ABSTRACT Management of painful peripheral neuropathies remains challenging, since patients with chronic pain respond poorly to the available pharmacopeia. In recent years, the G-protein-coupled receptor neurotensin (NT) type 2 (NTS2) emerged as an attractive target for treating transitory pain states. To date, however, there is no evidence for its role in the regulation of chronic peripheral neuropathies. Here, we found that NTS2 receptors were largely localized to primary afferent fibers and superficial dorsal horns. Changes in the time course of the gene expression profile of NT, NTS1, and NTS2 were observed over a 28-d period following the sciatic nerve constriction [chronic constriction injury (CCI) model]. We next determined the effects of central delivery of selective-NTS2 agonists to CCI-treated rats on both mechanical allodynia (evoked withdrawal responses) and weight-bearing deficits (discomfort and quality-of-life proxy). The NTS2 analogs JMv431, levocabastine, and β-lactotensin were all effective in reducing ongoing tactile allodynia in CCI-treated rats. Likewise, amitriptyline, pregabalin, and morphine significantly attenuated CCI-induced mechanical hypersensitivity. NTS2 agonists were also efficient in reversing weight-bearing and postural deficits caused by nerve damage, unlike reference analgesics currently used in the clinic. Thus, NTS2 agonists may offer new treatment avenues for limiting pain associated with peripheral neuropathies and improve functional rehabilitation and well-being.—Tétreault, P., Beaudet, N., Perron, A., Belleville, K., René, A., Cavelier, F., Martinez, J., Stroh, T., Jacobi, A. M., Rose, S. D., Behlke, M. A., Sarret, P. Spinal NTS2 receptor activation reverses signs of neuropathic pain. FASEB J. 27, 000–000 (2013). www.fasebj.org

Key Words: GPCR · allodynia · incapacity · weight-bearing deficits

Peripheral nerve injury leading to chronic neuropathic pain, arising from diverse etiologies like trauma, infection, disease, or chemotherapy, is one of the most difficult types of pain to manage [1]. To date, the pharmacological management of neuropathic pain is based on the use of antidepressants [e.g., amitriptyline (AMI)], antiepileptics [gabapentin, pregabalin (PGB)] and topical lidocaine as first-line treatment options, the use of opioid analgesics [e.g., morphine sulfate (MS)] and tramadol being recommended for second-line treatments (2–4). Nonetheless, no more than half of patients achieve clinically meaningful pain relief with the pharmacopoeia currently available (5, 6). Their ineffectiveness is mainly related to the fact that some forms of neuropathic pain are insensitive to classical analgesics or that adverse effects (e.g., constipation, drowsiness, and dizziness) drive an important number of patients to stop taking their medications (7–9, 10, 11). Thus, there is a continuing need to develop safe and more effective drugs to improve neuropathic pain management.

The key positioning and signaling properties of G-protein-coupled receptors (GPCRs) at the cellular membrane make them the most targeted protein families in pharmacology today. About one-third of currently marketed drugs rely on direct interaction with GPCRs or act through GPCR-associated mechanisms (12, 13). In recent years, the neurotensin (NT) tridecapeptide, exerting its biological effects by interacting with GPCRs or act through GPCR-associated mechanisms (12, 13). In recent years, the neurotensin (NT) tridecapeptide, exerting its biological effects by interacting with receptive sites on the peripheral sensory nerves, was identified to be a powerful pain reliever that is highly effective in treating chronic neuropathic pain. This finding has led to the development of selective GPCR agonists that can target the NTS2 receptor, which is highly expressed in the dorsal horn of the spinal cord. These agonists have been shown to significantly reduce mechanical and heat hyperalgesia, as well as thermal and mechanical allodynia in various rodent models of neuropathic pain. Therefore, NTS2 agonists offer a promising new target for the treatment of chronic pain, particularly in patients with chronic neuropathic pain. However, further research is needed to understand the mechanisms underlying the actions of NTS2 agonists and to identify optimal dosing and administration strategies for clinical use.

Abbreviations: AMI, amitriptyline; BL, baseline; BSA, bovine serum albumin; CCI, chronic constriction injury; DRG, dorsal root ganglion; DWB, dynamic weight bearing; GPCR, G-protein-coupled receptor; MS, morphine sulfate; NGS, normal goat serum; NPY, neuropeptide Y; NT, neurotensin; NTS1/2, neurotensin type 1/2; PB, phosphate buffer; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PGB, pregabalin; PWT, paw withdrawal threshold; qPCR, quantitative polymerase chain reaction

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with 2 subtypes of class A GPCRs, termed neurotensin type 1 and 2 (NTS1 and NTS2), has emerged as an important modulator of nociceptive transmission (14–16). Indeed, central delivery of stable NT analogs produces dose-dependent antinociception in response to different experimental pain modalities, including thermal, mechanical, and chemical stimuli (17–27). Accordingly, inhibition of NTS1 or NTS2 function by pharmacological NT receptor antagonists, antisense technologies, or generation of knockout animals supports the involvement of these receptors in the regulation of pain processing (19, 25, 28–34). In light of the fact that neuropathic pain conditions are only partially responsive to opioid therapy, it is also important to point out that NT exerts its analgesic effects at least in part through opioid-independent mechanisms (35, 36). Notably, it was pharmacologically demonstrated that the antinociceptive response induced by NT analogs could not be reversed by the opioid antagonists naltrexone and naloxone (37–40). Above all, it has also been recently demonstrated that NT agonists combined with opioids may act synergistically to reduce nociception (17, 41, 42).

