

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 A_5 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 α -adrenoreceptors in the DRt ($n = 4$ to 6 per group).

Results: pCREB expression significantly increased in the locus coeruleus and A_5 of SNI animals, and most noradrenergic DRt-projecting neurons expressed pCREB. In SNI animals, noradrenaline levels significantly increased on pinprick (mean \pm SD, $126 \pm 14\%$; $P = 0.025$ vs. baseline) and acetone stimulation (mean \pm SD, $151 \pm 12\%$; $P < 0.001$ vs. baseline), and clonidine infusion showed decreased α_2 -mediated inhibitory function. α_1 -adrenoreceptor blockade decreased nociceptive behavioral responses in SNI animals. α_2 -adrenoreceptor 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.¹ It is frequently treated using antidepressants that block noradrenaline reuptake at the spinal cord level, inhibiting the transmission of nociceptive information.^{2,3} 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.⁴⁻⁶ The main sources of noradrenergic innervation of the spinal cord are the brainstem noradrenergic nuclei, namely the locus coeruleus (LC) and the A_5 and A_7 cell groups.⁷ 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.⁸⁻¹¹

The dorsal reticular nucleus (DRt) plays a unique role in the facilitation of pain transmission at the spinal cord.¹² It is reciprocally connected with the spinal dorsal

What We Already Know about This Topic

- Antidepressants that inhibit norepinephrine reuptake exert analgesia in part by actions in the spinal cord, but their supraspinal actions are less well studied

What This Article Tells Us That Is New

- In rats, peripheral nerve injury-induced hypersensitivity was associated with evidence for activation of cells in the brainstem, noradrenergic locus coeruleus nucleus, including cells that project to the dorsal reticular nucleus, which provides descending pain facilitating input
- Pharmacologic studies suggest that the analgesic action of spinally released norepinephrine may be reduced by simultaneous activation of supraspinal facilitating systems dependent on α_1 -adrenoreceptors

horn through a direct and putative excitatory pathway that enhances descending pain facilitation from the DRt in response to local arrival of nociceptive input.¹² The DRt

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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.¹³ 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 pain¹⁴ and noradrenaline contributes to the pain facilitatory actions of the DRt in a model of inflammatory pain.¹⁵ Taking these findings into account along with the fact that chronic pain is associated with an increased descending facilitation,¹⁶ 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 α_2 -adrenoreceptor agonist. Finally, we determined the effects of neuropathic pain on the expression of α_2 -adrenoreceptor 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.¹⁷ 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.¹⁸ 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% finadyne (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 Watson¹⁹ (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- μ l 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.¹⁵

Behavioral Assessment

After SNI induction, the animals typically develop hypersensitivity to mechanical and cold stimuli on the injured paw.¹⁸ 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.²⁰ 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.²¹ 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.²²

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 α_2 -adrenoreceptor 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's solution (140.0 mM NaCl, 4.0 mM KCl, 1.2 mM CaCl_2 , 1.0 mM MgCl_2) perfusion at a flow rate of 2.0 $\mu\text{l}/\text{min}$, four microdialysate 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 microdialysate 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 five applications of 1 g von Frey monofilament, and then they were subjected to a pinprick stimulation during which the animals received a brief stimulation with a safety pin and finally to acetone stimulation during which of a drop of acetone was applied. The magnitude of behavioral withdrawal responses after the application of the aforementioned stimuli was not measured. After stimulation, microdialysate samples were then collected in 15-min intervals for 60 min. This timing was chosen based in our preliminary studies, showing that noradrenaline levels returned to baseline within 60 min after stimulation. Results were reported as mean percentage of baseline (*i.e.*, mean of four baseline collections).

