.t. administration of Chembridge-5861528

To assess time-course of the antihypersensitivity effect induced by acute pharmacological blocking of the spinal TRPA1, mechanical hypersensitivity was assessed on postoperative day 7 (D7) at various time points following administration of a single dose of Chembridge-5861528 (CHEM; a TRPA1 antagonist) in the group treated twice daily for six previous days with CHEM (group CHEM D0–D7). CHEM treatment had a significant acute effect on mechanical hypersensitivity (main effect of time in the CHEM D0–D7 group: $Fr = 11.9$, $P = 0.0026$), whereas in the group pretreated twice daily for the previous six days with vehicle, vehicle on the seventh treatment day failed to have an acute influence on mechanical hypersensitivity (main effect of time in the Veh group: $Fr = 3.0$; Fig. 3A). Post hoc testing indicated that the mechanical antihypersensitivity effect induced by CHEM was significant 30 min after administration of CHEM, and the antihypersensitivity effect was reduced to a non-significant level 60 min after CHEM administration (Fig. 3A).

To assess whether the seven-day treatment with twice daily administration of CHEM (10 μg) influences the acute antihypersensitivity effect of CHEM, the mechanical antihypersensitivity effect induced by CHEM (10 μg) was assessed in SNI groups treated the preceding seven days twice daily with vehicle (CHEM D7 group) or with CHEM (CHEM D0–D7 group). A reference group of SNI animals received vehicle both at the time of testing and twice daily during the preceding seven days. These assessments were performed on D7 before and at 15 min, 30 min and 60 min after drug/vehicle administration. Responses evoked by a low (2 g) and a high (15 g) test stimulus force (15 g) were evaluated separately.

On the seventh treatment day, the antihypersensitivity effects in the three drug treatment groups (CHEM D7, CHEM D0–D7, Veh) were significantly different when assessed at a low test stimulus force of 2 g ($KW = 14.3$, $P = 0.0002$; Fig. 3B). Post hoc testing indicated that on the seventh treatment day, the acute antihypersensitivity effect induced by CHEM was significant both in the CHEM D7 and the CHEM D0–D7 group when compared with the effect of saline administration in the Veh group (Fig. 3B). Moreover, post hoc testing indicated that the baseline hypersensitivity of SNI animals just before the last treatment dose on the seventh treatment day was not significantly different among the treatment groups ($KW = 1.4$; not shown).

When the antihypersensitivity effect induced by CHEM was assessed on the seventh treatment day using a test stimulus force of 15 g, the difference in the acute antihypersensitivity effect of drug treatments was

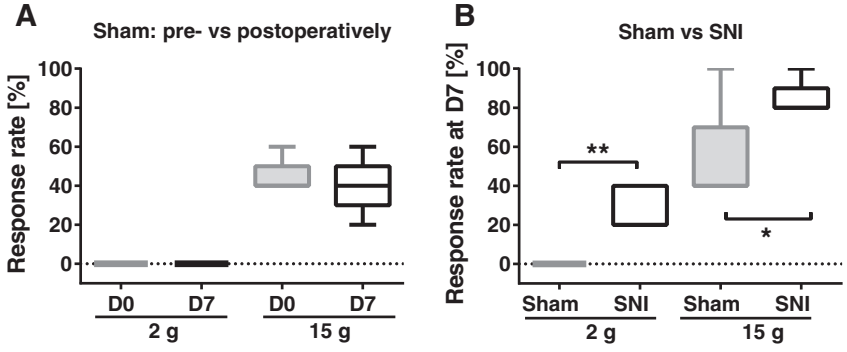

Fig. 2. A) Mechanical sensitivity in sham-operated vehicle-treated control animals preoperatively on day 0 (D0) and postoperatively on day 7 (D7). B) Comparison of mechanical sensitivity in animals with spared nerve injury (SNI) and sham operation (Sham) after a seven-day i.t. treatment with vehicle. The graphs show the limb withdrawal response rate to repeated monofilament stimulation at an innocuous test stimulus force of 2 g and a noxious test stimulus force of 15 g. Increase in the response rate indicates hypersensitivity. Graphs show medians, the boxes extend from the 25th to the 75th percentile, and the whiskers extend from the smallest to the largest value ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$ (Mann-Whitney *U* test).

