Increased Noradrenergic Neurotransmission to a Pain Facilitatory Area of the Brain Is Implicated in Facilitation of Chronic Pain

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ABSTRACT

Background: Noradrenaline reuptake inhibitors are known to produce analgesia through a spinal action but they also act in the brain. However, the action of noradrenaline on supraspinal pain control regions is understudied. The authors addressed the noradrenergic modulation of the dorsal reticular nucleus (DRt), a medullary pronociceptive area, in the spared nerve injury (SNI) model of neuropathic pain.

Methods: The expression of the phosphorylated cAMP response element-binding protein (pCREB), a marker of neuronal activation, was evaluated in the locus coeruleus and $\alpha_2$ noradrenergic neurons ($n = 6$ rats/group). pCREB was studied in noradrenergic DRt-projecting neurons retrogradely labeled in SNI animals ($n = 3$). In vivo microdialysis was used to measure noradrenaline release in the DRt on nociceptive stimulation or after DRt infusion of clonidine ($n = 5$ to 6 per group). Pharmacology, immunohistochemistry, and western blot were used to study $\alpha_1$-adrenoreceptors in the DRt ($n = 4$ to 6 per group).

Results: pCREB expression significantly increased in the locus coeruleus and $\alpha_2$ of SNI animals, and most noradrenergic DRt-projecting neurons expressed pCREB. In SNI animals, noradrenaline levels significantly increased on pinprick (mean ± SD, 126 ± 14%; $P = 0.025$ vs. baseline) and acetone stimulation (mean ± SD, 151 ± 12%; $P < 0.001$ vs. baseline), and clonidine infusion showed decreased $\alpha_1$-mediated inhibitory function. $\alpha_1$-adrenoceptor blockade decreased nociceptive behavioral responses in SNI animals. $\alpha_2$-adrenoceptor expression was not altered.

Conclusions: Chronic pain induces brainstem noradrenergic activation that enhances descending facilitation from the DRt. This suggests that antidepressants inhibiting noradrenaline reuptake may enhance pain facilitation from the brain, counteracting their analgesic effects at the spinal cord. (ANESTHESIOLOGY 2015; 123:642-53)

NEUROPATHIC pain is a disabling clinical condition. It is frequently treated using antidepressants that block noradrenaline reuptake at the spinal cord level, inhibiting the transmission of nociceptive information. Antidepressants do not act only at the spinal cord because systemic administration of antidepressants blocking noradrenaline reuptake leads to increase of noradrenaline levels in several brain areas. The main sources of noradrenergic innervation of the spinal cord are the brainstem noradrenergic nuclei, namely the locus coeruleus (LC) and the $\alpha_2$ and $\alpha_1$ cell groups. The role of these brainstem areas during neuropathic pain has started to be unraveled, namely for the LC. Chronic pain induced by peripheral nerve injury induces plastic changes in the LC, which may play a role in the maintenance of neuropathic pain.

The dorsal reticular nucleus (DRt) plays a unique role in the facilitation of pain transmission at the spinal cord. It is reciprocally connected with the spinal dorsal horn through a direct and putative excitatory pathway that enhances descending pain facilitation from the DRt in response to local arrival of nociceptive input.

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receives input from several brain areas, including noradrenergic cell groups in the brainstem, namely the LC and the A5 noradrenergic cell group. It is possible that these areas exert an effect in noradrenergic control of pain facilitation from the DRt because a decrease of noradrenaline levels in the DRt attenuates neuropathic pain and noradrenaline contributes to the pain facilitatory actions of the DRt in a model of inflammatory pain. Taking these findings into account along with the fact that chronic pain is associated with an increased descending facilitation, the goal of this study was to test the hypothesis that increased noradrenergic input to the DRt occurs on nociceptive stimulation in the spared nerve injury (SNI) model of neuropathic pain and contributes to increased pain facilitatory actions of the DRt. We determined whether there are changes in the activation of the main noradrenergic sources of the DRt, namely the LC and the A5 noradrenergic cell group, in the SNI model. We also evaluated whether noradrenergic DRt-projecting neurons from these areas are activated during neuropathic pain. Then we used in vivo microdialysis to monitor the extracellular levels of noradrenaline in the DRt in response to nociceptive stimuli of SNI rats and evaluated the effects of noradrenaline by pharmacologic manipulation of the DRt. We also used in vivo microdialysis to measure noradrenaline release in the DRt under the effect of local infusion of a α2-adrenoceptor agonist. Finally, we determined the effects of neuropathic pain on the expression of α2-adrenoceptor at the DRt.