In the present study, we sought to determine whether spinal NTS2 receptor activation could alleviate neuropathic pain symptoms. For this purpose, the effectiveness of different NTS2 agonists in reversing pain behaviors induced by a sciatic nerve constriction was evaluated over a 28-d period and tested in parallel to the actual prescribed drugs used as first- or second-line treatment options. Neuropathic rats were subjected to different behavioral tests, including evoked withdrawal responses (e.g., mechanical allodynia) and assessment of weight-bearing deficits and quality-of-life proxies (e.g., functional rehabilitation). Since a high degree of plasticity is observed in both sensory neurons and spinal cord in response to nerve injury (43–45), we also examined the time-course gene expression profiles of NT, NTS1, and NTS2 over the 28-d testing period.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (200–225 g; Charles River Laboratories, St-Constant, QC, Canada) were maintained on a 12-h light-dark cycle with access to food and water ad libitum. Rats were acclimatized 4 d to the animal facility before behavioral studies. The experimental procedures in this study were approved by the Animal Care Committee of the Université de Sherbrooke and were in accordance with policies and directives of the Canadian Council on Animal Care.

Chronic constriction injury (CCI) model

Following baseline measurements, adult rats underwent a surgical procedure to induce a chronic neuropathic pain state. Animals were randomly allocated to either of 2 surgical treatment groups, i.e., neuropathic pain or sham treatment. Neuropathic pain was induced through CCI of the sciatic nerve, as described previously by Bennett and Xie (46) with a modification in the suture used (5-0 Prolene; Ethicon, Somerville, NJ, USA) to minimize suture-induced inflammation. Briefly, while the animals were under isoflurane anesthesia (induction with 5% and maintained at 2.5%; Abbott Laboratories, Montreal, QC, Canada), the left sciatic nerve was loosely ligatured with 4 sutures distant by 1 mm upstream of the tibial, sural, and common peroneal nerve trifurcation. This left side was defined as the treated-ipsilateral limb, while the non-treated leg (right) represented the contralateral side. The muscle, conjunctive tissue, and skin were then closed with proper sutures and washed with 3% v/v hydrogen peroxide. Sham-treated rats received the same surgical procedure, except that the sciatic nerve was not ligated. Rats were housed individually for 24 h to recover from the surgery before being housed at 4 animals/cage.

Animal injection and drugs

Behavioral experiments aimed at establishing the effects of NTS2 analogs on neuropathic pain behaviors. To this end, rats were lightly anesthetized with 2% isoflurane (Abbott Laboratories) and injected intrathecally, at the L5–L6 intervertebral space, with either JVM-431 (3–90 µg/kg), levcobasentine (5 µg/kg; kindly provided by Janssen Research, Beerse, Belgium), or β-lactotensin (180 µg/kg; U.S. National Institute of Mental Health Chemical Synthesis and Drug Supply Program) diluted in physiological saline (0.9% NaCl), 18 min before behavioral testing. Control animals received physiological saline. Reference drugs were used as follows: MS (3 mg/kg, subcutaneous injection; Sandoz, Boucherville, Canada), AMI (10 mg/kg, intraperitoneal injection; Sigma-Alrich, St. Louis, MO, USA), and PGB (50 mg/kg, intraperitoneal injection; Pfizer, Sandwich, UK).

Behavioral studies

Pain-related behavioral parameters were examined at d 0 [presurgical baseline (BL)] and after nerve injury at 3, 7, 14, 21, and 28 d. Animals were habituated to the behavioral testing room at 8 AM. The behavioral assessment began 1 h later. Mechanical allodynia (e.g., pain produced by a non-noxious stimulus) and weight-bearing deficits (e.g., functional rehabilitation) were used as outcome measures of neuropathic pain development and as indicators of drug analgesic efficacy. Dynamic weight-bearing measurements were conducted immediately after performing the von Frey filament test.

To determine the presence of mechanical allodynia, animals were placed into compartment enclosures on an elevated wire mesh floor. A dynamic plantar aesthesiometer (Ugo Basile, Stoelting, IL, USA), consisting of a metal probe (0.5 mm diameter), was directed against the hind-paw pad, and an upward force was exerted (3.33 g/s). The force required to elicit a withdrawal response was measured in grams and automatically registered when the paw was withdrawn or the preset cutoff (50 g) was reached. Five values were taken alternately on both ipsilateral (treated side) and contralateral hind paws at 15-s intervals. Rats were acclimated to the enclosures for 2 d before testing.

The dynamic weight-bearing (DWB) device (Biosch, Boulogne, France) was used to evaluate the discomfort and quality-of-life proxies. This recently characterized apparatus (47) consists of a Plexiglas enclosure (22×22×30 cm) with a floor sensor composed of 44 × 44 capsors (10.89 mm²/captor). A camera was positioned to the side of the enclosure. The rat was allowed to move freely within the apparatus for 5 min while the pressure data and live recording were transmitted to a laptop computer via a USB interface handle. Raw pressure and visual data were colligated with the latest DWB software available at the time. A zone was considered valid.
when the following parameters were detected: ≥1 g on 1 captor with a minimum of 2 adjacent captors recording ≥1 g. For each time segment where the weight distribution was stable for >0.5 s, zones that met the minimal criteria were then validated and assigned as either right or left hind paw or front paw by an observer according to the video and the scaled map of activated captors. Other detected zones (tail, testicles, etc.) were also validated and analyzed. Finally, a mean value for the weight on every limb was calculated for the whole testing period and further reported on the animal weight to obtain the percentage of total body weight on each paw. The time corresponding to weight bore on each paw was also available in the final analysis, lending the possibility to assess the frequency of standing behaviors, such as rearing, exploring, and grooming. Animals were not aclimatized to the enclosure before the initial testing period to maximize exploration behaviors.

