Behavioral/Systems/Cognitive

Potent Spinal Analgesia Elicited through Stimulation of NTS2 Neurotensin Receptors

Philippe Sarret,[1] Michael J. Esdaile,[1] Amélie Perron,[1] Jean Martinez,[2] Thomas Stroh,[1] and Alain Beaudet[1]

[1]Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada H3A 2B4, and [2]Laboratoire des Aminoacides, Peptides et Protéines, Unité Mixte de Recherche 5810, Faculté de Pharmacie, Université de Montpellier I & II, 34093 Montpellier, France

Intrathecal administration of the neuropeptide neurotensin (NT) was shown previously to exert antinociceptive effects in a variety of acute spinal pain paradigms including hotplate, tail-flick, and writhing tests. In the present study, we sought to determine whether some of these antinociceptive effects might be elicited via stimulation of low-affinity NTS2 receptors. We first established, using immunoblotting and immunohistochemical techniques, that NTS2 receptors were extensively associated with putative spinal nociceptive pathways, both at the level of the dorsal root ganglia and of the superficial layers of the dorsal horn of the spinal cord. We then examined the effects of intrathecal administration of NT or selective NTS2 agonists on acute thermal pain. Both NT and NTS2 agonists, levocabastine and Boc-Arg-Arg-Pro-TyrΨ(CH_2NH)Ile-Leu-OH (JMV-431), induced dose-dependent antinociceptive responses in the tail-flick test. The effects of levocabastine and of JMV-431 were unaffected by coadministration of the NTS1-specific antagonist 2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxy-phenyl)pyrazol-3-yl)carboxylamino]tricyclo)3.3.1.1.^{3,7})-decan-2-carboxylic acid (SR48692), confirming that they were NTS2 mediated. In contrast, the antinociceptive effects of NT were partly abolished by coadministration of SR48692, indicating that NTS1 and NTS2 receptors were both involved. These results suggest that NTS2 receptors play a role in the regulation of spinal nociceptive inputs and that selective NTS2 agonists may offer new avenues for the treatment of acute pain.

Key words: **neuropeptide; GPCRs; antinociception; intrathecal; tail-flick; confocal microscopy**

Introduction

Neurotensin (NT) is a tridecapeptide that has been documented to play a role in sensory systems, including those mediating pain (Clineschmidt and McGuffin, 1977; Osbahr et al., 1981; Hylden and Wilcox, 1983; Pazos et al., 1984). Pharmacological studies have indicated that, in the rat, centrally administered NT exerts antinociceptive actions both spinally and supraspinally. These antinociceptive effects are naloxone insensitive, implying that they are not dependent on endogenous opioid mechanisms (Clineschmidt et al., 1979; Nemeroff et al., 1979; Osbahr et al., 1981; Behbehani and Pert, 1984; al-Rodhan et al., 1991).

At the spinal level, intrathecal administration of NT results in an increase in nociceptive threshold in the hotplate test and alters the tail-flick and acetic acid-induced writhing response latency (Martin et al., 1981; Clineschmidt et al., 1982; Martin and Naruse, 1982; Yaksh et al., 1982; Hylden and Wilcox, 1983; Spampinato et al., 1988). In support of a physiological counterpart to these effects of exogenous NT, high concentrations of NT-immunoreactive cell bodies and axon terminals have been detected in the superficial layers of the dorsal horn of the spinal cord (Uhl et al., 1979; Gibson et al., 1981; Hunt et al., 1981; Ninkovic et al., 1981; Seybold and Elde, 1982; Yaksh et al., 1982; Reinecke et al., 1983; Difiglia et al., 1984; Urban and Smith, 1993).

At supraspinal levels, microinjections of NT in the preoptic area, central nucleus of the amygdala, periaqueductal gray, and rostroventral medial medulla (RVM) induce antinociceptive responses in both acute and tonic pain models (Kalivas et al., 1982a,b; Urban and Smith, 1993; Benmoussa et al., 1996). Accordingly, NT-immunoreactive nerve cell bodies and/or fibers were detected in most of these structures, including the periaqueductal gray and the RVM, which have long been implicated in pain regulation (Uhl et al., 1979; Jennes et al., 1982; Kalivas et al., 1982b; Shipley et al., 1987; Urban and Smith, 1994; Li et al., 2001).

Three NT receptor subtypes have been cloned to date. Two of these, NTS1 and NTS2, correspond to G-protein-coupled receptors, whereas the third one, NTS3, is a single transmembrane spanning receptor that shares 100% homology with the sorting protein, gp95/sortilin (Hermans and Maloteaux, 1998; Vincent et al., 1999; Sarret and Beaudet, 2003). Although some of the antinociceptive effects of NT appear to be exerted through the high-affinity NTS1 receptor (Tyler et al., 1998; Pettibone et al., 2002), the fact that various NT analogs exhibit analgesic potencies that do not correlate with their binding affinity for NTS1 suggests that other receptors are also involved. Several lines of evidence point to the levocabastine-sensitive low-affinity NTS2 subtype as a pos-

Received March 1, 2005; revised July 15, 2005; accepted July 15, 2005.

This work was supported by Canadian Institutes of Health Research Grant MT-7366 to A.B. P.S. and A.P. were supported by fellowships from the Fonds de la Recherche en Santé du Québec. We thank Naomi Takeda for secretarial help.

Correspondence should be addressed to Dr. Alain Beaudet, Department of Neurology and Neurosurgery, Montreal Neurological Institute, 3801 University Street, Montreal, Quebec, Canada H3A 2B4. E-mail: alain.beaudet@mcgill.ca.

P. Sarret's present address: Department of Physiology and Biophysics, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec, Canada J1H 5N4.

DOI:10.1523/JNEUROSCI.0810-05.2005

 0270-6474/05/258188-09$15.00/0

sible antinociceptor (Sarret and Beaudet, 2003). Thus, the analgesic effects produced by intracerebroventricular administration of NT are not antagonized by the NTS1-specific antagonist 2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxy-phenyl)pyrazol-3-yl)carboxylamino]tricyclo)3.3.1.1.^{3,7})-decan-2-carboxylic acid (SR48692), but are blocked by SR142948A (2-([5-{2,6-dimethoxyphenyl}-1-{4-(*N*-[3-dimethylaminopropyl]-*N*-methylcarbamoyl)-2-isopropylphenyl}-1*H*-pyrazole-3-carbonyl]amino)-adamantane-2-carboxylic acid hydrochloride), which recognizes both NTS1 and NTS2 (Gully et al., 1993; Dubuc et al., 1994; Labbe-Jullie et al., 1994; Gully et al., 1997). Additionally, knock-down strategies, using either NTS2 antisense oligonucleotides (Dubuc et al., 1999) or NTS2-deficient mice, markedly inhibit NT-induced antinociception (Maeno et al., 2004). The presence of high levels of NTS2 mRNA and of strong NTS2 immunolabeling in regions implicated in pain regulation (such as the periaqueductal gray, nuclei raphe dorsalis, magnus, pallidus, and gigantocellularis, pars alpha) was also interpreted as circumstantial evidence for a role of NTS2 receptors in antinociception (Sarret et al., 1998, 2003; Walker et al., 1998).

The aim of this study was to take advantage of recently developed antibodies against rat NTS2 (Sarret et al., 2003) and stable NT analogs displaying preferential affinity for NTS2 (Labbe-Jullie et al., 1994; Dubuc et al., 1999) to investigate the distribution of NTS2 in spinal cord and DRGs and its implication in spinal analgesia. Indeed, whereas neurons expressing NTS1 mRNA had been reported in DRG neurons (Zhang et al., 1995) and high concentrations of NTS1 binding sites, and NTS1-immunoreactive nerve cell bodies and fibers had been detected in the substantia gelatinosa of the dorsal horn (Ninkovic et al., 1981; Kar and Quirion, 1995; Fassio et al., 2000), nothing was known about the distribution of NTS2 receptors in these two areas. Likewise, recent studies using knock-down strategies had suggested that NTS1 receptors may be implicated in spinal antinociception (Tyler et al., 1998; Pettibone et al., 2002), but whether NTS2 receptors were also involved in this process was still an open question.