Microdialysis during Infusion of the α_2 -Adrenoreceptor Agonist Clonidine

One week after stereotaxic surgery in five SNI and five sham rats, the stylet was replaced with a 2-mm open length microdialysis probe (molecular weight cutoff, 45 to 50 kDa; Brainlink BV). The probe was first perfused with Ringer's solution for 3 h followed by Ringer's supplemented with 10 μM of nomifensine for 45 min, as described in Microdialysis

during Nociceptive Stimulation. Four baseline microdialysates samples were then collected in 15-min intervals into the sample loop of an HPLC. After the last baseline sample had been collected, the probes continued to be perfused with Ringer's/nomifensine solution further supplemented with the α_2 -adrenoreceptor 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.¹⁵ 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 α -adrenoreceptor activation at the DRt of SNI animals by using the α_1 - and α_2 -adrenoreceptor 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.¹⁵ 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,^{9,23} by an experimenter blinded to the treatments. The results were reported as mean percentage of baseline (*i.e.*, before drug injection) \pm 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 α_2 -Adrenoreceptor. 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 α_{2A} -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 antigoat 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% H_2O_2 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. NIH Image J 1.52 software (National Institute of Health, USA) was used for densitometric quantification of α_{2A} labeling in six randomly taken microphotographs from the left and right DRt. Normalization of the background preceded computer generation of mean optical density. No differences were detected between the left and right DRt for either SNI or sham animals (data not shown); therefore, left and right mean optical densities were summed in each tissue section. The specificity of the α_{2A} -adrenoreceptor antibody was previously tested in α_{2A} knockout mice²⁴ and by absorption of the α_{2A} -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 α_{2A} -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 antiserum 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, coverslipped 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 A_5 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 (\pm SD), mean numbers of neurons double labeled for TH and pCREB (TH/pCREB) (\pm SD), and percentage of colocalization in the total neurons labeled for TH (\pm SD). For the second set of experiments, the results were reported as mean numbers of neurons FG/TH (\pm SD), mean numbers of neurons triple labeled for FG, TH, and pCREB (FG/TH/pCREB) (\pm SD), and percentage of colocalization of triple-labeled neurons in the total of neurons labeled with FG and TH (\pm SD).

Western Blot Analysis of α_2 -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 , from -5.60 to -4.68 mm relative to the interaural line.¹⁹ The DRt was extracted from eight sections and drawn up with a 2- μl Hamilton syringe. The tissue was homogenized with 50 μl of ice-cold lysis buffer (50 nM Tris, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing the protease inhibitor cocktail 2 (Sigma-Aldrich), sonicated, and centrifuged for 2 min at 10,000 rpm. Total protein concentration was determined by 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) and run on an any KD TGX Stain-Free pre-cast gels (Bio-Rad, USA) for 20 min at 300 V. Stain-free gels were activated according to the manufacturer instructions using ChemiDoc XRS (Bio-Rad). Sample

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 α_{2A} -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, Advanta, USA), performed on the ChemiDoc equipment. Total protein staining obtained from the TGX Stain-Free gels was used for normalization of the α_{2A} -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 μ l 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 α_{2A} -adrenoreceptor were compared

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 22047	Rat 22048	Rat 22051	Mean \pm SD
Locus coeruleus				
FG/TH/pCREB	67	48	70	61.6 \pm 11.9
FG/TH	78	59	78	71.6 \pm 11
% FG/TH/pCREB	85.9	81.3	89.7	85.6 \pm 4.2
A ₅ noradrenergic cell group				
FG/TH/pCREB	77	95	61	77.7 \pm 17
FG/TH	96	116	81	97.7 \pm 17.5
% FG/TH/pCREB	80.2	81.9	75.3	79.1 \pm 3.4

DRt = dorsal reticular nucleus; FG = fluorogold; pCREB = phosphorylated cAMP response element-binding protein; SNI = spared nerve injury; TH = tyrosine hydroxylase.

by unpaired *t* tests. Data in the text and table 1 are presented as mean \pm 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 ($t_{10} = -3.91$, $P = 0.003$; fig. 1B) and the A₅ noradrenergic cell group ($t_{10} = -7.77$, $P < 0.001$; fig. 1E). In the LC, SNI animals showed an average of 79.8 \pm 3.8% (mean \pm SD) of colocalization (241 \pm 39 double-labeled neurons; fig. 1, A and B), whereas sham animals showed an average of 64.3 \pm 9% of colocalization (163 \pm 32 double-labeled neurons; fig. 1, A and B). In the A₅ noradrenergic cell group, SNI animals showed an average of 77 \pm 6.5% of colocalization (160 \pm 44 double-labeled neurons; fig. 1, D and E), whereas sham animals showed an average of 50.6 \pm 5.2% of colocalization (107 \pm 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