**Material and Methods**

**Animals**

All procedures were approved by Institutional Animal Care and Use Committee of the College of Mathematics and Natural Sciences of the University of Groningen and were performed according to the ethical guidelines for pain investigation. Male Wistar rats (Harlan colony, The Netherlands) were individually housed in plastic cages (30 × 30 × 40 cm), at 22° ± 2°C and on a standard 12-h light/dark cycle with food and water available ad libitum. The animals were acclimated to the housing facility for at least 1 week before any treatment. No formal randomization procedures were used to assign animals to experimental groups. Briefly, on their arrival, the animals were arbitrarily housed in pairs, and then they were sequentially picked from the cage for each procedure. No a priori power analysis was conducted. The sample sizes were based on the previous experience. There were no missing data; all values from nonstereotaxically injected/implanted animals and from animals correctly injected/implanted into the DRt (as described in Histology) were available for the analysis.

**Induction of the SNI Model**

The SNI model of neuropathic pain was induced as described by Decosterd and Woolf. In brief, rats weighing 210 to 220 g were anesthetized with isoflurane, the tibial and common peroneal components of the left sciatic nerve were carefully isolated, tightly ligated and sectioned, while the sural nerve was left intact. In sham-operated rats, the sciatic nerve and its branches were exposed, but no lesion was performed. At the end of the procedure, the muscle and skin were closed in two layers. The animals were treated with 0.1% finadine (0.1 ml/kg, intraperitoneal) for postoperative analgesia.

**Stereotaxic Surgeries**

Fourteen days after SNI induction or sham surgery, rats weighing 285 to 315 g were deeply anesthetized as described in Induction of the SNI Model and placed on a stereotaxic frame for the injection of the retrograde tracer fluorogold (FG) or cannula implantation into the left DRt. At the end of the procedure, the animals were treated as described in Induction of the SNI Model for postoperative analgesia.

**Injection of FG.** A group of three SNI animals was injected with FG (Fluorochrome LLC, USA) at 2% in two rostro-caudal parts of the left DRt following the coordinates of the atlas as described in the study by Paxinos and Watson (first injection: anteroposterior, −6.0 mm; lateromedial, −1.4 mm; dorsoventral, −1.5 mm; second injection: anteroposterior, −6.4 mm; lateromedial: −1.3 mm; dorsoventral: −1.7 mm). A volume 0.3-μl per site was slowly injected (flow rate: 50 nl/min), and at the completion of the injections, the needle was left in place for 10 min before being slowly removed. The animals were killed 1 week later.

**Cannula Implantation.** A guide cannula was implanted in the left DRt for microdialysis (n = 22) or pharmacologic (n = 22) experiments following the coordinates and procedures as described previously.

**Behavioral Assessment**

After SNI induction, the animals typically develop hypersensitivity to mechanical and cold stimuli on the injured paw. To confirm the development of hypersensitivity, after a training period for habituation purposes, the rats were placed on an elevated transparent cage with a mesh wire bottom allowing the stimulation of the lateral plantar surface of the injured hind paw. Hypersensitivity to mechanical stimuli was assessed by using the von Frey and pinprick tests. The von Frey test was performed by the application of calibrated monofilaments (Stoelting, USA) in a sequence of increasing forces, and the threshold was considered the lowest force that evoked a brisk withdrawal response to one of five repetitive stimuli. The pinprick test was performed by applying a brief stimulation with a safety pin at intensity sufficient to indent but not penetrate the skin. The duration of the paw withdrawal was recorded with an arbitrary minimal time of 0.5 s. Hypersensitivity to a cold stimulus was assessed by the application of a drop of acetone and recording the duration of the withdrawal response with an arbitrary minimal value of 0.5 s.
All animals were behaviorally evaluated at the day before SNI induction or sham surgery and 14 days after surgery to confirm the development of mechanical and cold hypersensitivity. In addition, these animals were monitored for signs of sedation and locomotion impairments.

**Microdialysis Experiments**

*In vivo* microdialysis was performed to evaluate noradrenaline levels in the DRt immediately after nociceptive stimulation of the hind paw or during infusion of the α₂-adrenoceptor agonist clonidine.