Materials and Methods

Western blotting experiments

Adult Sprague Dawley rats (200–250 g; Charles River, St. Constant, Quebec, Canada) were decapitated. Lumbar spinal cords and dorsal root ganglia were quickly dissected, 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, Quebec, 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, Ontario, Canada), and transferred to nitrocellulose membranes (Bio-Rad, Mississauga, Ontario, Canada). Nonspecific sites were blocked by 0.1% Tween 20 and 10% milk powder (Carnation, Don Mills, Ontario, Canada) in PBS, pH 7.4, overnight at 4°C. Nitrocellulose membranes were then immunoblotted overnight at 4°C with the N-terminal-specific anti-NTS2 rabbit antibody [1:10,000; made on demand by Affinity BioReagents (Golden, CO)] in PBS with 1% BSA and 1% ovalbumin. After washing with PBS–Tween 20, blots were incubated for 1 h at room temperature (RT) with an HRP-conjugated goat anti-rabbit secondary antibody (1:4000; Amersham Biosciences, Baie d'Urfe, Quebec, Canada) in PBS with 5% milk powder, and proteins were visualized by using an enhanced chemiluminescent detection system (PerkinElmer, Boston, MA). The specificity of the antiserum was confirmed by preadsorption of the NTS2 antibody overnight with an excess of immunizing peptide (2 μg/ml adsorbing peptide at a final antibody dilution of 1:10,000) as shown previously (Sarret et al., 2003).

Immunolocalization of NTS2 receptor in rat spinal cord and dorsal root ganglia

Adult Sprague Dawley rats (200–250 g) were deeply anesthetized with 100 mg/kg sodium pentobarbital, administered intraperitoneally, and perfused transaortically with a freshly prepared solution of 4% paraformaldehyde 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 −40°C. Spinal cords were sectioned transversely at 30 μm on a freezing microtome, and DRGs were cut on a cryostat at 20 μm.

Light microscopic studies. Sections were incubated overnight at 4°C in anti-NTS2 rabbit antiserum diluted 1:10,000. Immunostaining was performed according to the avidin–biotin peroxidase method (Elite ABC kit; Vector Laboratories, Burlington, Ontario, Canada) using a nickel-intensified diaminobenzidine protocol to localize the horseradish peroxidase immunoreaction product as described previously (Sarret et al., 2003). For specificity control, sections were incubated overnight with primary antiserum preadsorbed with 2 μg/ml NTS2 amino-terminal peptide. Reacted sections were mounted on chrome alum/gelatin-coated slides, dehydrated in graded ethanols, defatted in xylene, and mounted with Permount (Fisher Scientific, Montreal, Quebec, Canada). Labeled structures were examined under bright-field illumination with a Leitz Aristoplan microscope (Leica, Dollard Desormeaux, Quebec, Canada), and digitized images were obtained with a Pulnix TMC-1000CL camera using VisionGauge software (VISIONx, Montreal, Quebec, Canada).

Confocal microscopic studies. To identify NTS2-expressing neurons in spinal cord and DRGs, sections were processed for double-immunofluorescence labeling using a mixture of anti-NTS2 and one of the following primary antibodies: anti-substance P (SP) (Chemicon, Temecula, CA) or anti-calcitonin gene-related peptide (CGRP) (Peninsula Laboratories, San Carlos, CA) to stain small and medium peptidergic neurons, or anti-neurofilament 200 (NF200) (Sigma, Oakville, Ontario, Canada) clone 52 to identify large ganglion cells. Small, nonpeptidergic neurons were identified using biotinylated isolectin B4 (IB4) (Sigma). Briefly, sections were treated for 30 min in 3% NGS in TBS and incubated overnight at 4°C with a mixture of primary antibodies in TBS containing 0.05% Triton X-100 and 0.5% NGS. For localization of NTS2 to small- and medium-sized peptidergic neurons, sections were incubated with a mixture of rabbit NTS2 antibody (1:10,000) and either SP (1:800) or CGRP (1:200) antibodies raised in guinea pig. After rinsing three times with TBS, bound primary antibodies were revealed by simultaneous incubation with goat anti-rabbit Alexa 594- and goat anti-guinea pig Alexa 488-conjugated secondary antibodies (1:500; both from Invitrogen), respectively, for 60 min at RT. To double-label large DRG cells for NTS2 and NF200, sections were incubated overnight with rabbit NTS2 antiserum (1:10,000) and mouse anti-neurofilament 200 (1:400). The two primary antisera were localized with an Alexa 594-coupled goat anti-rabbit diluted 1:500 and an Alexa 488-coupled goat anti-mouse diluted 1:750 (both from Invitrogen), respectively. For costaining with *Bandeiraca simplicifolia* IB4 lectin, sections were incubated with rabbit NTS2 antiserum (1:10,000) and 2 μg/ml biotinylated IB4 lectin and then revealed with an Alexa 594-coupled goat anti-rabbit (1:500) and an Alexa 488-conjugated streptavidin (1:750; Invitrogen). Sections were then rinsed and mounted with Aquamount. The absence of cross-reactivity of the secondary antibodies was verified by omitting one or both primary antibodies during the overnight incubation. Double-labeled sections were analyzed by confocal microscopy using a Zeiss (Toronto, Ontario, Canada) 510 laser-scanning microscope equipped with a Zeiss inverted microscope and argon (488 nm) and He/Ne (543 nm) lasers. Images were acquired simultaneously for both fluorophores by using the multitrack configuration mode and processed using the Zeiss 510 laser-scanning microscope software. Light-microscopic photomicrographs and color images from double-labeling experiments were adjusted for contrast and brightness using Adobe Photoshop 6.0 software (Adobe, San Jose, CA). The final composites were processed using Deneba's Canvas 7.0 imaging software (Deneba Software, Miami, FL) on an Apple PowerBook G3.

Quantification. To quantify the proportion of the various types of DRG cells expressing NTS2 receptors, lumbar dorsal root ganglia from three different rats were serially sectioned at 20 μm. Sections were dually

labeled simultaneously with anti-NTS2 and anti-SP, anti-CGRP, anti-NF200, or biotinylated lectin B4. For each rat and each pair of antibodies, four sections, 60 μm apart, were selected for quantification to avoid counting the same cells twice. Cells were counted on screen, from confocal images of whole sections. Only neurons with clearly visible nuclei were counted. Calculations and statistical analyses were performed using Excel 5.0 (Microsoft, San Francisco, CA) and Prism 3.02 (Graph Pad Software, San Diego, CA). Quantitative data are represented as means ± SEM of three independent experiments.

Behavioral studies

Animals. Adult male Sprague Dawley rats (200–250 g) were maintained on a 12 h light/dark cycle and allowed free access to food and water. The experimental procedures in this study were approved by the Animal Care Committee at McGill University and were in accordance with policies and directives of the Canadian Council on Animal Care. Rats were allowed to acclimate for at least 2 d before any behavioral tests.

Acute antinociceptive effects of NTS2 agonists. Antinociception was assessed using the tail-flick test. Thermal threshold latencies were determined every 10 min for up to 1 h after drug injection. Testing involved measuring the latency (in seconds) for the rat to withdraw its tail from a water bath maintained at 52°C. The tail was submerged in hot water up to 5 cm from the tip, and the latency to flick or curl the tail from the water was recorded. Three baseline readings were obtained and averaged before drug injection. Baseline responses were typically 3 s, and a cutoff was imposed at 10 s to prevent tissue damage. If the animal reached cutoff, the tail was removed from the water and the animal was assigned the maximum score. Tail-flick latencies were converted to percentage of maximum possible effect (MPE) according to the following formula: percentage of MPE = [(test latency) − (baseline latency)]/[(cutoff) − (baseline latency)] × 100.

A first set of experiments was aimed at establishing the effects of NT, levocabastine (kindly provided by Janssen Pharmaceutica, Beerse, Belgium), and the degradation-resistant NT analog Boc-Arg-Arg-Pro-TyrΨ(CH_2NH)Ile-Leu-OH (JMV-431) (synthesized by J. Martinez) (Labbe-Jullie et al., 1994) on thermal nociception. To this end, rats were anesthetized with halothane and injected intrathecally (at L5–L6) with NT (10, 100, 600, 1200, 2400 pmol), levocabastine (600, 1200, 2400 pmol), or JMV-431 (1, 2, 12, 24, 48 nmol) diluted in 30 μl of saline. Antinociceptive effects of intrathecal NT receptor agonists were assessed at 20 min after injection, and ED_{50} values, defined as the dose producing a half-maximal effect, were calculated from the theoretical curve. Doses for each drug were selected on the basis of their affinity for the NTS2 receptor subtype.

In a second set of experiments, NT (1200 pmol), levocabastine (1200 pmol), and JMV-431 (24 nmol) were administered in conjunction with the NTS1-preferring antagonist SR48692 (170 pmol, 1.7 nmol, 8.5 nmol; kindly supplied by Sanofi-Synthelabo, Toulouse, France) to tease out the role of NTS1 versus NTS2 in the observed antinociceptive effects. The final pH of all injected drug solutions was 7.4 to prevent any potential pH effects on peripheral nociception. Vehicles (0.9% saline) were prepared exactly as the corresponding drug solutions.