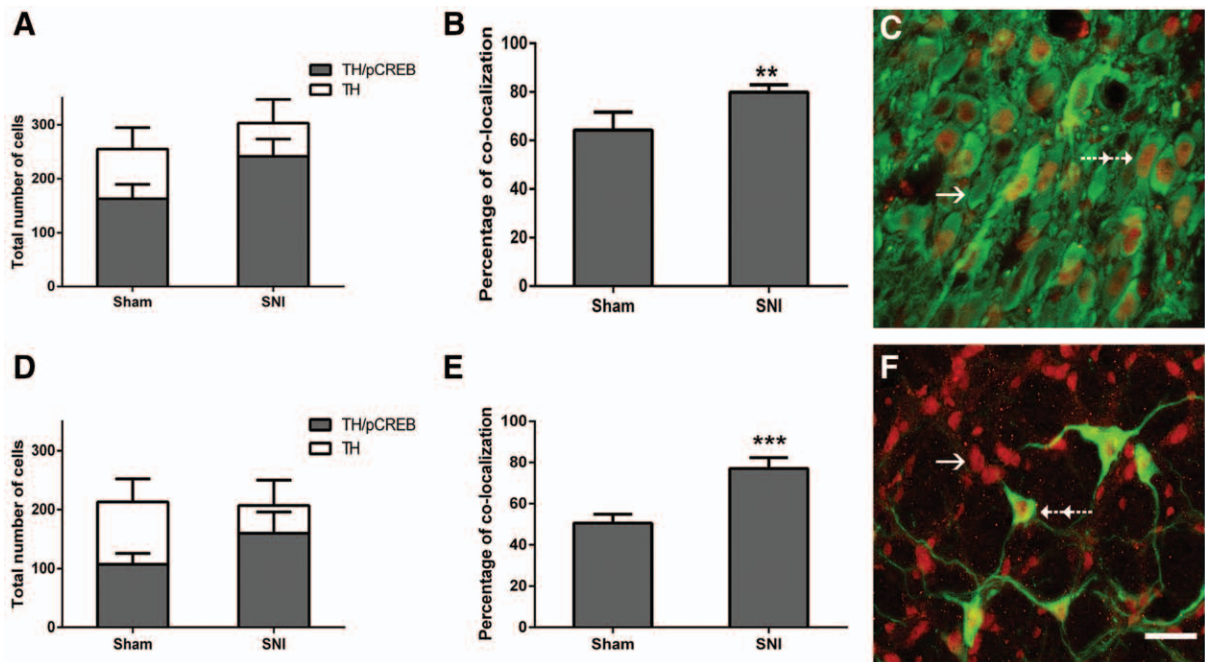


Fig. 1. Neuropathic pain increases noradrenergic activation. Total number of TH-IR neurons and number or percentage of TH-IR neurons that colocalize with pCREB in the locus coeruleus (A, B) and the A₅ noradrenergic cell group (D, E) of sham and SNI animals. Representative photomicrographs of single TH-IR 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 μ m (C is at the same magnification). (A, B, D, and E) Data are presented as mean \pm 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.