Western blotting experiments

Lumbar dorsal root ganglia (DRGs) and spinal cord isolated from adult Sprague-Dawley rats were homogenized separately with a polytron in 50 mM Tris-HCl (pH 7.0) and 4 mM EDTA with protease inhibitors (Complete protease inhibitor tablets; Roche Molecular Biochemicals, Laval, QC, Canada) and centrifuged at 4°C for 10 min at 46,000 rpm. The pellets were then resuspended in 50 mM Tris-HCl (pH 7.0) and 0.2 mM EDTA with protease inhibitors by vortexing and brief sonication. The membranes were subsequently denatured using Laemmli sample buffer, resolved using 8% Tris-glycine precast gels (Invitrogen, Burlington, ON, Canada), and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Mississauga, ON, Canada). Each lane represents the transfer of 60 μg of membrane protein. Nonspecific sites were blocked by 0.1% Tween 20 and 10% milk powder (Carnation, Don Mills, ON, Canada) in phosphate-buffered saline (PBS; pH 7.4) overnight at 4°C. Immunoblotting was performed as described previously (26, 48). Briefly, nitrocellulose membranes were incubated overnight at 4°C with the N-terminal specific anti-NTS2 rabbit antibody [1:10,000; made on demand by Affinity BioReagents (ABR), Golden, CO, USA] in PBS with 1% bovine serum albumin (BSA) and 1% ovalbumin. After being washed with PBS-Tween, blots were incubated for 60 min at room temperature with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:4000; Amersham Pharmacia Biotech, Baie d’Urfe, QC, Canada) in PBS with 5% milk powder, and proteins were visualized by using an enhanced chemiluminescent detection system (Perkin Elmer Life Sciences, Boston, MA, USA).

Light and confocal microscopic studies

Adult Sprague-Dawley rats were deeply anesthetized with ketamine (87 mg/kg) and xylazine (3 mg/kg) administered intramuscularly (300 μl) and perfused transaortically with a freshly prepared solution of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4). Tissues were rapidly removed, cryoprotected overnight in 0.1 M PB containing 30% sucrose at 4°C, and frozen for 1 min in isopentane at −60°C, transferred to liquid nitrogen for 1 min, and finally thawed in PB at room temperature. Thawed sections were preincubated for 30 min in 0.1 M TBS (pH 7.4) containing 3% normal goat serum (NGS) and then incubated for 48 h at 4°C with NTS2 antiserum (1:1800) in PBS containing 0.5% NGS, followed by 2 h at room temperature with colloidal gold (1 nm)-conjugated goat anti-rabbit antibody (1:50; Cedarlane Laboratories, Hornby, ON, Canada), diluted in PBS containing 0.2% gelatin and 0.8% BSA. After several washes in PBS, sections were fixed for 10 min in 2% glutaraldehyde in 0.1 M PB and rinsed in 0.2 M citrate buffer (pH 7.4). Immunogold particles were amplified through silver intensification by incubating the sections for 15 min with IntenSE M silver solution (Amersham Pharmacia Biotech). The reaction was stopped by washing in citrate buffer, and the sections were postfixed in 2% osmium tetroxide in 0.1 M PB for 40 min, dehydrated in graded ethanol, and infiltrated with propylene oxide followed by Epon 812 (1:1 then 1:3, respectively). The mixture was replaced by 100% Epon 812 and incubated overnight at 4°C, followed by placement between sheets of acetate at 60°C for 24 h for flat embedding. Ultrathin sections (80 nm thick) were collected and counterstained with uranyl acetate/lead nitrate and examined with a Jeol 100CX transmission electron microscope (MECA Ltée, Montreal, QC, Canada). Electron microscopic negatives were digitized using a high-resolution digital camera and processed using Adobe Photoshop (Adobe Systems, San Jose, CA, USA). Light microscopic photomicrographs and color images were adjusted for contrast and brightness using Adobe Photoshop CS4 software. The final composites were processed by using Deneba’s Canvas X imaging software (Deneba Software, Miami, FL, USA) on an Apple iMac (Apple Inc., Cupertino, CA, USA).

Electron microscopy

Sections were processed for electron microscopy by using a preembedding procedure, as described previously (49). Anesthetized Sprague-Dawley rats were transaortically perfused with a mixture of 3.75% acrolein and 2% PFA. Lumbar spinal cords were removed, postfixed in 2% PFA, and cut (50 μm thick) on a vibratome. Sections were then incubated in a solution of 1% sodium borohydride in PB for 30 min to neutralize free aldehyde groups. Following extensive rinsing with PB, sections were cryoprotected for 30 min by immersion in a mixture of 25% sucrose and 3% glycerol in 0.1 M PB, rapidly frozen in isopentane at −60°C, transferred to liquid nitrogen for 1 min, and finally thawed in PB at room temperature. Thawed sections were preincubated for 30 min in 0.1 M TBS (pH 7.4) containing 3% normal goat serum (NGS) and then incubated for 48 h at 4°C with NTS2 antiserum (1:1800) in PBS containing 0.5% NGS, followed by 2 h at room temperature with colloidal gold (1 nm)-conjugated goat anti-rabbit antibody (1:50; Cedarlane Laboratories, Hornby, ON, Canada), diluted in PBS containing 0.2% gelatin and 0.8% BSA. After several washes in PBS, sections were fixed for 10 min in 2% glutaraldehyde in 0.1 M PB and rinsed in 0.2 M citrate buffer (pH 7.4). Immunogold particles were amplified through silver intensification by incubating the sections for 15 min with IntenSE M silver solution (Amersham Pharmacia Biotech). The reaction was stopped by washing in citrate buffer, and the sections were postfixed in 2% osmium tetroxide in 0.1 M PB for 40 min, dehydrated in graded ethanol, and infiltrated with propylene oxide followed by Epon 812 (1:1 then 1:3, respectively). The mixture was replaced by 100% Epon 812 and incubated overnight at 4°C, followed by placement between sheets of acetate at 60°C for 24 h for flat embedding. Ultrathin sections (80 nm thick) were collected and counterstained with uranyl acetate/lead nitrate and examined with a Jeol 100CX transmission electron microscope (MECA Ltée, Montreal, QC, Canada). Electron microscopic negatives were digitized using a high-resolution digital camera and processed using Adobe Photoshop (Adobe Systems, San Jose, CA, USA). Light microscopic photomicrographs and color images were adjusted for contrast and brightness using Adobe Photoshop CS4 software. The final composites were processed by using Deneba’s Canvas X imaging software (Deneba Software, Miami, FL, USA) on an Apple iMac (Apple Inc., Cupertino, CA, USA).
Real-time quantitative polymerase chain reaction (qPCR)