Statistical analysis. Data are presented as means ± SEM. All calculations and statistical analysis were performed using Prism 3.02 (Graph Pad Software). One-way ANOVA followed by *post hoc* comparisons using Dunnett's multiple comparison test (MCT) were conducted. Asterisks denote statistical significance when compared with saline-treated controls ($^*p < 0.05$; $^{**}p < 0.001$).

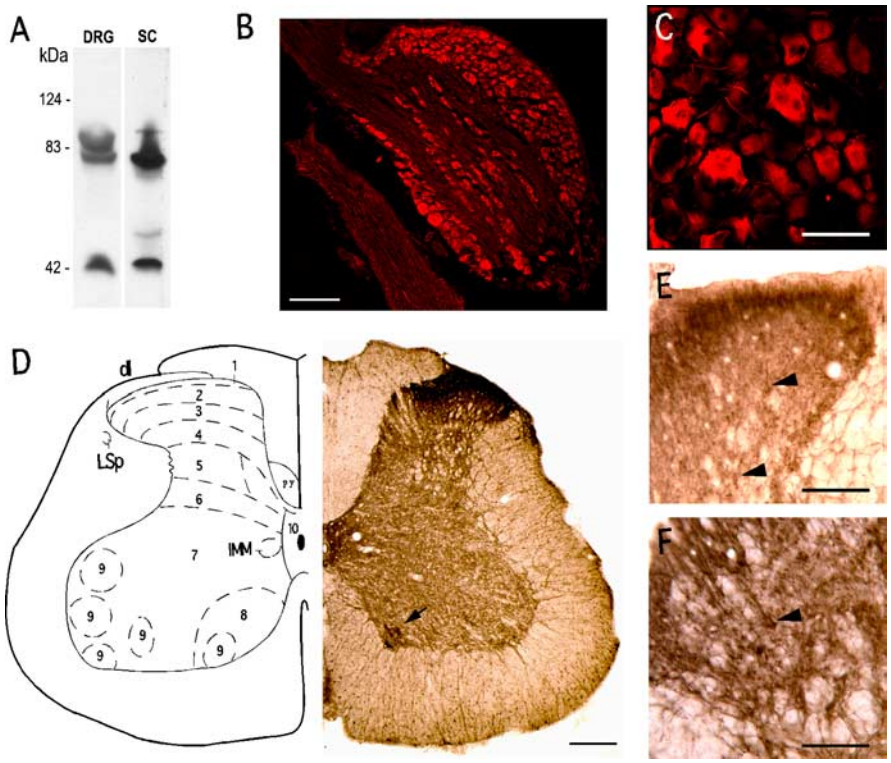

Figure 1. Expression of NTS2 receptors in sensory neurons and spinal cord. ***A***, Identification of endogenously expressed NTS2 receptors by Western blotting. Specific immunoreactive bands are detected at estimated molecular masses of 46 and 80–85 kDa in homogenates from both DRGs and lumbar spinal cord (SC). Each lane represents the transfer of 60 μg of membrane protein. Data are representative of four independent experiments. ***B***, Confocal laser microscopic analysis of NTS2 expression in primary afferent neurons. NTS2 is expressed in subpopulations of small and large ganglion cells. ***C***, High magnification of ***B***. ***D***, Schematic representation of a hemisection of rat lumbar spinal cord (left). Numbers 1–10, Spinal cord layers; dl, dorsolateral fasciculus; IMM, intermediomedial cell column; LSp, lateral spinal nucleus; py, pyramidal tract. Immunoperoxidase staining reveals the presence of NTS2-like immunoreactivity throughout the lumbar spinal cord (right). Immunostaining is most prominent in the superficial layers of the dorsal horn, around the central canal, and over motoneurons in lamina layer IX (arrow). ***E***, ***F***, At high magnification, dense NTS2 immunostaining is observed over laminas I and II of the dorsal horn. Immunoreactive nerve cell bodies are also visible in laminas III and IV (arrowheads). Scale bars: ***B***, 200 μm; ***C***, 80 μm; ***D***, 400 μm; ***E***, 250 μm; ***F***, 50 μm.

Results

Expression of NTS2 receptors in dorsal root ganglia and spinal cord

We first investigated, by Western blotting, the expression of NTS2 receptor proteins in DRGs and spinal cord. As demonstrated previously in membranes prepared from rat brain and cerebellum (Sarret et al., 2003), specific NTS2-immunoreactive bands were detected at estimated molecular weights of 46 and 80–85 kDa in homogenates from DRGs and spinal cords (Fig. 1*A*). The 46 kDa protein band likely corresponds to the monomeric form of the receptor, because it migrates at the theoretical molecular weight deduced from the cDNA sequence of NTS2. The higher molecular weight forms presumably correspond to multimeric species of NTS2 receptors. None of these bands was present when the blots were incubated with antiserum preadsorbed with the antigenic peptide (data not shown).

Cellular distribution of NTS2 receptors in primary afferent neurons

The distribution of NTS2 receptors in DRGs was then assessed by immunofluorescence using laser-scanning confocal microscopy. Strong labeling was evident in small and large ganglion cells (Fig.

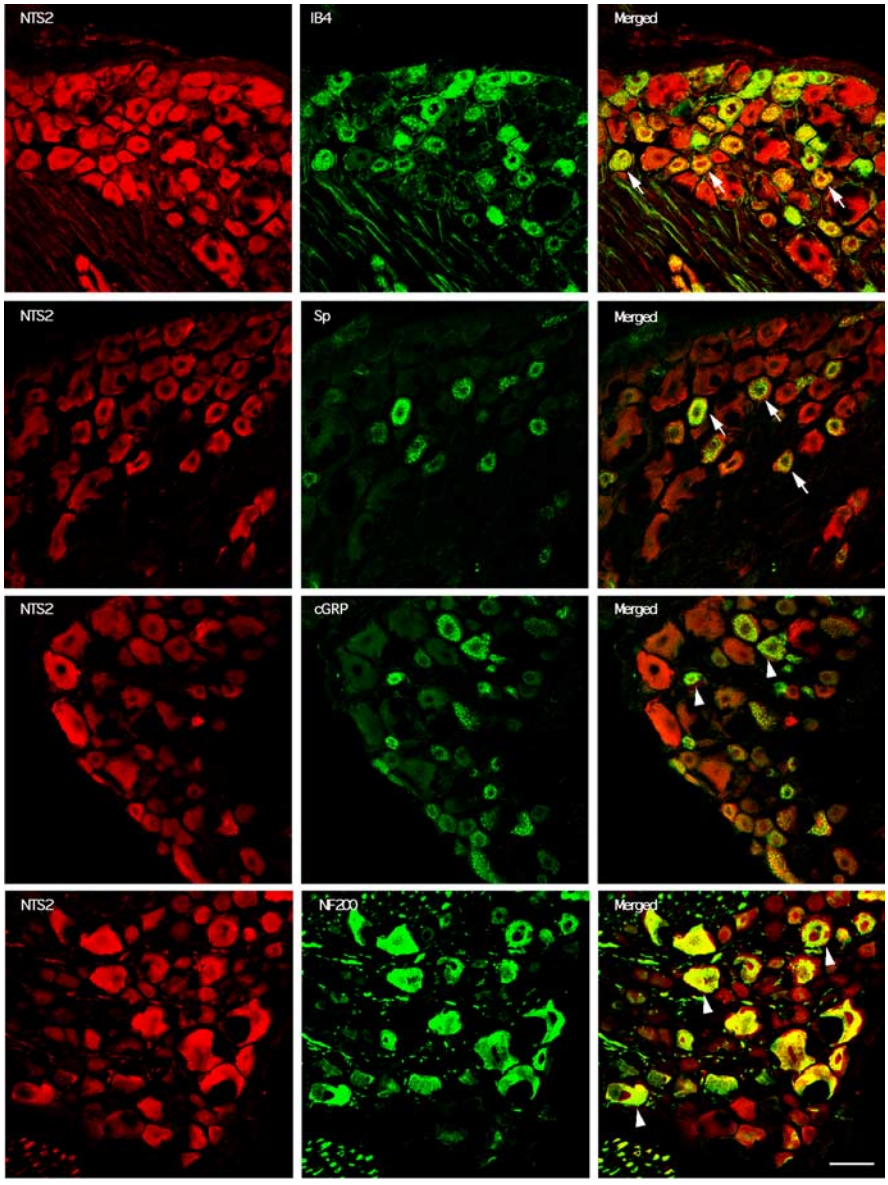

Figure 2. Cellular distribution of NTS2-IR in rat DRGs. Double immunofluorescence is used to colocalize NTS2 (red; left column) and lectin IB4, SP, CGRP, or NF200 (green; middle column). The merged images show dually labeled cells (yellow; right column). Numerous IB4- and SP-positive ganglion cells colocalize NTS2 (arrows). In contrast, CGRP-immunoreactive cells only rarely costain for NTS2 (arrowheads). Most large, NF200-positive ganglions cells colocalize NTS2 (arrowheads). Nonetheless, several NTS2-positive cells do not contain NF200. Scale bar, 50 μm.