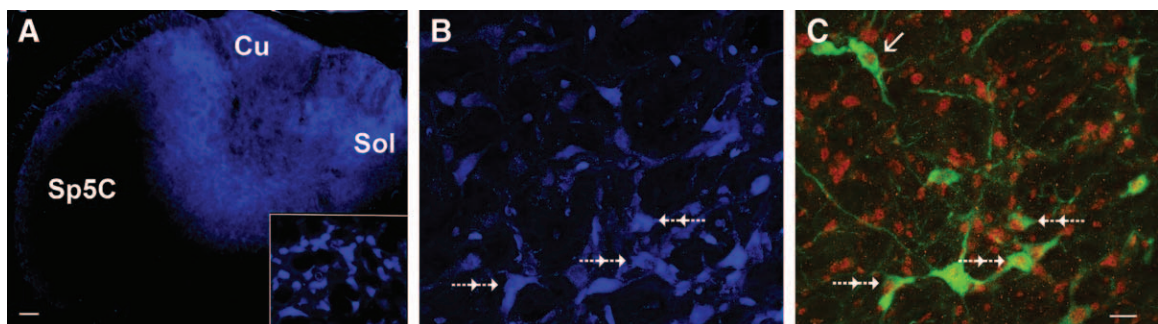


Fig. 2. Activated noradrenergic neurons project to the DRt. (A) Photomicrograph of a representative injection of FG in the DRt. The injection site is surrounded by FG⁺ neurons (better depicted at higher magnification in the insert). (B, C) Photomicrographs of FG⁺ neurons (blue, B) and double labeled 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 μ m (A) and 20 μ m (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.

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. FG injections were confined to the boundaries of the DRt (fig. 2A). The periphery of the injection site showed some perikarya labeled as a result of retrograde uptake (fig. 2A). The majority of FG/TH-labeled neurons colocalized with pCREB both at the LC (85.6 \pm 4.2%; table 1) and the A₅ noradrenergic cell group (79.1 \pm 3.4%; table 1; fig. 2, B and C).

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 ($F_{1,20} = 10.71$, $P = 0.004$). Before nomifensine, no significant differences were observed between SNI and sham animals (Sham: 1.8 ± 0.8 fmol/sample; SNI: 3 ± 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 ($F_{4,40} = 3.00$, $P < 0.029$). Pinprick stimulation in SNI rats increased noradrenaline levels to $126 \pm 14\%$ 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 ($F_{4,38} = 5.97$, $P = 0.0008$). Acetone stimulation in SNI rats increased noradrenaline levels to $151 \pm 12\%$ at 15 min and $124 \pm 15\%$ 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 α_2 -Adrenoreceptor Function at the DRt in the SNI Model of Neuropathic Pain

The effects of α -adrenoreceptor activation were studied by nociceptive behavioral evaluation after DRt microinjection of α antagonists and noradrenaline release on DRt infusion of the α_2 agonist clonidine. The analysis of the effects of α antagonist microinjection into the DRt revealed significant differences between the groups in both the pinprick ($F_{3,21} = 14.89$, $P < 0.001$) and acetone ($F_{3,21} = 8.99$, $P = 0.001$) tests. Microinjection of prazosin, a α_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 α_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