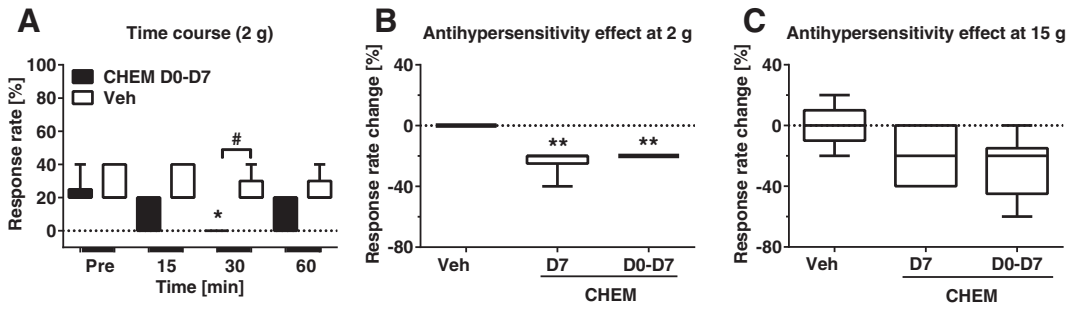

Fig. 3. Influence by prolonged intrathecal treatment with 10 μg of Chembridge-5861528 (CHEM; TRPA1 antagonist) or vehicle (Veh) on mechanical hypersensitivity in a spared nerve injury model of neuropathy. A) Time-course of the effect at a test stimulus force of 2 g. B) Response rate change before versus 30 min after drug administration tested at a stimulus force of 2 g. C) Response rate change before and 30 min after drug administration tested at a stimulus force of 15 g. In A, higher the response rate, stronger the hypersensitivity. In B and C, 0% represents the response rate before drug administration and response rate values <0% represent drug-induced antihypersensitivity effect. In group D0–D7, the animals received twice daily 10 μg of Chembridge-5861528 for seven days. In group D7, animals received twice daily vehicle, except that the last injection on day 7 was 10 μg of Chembridge-5861528. In Veh group, animals received twice daily vehicle for seven days. In all groups, hypersensitivity measurements were performed on the seventh treatment day (D7). Graphs show medians, the boxes extend from the 25th to the 75th percentile, and the whiskers extend from the smallest to the largest value (n = 6, except that $n_{Veh} = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunn's test; reference: the corresponding Pre-value in A, and Veh-value in B and C, unless defined otherwise).

close to significance among treatment groups ($KW = 5.1$, $P = 0.06$; Fig. 3C). On the seventh treatment day, the baseline (pre-drug) response rate to a test stimulus force of 15 g was not significantly different among the treatment groups ($KW = 5.3$; not shown).

3.3. Antihypersensitivity effect induced by i.t. administration of carbenoxolone

To assess time-course of the antihypersensitivity effect induced by acute pharmacological decoupling of gap junctions in the spinal dorsal horn (SDH), mechanical hypersensitivity was assessed at a force of 2 g on D7 at various time points following i.t. administration of 10 μg of carbenoxolone (a gap junction decoupler) in SNI animals that received the previous six days twice daily carbenoxolone (carbenoxolone D0–D7 group). Carbenoxolone treatment had a significant acute effect on hypersensitivity (main effect of time in the carbenoxolone D0–D7 group: $Fr = 14.3$, $P = 0.0004$; Fig. 4A), while vehicle treatment failed to influence hypersensitivity in the Veh group (main effect of time: $Fr = 3$). Post hoc tests indicated that carbenoxolone (10 μg i.t.) produced a significant mechanical antihypersensitivity effect only 15 min after its application (Fig. 4A). In further analyses, carbenoxolone-induced effects were assessed 15 min after its i.t. administration.

To assess whether the seven-day treatment with twice daily spinal administration of carbenoxolone (10 μg) influences acute antihypersensitivity effect of carbenoxolone, mechanical antihypersensitivity effect induced by carbenoxolone (10 μg) was assessed in SNI groups treated the preceding seven days twice daily with vehicle (carbenoxolone D7 group) or with carbenoxolone (carbenoxolone D0–D7 group). A reference group of SNI animals received vehicle at the test day as well as during the preceding seven days (Veh group).

Acute antihypersensitivity effect assessed at an innocuous stimulus force of 2 g on the seventh treatment day varied significantly among the three treatment groups ($KW = 8.2$, $P = 0.011$; Fig. 4B). Post hoc testing indicated that when compared with the vehicle treatment group, acute carbenoxolone treatment produced a significant antihypersensitivity effect in the carbenoxolone D0–D7 group but not in the carbenoxolone D7 group (Fig. 4B). On the seventh treatment day, the baseline (pre-drug) response rate to a test stimulus force of 2 g was not significantly different among the treatment groups ($KW = 2.8$; not shown).

Acute effects of carbenoxolone/vehicle on mechanical hypersensitivity assessed at a noxious stimulus force of 15 g on the seventh treatment day were not significantly different among the three treatment groups ($KW = 3.0$; Fig. 4C); i.e.; carbenoxolone failed to produce an acute antihypersensitivity effect at test force 15 g either in the carbenoxolone D7 or carbenoxolone D0–D7 group. On the seventh treatment day, the baseline (pre-drug) response rate to a test stimulus force of 15 g was not significantly different among the three treatment groups ($KW = 4.2$; not shown).