1*B*,*C*). This labeling was completely prevented by preincubation with the cognate peptide (data not shown). To identify the phenotype of NTS2-expressing cells, free-floating sections were processed for double-labeling immunohistochemistry combining NTS2 antibodies with classical neuronal markers. Quantitative analysis indicated that 42 and 37% of NTS2-positive cells in lumbar DRGs colocalized with the small ganglion cell markers lectin IB4 and SP, respectively (Figs. 2, 3*A*). Conversely, 60% of lectin IB4- and 85% of SP-positive neurons contained NTS2 (Fig. 3*B*). Approximately 19% of NTS2-positive cells displayed CGRP immunoreactivity (IR), and, conversely, 25% of CGRP-positive neurons immunostained for NTS2 (Fig. 3*A*,*B*), indicating limited expression of NTS2 by medium DRG neurons (Fig. 2, arrowheads). Finally, 27% of NTS2-immunoreactive cells expressed the large ganglion cell marker NF200 (Fig. 3*A*), and, conversely, 90% of large NF200-positive ganglion cells exhibited NTS2-IR (Figs. 2, 3*B*).

Localization of NTS2 receptors in lumbar spinal cord

In rat lumbar spinal cord, immunoperoxidase staining revealed intense NTS2-like immunoreactivity within the superficial layers of the dorsal horn (Fig. 1*D*). A dense plexus of NTS2-immunoreactive neuronal processes, as well as limited numbers of NTS2-expressing cells, were observed in laminas I and II (Fig. 1*E*,*F*). In addition, more sparsely distributed NTS2-immunoreactive perikarya and processes were detected in the deeper layers of the dorsal horn, around the central canal, and throughout the gray matter of the ventral horn (Fig. 1*D*,*E*). Large, intensely labeled motoneurons were also evident in lamina IX (Fig. 1*D*). Preadsorption of the primary antiserum with an excess of the immunogenic peptide completely abolished NTS2 immunostaining (data not shown).

To investigate whether NTS2-immunoreactive fibers detected in the superficial laminas of the dorsal horn corresponded to primary afferent axons, spinal cord sections were dually stained for NTS2 and for SP, CGRP, or the biotinylated lectin IB4. Confocal microscopy revealed the presence of a dense band of NTS2-immunoreactive processes in lamina II of the dorsal horn (Fig. 4*A*,*E*,*I*). In addition, sparse transversely orientated NTS2-immunoreactive nerve fibers were observed in lamina I (Fig. 4*A*). NTS2-IR colocalized with IB4 staining in the inner portion of lamina II, as visible at both low (Fig. 4*C*) and high (Fig. 4*D*) magnification. There was also a partial overlap of NTS2-IR with CGRP immunostaining in the outer portion of lamina II, in which part of Aδ primary afferents arborize (Fig. 4*G*,*H*). In contrast, there was no apparent overlap between SP and NTS2 immunostaining in either lamina I or the outer portion of lamina II, in which most peptide-containing afferents terminate. In fact, NTS2 immunostaining was most heavily concentrated in lamina III, in which SP-IR is sparse (Fig. 4*K*,*L*). However, numerous NTS2-immunoreactive nerve cell bodies, identified among the plexus of processes pervading laminas III–V of the dorsal horn, colocalized SP (Fig. 4*L*).

Effect of intrathecal injection of NT and NTS2-preferring agonists in a model of acute thermal pain

In the tail-flick test, intrathecal administration of NT in rats elicited a dose-dependent antinociceptive effect, characterized by an increase in response latency compared with baseline values (Fig. 5). Peak antinociceptive responses consistently occurred 20 min after injection of NT (Fig. 5*A*). Response latencies returned to baseline values by 40 min except with the highest dose tested (1200 pmol; Fig. 5*A*). Comparison of percentage of MPE at 20 min after injection showed significant effects for each dose, with

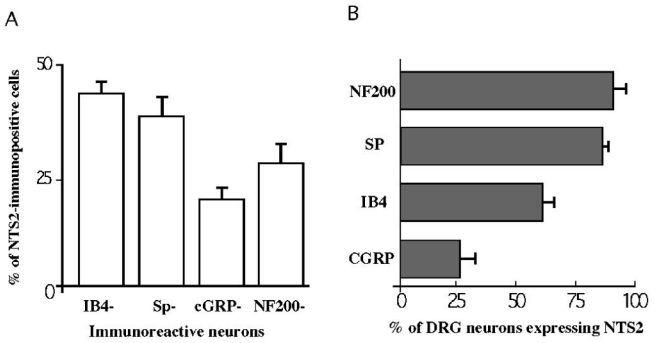

Figure 3. Histochemical identification of NTS2-immunoreactive neurons in L5 DRG. Quantitative analysis of the proportion of dually labeled cells. ***A***, Percentage of NTS2-positive ganglion cells immunostained for IB4, SP, CGRP, and NF200. ***B***, Percentage of NF200-, SP-, IB4-, and CGRP-containing DRG neurons expressing NTS2 (mean ± SEM). The data are from three rats (4 sections per rat).

the highest dose inducing maximal elevation of the tail-flick threshold (54.5 ± 8.1%; $p < 0.001$) (Fig. 5*B*). A dose–response curve, generated by plotting the percentage of MPE at the time of peak antinociceptive response for each dose of NT, yielded an ED_{50} value of 1 nmol (Fig. 5*C*).

To test whether NTS2 receptors were involved in NT-induced analgesia, we examined, in the same acute pain paradigm, the antinociceptive effects of two NTS2-specific agonists, levocabastine and JMV-431, at doses comparable with those of NT in terms of their affinity for the NTS2 receptor. Intrathecal levocabastine (Fig. 6) and JMV-431 (Fig. 7) both increased the nociceptive threshold in the tail-flick test. However, the analgesic efficacy of JMV-431, expressed as percentage of MPE 20 min after drug delivery (92.3 ± 6.3%; $p < 0.001$) (Fig. 7*B*), was considerably higher than that of either levocabastine (33.5 ± 5.4%; $p < 0.001$) (Fig. 6*B*) or NT (54.5 ± 8.1%; $p < 0.001$) (Fig. 5*B*). The analgesic effects obtained with the two highest doses of JMV-431 (24 and 48 nmol) were also maintained over longer periods of time than those recorded after either levocabastine (Fig. 6*A*) or the lower dose of JMV-431 (Fig. 7*A*). The JMV-431 dose–response curve was both steeper and shifted to the right, compared with that of NT (Fig. 7*C*). The resulting ED_{50} value was estimated at 10 nmol.

Implication of NTS1 versus NTS2 in the antinociceptive effects of intrathecally injected NT

To tease out the respective roles of NTS1 and NTS2 receptors in NT-induced spinal analgesia, we repeated the above experiments in the presence of the NTS1 receptor antagonist SR48692. Because SR48692, while competitively inhibiting ^{125}I-NT binding to NTS1 with an affinity of 0.5–4 nM, also recognizes NTS2 receptors at high concentration (IC_{50}, 150–300 nM) (Gully et al., 1993), we first determined at which dose SR48692 would affect NTS2-induced analgesia in the present experimental paradigm. For this purpose, we administered the NTS2-selective drug levocabastine with increasing concentrations of SR48692. As shown in Figure 8, low (170 pmol) and intermediate (1.7 nmol) doses of SR48692 had no effect on the levocabastine-induced nociceptive responses. However, at a higher dose of SR48692 (8.5 nmol), the response generated by levocabastine was significantly reduced (Fig. 8*A*,*B*), indicating that, at a high concentration, SR48692 affected levocabastine-induced antinociception through interaction with NTS2 receptors. When SR48692 was injected alone, no significant elevation of the tail-flick latency was detected (data not shown).