**Microdialysis during Nociceptive Stimulation**

One week after stereotaxic surgery, SNI and sham animals (n = 6 each) were placed on the elevated transparent cage with wire mesh bottom; the stylet was replaced by a microdialysis probe (polyacrylonitrile membrane with 2-mm open length, molecular weight cutoff, 45 to 50 kDa; Brainlink BV, The Netherlands); and after 3 h of Ringer solution (140.0 mM NaCl, 4.0 mM KCl, 1.2 mM CaCl₂, 1.0 mM MgCl₂) perfusion at a flow rate of 2.0 μl/min, four microdialysates samples were collected in 15-min intervals into the sample loop of a high-performance liquid chromatography (HPLC). Noradrenaline levels in these samples approached the detection limit (approximately 2 fmol per sample). Herein, to improve the detection of noradrenaline, the noradrenaline reuptake inhibitor nomifensine (10 μM) was added to the perfusion fluid. Forty-five minutes after stabilization, four additional baseline microdialysates samples were collected on-line in 15-min intervals. After collection of the last baseline sample, the animals received three different stimuli to evaluate mechanical and cold hypersensitivity. Each stimulus was applied three times every 5 min on the injured paw, keeping a time interval of 60 min. The animals were first subjected to a pinprick stimulation during which they were subjected to a pinprick stimulation during which a drop of acetone was applied. The magnitude of behavioral withdrawal responses after the application of the aforementioned stimuli was not measured. After stimulation, microdialysates samples were then collected in 15-min intervals for 60 min. This timing was chosen based on our preliminary studies, showing that noradrenaline levels returned to baseline within 60 min. When the last baseline sample had been collected, the probes continued to be perfused with Ringer’s/nomifensine solution further supplemented with the α₂-adrenoceptor agonist clonidine (Sigma-Aldrich, USA) at increasing concentrations (0.01, 0.1, 1, and 10 μM). Each concentration of clonidine was perfused for 1 h, and four microdialysates samples were collected on-line in 15-min intervals. Results were reported as mean percentage of baseline (i.e., mean of four baseline collections).

**Noradrenaline Quantification**

Noradrenaline was quantitated by HPLC with electrochemical detection according to the procedures described previously. The detection limit of the assay was approximately 2 fmol per sample (on-column).

**Pharmacologic Experiments**

These experiments were performed to test the effects of α₂-adrenoceptor activation at the DRt of SNI animals by using the α₂- and α₁-adrenoceptor antagonists prazosin hydrochloride (n = 5; Tocris Bioscience, United Kingdom) and atipamezole hydrochloride (n = 6; Sigma-Aldrich). Both drugs were injected at the dose of 5 μg in a 0.5-μl injection volume following the procedures described previously. Atipamezole was dissolved in saline, and prazosin was dissolved in a 1:1 (volume:volume) mixture of dimethyl sulfoxide and saline. The respective control groups were infused with dimethyl sulfoxide/saline (n = 5) or saline (n = 6).

The behavioral tests to evaluate mechanical and cold hypersensitivity were performed before and at 30 min after drug injection, which is the time of maximal effects of both drugs, by an experimenter blinded to the treatments. The results were reported as mean percentage of baseline (i.e., before drug injection) ±SD.

**Tissue Preparation and Immunohistochemistry**

Three weeks after SNI or sham surgery, rats were deeply anaesthetized with an overdose of sodium pentobarbital (70 mg/kg intraperitoneal) and perfused through the ascending aorta with 100 ml of calcium-free Tyrode’s solution, followed by 750 ml of a fixative solution containing 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.2. The brainstems were removed, immersed in fixative for 4 h followed by 30% sucrose in 0.1 M phosphate-buffered saline (PBS) overnight, at 4°C, and sliced at 40 μm in coronal orientation in a freezing microtome.

**Immunodetection of α₂-Adrenoceptor.** One in every fourth section encompassing the DRt from sham or SNI animals (n = 5 each) was incubated for 2 h in a blocking solution containing 0.1 M glycine and 10% normal swine serum in 0.1 M PBS containing 0.3% Triton X-100 (PBS-T) followed by an incubation for 48 h, at 4°C, in a goat polyclonal
antibody against α2A-adrenoreceptor (ref: sc-1478; Santa Cruz Biotechnology, Inc., USA), diluted at 1:500 in PBS-T containing 2% normal swine serum. After washing with PBS-T, the sections were incubated for 1 h with a horse biotinylated antigen serum at 1:200 (Dako, Denmark), diluted in PBS-T containing 2% swine normal serum. Sections were washed again and incubated for 1 h in PBS-T containing the avidin–biotin complex (1:200; Vector Laboratories, USA). After washing in 0.05 M Tris-HCl, pH 7.6, bound peroxidase was revealed using 0.0125% 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and 0.025% H2O2 in the same buffer. After mounting and coverslipping, the sections were observed in a light microscope (Axioskop 40; Carl Zeiss, Germany), and the images were acquired using a high-resolution digital camera coupled to a computer.

The specificity of the α2A-adrenoreceptor antibody was previously tested in α2A knockout mice24 and by absorption of the α2A-adrenoreceptor.25 We also obtained evidences to support the specificity of the antibody by immunoblotting using DRt extracts and showing that the primary antibody binds to a single protein of 70 KDa, which corresponds to the molecular weight of the α2A-adrenoreceptor. We further tested antibody specificity by performing negative controls with omission of either the primary or the secondary antibodies that blocked all the immunostaining.

Double Immunodetection of Tyrosine Hydroxylase and Phosphorylated cAMP Response Element-binding Protein.