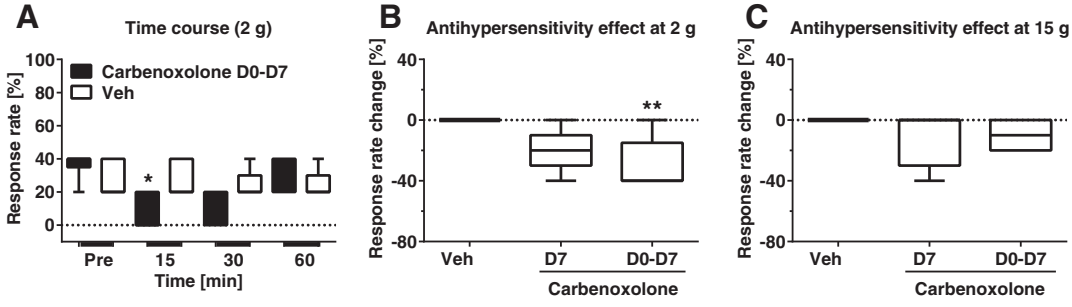

Fig. 4. Influence by prolonged intrathecal treatment with 10 μg of carbenoxolone (gap junction decoupler) or vehicle (Veh) on mechanical hypersensitivity in a spared nerve injury model of neuropathy. A) Time-course of the effect at a test stimulus force of 2 g. B) Response rate change before versus 15 min after drug administration at a test stimulus force of 2 g. C) Response rate change before versus 15 min after drug administration at a test stimulus force of 15 g. In A, higher the response rate, stronger the hypersensitivity. In B and C, 0% represents the response rate before drug administration and response rate values <0% represent drug-induced antihypersensitivity effect. In group D0–D7, the animals received twice daily 10 μg of carbenoxolone for seven days. In group D7, animals received twice daily vehicle, except that the last injection on day 7 was 10 μg of carbenoxolone. In the Veh group, animals received twice daily vehicle for seven days. In all groups, hypersensitivity was assessed on the seventh treatment day (D7). Graphs show medians, the boxes extend from the 25th to the 75th percentile, and the whiskers extend from the smallest to the largest value ($n_{D1–D7} = 6$, $n_{D7} = 5$, $n_{Veh} = 5$). $^{*}P < 0.05$ (Dunn's test; reference: the corresponding Pre-value in A, and the corresponding Veh value in B).

3.3.1. Attempt to attenuate TRPA1 agonist-induced hypersensitivity by carbenoxolone in healthy controls

To study further whether a possible interaction of astrocytes with TRPA1 might contribute to the present results, we induced mechanical hypersensitivity in healthy control animals by administering i.t. 10 μg of cinnamaldehyde, a TRPA1 agonist, and attempted to prevent the cinnamaldehyde-induced hypersensitivity by pretreating the animal with 10 μg of carbenoxolone. In vehicle pretreated animals, cinnamaldehyde increased the median withdrawal response rate evoked by an innocuous stimulus force of 2 g from 0% to 20%, and that evoked by a noxious stimulus force of 15 g from 70% to 100% (n = 6). I.t. pretreatment with 10 μg of carbenoxolone failed to attenuate the cinnamaldehyde-induced hypersensitivity at an innocuous ($U = 8$; Fig. 5A) or a noxious stimulus range ($U = 14.5$; Fig. 5B).

3.4. Antihypersensitivity effect induced by i.t. administration of gabapentin

For comparison, antihypersensitivity effect induced by gabapentin, a clinically used analgesic compound, was assessed. Mechanical hypersensitivity determined at an innocuous force of 2 g was significantly influenced by gabapentin/vehicle treatments ($KW = 12.3$, $P = 0.0002$; Fig. 5C). Post hoc testing indicated that 50 μg of gabapentin produced a significant antihypersensitivity effect as assessed at the innocuous force of 2 g independent whether it had been administered twice daily for seven previous days or whether it was administered only once after a seven-day vehicle treatment period (groups gabapentin D0–D7 and gabapentin D7, respectively; Fig. 5C). However, when the antihypersensitivity effect of gabapentin was assessed at the noxious stimulus force of 15 g, gabapentin/vehicle treatments failed to have a significant acute effect on hypersensitivity ($KW = 3.3$; Fig. 5D).

3.4.1. Expression of TRPA1 mRNA in animals treated with Chembridge-5861528

The assessment of the dorsal root ganglion (DRG) in CHEM/vehicle-treated SNI animals and sham-operated controls indicated that the expression of TRPA1 mRNA was influenced by the experimental conditions ($KW = 11.8$, $P = 0.008$). Post hoc tests indicated that when compared with the DRG of sham-operated animals, the expression of TRPA1 mRNA was increased in the DRG of SNI animals treated for seven days with vehicle or with CHEM (CHEM D0–D7 group), while there was no significant difference of the TRPA1 mRNA expression in the DRG among the three different treatment groups of SNI animals (Vehicle, CHEM D7, CHEM D0–D7; Fig. 6A). In the spinal dorsal horn (SDH), the expression of TRPA1 mRNA was not influenced by the experimental conditions ($KW = 0.4$, $P = 0.9$; Fig. 6B).

3.4.2. Expression of TRPA1 mRNA in animals treated with carbenoxolone

The assessment of the DRG in carbenoxolone/vehicle-treated SNI animals and sham-operated controls indicated that the expression of TRPA1 mRNA was influenced by the experimental conditions ($KW =$