Lumbar DRGs (L4 to L6) and spinal cord bulge located in the thoracic region were harvested at specific time points (BL and 3, 7, 14, and 28 d post-surgery) and immediately put in a volume of RNAlater solution (Invitrogen) sufficient to cover the tissue (~100 μl for DRGs and 150–300 μl for spinal cord section). RNAlater-stored tissues were conserved according to manufacturer’s recommendations. RNA was further extracted using QiAqol Lysis Reagent and protocol (Qiagen, Valencia, CA, USA) with the use of TissueLyser II (Qiagen) to insure uniform homogenization. Samples were then DNase treated using Turbo DNase (Applied Biosystems, Carlsbad, CA, USA). All samples were run on Experion (Bio-Rad) to validate RNA quantity and quality. Reverse transcription was carried out on 1 μg RNA with 160 U Superscript II (Invitrogen), primed with oligo dT and random hexamers (Integrated DNA Technologies, Coralville, IA, USA). Samples were then loaded onto 384-well plates with Janus Automated Workstation (Perkin Elmer), and real-time PCR was further performed in triplicate for each cDNA sample on a 7900HT Fast Real-Time PCR system (Applied Biosystems) using 0.8 U Immolsase DNA polymerase (Bioline Taunton, MA, USA). The average NT, NTS1/2, and neuropeptide Y (NPY) expression was normalized to housekeeping gene and compared with healthy animals. Each value represents mean ± SEM of 5–6 samples. DNA oligonucleotides and probes used in the qPCR assay are listed in Table 1.

Statistical analysis

Data were plotted as means ± SEM for all curves and bar graphs. Two-way ANOVA, followed by Bonferroni’s post hoc test was performed for all curves. Graph bars were analyzed with a Kruskal-Wallis, followed by Dunn’s post hoc test. Prism 5 software (GraphPad software, San Diego, CA USA) was used for all statistical calculations and analyses. The percentage of antiallodynia was calculated with the area under the curve of every treatment for the time period comprised between 3 and 28 d, with the use of the following equation: percentage antiallodynia = 100 × [(CCI + drug) – CCI control]/(sham – CCI control). From the latter formula, 0% represents no antiallodynic effect of the compound, while 100% corresponds to a complete relief of mechanical hypersensitivity. We used the same formula to calculate the percentage of rehabilitation, weight recovery, and compensation reversal. Values of P < 0.05 indicate significant differences between groups. For the quantitative real-time PCR experiments, statistical analyses were performed by 1-way ANOVA followed by Bonferroni post hoc tests.

RESULTS

NTS2 receptor protein distribution in rat DRGs and spinal cord

The presence of NTS2 receptors in lumbar DRGs and spinal cord tissues isolated from healthy rats was first assessed by Western blotting. In both structures, the NTS2 antiserum specifically recognized a protein band at 46 kDa, consistent with the molecular mass of the monomeric form of the receptor, as deduced from its cDNA sequence (Fig. 1A, arrowhead). As previously reported (48), high-molecular-mass complexes, consistent with receptor dimerization, were also detected at ~80–85 kDa in homogenates from both DRGs and spinal cord (Fig. 1A). Accordingly, intense NTS2-like immunoreactivity was observed within the superficial layers of the spinal dorsal horn and in subsets of primary sensory neurons (Fig. 1B–D). In the dorsal horn, the NTS2-like immunolabeling was prominently concentrated over neuronal processes and was particularly intense over the substantia gelatinosa, which receives many synaptic terminations from C and Aδ primary afferent fibers (Fig. 1B, D). Accordingly, strong NTS2 labeling was evident in different subpopulations of primary sensory neurons (Fig. 1C, arrowheads). In somata of small to large ganglion cells, the staining was found to be associated with punctate cytoplasmic vesicle-like structures. Confocal microscopy also revealed the presence of a dense network of NTS2-immunoreactive dendrites pervading the deeper layers of the dorsal horn (Fig. 1D, arrowheads).