One in every fourth brainstem section was incubated for 30 min in sodium borohydride solution at 1%, followed by an incubation for 2 h in PBS-T containing 0.1 M glycine and 10% normal swine serum. After washing, the sections were incubated for 48 h at 4°C with a rabbit antiseraum against phosphorylated cAMP response element-binding protein (pCREB; Merck Millipore, Germany) at 1:1000, diluted in PBS-T and 2% of normal swine serum. After washing in PBS-T, the sections were incubated for 1 h with an Alexa 594 donkey antirabbit antibody (Molecular Probes, The Netherlands), at 1:1000, diluted in PBS-T. After repeated washes, the sections were incubated for 24 h at 4°C with a mouse monoclonal antibody against tyrosine hydroxylase (TH; Sigma-Aldrich), diluted at 1:4000 in a PBS-T solution containing 2% of normal swine serum. After washing, the sections were incubated for 1 h in a horse biotinylated antimouse serum, at 1:200 (Dako) diluted in PBS-T and containing 2% of normal swine serum, and washed again and incubated for 1 h with an Alexa Streptavidin 488 (Molecular Probes), at 1:1000 diluted in PBS-T.

The sections were mounted on gelatin-coated slides, cover-slipped with buffered glycerol, and analyzed using an ApoTome microscope (Carl Zeiss, Germany).

In one set of experiments, we used sections from SNI and sham animals (n = 6 each) to evaluate the numbers of neurons single labeled for TH and double labeled for TH and pCREB. In a second set of experiments, we used sections from SNI animals injected with FG into the DRt and killed 7 days later (n = 3) to evaluate the colocalization of TH-positive neurons that project to the DRt (FG/TH double labeling) with pCREB (FG/TH/pCREB triple labeling). The numbers of neurons immunoreactive for TH, double immunoreactive for TH and pCREB (TH/pCREB) or FG and TH (FG/TH), and triple immunoreactive for FG, TH, and pCREB (FG/TH/pCREB) were counted using the NIH Image J 1.52 software (National Institutes of Health, USA) in 15 photomicrographs taken from sections encompassing the LC and the A1 noradrenergic cell group. The images were acquired using a high-resolution digital camera coupled to a computer. Serial optical planes were collected in the z-axis, and merged images were obtained using the ApoTome software (Carl Zeiss). No differences were detected between the left and right side of each area for either SNI or sham animals (data not shown); therefore, left and right cell profile counts were summed in each tissue section. Cell counting was performed by an experimenter blinded to the experimental groups. For the first set of experiments, the results were reported as mean numbers of neurons labeled for TH (±SD), mean numbers of neurons double labeled for TH and pCREB (TH/pCREB) (±SD), and percentage of colocalization in the total neurons labeled for TH (±SD). For the second set of experiments, the results were reported as mean numbers of neurons FG/TH (±SD), mean numbers of neurons triple labeled for FG, TH, and pCREB (FG/TH/pCREB) (±SD), and percentage of colocalization of triple-labeled neurons in the total of neurons labeled with FG and TH (±SD).

Western Blot Analysis of α2A-Adrenoreceptor

Three weeks after SNI or sham surgery (n = 4 each), rats were deeply anaesthetized with an overdose of sodium pentobarbital (70 mg/kg intraperitoneal) and killed by decapitation. The brains were harvested and immediately stored at −80°C. The brainstems were cut in a freezing microtome at 100 μm, and three pieces were collected in the z-axis, and merged images were obtained using the Bradford method. A total of 15 μg of protein was heated at 65°C for 5 min in sample buffer (containing 100 mM dithiothreitol, 2-Adrenoreceptor (ref: sc-1478; Santa Cruz Biotechnology, Inc., USA), diluted at 1:500 in PBS-T containing 2% normal swine serum. After washing with PBS-T, the sections were incubated for 1 h with a horse biotinylated antimouse serum, at 1:200 (Dako) diluted in PBS-T and containing 2% of normal swine serum, and washed again and incubated for 1 h with an Alexa Streptavidin 488 (Molecular Probes), at 1:1000 diluted in PBS-T.
proteins were then transferred onto nitrocellulose membranes using a Trans-Blot Turbo Transfer System (Bio-Rad). Subsequently, membranes were imaged, blocked in 4% bovine serum albumin, and incubated overnight at 4°C, with the antibody against α2A-adrenoreceptor referred in the Tissue preparation and immunohistochemistry (1:500). Membranes were then washed and incubated with an HRP-conjugated secondary antibody raised in rabbit (1:10000; GE Healthcare, USA) for 1 h followed by signal detection with a sensitive chemiluminescence reagent (WesternBright Quantum, Advansta, USA), performed on the ChemiDoc equipment. Total protein staining obtained from the TGX Stain-Free gels was used for normalization of the α2A-adrenoreceptor band intensities, and the densitometric analyses were performed using the Image Lab Software (Bio-Rad).26–28

**Histology**

At the completion of the experiments, the animals used in microdialysis were killed by vascular perfusion, whereas the animals used in pharmacologic experiments were injected with 0.5 μl of 0.6% Chicago sky blue dye (Sigma-Aldrich) and killed by decapitation for verification of probe or injection location following the procedures described previously.15 Only animals with probe or cannula placement centered in the DRt were included in data analysis.