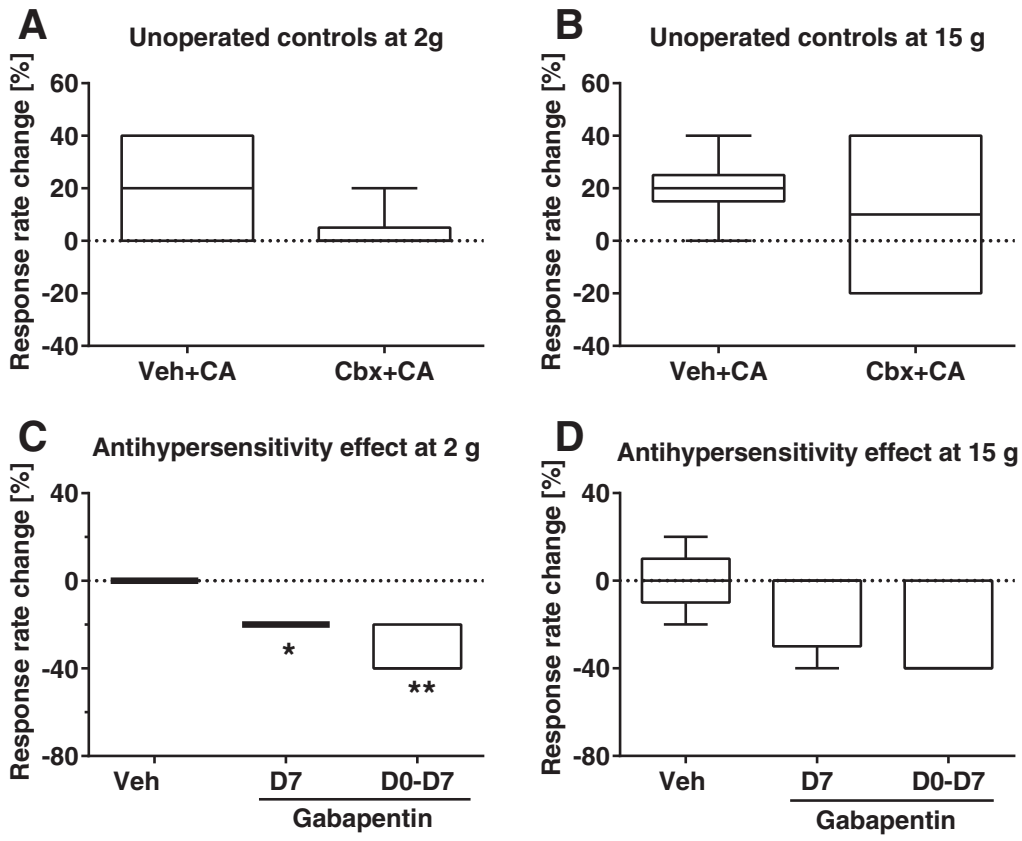

Fig. 5. Attempt to prevent TRPA1 agonist-induced hypersensitivity by pretreatment with carbenoxolone in healthy controls (A, B), and the influence by prolonged treatment with gabapentin in the spared nerve injury model of neuropathy (C, D). Change in response rate before versus after drug administrations tested at an innocuous force of 2 g (A and C) or a noxious force of 15 g (B and D). Testing performed in unoperated controls (A and B) or in animals with a spared nerve injury (C and D). Response rate 0% represents the corresponding pre-drug value, response rate value >0% represent drug-induced increase in hypersensitivity, whereas values <0% represent an antihypersensitivity effect. In A and B, animals received intrathecally vehicle (Veh) or carbenoxolone (Cbx, a gap junction decoupler; 10 μg) 15 min before post-drug testing followed by cinnamaldehyde (CA, a TRPA1 agonist; 10 μg) 2 min before testing. In C and D, the animals in group D0–D7 received twice daily intrathecally 50 μg of gabapentin for seven days, while in group D7 animals received once intrathecally 50 μg of gabapentin at postoperative day 7, and in the Veh group animals received twice daily intrathecally vehicle for seven days. In C and D, hypersensitivity was assessed on the seventh treatment day (D7). Graphs show medians, the boxes extend from the 25th to the 75th percentile, and the whiskers extend from the smallest to the largest value (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunn's test; reference: the corresponding Veh value).