We next examined the subcellular distribution of NTS2 receptors in rat spinal substantia gelatinosa by electron microscopy (Fig. 1E–H). At the ultrastructural level, the NTS2 silver-intensified gold labeling was primarily associated with large dendritic profiles, probably originating from spinal neurons localized in lami-

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### Table 1. Primers

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nae IV–V (Fig. 1). Within these dendrites, NTS2-like immunoreactivity was mainly present over intracellular organelles. Only few immunogold-receptor complexes were detected at the plasma membranes (Fig. 1). A strong immunolabeling was also observed over small dendritic profiles, probably stemming from distal dendrites in laminae I and II (Fig. 1G). Only few immunolabeled NTS2 receptors were detected in association with synaptic terminals (Fig. 1G, H; white arrowheads). A small number of these terminals appeared to be peptidergic, as evidenced by the presence of a few large dense core vesicles (Fig. 1G, black arrowhead) in addition to synaptic vesicles. Remarkably, within synaptic terminals, NTS2 immunolabeling was quite frequently associated with the plasma membrane (Fig. 1G, white arrowheads). In addition, sparse NTS2 immunoreactivity was also detected on lightly myelinated axons coursing to the neuropil (Fig. 1H).

**Temporal changes of NT and NT receptor mRNA expression in DRGs and dorsal spinal cord following induction of neuropathic pain**

The peripheral and central neural networks involved in the processing of nociceptive information showed extensive plasticity in pathological pain states. Here, we examined whether there was a differential mRNA expression profile of the neurotensinergic system in rat DRGs and superficial dorsal horns following nerve injury. Quantitative real-time RT-PCR was used to evaluate the temporal pattern (3, 7, 14 and 28 d postinjury) of NT, NTS1, and NTS2 mRNA expression. The data were normalized at every time point to the Hprt1 housekeeping gene stably expressed over the whole experimental period. The expression of NPY was used as a positive control since it was previously reported as up-regulated following nerve injury (50). We found that the CCI of the sciatic nerve (CCI model) time dependently altered NT, NTS1, and NTS2 mRNA expression levels in both DRG and spinal cord (Table 2). A 1-way ANOVA analysis revealed that NT mRNA levels isolated from DRG and spinal cord dorsal horn of injured rats were significantly higher than those measured in noninjured and sham-treated control animals. At all postoperative time points, NT mRNA levels were significantly increased by 3- to 4-fold in the ipsilateral (L) and superficial dorsal horns following nerve injury. Quantiative real-time RT-PCR was used to evaluate the temporal pattern (3, 7, 14 and 28 d postinjury) of NT, NTS1, and NTS2 mRNA expression. The data were normalized at every time point to the Hprt1 housekeeping gene stably expressed over the whole experimental period. The expression of NPY was used as a positive control since it was previously reported as up-regulated following nerve injury (50). We found that the CCI of the sciatic nerve (CCI model) time dependently altered NT, NTS1, and NTS2 mRNA expression levels in both DRG and spinal cord (Table 2). A 1-way ANOVA analysis revealed that NT mRNA levels isolated from DRG and spinal cord dorsal horn of injured rats were significantly higher than those measured in noninjured and sham-treated control animals. At all postoperative time points, NT mRNA levels were significantly increased by 3- to 4-fold in the ipsilateral (L) and superficial dorsal horns following nerve injury.

**Figure 1.** NTS2 receptor protein distribution in rat DRG and spinal cord. A) Identification of endogenously expressed NTS2 receptors by Western blotting. Specific immunoreactive bands at 46 kDa (arrowhead) and 80–85 kDa are detected in homogenates from both DRG and spinal cord (SC). B) Immunoperoxidase staining reveals the presence of NTS2-like immunoreactivity throughout the dorsal horn of the spinal cord. A dense NTS2-like immunolabeling is observed in the superficial layers of the dorsal horn. C) As observed by confocal microscopy, NTS2 receptors are present in all subtypes of sensory neurons, notably in small/medium-sized ganglion cells innervating laminae I and II of the dorsal horn (arrowheads). D) NTS2-like immunolabeling is most prominent over the substantia gelatinosa. Also note that NTS2-immunoreactive dendritic profiles originating in the nucleus proprius radiate into the superficial layers of the dorsal horn and can make synaptic contact with primary afferent fibers (arrowheads). E, F) At the ultrastructural level, immunoreactive NTS2 is most frequently found in large dendritic profiles (D). Within these profiles, the silver-enhanced gold particles are typically associated with intracellular organelles. Occasionally, immunoreactive NTS2 is associated with the plasma membrane. These large dendrites are decorated with numerous synaptic terminals, which are, however, typically devoid of NTS2. G, H) NTS2-like immunolabeling is also found within smaller dendritic profiles (D), which are abutted by synaptic terminals (T) as well. G) Infrequently, these terminals are NTS2 immunopositive, albeit lightly as compared with dendritic labeling (white arrowheads). Immunoreactive NTS2 in synaptic terminals is frequently associated with the plasma membrane (white arrowheads). Few large dense core vesicles were also present (black arrowhead), suggesting that some of these terminals are peptidergic. H) In addition, NTS2 immunolabeling is found in association with lightly myelinated axons (A). Scale bars = 400 μm (B); 50 μm (C); 200 μm (D); 500 nm (E–G); 750 nm (H).
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<td>&gt;10 ± 2.76**</td>
<td>&gt;100 ± 8.40***</td>
<td>&gt;100 ± 10.23***</td>
<td>&gt;50 ± 5.90***</td>
<td>1.21 ± 0.19</td>
<td>0.86 ± 0.21</td>
<td>0.80 ± 0.12</td>
<td>2.03 ± 0.32***</td>
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<td>2.16 ± 0.17*</td>
<td>2.05 ± 0.13*</td>
<td>2.00 ± 0.35*</td>
<td>2.22 ± 0.17**</td>
<td>2.15 ± 0.24*</td>
<td>1.71 ± 0.18</td>
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<td>0.86 ± 0.09</td>
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<td>1.70 ± 0.13***</td>
<td>1.50 ± 0.15</td>
<td>1.29 ± 0.14</td>
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*P < 0.05, **P < 0.01, ***P < 0.001 vs. control group.
NTS2 AGONISTS REDUCE PAIN INDUCED BY NERVE INJURY