**Statistical Analysis**

Noradrenaline release during nociceptive stimulation was analyzed by two-way mixed ANOVA for repeated measurements. In case of a significant interaction between group and time, we proceeded with pairwise comparisons using Tukey correction to adjust P values for multiple testing. Baseline noradrenaline levels between sham and SNI animals before and after nomifensine addition was analyzed by two-way ANOVA followed, in case of significant interaction between group and treatment, by pairwise comparisons using Tukey correction. The effects of the drugs (prazosin and atipamezole) on the von Frey, pinprick, and acetone tests were analyzed by one-way ANOVA followed by pairwise comparisons, using Tukey correction.

Noradrenaline response to the different doses of clonidine was expressed as the percentage of baseline. The comparisons between the sham and SNI groups were performed with a three-way ANOVA by taking into account the repeated measurements on the same animal for the different doses of clonidine and, within each dose, the four measurements performed at 15, 30, 45, and 60 min after the dose administration. The pairwise comparisons between the group and dosage levels were performed using Tukey correction. The results are also presented graphically, but the four repeated measurements of noradrenaline in each dose were summarized by their mean and CI estimated using a robust SE to correct for the repeated measurements. The percentages of colocalizations (TH/pCREB or FG/TH/pCREB) and expression levels of the α2A-adrenoreceptor were compared by unpaired t tests. Data in the text and table 1 are presented as mean ± SD. In the plots, the error bars represent the 95% CIs for the means. The statistical analysis was performed by SAS v9.2 (SAS Institute Inc., USA) and SPSS v19.1 (IBM Corporation, USA). The significance level was set at 0.05, and all statistical tests were two tailed.

**Results**

**Induction of the SNI Model**

To determine whether the induction of the SNI model of neuropathic pain increases the activity of neurons at the main noradrenergic afferents of the DRt,13 we quantified the colocalization of TH with pCREB in the LC and the A5 noradrenergic cell group. Figure 1 shows the total number of neurons single labeled for TH and double labeled for TH and pCREB (fig. 1, A, C, D, and F) and the percentage of colocalization in SNI and sham animals (fig. 1, B and E). The percentage of double labeled neurons was significantly higher in SNI animals both in the LC (t16 = −3.911, P = 0.003; fig. 1B) and the A5 noradrenergic cell group (t10 = −7.77, P < 0.001; fig. 1E). In the LC, SNI animals showed an average of 79.8 ± 3.8% (mean ± SD) of colocalization (241 ± 39 double-labeled neurons; fig.1, A and B), whereas sham animals showed an average of 64.3 ± 9% of colocalization (163 ± 32 double-labeled neurons; fig. 1, A and B). In the A5 noradrenergic cell group, SNI animals showed an average of 77 ± 6.5% of colocalization (160 ± 44 double-labeled neurons; fig. 1, D and E), whereas sham animals showed an average of 50.6 ± 5.2% of colocalization (107 ± 23 double-labeled neurons; fig. 1, D and E). No significant differences were observed in the total number of neurons single labeled for TH between SNI and sham animals either in the LC or the A5 noradrenergic cell group (fig. 1, A and D).

**Activated Noradrenergic Neurons Project to the DRt**

To determine whether activated noradrenergic neurons project to the DRt, a group of SNI animals was injected with

| Table 1. Numbers and Percentages of Noradrenergic Neurons Projecting to the DRt (FG/TH) that Colocalize with pCREB (FG/TH/pCREB) in the Locus Coeruleus and the A5 Noradrenergic Cell Group of SNI Animals |
|-----------------|--------|--------|--------|-------|
|                  | Rat    | Rat    | Rat    | Mean ± SD |
|                  | 22047  | 22048  | 22051  |       |
| Locus coeruleus |        |        |        |       |
| FG/TH/pCREB     | 67     | 48     | 70     | 61.6 ± 11.9 |
| FG/TH           | 78     | 59     | 78     | 71.6 ± 11 |
| % FG/TH/pCREB   | 85.9   | 81.3   | 89.7   | 85.6 ± 4.2 |
| A5 noradrenergic cell group |        |        |        |       |
| FG/TH/pCREB     | 77     | 95     | 61     | 77.7 ± 17 |
| FG/TH           | 96     | 116    | 81     | 97.7 ± 17.5 |
| % FG/TH/pCREB   | 80.2   | 81.9   | 75.3   | 79.1 ± 3.4 |

DRt = dorsal reticular nucleus; FG = fluorogold; pCREB = phosphorylated cAMP response element-binding protein; SNI = spared nerve injury; TH = tyrosine hydroxylase.
the retrograde tracer FG into the DRt, and the colocalization of FG/TH with pCREB was evaluated at the LC and the A5 noradrenergic cell group (D, E) of sham and SNI animals. Representative photomicrographs of single TH-IR neurons (green and arrow, C), single pCREB nuclear labeling (red and arrow, F), and double-labeled neurons (double arrows, C and F) in the locus coeruleus (C) and the A5 noradrenergic cell group (F). Scale bar: 20 μm (C is at the same magnification). (A, B, D, and E) Data are presented as mean ± 95% CIs (n = 6/group). **P < 0.01, ***P < 0.001 versus sham group. pCREB = phosphorylated cAMP response element-binding protein; SNI = spared nerve injury; TH-IR = tyrosine hydroxylase immunoreactive.