We then investigated whether the analgesic effects of NT (1200 pmol) or JMV-431 (24 nmol) would be affected if the drugs were administered in conjunction with SR48692, at a dose (170 pmol) at which the antagonist did not perturb NTS2-mediated analgesia. As shown in Figure 9, SR48692 reversed by 55 ± 4.2% the analgesia elicited by 1200 pmol of NT, suggesting that NT-induced effects were partly exerted through interaction of the peptide with NTS1 (Fig. 9*A*). However, this dual treatment strategy failed to modify the JMV-431-induced increase in tail-flick latency, indicating that JMV-431 effects were selectively NTS2 mediated (Fig. 9*B*).

Discussion

The present study demonstrates that NTS2 receptors are expressed by neurons associated with nociceptive pathways in rat spinal cord and dorsal root ganglia and provides the first evidence for an implication of this receptor subtype in neurotensin-induced analgesia at the spinal level. Our results also suggest that selective NTS2 agonists may represent a new class of potent spinal analgesics.

The association of NTS2 receptor mRNAs with primary nocicep-

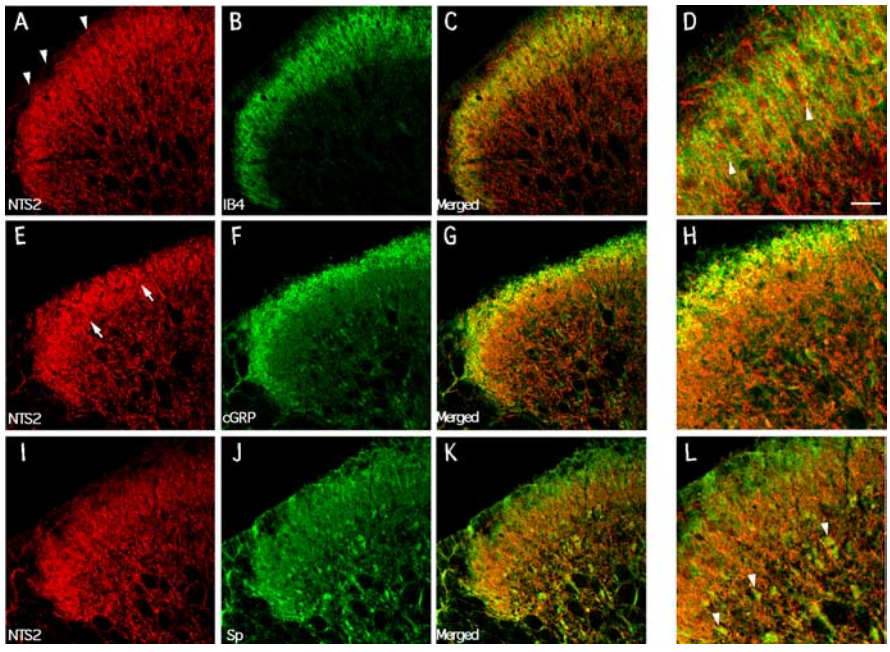

Figure 4. Immunohistochemical distribution of NTS2 receptors in the superficial dorsal horn of the lumbar spinal cord. ***A***–***C***, Colocalization of NTS2-IR (red) and lectin IB4 staining (green). A dense network of NTS2-immunopositive processes is visible in lamina II. More sparse, transversely orientated NTS2-immunoreactive fibers are also evident in lamina I (arrowheads). NTS2-IR overlaps extensively with lectin IB4 staining in the inner portion of lamina II. ***D***, At high magnification, individual dually labeled fibers (in yellow) are evident among singly labeled ones (arrowheads). ***E***–***G***, Colocalization of NTS2 and CGRP immunoreactivity. Restricted overlap of the two markers is visible in the outer segment of lamina II (arrows). ***H***, Higher magnification of ***G***. ***I***–***K***, Dual NTS2 and SP immunostaining. No overlap between NTS2 and SP immunostaining is apparent in laminas I and II. However, numerous SP-immunoreactive cells in laminas III–V colocalize NTS2 (arrowheads in ***L***). Scale bar: (in ***D***) ***A***–***C***, 15 μm; ***D***, 50 μm; ***E***–***G***, ***I***–***K***, 25 μm; ***H***, ***L***, 30 μm.

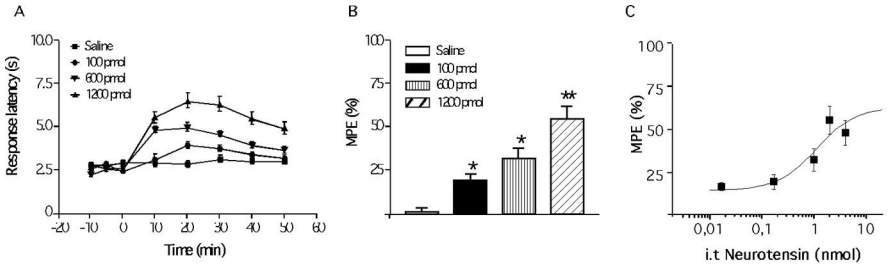

Figure 5. Effect of acute intrathecal (i.t.) injection of NT on tail-flick latencies in rats. ***A***, Time and dose dependency of the antinociceptive effects of NT. Baseline latencies were measured three times before drug injection. Latencies were determined every 10 min for up to 1 h subsequent to intrathecal administration of NT. ***B***, Percentage of MPE ± SEM calculated at the time of peak antinociceptive response (20 min) for each dose (100, 600, and 1200 pmol). Asterisks denote a statistically significant increase compared with saline-treated controls ($n = 10$ per group; $^*p < 0.05$; $^{**}p < 0.001$; ANOVA followed by Dunnett's MCT). ***C***, Antinociceptive NT dose–response curve generated by plotting the percentage of MPE at the time of peak antinociceptive response for each dose of NT. Error bars indicate SEM.

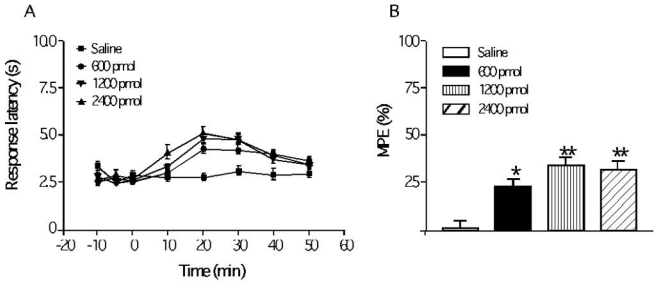

Figure 6. Antinociceptive effects of acute intrathecal injections of levocabastine on tail-flick latencies in rats. ***A***, Time course of antinociceptive effects of levocabastine. Baseline latencies were measured three times before drug injection and every 10 min for up to 1 h subsequent to intrathecal administration of the drug. ***B***, Percentage of MPE ± SEM determined at 20 min after injection for each dose. Asterisks denote a statistically significant increase compared with saline-treated controls ($n = 10–12$ per group; $^*p < 0.05$; $^{**}p < 0.001$; ANOVA followed by Dunnett's MCT). Error bars indicate SEM.

tive pathways was initially suggested by a cDNA microarray approach in a rat peripheral axotomy model of neuropathic pain (Xiao et al., 2002; Yang et al., 2004). In the present study, we demonstrated the presence of NTS2 receptor proteins in spinal cord and DRGs by Western blot and immunohistochemistry. In each structure, the receptor was detected by Western blot as low molecular weight (46 kDa), presumptively monomeric, and higher molecular weight (80–85 kDa), presumptively multimeric forms. These molecular forms were similar in size to those detected previously in membrane preparations from rat brain and cerebellum (Sarret et al., 2003). Recent studies have shown that the NTS2 receptor was expressed as both full-length and five-transmembrane domain variant (vNTS2) isoforms in rodent brain, but that the shorter isoform did not exist in monomeric form (Botto et al., 1997; Perron et al., 2005). Accordingly, the size of the low molecular weight form of the receptor, detected here in spinal cord and DRGs, conforms to that of the monomeric full-length receptor, as deduced from its amino acid composition (Chalon et al., 1996). However, both short and long NTS2 isoforms can heterodimerize *in vitro* (Perron et al., 2005), suggesting that the higher molecular species of the receptor could correspond to both NTS2 homodimeric and vNTS2/NTS2 heterodimeric species.