Figure 2. Effect of NTS2 agonists on mechanical allodynia induced by CCI of the sciatic nerve in rats. PWT following automated von Frey hair stimulation was measured at various time points following induction of neuropathic pain. A, B) PWTs were determined following acute intrathecal injection of three doses of JMV431 (3, 30 and 90 μg/kg; A), levocabastine (lev; 5 μg/kg; B), and β-lactotensin (β-lacto; 180 μg/kg; B) at d 3, 7, 14, 21, and 28 postsurgery. BL indicates BL before CCI surgery. Statistical analyses were performed with a 2-way ANOVA followed by a Bonferroni’s posttest, comparing NTS2 agonist treatments to corresponding saline-treated control at each time point. C) Calculations of antiallodynic effects from testing time points d 3–28 showed that all tested compounds attenuated the development of hypersensitivity to mechanical stimulus. One-way ANOVA followed by a Bonferroni’s posttest was performed to detect differences between groups of CCI rats treated spinally with either saline or NTS2 agonists. Data are shown as means ± SEM (6–8 rats/group for NT compounds, 10–15 rats/group for CCI and sham treatment). **P < 0.01, ***P < 0.001 vs. CCI saline-treated group.

Figure 3. Effects of systemic administration of AMI, PGB, and MS on mechanical sensitivity in neuropathic rats. Mechanical hypersensitivity was observed by a decreased PWT of the ipsilateral hind paw in response to an innocuous tactile stimulus. A) Time course of mechanical allodynia was examined in CCI- and sham-treated rats during the 4 wk period after the surgery. Data were analyzed statistically using a 2-way ANOVA followed by a Bonferroni’s posttest, comparing AMI (10 mg/kg), PGB (30 mg/kg), or MS (3 mg/kg) to corresponding vehicle thresholds at each time point. N.S., not significant. **P < 0.01, ***P < 0.001. B) Both first- and second-line drugs were effective in reversing mechanical allodynia in nerve-injury rats. One-way ANOVA followed by a Bonferroni’s posttest was used to evaluate the drug treatment effectiveness. Data are shown as means ± SEM (6–8 rats/group for reference compounds, 10–15 rats/group for CCI and sham treatment). ***P < 0.001.

Figure 4. Effect of NTS2 agonists on mechanical allodynia induced by CCI of the sciatic nerve in rats. PWT following automated von Frey hair stimulation was measured at various time points following induction of neuropathic pain. A) PWTs were determined following acute intrathecal injection of three doses of JMV431 (3, 30 and 90 μg/kg; A), levocabastine (lev; 5 μg/kg; B), and β-lactotensin (β-lacto; 180 μg/kg; B) at d 3, 7, 14, 21, and 28 postsurgery. BL indicates BL before CCI surgery. Statistical analyses were performed with a 2-way ANOVA followed by a Bonferroni’s posttest, comparing AMI (10 mg/kg), PGB (30 mg/kg), or MS (3 mg/kg) to corresponding vehicle thresholds at each time point. N.S., not significant. *P < 0.05, **P < 0.01, ***P < 0.001. B) Both first- and second-line drugs were effective in reversing mechanical allodynia in nerve-injury rats. One-way ANOVA followed by a Bonferroni’s posttest was used to evaluate the drug treatment effectiveness. Data are shown as means ± SEM (6–8 rats/group for reference compounds, 10–15 rats/group for CCI and sham treatment). ***P < 0.001.

Figure 5. Effects of systemic administration of AMI, PGB, and MS on mechanical sensitivity in neuropathic rats. Mechanical hypersensitivity was observed by a decreased PWT of the ipsilateral hind paw in response to an innocuous tactile stimulus. A) Time course of mechanical allodynia was examined in CCI- and sham-treated rats during the 4 wk period after the surgery. Data were analyzed statistically using a 2-way ANOVA followed by a Bonferroni’s posttest, comparing AMI (10 mg/kg), PGB (30 mg/kg), or MS (3 mg/kg) to corresponding vehicle thresholds at each time point. N.S., not significant. **P < 0.01, ***P < 0.001. B) Both first- and second-line drugs were effective in reversing mechanical allodynia in nerve-injury rats. One-way ANOVA followed by a Bonferroni’s posttest was used to evaluate the drug treatment effectiveness. Data are shown as means ± SEM (6–8 rats/group for reference compounds, 10–15 rats/group for CCI and sham treatment). ***P < 0.001.

NTS2 AGONISTS REDUCE PAIN INDUCED BY NERVE INJURY

We first evaluated in CCI-treated rats the effectiveness of drug treatment in reversing the decrease in the use of the affected limb (Fig. 4). Drug effects were expressed as the percentage of rehabilitation over the 28-d period, where 100% corresponds to a complete recovery of the injured paw utilization. Our results revealed that both JMV-431 and levocabastine significantly improved the time of ambulation spent on the injured paw, rehabilitating the functionality of the limb to 74.8 ± 15.9 and 87.6 ± 5.0%, respectively (P < 0.05; Fig. 4A). At the dose used, β-lactotensin was unable to restore the functionality. Using the same paradigm, we also demonstrated that AMI increased the use of the injured limb (82.6 ± 7.9% reversal; P < 0.01), whereas PGB and MS were mostly ineffective (Fig. 4B).