**Fig. 2.** Activated noradrenergic neurons project to the DRt. (A) Photomicrograph of a representative injection of FG in the DRt. The injection site is surrounded by FG+ neurons (better depicted at higher magnification in the insert). (B, C) Photomicrographs of FG+ neurons (blue, B) and double labeled for TH and pCREB (TH = green; pCREB = red, arrow, C) in the A5 noradrenergic cells group. Triple-labeled neurons (FG/TH/pCREB) are marked by double arrows (B, C). Scale bars: 100 μm (A) and 20 μm (C; B is at the same magnification). Cu = cuneate nucleus; DRt = dorsal reticular nucleus; FG = fluorogold; pCREB = phosphorylated cAMP response element-binding protein; Sol = nucleus of the solitary tract; Sp5C = spinal trigeminal nucleus, pars caudalis; TH = tyrosine hydroxylase.

**Pinprick and Acetone Stimulation Increase Noradrenaline Release into the DRt of Neuropathic Animals**

To determine the effect of nociceptive stimulation on noradrenaline release at the DRt, we evaluated locally the extracellular levels of noradrenaline on the application of von Frey, pinprick, and acetone stimuli by in vivo microdialysis. All SNI animals used in these experiments, unlike sham control animals, showed a marked hypersensitivity to von Frey, pinprick, and acetone application, which was similar to the previous studies.14,18
The analysis of baseline levels of noradrenaline (i.e., mean of four baseline collections) before and after nomifensine addition revealed a significant interaction between groups (sham vs. SNI) and treatment (F1,20 = 10.71, P < 0.004). Before nomifensine, no significant differences were observed between SNI and sham animals (Sham: 1.8 ± 0.8 fmol/sample; SNI: 1.9 ± 1.9 fmol/sample; P = 0.83). After nomifensine addition, baseline levels increased significantly, but this increase was more important in sham animals with a 11.3-fold increase to 20.3 ± 2.3 fmol/sample than in SNI animals with a 4.8-fold increase to 14.6 ± 3.6 fmol/sample (P < 0.006).

Noradrenaline levels were not significantly affected by von Frey stimulation in SNI or sham animals (fig. 3A). The analysis of noradrenaline levels after pinprick stimulation revealed a significant interaction between groups and time points (F4,40 = 3.00, P < 0.029). Pinprick stimulation in SNI rats increased noradrenaline levels to 126 ± 14% at 15 min (fig. 3B); this increase was statistically significant compared with baseline (P < 0.025) and was marginally significant compared with sham values (P = 0.053). Noradrenaline returned to baseline levels within 30 min (fig. 3B). In sham rats, noradrenaline levels were not altered by the pinprick stimulation (fig. 3B). The analysis of noradrenaline levels after acetone application revealed a significant interaction between groups and time points (F4,38 = 5.97, P < 0.0008). Acetone stimulation in SNI rats increased noradrenaline levels to 151 ± 12% at 15 min and 124 ± 15% at 30 min (fig. 3C). At 15 min, increased levels were statistically significant compared with both baseline and sham (P < 0.001) values. At 30 min, noradrenaline levels were statistically significant compared with sham values (P = 0.034). Noradrenaline returned to baseline levels within 45 min (fig. 3C). In sham rats, noradrenaline levels were not significantly altered by the acetone stimulation (fig. 3C).

### Impairment of $\alpha_2$-Adrenoreceptor Function at the DRt in the SNI Model of Neuropathic Pain

The effects of $\alpha_2$-adrenoreceptor activation were studied by nociceptive behavioral evaluation after DRt microinjection of $\alpha$ antagonists and noradrenaline release on DRt infusion of the $\alpha_2$ agonist clonidine. The analysis of the effects of $\alpha$ antagonist microinjection into the DRt revealed significant differences between the groups in both the pinprick (F2,21 = 14.89, P < 0.001) and acetone (F4,21 = 8.99, P = 0.001) tests. Microinjection of prazosin, a $\alpha_1$-adrenoreceptor antagonist, induced a significant decrease of the withdrawal responses in both the pinprick and acetone tests compared with vehicle (pinprick and acetone: P = 0.028; fig. 4, A and B). Microinjection of atipamezole, a $\alpha_2$-adrenoreceptor antagonist, induced a significant increase of withdrawal responses in the pinprick test compared with saline (P = 0.002; fig. 4A) but did not produce a significant effect in the acetone test (fig. 4B). In the von Frey test, no significant effects were induced by both drugs.