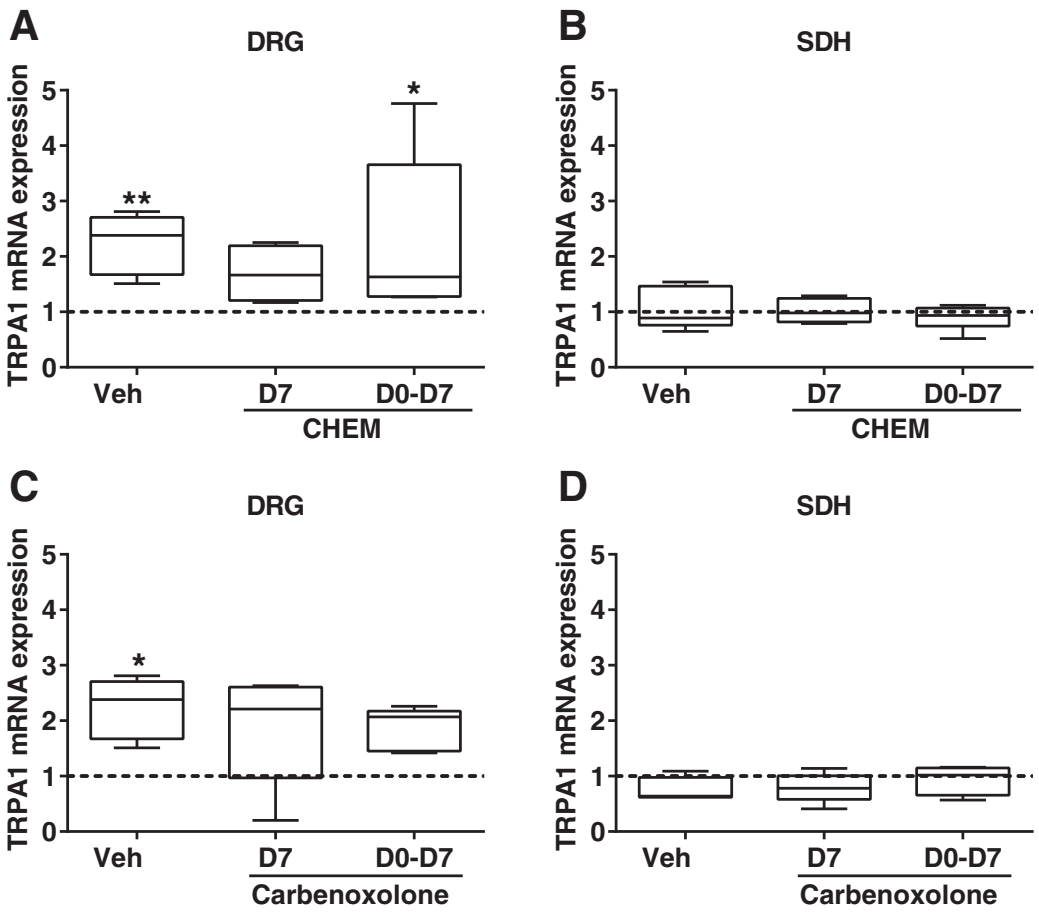

Fig. 6. Expression of TRPA1 mRNA in the dorsal root ganglion (DRG; A & C) and the spinal dorsal horn (SDH; B & D) following prolonged intrathecal treatment with 10 μg of Chembridge-5861528 (CHEM; A & B), carbenoxolone (C & D) or vehicle (Veh; A–D). In groups D0–D7, the animals received twice daily 10 μg of Chembridge-5861528 or carbenoxolone for seven days. In group D7, animals received twice daily vehicle, except that the last injection on day 7 was 10 μg of Chembridge-5861528 or carbenoxolone. In the Veh group, animals received twice daily vehicle for seven days. The samples were taken about 90 min after the last drug injection. The dotted horizontal lines (1.0) represent mRNA expression in vehicle-treated sham-operated control animals. Graphs show medians, the boxes extend from the 25th to the 75th percentile, and the whiskers extend from the smallest to the largest value. In each group, n = 4–6. $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunn's test; reference: the corresponding value in the sham control group).

9.3, $P = 0.026$; Fig. 6C). Post hoc tests indicated that when compared with the DRG of sham-operated controls, the expression of TRPA1 mRNA was increased in the vehicle-treated SNI group, while there were no significant differences among vehicle- and carbenoxolone-treated SNI groups (Fig. 6C). In the SDH, the expression of TRPA1 mRNA was not influenced by the experimental conditions ($KW = 3.9$, $P = 0.3$; Fig. 6 D).

3.4.3. Expression of DAAO mRNA in animals treated with Chembridge-5861528

The assessment of the DRG in CHEM/vehicle-treated SNI animals and sham controls indicated that the expression of DAAO mRNA was influenced by the experimental conditions ($KW = 15.0$, $P = 0.0018$; Fig. 7A). Post hoc tests indicated that when compared with the DRG of sham controls, the expression of DAAO mRNA was increased in both of the CHEM-treated SNI groups (D7 and D0–D7), while there were no significant differences in DAAO mRNA expression among vehicle- and CHEM-treated SNI groups (Fig. 7A). In the SDH, the expression of DAAO mRNA was not influenced by the experimental conditions ($KW = 4.2$, $P = 0.24$; Fig. 7B).

3.4.4. Expression of DAAO mRNA in animals treated with carbenoxolone

The assessment of the DRG in carbenoxolone/vehicle-treated SNI animals and sham controls indicated that the expression of DAAO mRNA was influenced by the experimental conditions ($KW = 11.9$, $P = 0.0011$; Fig. 7C). Post hoc tests indicated that when compared with the DRG of sham controls, the expression of DAAO mRNA was increased in one of the carbenoxolone-treated SNI groups (D7 group), while there were no significant differences in DAAO mRNA expression among vehicle- and carbenoxolone-treated SNI groups (Fig. 7C). In the SDH, the expression of DAAO mRNA was not influenced by the experimental conditions ($KW = 1.3$, $P = 0.7$; Fig. 7D).

4. Discussion

4.1. Antihypersensitivity effect induced by spinal administration of a TRPA1 antagonist or a gap junction decoupler

Acute spinal administration of a selective TRPA1 antagonist attenuated maintenance of neuropathic pain hypersensitivity in the SNI model of neuropathy, which finding is in line with earlier results in spinal nerve ligation- and diabetes-induced models of experimental neuropathy (Wei et al. 2010a, 2011). Antihypersensitivity effect induced by acute pharmacological blocking of the spinal TRPA1 has earlier been shown also in various other models of pain hypersensitivity, such as that induced by persistent inflammation induced by Complete Freund's Adjuvant (da Costa et al., 2010), skin incision (Wei et al., 2012), neurogenic inflammation (Wei et al., 2010a), spinal nerve ligation, and sleep-deprivation (Wei et al., 2011). Additionally, spinal TRPA1 may contribute to nociception also in physiological conditions