We next measured the weight-bearing deficits across the 4 limbs in nonrestrained neuropathic rats (Fig. 5). The load on the affected limb was, on average, decreased by 55 ± 3.0% from d 3 to 28 and compensated by a weight shift to the forepaws and contralateral hind paw (not shown). Intrathecal delivery of JMV-431 and levocabastine both reversed CCI-induced ipsilateral paw weight load deficits, leading to a gain of 25–30% of weight recovery (P < 0.05; Fig. 5A). However, only JMV-431 was effective in reducing the compensatory shift on the other limbs (35.3 ± 10.8% reversal, P < 0.05; Fig. 5C). In contrast, β-lactotensin and all reference drugs used in this study were inefficient in improving these quality-of-life proxies (Fig. 5B–D).

DISCUSSION

Chronic neuropathic pain is a common clinical problem worldwide, underdiagnosed and undertreated,
leading to suffering, disability, impairment of quality of life, and increased healthcare use and costs (1–3). Thus, the development of new pharmacological painkillers stays at the forefront of pain research. According to Overington et al. (57), of the 266 human targets of FDA-approved drugs, 27% correspond to the rhodopsin-like family of GPCRs. There is therefore a broad scientific consensus that GPCRs will remain the preeminent class of druggable targets and clearly one of most important therapeutic targets in pain medicine (13).

In the past few years, we and others have provided compelling evidence that the neurotensinergic system is involved in the regulation of pain transmission (14–16). Although there are now clear indications of the contribution of NTS1 and NTS2 receptor subtypes in regulating the nociceptive processes at both spinal and supraspinal levels, their potential role in reducing chronic pain remains mainly unsupported. The present study aimed at investigating the role of NTS2 receptors in the regulation of neuropathic pain symptoms. We first demonstrated that the NTS2 receptor is highly expressed in sensory neurons and in the superficial layers of the spinal dorsal horn. Within DRGs, the immunostaining for NTS2 was found in association with different subpopulations of primary sensory neurons. This result is consistent with previous findings demonstrating the presence of immunoreactive NTS2 receptors in a subset of sensory neurons carrying primary nociceptive inputs, as well as in NF200-positive large Aβ fibers, which are believed to mediate allodynia (26). NTS2-immunoreactive fibers were also particularly dense over the substantia gelatinosa and in periphery to lamina II, thus suggesting that a certain proportion of NTS2 receptors may be anterogradely transported to the central terminals of sensory neurons. In the superficial layers of the dorsal horn, immunolabeled NTS2 receptors, detected by immunogold electron microscopy, were mainly observed over dendritic profiles. An important fraction of these NTS2 receptors had an intracellular distribution, indicating that neurons expressing NTS2 hold large pools of receptors. Accordingly, we previously demonstrated using in vivo experiments that NTS2 receptors externalized on sustained NT stimulation to reach the dendritic plasma membranes of dorsal horn neurons (58). Conversely, the subcellular distribution of NTS2 receptors revealed that a high proportion of NTS2, probably originating from primary afferents, was associated with the plasma membrane in synaptic terminals.

In the present study, we also demonstrated that the mRNA expression profile of the neurotensinergic system was affected following damage to the peripheral nervous system. Notably, the increased expression of NT found here corroborates previous findings demonstrating that the NT peptide is up-regulated in the lumbar spinal cord, 7 d following induction of neuropathic pain by sciatic nerve cuff implantation (59). Consistently, an increase in NT-like immunoreactivity was also observed in large sensory neurons following peripheral axotomy (60). According to the gene expression profiles of NT receptors, our data reinforce previous cDNA microarray studies reporting changes in NTS2 expression at both DRG and dorsal spinal horn 14 d after peripheral axotomy (61–63). Overall, our results indicate that the expression patterns of NT, NTS1, and NTS2 are dynamically regulated in both DRGs and spinal cord after sciatic nerve constriction.

Over the past 25 yr, several preclinical animal models of neuropathic pain have been developed to mimic human disease conditions (64, 65). These rodent models of neuropathy have been helpful for elucidating the peripheral and central pathophysiological mechanisms underlying neuropathic pain. They have also been successful for identifying potential new therapeutic targets for the management of neuropathic pain. Among the most commonly used models of peripheral neuropathy, the CCI model, developed by Bennett and Xie (46), has been validated in terms of clinical relevance and efficiency for drug screening (66–68). Indeed, this model reproduces sensory deficits seen in patients with neuropathic pain, including the development of allodynia against mechanical stimuli and the occurrence of spontaneous painful sensation (intermit-
NTS2 agonists reduce pain induced by nerve injury

Figure 5. Evaluation of neuropathic pain-induced weight-bearing deficits and weight compensation after the administration of NTS2 agonists or first- and second-line treatments. Weight recovery (A, B) or compensation reversal (C, D) was assessed with the DWB device over the 28-d period. Treatment effectiveness was determined by measuring the percentage of weight reported on the ipsilateral paw and compensation shifts to the front paws and other anatomical zones, with 100% corresponding to a full recovery in the weight borne on the injured paw or complete weight redistribution to initial baseline values over the 4 limbs. Acute injection of JMV-431 (90 μg/kg) or levocabastine (5 μg/kg) induced a significant weight recovery (A), while only JMV-431 (90 μg/kg) reversed the weight compensation occurring in the CCI model (C). The clinically used analgesics AMI (10 mg/kg), PGB (30 mg/kg), and MS (3 mg/kg) were unable to improve these quality-of-life proxies (B, D). One-way ANOVA followed by a Bonferroni’s posttest was used to compare rats receiving drugs and CCI-treated rats receiving saline. Data are shown as means ± SEM (6–8 rats/group for all drugs, 10–15 rats/group for CCI and sham treatment). *P < 0.05.