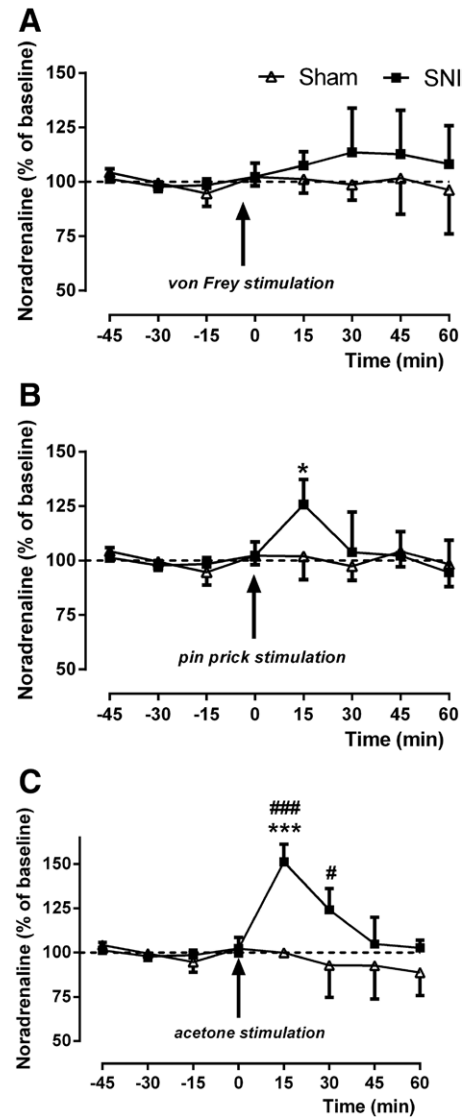


Fig. 3. Nociceptive stimulation increases noradrenaline release into the dorsal reticular nucleus of neuropathic animals. Extracellular levels of noradrenaline collected in 15-min intervals from 45 min before and until 60 min after von Frey (A), pinprick (B), and acetone (C) stimulation 3 weeks after sham or SNI surgery. Each stimuli was applied three times (5 min apart) starting at time 0 min. Data are expressed as percentage of baseline levels and are presented as mean \pm 95% CIs ($n = 6$ /group). * $P < 0.05$, *** $P < 0.001$ versus baseline values; # $P < 0.05$, ### $P < 0.001$ versus sham group. SNI = spared nerve injury.

manner ($F_{3,24} = 97.2$, $P < 0.001$, fig. 5). Clonidine exerted less of an inhibitory effect on noradrenaline release in SNI animals ($F_{1,120} = 69.4$, $P < 0.001$) and consistently for all doses, *i.e.*, no evidence of interaction between dose and group ($F_{3,120} = 1.6$, $P = 0.194$).

Expression Levels of α_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 α_{2A} -adrenoreceptor subtype, which is the most predominant

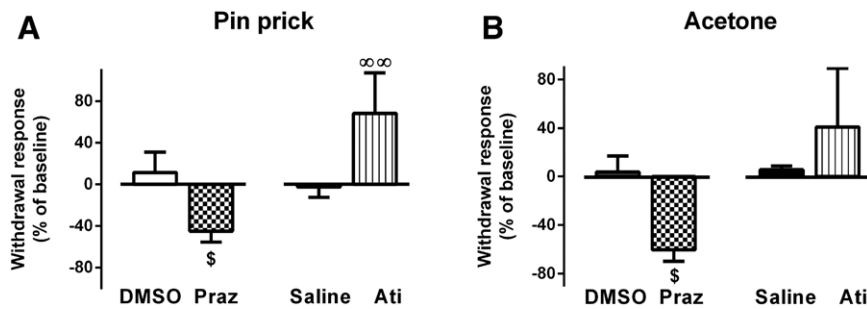


Fig. 4. Effects of the α_1 - and α_2 -adrenoreceptor antagonists prazosin (Praz) and atipamezole (Ati), respectively, on mechanical hypersensitivity assessed by the pinprick test (A) and on cold hypersensitivity assessed by the acetone test (B). Prazosin attenuates mechanical and cold hypersensitivity 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 \pm 95% CIs (atipamezole and saline: $n = 6$ /group; prazosin and DMSO: $n = 5$ /group). $^{\S}P < 0.05$ versus DMSO; $^{\infty}P < 0.01$ versus saline. DMSO = dimethyl sulfoxide.

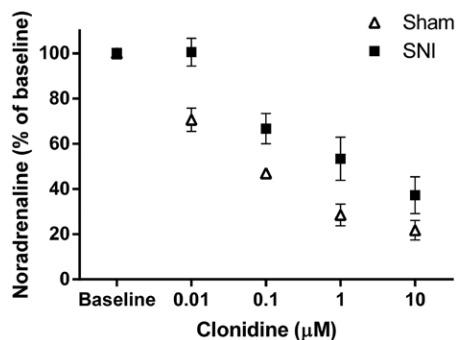


Fig. 5. Impairment of the α_2 -adrenoreceptor inhibitory function on the release of noradrenaline in the DRt. Noradrenaline release in response to increasing concentrations (baseline, 0.01 to 10 μ M) of the α_2 agonist clonidine. Clonidine was infused at the DRt by reverse dialysis 3 weeks after sham or SNI surgery. Data are expressed as mean percentage of baseline levels across four measurements at each dose (15, 30, 50, and 60 min after administration), with the respective 95% CIs ($n = 5$ /group). DRt = dorsal reticular nucleus; SNI = spared nerve injury.

subtype in the brainstem,²⁹ by western blot and 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 A_5 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, probably enhancing pain facilitation. This study also suggests impairments of the feedback inhibitory function of α_2 -autoreceptors in SNI animals, further enhancing the noradrenergic input to the DRt during chronic pain.