Clonidine infusion at the DRt induced a decrease of extracellular levels of noradrenaline in a concentration-dependent manner (F3,24 = 97.2, P < 0.001, fig. 5). Clonidine exerted less of an inhibitory effect on noradrenaline release in SNI animals (F1,120 = 69.4, P < 0.001) and consistently for all doses, i.e., no evidence of interaction between dose and group (F3,120 = 1.6, P = 0.194).

### Expression Levels of $\alpha_2$-Adrenoreceptor at the DRt Are Not Altered in the SNI Model of Neuropathic Pain

We studied the effect of neuropathic pain on the expression of $\alpha_2$-adrenoreceptor subtype, which is the most predominant...
Immunohistochemistry. No significant changes were detected between sham and SNI rats by both techniques (fig. 6).

Discussion

This study shows that the SNI model of neuropathic pain increases neuronal activation in the LC and A5 noradrenergic cell group. We further show that the majority of noradrenergic DRt-projecting neurons in SNI animals are activated. This study also shows that noradrenaline levels increase at the DRt in response to nociceptive stimulation in SNI animals. Further activation of these areas is shown in several brain areas, including noradrenergic nuclei, in a model of constriction peripheral mononeuropathy.34 This bilateral pattern of activity might be explained by the bilateral spinal projections to the brain.35 The basal levels of pCREB found in our study are relatively high, but the significance of this finding is hard to compare with previous studies. Another study examining pCREB expression at the LC showed lower levels, but the expression was evaluated on innocuous tactile stimulation and used another neuropathic pain model,8 which impairs solid comparison.

This study shows that (1) noradrenaline is released at the DRt in response to nociceptive stimuli of neuropathic animals and (2) blockade of the noradrenergic input decreases mechanical and cold hypersensitivity. The magnitude of noradrenaline release on each type of nociceptive stimuli (pinprick and acetone) matches the results obtained with the α2-adrenoceptor antagonist prazosin (Praz) and atipamezole (Ati), respectively, on mechanical hypersensitivity assessed by the pinprick test (A) and on cold hypersensitivity assessed by the acetone test (B). Prazosin attenuates mechanical and cold hypersensitivity as shown by the significant decrease of withdrawal in response to pinprick and acetone stimulation, respectively. Atipamezole increased mechanical hypersensitivity as shown by the significant increase of withdrawal in response to pinprick stimulation. The drugs were administered 3 weeks after spared nerve injury induction, and their effects were tested before and 30 min after injection. Data are expressed as percentage of change from baseline (i.e., before drug injection) and are presented as mean ± 95% CIs (atipamezole and saline: n = 6/group; prazosin and DMSO: n = 5/group). $P < 0.05$ versus DMSO; $\cdots \cdots P < 0.01$ versus saline. DMSO = dimethyl sulfoxide.
antagonist, with stronger effects obtained on acetone stimulation than on pinprick. No increases of noradrenaline levels were detected on application of the 1 g von Frey filament to which SNI animals respond with a withdrawal. These results suggest that the noradrenergic input to the DRt exhibits sensory modality specificity, which is in agreement with previous studies showing modality-specific effects of noradrenaline at the DRt14 and other supraspinal pain control areas.9,36,37

We propose that the input from the LC and the A5 noradrenergic cell group to the DRt is facilitatory, in opposition to the inhibitory input of those areas to the spinal cord.7,37 Studies from other groups corroborate the existence of facilitatory pathways from the LC.8,38 Thus, the LC exerts a bidirectional modulation of nociception with a direct antinociceptive action mediated by its spinal noradrenergic projections and an indirect pronociceptive effect because of its projections to the DRt (current results). Although there is no direct evidence for bidirectional effects of the A5 noradrenergic cell group, it is likely that it might also be involved in pain facilitation because hypersensitivity is triggered on local pharmacologic manipulation.39

