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Inhibition of cardiac baroreflex by noxious thermal stimuli: A key role for lateral paragigantocellular serotonergic cells

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ABSTRACT

The present study was designed to identify the neuronal mechanisms causing cardiac baroreflex inhibition associated with thermal nociception in rats. Under urethane-anesthesia, noxious thermal stimuli $\geqslant$48 °C were found to inhibit the cardiac baroreflex, whereas noxious stimuli $\leqslant$46 °C had no effect. Using double immunohistochemical labeling, noxious stimuli $\geqslant$48 °C were found to evoke primarily a strong expression of Fos protein (Fos) encoded by *c-fos* gene in serotonergic neurons of lateral paragigantocellular reticular nucleus (LPGi). Noxious stimuli $\leqslant$46 °C did not evoke Fos expression in any serotonergic neurons of the brainstem. Local blockade of neuronal activity by bilateral microinjections of fluorescent muscimol (a $GABA_A$ receptor agonist tagged with a fluorophore that allowed visualization of the injections) into both the LPGi and the raphe magnus nucleus prevented the inhibitory effect of noxious stimuli $\geqslant$48 °C on the cardiac baroreflex. Bilateral microinjections of granisetron (a 5-HT_3 antagonist) within the nucleus tractus solitarius also prevented the inhibition of cardiac baroreflex elicited by noxious stimuli $\geqslant$48 °C. These results show that activation of serotonergic cells in the LPGi is critical to trigger nucleus tractus solitarius-mediated cardiac baroreflex inhibition elicited by intense thermal noxious stimuli.

1. Introduction

Cardiovascular changes such as increases in blood pressure, heart rate, and sympathetic nerve activity can be elicited by noxious visceral and cutaneous stimuli [37,41,44,53,73]. Furthermore, the blood pressure increase is constantly accompanied by an inhibition of the baroreflex in case of cutaneous [11,57,63] as well as visceral [35,56,59] nociception. Because the baroreflex is a mechanism that maintains blood pressure constant, its inhibition is needed to get an increase of cardiovascular parameters [55] and allow an adequate response to noxious stimuli.

Interestingly, the defense reaction triggered by direct stimulation of the dorsal periaqueductal gray (dPAG) [3,25,40] includes, similarly to nociception, an inhibition of the cardiac baroreflex [26,29,58]. The latter inhibition is mediated by serotonin (5-HT) through the activation of 5-HT_3 receptors heavily concentrated in the nucleus tractus solitarius (NTS) [16,19,31,33,47,48,67], which plays a key role in the integration of baroreceptor messages [30,34,61,65]. Recently, we demonstrated that the source of 5-HT responsible for the inhibition of cardiac baroreflex is located in the B3 serotonergic group that comprises the raphe magnus (RMg) and the lateral paragigantocellular reticular (LPGi) nuclei [6].

The aforementioned studies suggest that the cardiac baroreflex inhibition triggered by noxious stimuli could be underlain by a similar serotonergic mechanism. To address this question, we used graded thermal stimuli with the aim of determining the intensity of noxious stimuli needed to inhibit the cardiac component of baroreflex in urethane-anesthetized rats. Then we used double immunolabeling of Fos protein (Fos) + 5-HT to visualize the populations of 5-HT cells activated by noxious stimuli that potentially inhibit the baroreflex. Finally, we examined the possible involvement of these cells (identified previously in the B3 group) and the serotonergic 5-HT_3 receptors (within the NTS) in the inhibition of the cardiac baroreflex triggered by noxious stimulation. For this purpose, we

Abbreviations: 3, 7, 10 and 12, nuclei of 3th, 7th, 10th and 12th nerves; 4v, 4th ventricle; 5-HT, 5-hydroxytryptamine (serotonin); 6n, 7n, 8n, 6th, 7th, 8th nerves; acs 7, accessory nucleus of 7th nerve; B3, 3rd serotonergic group (including the RMg and the LPGi); BCR, baroreflex cardiac response; Fos, Fos protein (encoded by *c-fos* gene); DAB, 3,3′-diaminobenzidine; DCDp, dorsal cochlear nucleus deep core; dPAG, dorsal PAG; ECG, Electrocardiogram; Gi, gigantocellular reticular nucleus; HR, heart rate; icp, inferior cerebellar peduncle; LPGi, lateral paragigantocellular reticular nucleus; ml, medial lemniscus; mlf, medial longitudinal fasciculus; NGS, normal goat serum; NTS, nucleus tractus solitarius; PAG, periaqueductal gray matter; PBS, phosphate buffered saline; PCRt, parvicellular reticular nucleus; PE, phenylephrine; Pr, prepositus nucleus; Pr5, principal sensory trigeminal nucleus; py, pyramidal tract; RDr, raphe dorsalis nucleus; RMg, raphe magnus nucleus; ROb, raphe obscurus nucleus; RPa, raphe pallidus nucleus; RVLM, rostroventrolateral medulla; sol, tractus solitarius; sp5, spinal trigeminal tract; Sp5, trigeminal nucleus; SO, superior olivary nucleus; VC, ventral cochlear nucleus; Ve, vestibular nuclei.