Neuroanatomic tract tracing techniques showed that the LC and the A_5 noradrenergic cell group are the main

noradrenergic brain areas projecting to the DRt,¹³ which was confirmed by the retrograde transport from the DRt of a herpes simplex virus-1 viral vector.^{14,15,30} In this study, the high percentages of noradrenergic neurons expressing pCREB at the LC and the A_5 noradrenergic cell group of SNI animals suggests that neuropathic pain potentiates the activity of these areas. This agrees with reports showing increased brainstem noradrenergic activation in several neuropathic pain models.^{8,11,31–33} The increased neuronal activation at the LC could be related to its role in chronic pain maintenance.^{8–11} The DRt could represent one pathway through which the LC exerts this effect because the majority of LC neurons projecting to the DRt in SNI animals are activated, and noradrenaline is released at the DRt on stimulation, inducing pronociceptive effects. This hypothesis and a possible role of the A_5 noradrenergic cell group in chronic pain maintenance need to be explored. We found no lateralization of pCREB labeling in SNI animals. This agrees with another study showing similar pCREB levels in the ipsilateral and contralateral LC in SNI rats.⁸ Higher neuronal activity was also shown bilaterally in several brain areas, including noradrenergic nuclei, in a model of constriction peripheral mononeuropathy.³⁴ That bilateral pattern of activation 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 α_1 -adrenoreceptor

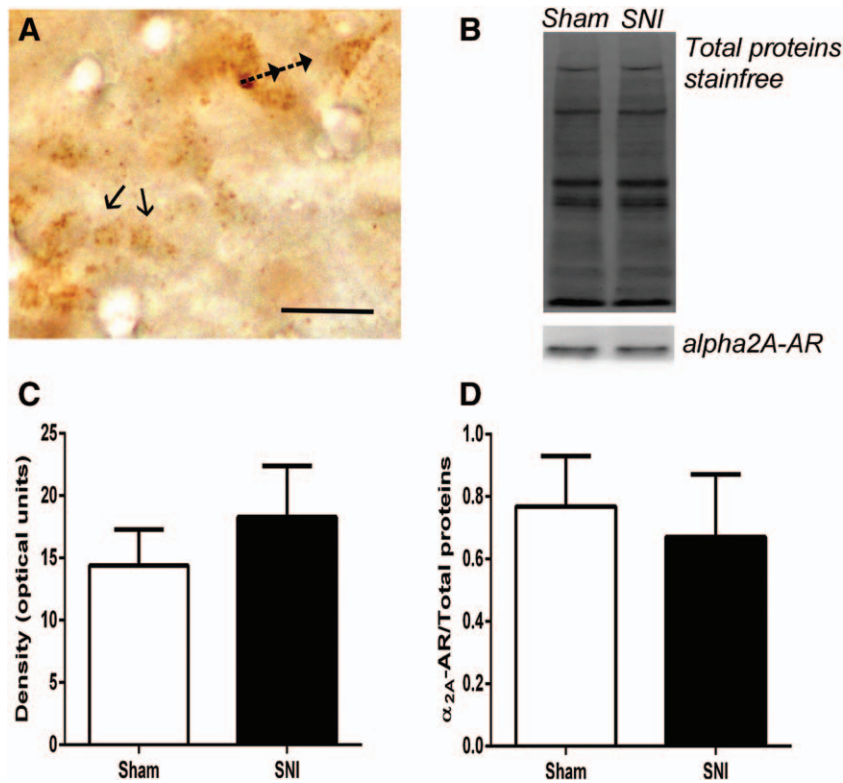


Fig. 6. α_{2A} -Adrenoreceptor expression is not altered during neuropathic pain. (A) Representative photomicrograph of α_{2A} -adrenoreceptor labeling in the dorsal reticular nucleus (DRt) of a SNI animal. The immunoreactivity is characterized by punctate labeling of the pericarya (single arrows) and dendritic labeling in some neurons (double arrow). Scale bar: 50 μ m. (B) Representative western blots of α_{2A} -adrenoreceptor from the DRt of a sham and SNI animal. (Top) Total protein bands in Stain-Free gel are shown. (C) Densitometric quantification of α_{2A} -adrenoreceptor immunolabeling in the DRt of sham and SNI animals ($n = 5$ each). (D) Mean amount of α_{2A} -adrenoreceptor relative to total proteins from sham and SNI animals ($n = 4$ each). (C, D) Data are presented as mean \pm 95% CIs. AR = adrenoreceptors; SNI = spared nerve injury.