tent or continuous) (64, 69). Furthermore, the CCI symptoms are also responsive to drugs, which are clinically used for the management of neuropathic pain (51–53, 66–68). Accordingly, we found here that MS, PGB, and AMI were effective in reducing mechanical allodynia in CCI-treated rats. More important, the present results demonstrate for the first time the critical role played by NTS2 receptors in the regulation of neuropathic pain hypersensitivity. As we previously demonstrated following administration of the NT native peptide (21), all NTS2 receptor agonists exerted antiallodynic activity in sciatic nerve-injured animals following spinal delivery. These results strengthened previous studies demonstrating the implication of NTS2 receptors in the analgesic effects of NT in acute and tonic pain paradigms. Thus, intracerebroventricular or intrathecal injection of the NTS2-selective agonist JMV-431 induced strong antinociceptive responses in acute, visceral, and formalin pain tests in rodents (24, 26, 30). Likewise, intrathecal or intraretroventral medullary administration of both NTS2 analogs β-lactotensin and levocabastine induced analgesia in rats, as measured in the tail-flick test (20, 26, 70, 71). Finally, the NT79 compound, which exhibits higher selectivity to NTS2, also attenuated both formalin-induced nociceptive behaviors and acetic acid-induced writhing responses (17, 27, 72). In line with our previous findings demonstrating that NTS1 agonists produced potent antiallodynic effects in nerve-injured rats (21), we can thus conclude that the recruitment of both NTS1 and NTS2 receptors mediates the analgesic actions of NT in chronic neuropathic pain conditions. However, since NTS1 agonists were found to induce blood pressure changes and hypothermia, whereas NTS2-selective analogs did not (30, 34, 72–75), NTS2 analogs may therefore represent a better treatment option for painful neuropathies with a reduced side-effect profile.

Despite substantial investments by the pharmaceutical industry over the past 50 yr, the currently available pharmacopoeia for neuropathic pain management is limited in terms of drug efficacy and undesired effects. Although many potential targets have been identified so far, little progress has been made in developing new, effective, and safe analgesics (76). Indeed, screening of new painkillers at the preclinical level remains limited and often leads to clinical assay abortion. Most successful analgesic development activity has been confined to the production of congeners and reformation of existing drugs or introduction of drugs designed initially to treat another disease, such as gabapentin, lidocaine, and ketamine (77, 78). Many in the field believe that the problem may lie in part in the poorly predictable techniques used to assess painful behaviors supposed to mimic the clinical reality (54, 55, 79). Indeed, most of the tests that are used to evaluate potential analgesics rely on the measurement of evoked hypersensitivity (80). However, evoked withdrawal responses do not measure pain itself but rather the hypersensitivity (e.g., allodynia) that often accompanies pain. Notably, even if a significant number of patients with neuropathic pain exhibit mechanical hypersensitivity (evoked pain), continuous or paroxysmal spontaneous (nonevoked) pain is more reflective of overall pain rating and is of much greater prevalence in people.
experiencing chronic pain (81). Finally, chronic pain has a negative effect on quality of life, leading to disturbed sleep and attention, anxiety, depression, and functional impairment. Therefore, assessment of well-being and functional rehabilitation should also be included in the screening process of analgesic drugs at the preclinical level. Accordingly, improved but more complex approaches have recently been proposed to measure pain and its effects, like conditioned place preference/aversion paradigms (53, 82–87, 88), rat grimace scale (89), or spontaneous innate behaviors (e.g., burrowing; refs. 90, 91).

In the present study, we therefore examined the effects of NTS2 putative analogues and actual prescribed drugs on different quality-of-life proxies in freely moving rats. For this purpose, we used a recently validated DWB device allowing the assessment of the time of use of the injured limb, weight load deficits, and compensatory shifts in weight distribution (47, 56). The results obtained with this behavioral device revealed that NTS2 agonists (with the exception of β-lactotensin) improved the time of ambulation spent on the injured paw and reversed the weight-bearing deficits induced by the peripheral neuropathy. Interestingly, our findings also demonstrate that both MS and PGB, which were effective in reducing tactile allodynia, were mostly inefficient in improving these quality-of-life parameters. Besides, AMI displayed a mixed profile, allowing recovery of the injured paw utilization but having no effect on neuropathy-induced weight-bearing deficits. The effects of AMI on rehabilitation do not seem to be related to hyperlocomotion (92, 93). Furthermore, previous studies assessing the effects of analogues on nonvoked pain behaviors, such as conditioned place preference/aversion tests (82, 86, 94), foot posture, and weight load (95), revealed that opioid agonists were not effective in reversing pain-related behaviors. These results may, in part, explain why these analogues do not provide adequate pain relief of continuous spontaneous (nonvoked) pain in patients with neuropathic pain. They also reinforce the idea of assessing affective and health-related quality-of-life components rather than purely sensory components of pain. Thus, development of new painkillers and screening of existing compound libraries should always include outcome measures capturing the multidimensional nature of pain.

In summary, these results demonstrate for the first time that NTS2 agonists are able to modulate pain sensation in the context of neuropathic pain, relieving touch-evoked allodynia and improving quality-of-life proxies. Notably, since NT mediates analgesia at least in part through opioid-independent mechanisms, these findings support the idea that NTS2 agonists represent an attractive alternative for treating painful neuropathies, while decreasing the side-effect charge of opioid medications. Alternatively, the development of concomitant therapy such as NTS2 agonists with existing reference drugs should rely on the gain of efficacy of each drug on both nonvoked pain and health-related quality-of-life components.

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Spinal NTS2 receptor activation reverses signs of neuropathic pain

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