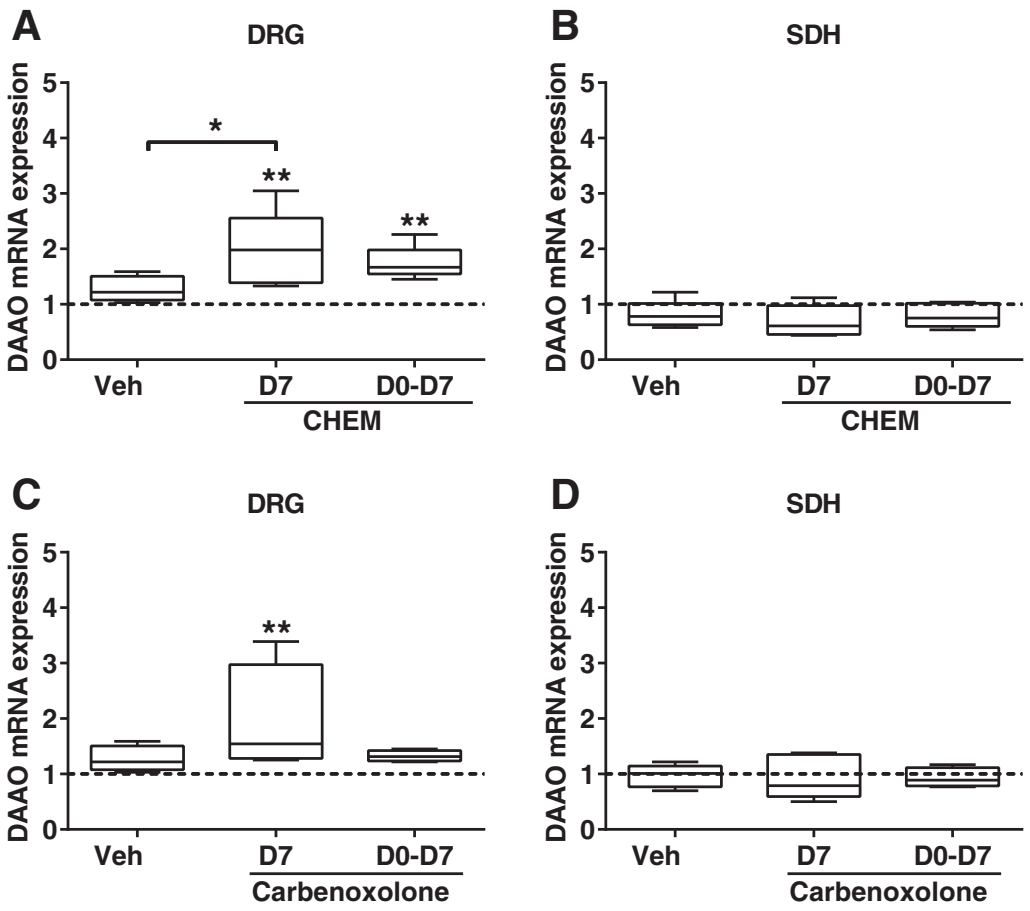

Fig. 7. Expression of D-amino acid oxidase (DAAO) mRNA in the dorsal root ganglion (DRG; A & C) and the spinal dorsal horn (SDH; B & D) following prolonged intrathecal treatment with 10 μg of Chembridge-5861528 (CHEM; A & B), carbenoxolone (C & D) or vehicle (Veh; A–D). In group D0–D7, the animals received twice daily 10 μg of Chembridge-5861528 or carbenoxolone for seven days. In group D7, animals received twice daily vehicle, except that the last injection on day 7 was 10 μg of Chembridge-5861528 or carbenoxolone. In the Veh group, animals received twice daily vehicle for seven days. The samples were taken about 90 min after the last drug injection. The dotted horizontal lines (1.0) represent mRNA expression in vehicle-treated sham-operated control animals. Graphs show medians, the boxes extend from the 25th to the 75th percentile, and the whiskers extend from the smallest to the largest value. In each group, n = 4–6. $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunn's test; reference: unless specified, the corresponding value in sham controls).

(Miyakawa et al., 2014). Importantly, the present study extended these earlier findings by showing that the magnitude of the antihypersensitivity effect induced by the spinally administered TRPA1 antagonist was not reduced when the treatment period was prolonged to a week, which contrasts with the development of antinociceptive tolerance following prolonged treatment e.g. with morphine (Trang et al., 2015), in which phenomenon spinal TRPA1 may be partly involved in (Wei et al., 2016). At a dose producing a significant antihypersensitivity effect, the currently used TRPA1 antagonist has not influenced locomotor activity in the Rotarod test (Wei et al., 2009) or in the open-field test (Wei et al., 2012). These earlier findings indicate that the TRPA1 antagonist-induced suppression of pain hypersensitivity may not be explained by action on the motor system.

Prolonged twice daily spinal administrations of a TRPA1 antagonist or a gap junction decoupler failed to prevent development of hypersensitivity in the present study as indicated by the finding that the baseline responses to mechanical stimulation assessed before administration of the last drug dose (i.e., assessed on the seventh treatment day at a time point when the acute pharmacological effect of the previous drug dose was over) were not significantly different from those in vehicle-treated controls, although the first drug doses of the prolonged treatment period were given prior to nerve injury. In contrast, e.g. a single dose of an *N*-methyl-D-aspartate antagonist administered systemically 15 min prior to but not 15 min after spinal nerve ligation has attenuated development of hypersensitivity indicating a pre-emptive analgesic effect (Wei and Pertovaara, 1999). On the other hand, repeated systemic administration of a TRPA1 antagonist has attenuated not only maintenance but also development of baseline hypersensitivity in diabetic neuropathy (Koivisto et al., 2012). It still remains to be studied whether repeated systemic (unlike spinal) administrations of a TRPA1 antagonist or a gap junction blocker might suppress development of the nerve ligation-induced as well as diabetic hypersensitivity.