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 hyperalgesia is triggered on local pharmacologic manipulation.³⁹

The pronociceptive effects of noradrenaline are likely mediated through α_1 -adrenoreceptor because the administration of the α_1 -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 μ g), 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 α_1 -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 α_1 -adrenoreceptor.^{40,41} Our data further show that noradrenaline also acts at the DRt through the activation of α_2 -adrenoreceptor, although its effects are less marked because the local administration of the α_2 antagonist atipamezole increased pinprick hypersensitivity but had no significant effects on cold hypersensitivity. In previous studies, infusion of the α_2 agonist clonidine at the DRt-induced sedative effects, precluding behavioral analysis.¹⁴ Herein, we also show no alterations on α_2 -adrenoreceptor expression. We cannot exclude an effect of neuropathic pain on a loss

of postsynaptic α_2 -adrenoreceptor sensitivity because we observed an impairment of α_2 -autoreceptors function into the feedback inhibition of noradrenaline release. However, it seems more likely that the contribution of α_2 -adrenoreceptor in the mediation of noradrenaline effects at the DRt is weaker than the contribution of α_1 -adrenoreceptor based on the attenuation of mechanical and cold hypersensitivity on herpes simplex virus-1-mediated decrease of noradrenaline at the DRt¹⁴ and the lack of effects of atipamezole injection into the DRt on the formalin pain model.¹⁵

To evaluate the integrity of α_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 α_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 α_1 -adrenoreceptor activation. The impairment of α_2 -autoreceptors could be due to down-regulation of α_2 -adrenoreceptor. Nonetheless, the expression of the α_{2A} -adrenoreceptor, which is the main α_2 -adrenoreceptor subtype expressed in the DRt and also on α_2 -autoreceptors,^{29,42} 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 α_2 -autoreceptors is most likely due to desensitization of the receptor, which is a phenomenon observed after enhancement of extracellular noradrenaline^{43,44} and can occur without concomitant decrease in the density of α_2 -autoreceptors.⁴³ It should be noted that chronic exposure to antidepressants induces desensitization of α_2 -autoreceptors.⁴⁵ In the case of nomifensine, which was used to improve the detection of noradrenaline, the acute treatment does not induce desensitization,⁴⁶ which thus discards nomifensine interaction. The desensitization of the α_2 -autoreceptor was shown to be associated with decreased expression of G α i proteins through which the receptor mediates intracellular signaling.⁴³

In microdialysis studies, we used nomifensine, which increases extracellular levels of noradrenaline by inhibiting its reuptake without affecting its release.⁴⁷⁻⁴⁹ The pattern of noradrenaline release in other brains areas during other behavioral conditions was not altered by nomifensine infusion.⁵⁰⁻⁵² Accordingly, noradrenaline release in the DRt on formalin injection is similar in the absence¹⁵ or presence of nomifensine (our own unpublished microdialysis experiments). Nomifensine also inhibits dopamine reuptake.⁵³ 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,¹³ and dopamine levels at the DRt are unaffected during the formalin test.¹⁵

Nomifensine infusion into the DRt was shown to increase nociceptive behaviors.¹⁵ 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 cord⁵⁴ and the LC.⁵⁵ 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 α_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.^{3,56}

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Competing Interests

The authors declare no competing interests.

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Address correspondence to Dr. Tavares: Departamento de Biologia Experimental, Faculdade de Medicina do Porto, Alameda Prof. Hernani Monteiro, 4200-319 Porto, Porto, Portugal. isatav@med.up.pt. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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