The pronociceptive effects of noradrenaline are likely mediated through α1-adrenoreceptor because the administration of the α1-adrenoreceptor antagonist prazosin at the DRt decreases the hypersensitivity to pinprick and acetone stimulation. In our previous work, prazosin injection into the DRt did not induce behavioral alterations,14 which might be explained by the smaller dose (2.5 μg), half the dose used in this study. In support of this proposal, we showed that prazosin injected into the DRt at the dose used in the current study also decreased nociceptive behavior in an inflammatory pain model.15 Given this pronociceptive role of α1-adrenoreceptor in the DRt, it is important to evaluate whether chronic pain induction alters its expression. However, studies comparing sham and SNI rats cannot be performed by immunohistochemistry or western blot because of lack of specificity of several commercially available antibodies against α1-adrenoreceptor.40,41 Our data further show that noradrenaline also acts at the DRt through the activation of α2-adrenoreceptor, although its effects are less marked because the local administration of the α1 antagonist atipamezole increased pinprick hypersensitivity but had no significant effects on cold hypersensitivity. In previous studies, infusion of the α2 agonist clonidine at the DRt-induced sedative effects, precluding behavioral analysis.14 Herein, we also show no alterations on α2-adrenoreceptor expression. We cannot exclude an effect of neuropathic pain on a loss
of postsynaptic $\alpha_2$-adrenoreceptor sensitivity because we observed an impairment of $\alpha_2$-autoreceptors function into the feedback inhibition of noradrenaline release. However, it seems more likely that the contribution of $\alpha_2$-adrenoreceptor in the mediation of noradrenaline effects at the DRt is weaker than the contribution of $\alpha_1$-adrenoreceptor based on the attenuation of mechanical and cold hypersensitivity on herpes simplex virus-1-mediated decrease of noradrenaline at the DRt and the lack of effects of atipamezole injection into the DRt on the formalin pain model. Herein, we did not evaluate whether $\alpha_2$-autoreceptors function into the DRt on the formalin pain model. 

To evaluate the integrity of $\alpha_2$-autoreceptors function into the feedback inhibition of noradrenaline release at the DRt, clonidine was locally administered. Our results indicate an impairment of the function of $\alpha_2$-autoreceptors in the DRt during neuropathic pain. A direct consequence of this impairment is the disinhibition of noradrenergic transmission to the DRt, which might further enhance pain facilitation by reinforcing $\alpha_1$-adrenoreceptor activation. The impairment of $\alpha_2$-autoreceptors could be due to down-regulation of $\alpha_2$-adrenoreceptor. Nonetheless, the expression of the $\alpha_2$-adrenoreceptor subtype expressed in the DRt and also on $\alpha_2$-autoreceptors was not altered as demonstrated by either western blot or immunohistochemistry. It could be argued that the inability of those techniques to analyze separately the presynaptic (autoreceptors) from postsynaptic receptors may account for those results. Impairment of $\alpha_2$-autoreceptors is most likely due to desensitization of the receptor, which is a phenomenon observed after enhancement of extracellular noradrenaline and can occur without concomitant decrease in the density of $\alpha_2$-autoreceptors. It should be noted that chronic exposure to antidepressants induces desensitization of $\alpha_2$-autoreceptors. In the case of nomifensine, which was used to improve the detection of noradrenaline, the acute treatment does not induce desensitization, which thus discards nomifensine interaction. The desensitization of the $\alpha_2$-autoreceptor was shown to be associated with decreased expression of G$\alpha$-proteins through which the receptor mediates intracellular signaling. 

In microdialysis studies, we used nomifensine, which increases extracellular levels of noradrenaline by inhibiting its reuptake without affecting its release. The pattern of noradrenaline release in other brains areas during other behavioral conditions was not altered by nomifensine infusion. Accordingly, noradrenaline release in the DRt on formalin injection is similar in the absence or presence of nomifensine (our own unpublished microdialysis experiments). Nomifensine also inhibits dopamine reuptake. We did not measure dopamine levels, but it is unlikely that they might be affected because the dopaminergic innervation of the DRt is scarce; there are no local dopaminergic neurons, and dopamine levels at the DRt are unaffected during the formalin test. Nomifensine infusion into the DRt was shown to increase nociceptive behaviors. Herein, we did not evaluate whether nomifensine increased nociceptive responses in SNI rats, but our results suggest that comparisons of behavioral responses should be performed before and after nomifensine infusion and not between sham and SNI animals because nomifensine induced higher increase of noradrenaline in sham than in SNI rats. The up-regulation of the noradrenaline transporter in SNI animals would explain the less efficient inhibition of noradrenaline recapture. This is consistent with studies in models of neuropathic pain showing up-regulation of the noradrenaline transporter at the spinal cord and the LC. The function of the noradrenaline transporter might be altered in SNI animals probably to compensate the increased noradrenaline release in the neuropathic pain condition. The dysregulation of the noradrenaline transporter together with an impairment of the function of the $\alpha_2$-autoreceptors should alter the tonic (basal) release of noradrenaline in SNI rats. Basal levels of noradrenaline in sham and SNI animals before nomifensine were not significantly different. Nonetheless, the basal levels approached the detection limit of our analytical system, which likely prevented the accurate detection of differences and draw conclusions.

In summary, the current study proposes increased noradrenergic neurotransmission from brainstem noradrenergic neurons to the DRt during chronic pain, which enhances pain facilitation from the DRt. It is possible that antidepressants inhibiting the recapture of noradrenaline may contribute to enhance DRt pain facilitation. Pain facilitatory actions of noradrenaline in the brain would explain the incomplete or transient analgesic effects often observed in patients treated with antidepressants.

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Competing Interests
The authors declare no competing interests.

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