In the spinal dorsal horn, TRPA1 is expressed on central terminals of nociceptive primary afferent fibers where it amplifies glutamatergic transmission (Kosugi et al., 2007). On the other hand, peripheral injury discharge leads to spinal increase of reactive oxygen species (Park et al., 2006), which are TRPA1 agonists (Andersson et al., 2008) that potentially contribute to the TRPA1-mediated spinal facilitation of pain. Consequently, blocking the TRPA1 on central terminals of primary afferent fibers is expected to attenuate pain-related responses providing one although not necessarily the only plausible explanation for the antihypersensitivity effect following block of the spinal TRPA1 in the present study.

Astrocytes, a subgroup of glial cells, is another cell type in the nervous system expressing TRPA1 (Shigetomi et al., 2011). Injury-induced activation of astrocytes is known to contribute to long-term pain though various actions (Hansson, 2010). In line with this, earlier studies together with the present findings indicate that spinally administered carbenoxolone, a gap junction blocker inhibiting activated astrocytes, attenuates neuropathic pain hypersensitivity (Spataro et al., 2004; Roh et al., 2010; Wang et al., 2014; Yoon et al., 2013). Among multiple mechanisms through which injury-induced activation of spinal astrocytes

have been described to have a pronociceptive effect is upregulation of astroglial D-amino acid oxidase (DAAO) that catalyzes oxidation of D-amino acids to hydrogen peroxidase (H_2O_2). This is indicated by the previous findings that injury induced pain behavior that was associated with upregulation of DAAO mRNA and increase of DAAO in the SDH, while knockout of DAAO gene or administration of DAAO inhibitor suppressed pain hypersensitivity in injured or sleep-deprived intact animals (Chen et al., 2012; Gong et al., 2011; Hopkins et al., 2013; Lu et al., 2012; Wei et al., 2013; Zhao et al., 2010). It is noteworthy that the end product of the astrocyte-DAAO pathway, H_2O_2 is a TRPA1 agonist (Andersson et al., 2008). Therefore, it may be speculated that H_2O_2 generated by activation of the astrocyte-DAAO pathway is among underlying TRPA1-mediated pronociceptive mechanisms in neuropathy through a direct H_2O_2-driven action on TRPA1 on central terminals of primary afferent nerve fibers that leads to amplification of nociceptive transmission. The finding that a spinally administered gap junction blocker failed to produce a significant prevention of the TRPA1 agonist-induced hypersensitivity does not support the hypothesis that H_2O_2 generated by astroglial DAAO provides a feedforward mechanism by acting on TRPA1 on astrocytes, thereby upregulating the astrocyte-DAAO-H_2O_2 pathway. Thus, neuropathy may have induced activation of astrocytes, at least partly, through mechanisms that may not involve TRPA1. This explanation does not exclude the possibility that TRPA1 (e.g. on the central endings of primary afferent nociceptors) is among final pronociceptive targets in the pronociceptive pathway driven by activated astrocytes.

The present finding that prolonged as well as acute spinal administration of carbenoxolone, a gap junction decoupler that reduces activation of astrocytes, produced an antihypersensitivity effect is in line with earlier evidence suggesting that the astrocyte-DAAO-H_2O_2 pathway contributes to neuropathic hypersensitivity (Zhao et al., 2010) and with the hypothesis that at least partly this pathway promotes neuropathic pain due to action on TRPA1. Carbenoxolone as well as Chembridge-5861528 preferentially suppressed hypersensitivity at an innocuous stimulus force (2 g), while at the currently used doses these compounds failed to produce a significant antihypersensitivity effect at a noxious stimulus force (15 g).

In contrast to the evidence indicating that activation of the astrocyte-DAAO pathway promotes pain, there are also studies suggesting that inhibition of DAAO has a pronociceptive effect (e.g., Dieb and Hafidi, 2013; Miraucourt et al., 2011; Wake et al., 2001), possibly due to accumulation of D-serine, an endogenous agonist on the glycine-binding B site of the pronociceptive *N*-methyl-D-aspartate receptor (Schell et al., 1995). In an earlier study, however, inhibition of spinal DAAO was accompanied by suppression of sustained formalin-induced pain behavior but not with a significant elevation of spinal D-serine level (Lu et al., 2012) which finding supports the proposal that spinal DAAO has pro- rather than antinociceptive action. It remains to be studied which experimental factors determine whether DAAO enhances or suppresses pain. Moreover, further studies are needed to assess whether TRPA1 on oligodendrocytes, a third type of cell in the nervous system expressing TRPA1 (Hamilton et al., 2016), contributes to the maintenance of neuropathic pain.

Spinal administration of the comparison drug, gabapentin, suppressed effectively (tactile allodynia-like) hypersensitive responses assessed at an innocuous mechanical stimulus force even after single treatment which is line with earlier results (Cheng et al., 2000). Previously, (mechanical hyperalgesia-like) hypersensitive responses evoked by a noxious stimulus force were only weakly suppressed by acute spinal administration of gabapentin, whereas following a five-day treatment the antihyperalgesic effect of gabapentin was significantly enhanced (Patel et al., 2001). Partly in line with this, acute spinal administration of gabapentin at an antiallodynic dose failed to reduce mechanical hyperalgesia in the present study. However, prolongation of the treatment period by several days failed to enhance the mechanical antihyperalgesia effect, as described before (Patel et al., 2001).