Within DRGs, NTS2-IR was found in association with both substance P- and lectin B4-containing small ganglion cells, indicating that the receptor is expressed within a subset of neurons documented to carry primary nociceptive inputs. In addition, immunoreactive NTS2 receptors were associated with large DRG neurons immunopositive for anti-neurofilament 200, suggesting that NTS2 is also involved in the regulation of other sensory modalities. In contrast, NTS1 receptors, as detected using *in situ* hybridization, appear to be expressed mainly by small sensory neurons, at least at L4 and L5 levels in the rat (Zhang et al., 1995). Electrophysiological studies support the concept of a differential expression of NTS1 and NTS2 receptor subtypes by rat DRG neurons. Indeed, under voltage-clamp conditions, the effect of NT on small DRG cells is mainly inhibitory via an outward current, whereas on large DRG neurons it is mainly excitatory, evoking an inward current (Zhang et al., 1995, 1996; Xu et al., 1997; Kawarada et al., 2000). After axotomy, the current elicited by NT on small DRG neurons is shifted from outward to inward (Xu et al., 1997), whereas NTS1, but not NTS2, receptors are downregulated in the same cells (Zhang et al., 1995). Therefore, it is tempting to speculate that NTS1 is responsible for the inhibitory effects of NT on small ganglion cells, whereas NTS2 mediates the excitatory effects of NT on both small and large DRG neurons.

Within the spinal cord, NTS2-immunoreactive nerve cell bodies and processes were detected throughout the gray matter of dorsal and ventral horns. Fiber networks were particularly dense in the superficial layers of the dorsal horn, especially within the zones bordering lamina II, which are known to be involved in the processing of nociceptive stimuli. Numerous NTS2-positive nerve cell bodies were also observed in layers III–V, in which large myelinated primary afferents terminate, as well as in the areas immediately surrounding the central canal, which, like the superficial laminas of the dorsal horn, play a role in the processing of nociceptive inputs. This distribution is considerably more widespread than that of NTS1 receptors, as described using either receptor autoradiography or immunohistochemistry. By autoradiography, high-affinity (i.e., NTS1) NT receptors were localized to lamina II of the dorsal horn, and most prominently in the deeper inner segment III (Ninkovic et al., 1981; Young and Kuhar, 1981; Uhl, 1982; Faull et al., 1989; Kar and Quirion, 1995). Likewise, by immunohistochemistry, a very dense plexus of NTS1-immunoreactive fibers was detected in the substantia gelatinosa of the dorsal horn, and NTS1-immunoreactive nerve cell bodies were observed in the deeper part of layer II (Fassio et al., 2000). Thus, anatomical localization studies suggest that if both NTS1 and NTS2 receptors are ideally poised to modulate incoming nociceptive inputs, NTS1 may do so in a more selective manner than NTS2.

The fact that NTS2 receptors are expressed both in DRGs and in the terminal fields of primary sensory neurons in the superficial dorsal horn suggests that at least a contingent of NTS2 receptors in the dorsal horn may be associated with primary afferent terminals. Although deafferentation studies will be needed to confirm whether or not primary afferent axons are endowed with NTS2 receptors, the present dual-labeling studies suggest that only a small proportion, if any, of primary nociceptive afferents contain NTS2. Indeed, NTS2-IR only partially overlapped with IB4-positive or CGRP-positive axonal fields and showed no overlap with SP-immunoreactive terminal arbors in the dorsal horn of the spinal cord. Likewise, few if any NTS1 receptors appear to

be associated with primary nociceptive fibers, because no change in the density of radiolabeled NTS1 receptors was observed in the dorsal horn after dorsal rhizotomy (Ninkovic et al., 1981). It would appear, therefore, that the bulk of the spinal nociceptive effects of NT are exerted postsynaptically in the dorsal horn of the spinal cord. If this is the case, one has to question the role of somatic receptors expressed in DRG neurons, because these would not be meant for delivery to the spinal cord. On the one hand, these receptors may be transported through the peripheral branch of DRG neurons for modulation of the peripheral effects of NT. However, they may also serve locally, to transduce the effects of NT circulating in the CSF, because NT does not appear to be synthesized within DRGs themselves (Zhang et al., 1993). Somatic DRG receptors may be involved, therefore, in mediating the effects of intrathecally injected NT agonists.

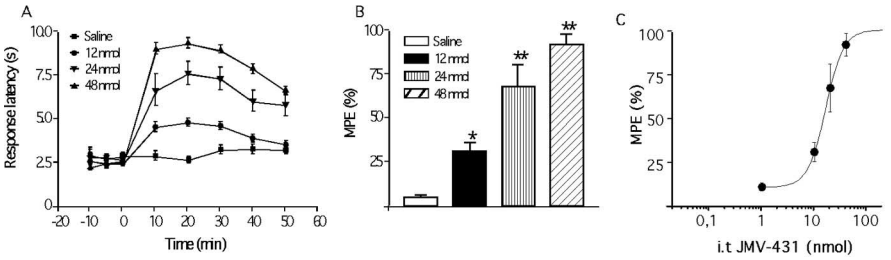

Figure 7. Antinociceptive response to JMV-431 in the tail-flick test. ***A***, Dose- and time-dependent antinociceptive effects of JMV-431. ***B***, Percentage of MPE of JMV-431. Each bar represents the percentage of MPE ± SEM calculated 20 min after drug delivery for each dose ($n = 10–12$ for each group). Note that the analgesic effects of the two highest doses of JMV-431 are maintained over a longer period of time than observed after the lower dose. *,**Significantly different from saline-injected rats with $p < 0.05$ and $p < 0.001$, respectively (ANOVA followed by Dunnett's MCT). ***C***, Antinociceptive JMV-431 dose–response curve generated by plotting the percentage of MPE at the time of peak antinociceptive response for each dose of the drug. Error bars indicate SEM.

Previous studies had shown that intrathecal injection of NT induces antinociception in a variety of analgesic tests including hotplate, tail-flick, and writhing tests (Clineschmidt et al., 1982; Martin and Naruse, 1982; Yaksh et al., 1982; Hylden and Wilcox, 1983; Spampinato et al., 1988). Some of these effects are likely mediated via NTS1, because mice deficient in NTS1 expression exhibited reduced antinociception in the hotplate test (Tyler et al., 1998; Pettibone et al., 2002). However, the extensive anatomical association found here between NTS2 and spinal nociceptive pathways, together with the evidence for a role of NTS2 in the mediation of the supraspinal analgesic actions of NT (Dubuc et al., 1999; Maeno et al., 2004), prompted us to investigate whether this receptor subtype might also be involved in the mediation of the antinociceptive effects of NT at the spinal level. We found that NT, as well as the selective NTS2 agonists levocabastine and JMV-431, elicited a dose-dependent analgesia in the tail-flick test when injected intrathecally in the rat. The antinociceptive effects of levocabastine and JMV-431 were clearly mediated via NTS2, because they were unaffected when the drugs were injected together with the selective NTS1 antagonist SR48692 (Gully et al., 1993) at a dose at which the latter did not affect NTS2. In contrast, coinjection of SR48692 partly reduced the analgesic effect elicited by NT, suggesting that both NTS1 and NTS2 receptors were involved in mediating the analgesic effects of the native peptide.

A striking finding of the present study was that the antinociceptive effects of JMV-431 were both more efficient and longer lasting than those elicited by either levocabastine or NT. These greater antinociceptive effects of JMV-431 may be attributed to the resistance of this drug to metabolic degradation, and hence to its longer half-life in the subarachnoid space (Labbe-Jullie et al., 1994). It is also possible that the greater efficacy of JMV-431 is attributable to the fact that it exerts its effects through selective stimulation of NTS2 receptors, whereas NT acts jointly via NTS1 and NTS2. This interpretation would be coherent with the differential electrophysiological effects of NT on large (expressing NTS2) versus small (expressing both NTS1 and NTS2) DRG neurons (Zhang et al., 1995, 1996; Xu et al., 1997; Kawarada et al., 2000). It would also conform to the bivalent antinociceptive effects of NT, facilitatory at low doses and inhibitory at high concentrations, observed after local injection of the drug in the rostroventral medulla (Smith et al., 1997; Urban and Gebhart, 1997; Urban et al., 1999; Gui et al., 2004). Whatever the mechanisms for the differential effects of the drugs, the present data demonstrate that selective, metaboloresistant NTS2 agonists can exert potent analgesic effects in acute spinal pain.