4.2. Expression of TRPA1 mRNA or DAAO mRNA in the DRG and SDH

Studies assessing TRPA1 expression in the dorsal root ganglion (DRG) following nerve injury have given variable results. In one study, immunohistochemistry revealed increased TRPA1-like labeling in the DRG of injured nerve and this was accompanied by an increased percentage of DRG neurons responding to a TRPA1 agonist (Ji et al., 2008). In line with this, increased TRPA1 mRNA has been reported in the DRG of the injured nerve (Frederick et al., 2007; Ta et al., 2010) or only in the DRG of the adjacent nerve (Obata et al., 2005). In contrast, there are also studies reporting only nerve injury-induced downregulation of TRPA1 mRNA (Caspani et al., 2009; Persson et al., 2010; Staaf et al., 2009) accompanied by a reduced DRG response to a TRPA1 agonist (Caspani et al., 2009).

In the present study, TRPA1 mRNA was increased in the DRG of SNI animals of the present study, which finding is in line with the results of some (Frederick et al., 2007; Michot et al., 2014; Ta et al., 2010; Zhou et al., 2013) although not all previous studies (Caspani et al., 2009; Persson et al., 2010; Staaf et al., 2009). While we studied DRG of the injured nerve, the increase of TRPA1 mRNA might have been more prominent in the DRG of an adjacent intact nerve as suggested by an earlier investigation (Obata et al., 2005). No overexpression of TRPA1 mRNA was observed in the SDH, although this finding does not exclude possible changes in spinal TRPA1 protein expression. Indeed, upregulation of TRPA1 protein in the SDH has been described in a model of peripheral nerve injury (Zhang et al., 2014). Spinal treatments with a TRPA1 antagonist or a gap junction decoupler failed to influence TRPA1 mRNA expression in the DRG or SDH of SNI animals. However, one should be cautious with interpretations of these findings since spinally administered drugs may have had a limited spread to the DRG. Moreover, the sensitivity of the assay might not have been sufficient to detect a potential drug-induced downregulation of the baseline level of TRPA1 mRNA in the SDH.

DAAO mRNA was increased in the DRG of SNI animals. Whether or not the slight upregulation of DAAO mRNA in the DRG is causally related to neuropathic symptoms, remains to be studied. In the present study, the spinal drug-induced antihypersensitivity effect may not allow meaningful comparisons to the DAAO mRNA expression in the DRG, since spinally administered drugs have a limited access to the DRG. In the SDH, upregulation of DAAO mRNA has been described in the spinal nerve ligation-induced model of neuropathy (Zhao et al., 2010) and in some other experimental conditions such as spinal morphine tolerance (e.g., Wei et al., 2016). Upregulation of DAAO mRNA was not observed in the SDH of SNI animals of the present study, unlike earlier in the spinal nerve ligation-induced neuropathy model (Zhao et al., 2010) that produces a more extensive surgical trauma than SNI, which possibly explains the difference. As with TRPA1 mRNA in the SDH, the sensitivity of the assay might not have been sufficient to detect a potential drug-induced downregulation of the baseline level of DAAO mRNA in the SDH.

4.3. Limitations of the study

Among limitations of the present study is that the assessment of hypersensitivity was not blinded, although our previous study by the same experimenter showed that the drug-induced mechanical antihypersensitivity effect using the same test stimulus procedure in the rat was of identical magnitude with and without formal blinding (Wei et al., 2012). Another limitation is that due to the normalization of the control mRNA data, the control group lacks variability that may have biased statistical analyses of the mRNA data. Therefore, one should be cautious with interpretations of the differences in mRNA results among the groups. A potential limitation concerning the antihypersensitivity effect obtained with the currently used TRPA1 antagonist is that although Chembridge-5861528 is a highly selective compound (Koivisto and Pertovaara, 2015), the result had been strengthened if the experiments

had been replicated with another selective TRPA1 antagonist having a different structure.

4.4. Conclusions

In nerve-injured animals, prolonged as well as acute spinal administration of a TRPA1 antagonist effectively attenuated maintenance of mechanical hypersensitivity predominantly at an innocuous test stimulus range. This finding suggests that spinally administered TRPA1 antagonists may provide a clinically applicable treatment alternative for attenuating tactile allodynia-like hypersensitivity in chronic neuropathy. Repeated spinal administration of a gap junction decoupler that presumably inhibited astrocyte activation also proved to be effective in attenuating hypersensitivity to innocuous test stimuli. However, neither prolonged spinal administration with a TRPA1 antagonist nor with a gap junction decoupler prevented the development of neuropathic hypersensitivity, although prolonged treatments were started as early as at time of the nerve injury. Upregulations of the TRPA1 and DAAO mRNA in the DRG but not SDH were associated with the SNI model of neuropathy, although the potential causal relationships of these minor upregulations to neuropathic symptoms still remain to be studied.

Acknowledgments

This study was supported by the Sigrid Jusélius Foundation and the Academy of Finland (no. 137795), Helsinki, Finland, and the National Natural Science Foundation of China (no. 81072623), the Shanghai Innovation Project (nos. 11ZR1416400, 12JC1404800), and the Industrial and Translational Project (no. 12431900603), China.

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