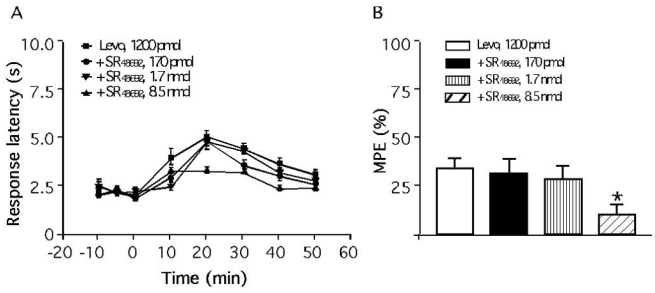

Figure 8. Influence of the NTS1 antagonist SR48692 on the tail-flick response to an antinociceptive dose of levocabastine. ***A***, Time course of antinociceptive effects of levocabastine administered alone (1200 pmol) versus levocabastine in combination with various doses (170 pmol to 8.5 nmol) of SR48692. Behavioral responses were measured every 10 min after intrathecal administration of the drug(s) ($n = 10$ for each group). ***B***, Percentage of MPE ± SEM calculated 20 min after intrathecal injection of the drug(s). No change in tail-flick latencies was seen after administration of SR48692 alone (data not shown). The addition of 170 pmol or 1.7 nmol SR48692 did not modify antinociceptive responses to intrathecal levocabastine. However, an attenuation of the response to levocabastine is observed when the drug is coinjected with 8.5 nmol of SR48692. *Significantly different from levocabastine alone with $p < 0.05$ (ANOVA followed by Dunnett's MCT). Error bars indicate SEM.

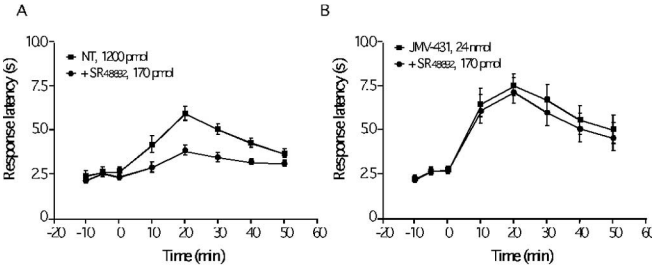

Figure 9. Effect of SR48692 on NT- and JMV-431-induced antinociceptive responses. ***A***, In the presence of 170 pmol of SR48692, the tail-flick response to intrathecal NT (1200 pmol) is significantly reduced at all time points after injection of the drug. ***B***, In similar conditions, SR48692 does not affect the time course of JMV-431-elicited antinociceptive responses. Error bars indicate SEM.

In summary, the present study demonstrates that the antinociceptive actions of NT at the spinal level are mediated via both NTS1 and NTS2 receptor subtypes, and provides the first evi-

dence for a role of NTS2 in the regulation of spinal nociceptive inputs. It also suggests that the use of selective NTS2 agonists may provide new avenues for the treatment of acute pain, without the disadvantages associated with the use of opioids.

References

al-Rodhan NR, Richelson E, Gilbert JA, McCormick DJ, Kanba KS, Pfenning MA, Nelson A, Larson EW, Yaksh TL (1991) Structure-antinociceptive activity of neurotensin and some novel analogues in the periaqueductal gray region of the brainstem. Brain Res 557:227–235.

Behbehani MM, Pert A (1984) A mechanism for the analgesic effect of neurotensin as revealed by behavioral and electrophysiological techniques. Brain Res 324:35–42.

Benmoussa M, Chait A, Loric G, de Beaurepaire R (1996) Low doses of neurotensin in the preoptic area produce hyperthermia. Comparison with other brain sites and with neurotensin-induced analgesia. Brain Res Bull 39:275–279.

Botto JM, Sarret P, Vincent JP, Mazella J (1997) Identification and expression of a variant isoform of the levocabastine-sensitive neurotensin receptor in the mouse central nervous system. FEBS Lett 400:211–214.

Chalon P, Vita N, Kaghad M, Guillemot M, Bonnin J, Delpech B, Le Fur G, Ferrara P, Caput D (1996) Molecular cloning of a levocabastine-sensitive neurotensin binding site. FEBS Lett 386:91–94.

Clineschmidt BV, McGuffin JC (1977) Neurotensin administered intracisternally inhibits responsiveness of mice to noxious stimuli. Eur J Pharmacol 46:395–396.

Clineschmidt BV, McGuffin JC, Bunting PB (1979) Neurotensin: antinocisponsive action in rodents. Eur J Pharmacol 54:129–139.

Clineschmidt BV, Martin GE, Veber DF (1982) Antinocisponsive effects of neurotensin and neurotensin-related peptides. Ann NY Acad Sci 400:283–306.

Difiglia M, Aronin N, Leeman SE (1984) Ultrastructural localization of immunoreactive neurotensin in the monkey superficial dorsal horn. J Comp Neurol 225:1–12.

Dubuc I, Costentin J, Terranova JP, Barnouin MC, Soubrie P, Le Fur G, Rostene W, Kitabgi P (1994) The nonpeptide neurotensin antagonist, SR 48692, used as a tool to reveal putative neurotensin receptor subtypes. Br J Pharmacol 112:352–354.

Dubuc I, Sarret P, Labbe-Jullie C, Botto JM, Honore E, Bourdel E, Martinez J, Costentin J, Vincent JP, Kitabgi P, Mazella J (1999) Identification of the receptor subtype involved in the analgesic effect of neurotensin. J Neurosci 19:503–510.

Fassio A, Evans G, Grisshammer R, Bolam JP, Mimmack M, Emson PC (2000) Distribution of the neurotensin receptor NTS1 in the rat CNS studied using an amino-terminal directed antibody. Neuropharmacology 39:1430–1442.

Faull RL, Villiger JW, Dragunow M (1989) Neurotensin receptors in the human spinal cord: a quantitative autoradiographic study. Neuroscience 29:603–613.

Gibson SJ, Polak JM, Bloom SR, Wall PD (1981) The distribution of nine peptides in rat spinal cord with special emphasis on the substantia gelatinosa and on the area around the central canal (lamina X). J Comp Neurol 201:65–79.

Gui X, Carraway RE, Dobner PR (2004) Endogenous neurotensin facilitates visceral nociception and is required for stress-induced antinociception in mice and rats. Neuroscience 126:1023–1032.

Gully D, Canton M, Boigegrain R, Jeanjean F, Molimard JC, Poncelet M, Gueudet C, Heaulme M, Leyris R, Brouard A, Pelaprat D, Labbe-Jullie C, Mazella J, Soubrie P, Maffrand J, Rostene W, Kitabgi P, Fur GL (1993) Biochemical and pharmacological profile of a potent and selective nonpeptide antagonist of the neurotensin receptor. Proc Natl Acad Sci USA 90:65–69.

Gully D, Labeeuw B, Boigegrain R, Oury-Donat F, Bachy A, Poncelet M, Steinberg R, Suaud-Chagny MF, Santucci V, Vita N, Pecceu F, Labbe-Jullie C, Kitabgi P, Soubrie P, Le Fur G, Maffrand JP (1997) Biochemical and pharmacological activities of SR 142948A, a new potent neurotensin receptor antagonist. J Pharmacol Exp Ther 280:802–812.

Hermans E, Maloteaux JM (1998) Mechanisms of regulation of neurotensin receptors. Pharmacol Ther 79:89–104.

Hunt SP, Kelly JS, Emson PC, Kimmel JR, Miller RJ, Wu JY (1981) An immunohistochemical study of neuronal populations containing neuropeptides or gamma-aminobutyrate within the superficial layers of the rat dorsal horn. Neuroscience 6:1883–1898.

Hylden JL, Wilcox GL (1983) Antinociceptive action of intrathecal neurotensin in mice. Peptides 4:517–520.

Jennes L, Stumpf WE, Kalivas PW (1982) Neurotensin: topographical distribution in rat brain by immunohistochemistry. J Comp Neurol 210:211–224.

Kalivas PW, Gau BA, Nemeroff CB, Prange Jr AJ (1982a) Antinociception after microinjection of neurotensin into the central amygdaloid nucleus of the rat. Brain Res 243:279–286.

Kalivas PW, Jennes L, Nemeroff CB, Prange Jr AJ (1982b) Neurotensin: topographical distribution of brain sites involved in hypothermia and antinociception. J Comp Neurol 210:225–238.

Kar S, Quirion R (1995) Neuropeptide receptors in developing and adult rat spinal cord: an in vitro quantitative autoradiography study of calcitonin gene-related peptide, neurokinins, μ-opioid, galanin, somatostatin, neurotensin and vasoactive intestinal polypeptide receptors. J Comp Neurol 354:253–281.

Kawarada S, Unno T, Ohashi H, Komori S (2000) Neurotensin-induced Cl^- current in guinea-pig dorsal root ganglion cells. Eur J Pharmacol 404:69–78.

Labbe-Jullie C, Dubuc I, Brouard A, Doulut S, Bourdel E, Pelaprat D, Mazella J, Martinez J, Rostene W, Costentin J, Kitabgi P (1994) In vivo and in vitro structure-activity studies with peptide and pseudopeptide neurotensin analogs suggest the existence of distinct central neurotensin receptor subtypes. J Pharmacol Exp Ther 268:328–336.

Li AH, Hwang HM, Tan PP, Wu T, Wang HL (2001) Neurotensin excites periaqueductal gray neurons projecting to the rostral ventromedial medulla. J Neurophysiol 85:1479–1488.

Maeno H, Yamada K, Santo-Yamada Y, Aoki K, Sun YJ, Sato E, Fukushima T, Ogura H, Araki T, Kamichi S, Kimura I, Yamano M, Maeno-Hikichi Y, Watase K, Aoki S, Kiyama H, Wada E, Wada K (2004) Comparison of mice deficient in the high- or low-affinity neurotensin receptors, Ntsr1 or Ntsr2, reveals a novel function for Ntsr2 in thermal nociception. Brain Res 998:122–129.

Martin GE, Naruse T (1982) Differences in the pharmacological actions of intrathecally administered neurotensin and morphine. Regul Pept 3:97–103.

Martin GE, Naruse T, Papp NL (1981) Antinociceptive and hypothermic actions of neurotensin administered centrally in the rat. Neuropeptides 1:447–454.

Nemeroff CB, Osbahr III AJ, Manberg PJ, Ervin GN, Prange Jr AJ (1979) Alterations in nociception and body temperature after intracisternal administration of neurotensin, beta-endorphin, other endogenous peptides, and morphine. Proc Natl Acad Sci USA 76:5368–5371.

Ninkovic M, Hunt SP, Kelly JS (1981) Effect of dorsal rhizotomy on the autoradiographic distribution of opiate and neurotensin receptors and neurotensin-like immunoreactivity within the rat spinal cord. Brain Res 230:111–119.

Osbahr III AJ, Nemeroff CB, Luttinger D, Mason GA, Prange Jr AJ (1981) Neurotensin-induced antinociception in mice: antagonism by thyrotropin-releasing hormone. J Pharmacol Exp Ther 217:645–651.

Pazos A, Lopez M, Florez J (1984) Different mechanisms are involved in the respiratory depression and analgesia induced by neurotensin in rats. Eur J Pharmacol 98:119–123.

Perron A, Sarret P, Gendron L, Stroh T, Beaudet A (2005) Identification and functional characterization of the 5-transmembrane domain variant of the NTS2 neurotensin receptor in the rat central nervous system. J Biol Chem 280:10219–10227.

Pettibone DJ, Hess JF, Hey PJ, Jacobson MA, Leviten M, Lis EV, Mallorga PJ, Pascarella DM, Snyder MA, Williams JB, Zeng Z (2002) The effects of deleting the mouse neurotensin receptor NTR1 on central and peripheral responses to neurotensin. J Pharmacol Exp Ther 300:305–313.

Reinecke M, Forssmann WG, Thiekotter G, Triepel J (1983) Localization of neurotensin-immunoreactivity in the spinal cord and peripheral nervous system of the guinea pig. Neurosci Lett 37:37–42.

Sarret P, Beaudet A (2003) Neurotensin receptors in the central nervous system. In: Handbook of chemical neuroanatomy, Vol 20, Peptide receptors, Pt II (Quirion R, Björklund A, Hökfelt T, eds), pp 323–400. Amsterdam: Elsevier.

Sarret P, Beaudet A, Vincent JP, Mazella J (1998) Regional and cellular dis-

tribution of low affinity neurotensin receptor mRNA in adult and developing mouse brain. J Comp Neurol 394:344–356.
Sarret P, Perron A, Stroh T, Beaudet A (2003) Immunohistochemical distribution of NTS2 neurotensin receptors in the rat central nervous system. J Comp Neurol 461:520–538.
Seybold VS, Elde RP (1982) Neurotensin immunoreactivity in the superficial laminae of the dorsal horn of the rat: I. Light microscopic studies of cell bodies and proximal dendrites. J Comp Neurol 205:89–100.
Shipley MT, McLean JH, Behbehani MM (1987) Heterogeneous distribution of neurotensin-like immunoreactive neurons and fibers in the midbrain periaqueductal gray of the rat. J Neurosci 7:2025–2034.
Smith DJ, Hawranko AA, Monroe PJ, Gully D, Urban MO, Craig CR, Smith JP, Smith DL (1997) Dose-dependent pain-facilitatory and -inhibitory actions of neurotensin are revealed by SR 48692, a nonpeptide neurotensin antagonist: influence on the antinociceptive effect of morphine. J Pharmacol Exp Ther 282:899–908.
Spampinato S, Romualdi P, Candeletti S, Cavicchini E, Ferri S (1988) Distinguishable effects of intrathecal dynorphins, somatostatin, neurotensin and s-calcitonin on nociception and motor function in the rat. Pain 35:95–104.
Tyler BM, McCormick DJ, Hoshall CV, Douglas CL, Jansen K, Lacy BW, Cusack B, Richelson E (1998) Specific gene blockade shows that peptide nucleic acids readily enter neuronal cells in vivo. FEBS Lett 421:280–284.
Uhl GR (1982) Distribution of neurotensin and its receptor in the central nervous system. Ann NY Acad Sci 400:132–149.
Uhl GR, Goodman RR, Snyder SH (1979) Neurotensin-containing cell bodies, fibers and nerve terminals in the brain stem of the rat: immunohistochemical mapping. Brain Res 167:77–91.
Urban MO, Gebhart GF (1997) Characterization of biphasic modulation of spinal nociceptive transmission by neurotensin in the rat rostral ventromedial medulla. J Neurophysiol 78:1550–1562.
Urban MO, Smith DJ (1993) Role of neurotensin in the nucleus raphe magnus in opioid-induced antinociception from the periaqueductal gray. J Pharmacol Exp Ther 265:580–586.
Urban MO, Smith DJ (1994) Localization of the antinociceptive and antianalgesic effects of neurotensin within the rostral ventromedial medulla. Neurosci Lett 174:21–25.
Urban MO, Coutinho SV, Gebhart GF (1999) Biphasic modulation of visceral nociception by neurotensin in rat rostral ventromedial medulla. J Pharmacol Exp Ther 290:207–213.
Vincent JP, Mazella J, Kitabgi P (1999) Neurotensin and neurotensin receptors. Trends Pharmacol Sci 20:302–309.
Walker N, Lepee-Lorgeoux I, Fournier J, Betancur C, Rostene W, Ferrara P, Caput D (1998) Tissue distribution and cellular localization of the levocabastine-sensitive neurotensin receptor mRNA in adult rat brain. Brain Res Mol Brain Res 57:193–200.
Xiao HS, Huang QH, Zhang FX, Bao L, Lu YJ, Guo C, Yang L, Huang WJ, Fu G, Xu SH, Cheng XP, Yan Q, Zhu ZD, Zhang X, Chen Z, Han ZG (2002) Identification of gene expression profile of dorsal root ganglion in the rat peripheral axotomy model of neuropathic pain. Proc Natl Acad Sci USA 99:8360–8365.
Xu ZQ, Zhang X, Grillner S, Hokfelt T (1997) Electrophysiological studies on rat dorsal root ganglion neurons after peripheral axotomy: changes in responses to neuropeptides. Proc Natl Acad Sci USA 94:13262–13266.
Yaksh TL, Schmauss C, Micevych PE, Abay EO, Go VL (1982) Pharmacological studies on the application, disposition, and release of neurotensin in the spinal cord. Ann NY Acad Sci 400:228–243.
Yang L, Zhang FX, Huang F, Lu YJ, Li GD, Bao L, Xiao HS, Zhang X (2004) Peripheral nerve injury induces trans-synaptic modification of channels, receptors and signal pathways in rat dorsal spinal cord. Eur J Neurosci 19:871–883.
Young III WS, Kuhar MJ (1981) Neurotensin receptor localization by light microscopic autoradiography in rat brain. Brain Res 206:273–285.
Zhang X, Nicholas AP, Hokfelt T (1993) Ultrastructural studies on peptides in the dorsal horn of the spinal cord—I. Co-existence of galanin with other peptides in primary afferents in normal rats. Neuroscience 57:365–384.
Zhang X, Xu ZQ, Bao L, Dagerlind A, Hokfelt T (1995) Complementary distribution of receptors for neurotensin and NPY in small neurons in rat lumbar DRGs and regulation of the receptors and peptides after peripheral axotomy. J Neurosci 15:2733–2747.
Zhang X, Bao L, Xu ZQ, Diez M, Frey P, Hokfelt T (1996) Peripheral axotomy induces increased expression of neurotensin in large neurons in rat lumbar dorsal root ganglia. Neurosci Res 25:359–369.