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analyzed first the effect of inactivation of the B3 region on noxious stimulus-induced suppression of the cardiac baroreflex. Inactivation of the B3 region was achieved by local microinjections of fluorescent muscimol (a $GABA_A$ receptor agonist, which produces a local blockade of synaptic transmission and the injection of which is visualized by fluorescence). In a second step, using bilateral microinjections of granisetron (a selective 5-HT_3 receptor antagonist [27]) into the NTS, we investigated the effect of NTS 5-HT_3 receptor blockade on noxious stimulus-induced baroreflex inhibition.

2. Materials and methods

2.1. General procedures

Experiments were performed on male Sprague–Dawley rats weighing 330–370 g. Procedures involving animals and their care were conducted in conformity with the institutional guidelines in compliance with the Council Directive No. 87–848 of the Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale (Permissions No. 75–148 to J.F.B., No. 75–116 to M.H., No. 75–117 to R.L. and No. 75–855 to C.S.C.).

Rats were anesthetized with urethane (1.5 g/kg, i.p.). Systemic and mean blood pressures were monitored (Pressure Processor and DC Amplifier, Gould, Courtaboeuf, France) through a catheter inserted into the femoral artery. Electrocardiogram (ECG) was recorded using stainless steel pins placed subcutaneously into fore- and hindpaws; signals were amplified and filtered (Universal Amplifier, Gould). Heart rate was computed from R wave pulses and displayed as mean frequency per minute (bin size = 1 s). Rectal temperature was maintained at 37 °C with a thermostatically controlled heating blanket. The animals were pretreated with i.v. injection of a beta-blocker (propranolol: 0.4 mg/kg, in some preliminary experiments, or atenolol: 1 mg/kg, in the core of experiments) to suppress the cardiac sympathetic response (tachycardia) induced by noxious stimuli.

The baroreflex response was triggered by intrafemoral injection of phenylephrine (PE) (10–15 μg in 0.1 ml of saline, duration = 1 s) immediately followed by intrafemoral rinsing with saline (0.1 ml, duration = 1 s). The baroreflex cardiac response (BCR) was defined as the ratio (%) of the maximal decrease in heart rate (ΔHR) over the heart rate baseline (HRb) value (BCR = [ΔHR/HRb] × 100). The gain (G) of baroreflex was defined as the ratio of BCR over the maximal increase of mean blood pressure (ΔMBP, in mmHg): G = [BCR/ΔMBP] $mmHg^{-1}$. Note that G is a negative number because ΔHR, which expresses a bradycardia, is negative. However, comparisons about G refer to the absolute value of G.

2.2. Experiment 1 – effects of graded thermal stimulation on the cardiac component of baroreflex

Noxious thermal stimuli consisted of immersing a hindpaw into a waterbath at different calibrated temperatures (neutral temperature: 33 °C; noxious temperatures: 44, 46, 48, 50 and 52 °C) during 20 or 30 s. The experimental BCR was tested as follows: the hindpaw was dipped into the waterbath at a given temperature and after ≈3 s delay PE was injected through the catheter. In each case, a control BCR was tested 15 min before and after determination of experimental BCR at a given temperature. Control BCR consisted of PE injection only.

2.3. Experiment 2 – immunohistochemical labeling of the Fos expression evoked in serotonergic cells by noxious thermal stimulation

2.3.1. Noxious thermal stimulation

The first experimental series (above) permitted us to choose 33 °C neutral temperature (used as control, $n = 9$) and 46, 48 and 52 °C noxious temperatures ($n = 5$, 5 and 10, respectively), as relevant stimuli for the modulation of BCR (see Section 3).

For each animal, the hindpaw was dipped into the waterbath at one controlled temperature during 30 s, then gently dried and let at room temperature during 90 s. This 2 min cycle was repeated 10 times (20 min total time). A notable inflammation arose in the hindpaw after the 52 °C cycle of noxious stimulation whereas no obvious damage was observed with 46 and 48 °C cycles of noxious stimulation.

2.3.2. Perfusion

Two hours after the end of the thermal stimulation, the anesthetized animal was perfused through the heart with: (1) warm (37 °C) heparinized saline (0.9% NaCl) solution (during 3 min), followed by (2) cold (10 °C) phosphate (0.12 M) buffered (pH 7.4) saline solution (PBS) containing 4% paraformaldehyde, 0.1% glutaraldehyde and 0.05% picric acid (during 24 min), and finally by (3) 20% sucrose solution (during 8 min). The brain was removed and cryoprotected in a 20% sucrose solution overnight. Coronal frozen sections (50 μm thick) of the whole brainstem were collected in three containers filled with PBS, allowing their parallel processing as three serial groups of floating sections.

2.3.3. Immunohistochemistry

To label Fos, two series of sections were pre-incubated for 2 h in PBS containing 0.4% Triton X-100 and 1% normal goat serum (NGS). They were then incubated overnight at room temperature with the primary Fos antibody (1/8000, polyclonal IgG made in rabbit against the amino acids 115–165 of human Fos; Santa Cruz Biotechnology Inc, Santa Cruz, CA; Refs. [K-25]: sc-253, batch D0804) in PBS containing 0.4% Triton X-100 and 1% NGS. Using Western blot, Santa Cruz Biotechnology confirmed that the protein recognized by this antibody has the expected molecular weight of Fos ≈62 kDa (personal communication). Furthermore, we controlled in our laboratory that in response to noxious stimuli, the Fos labeling markedly increased in the dorsal horn of the spinal cord, in agreement with consensus data [12,28,46]. After incubation with Fos antibody, sections were rinsed for 30 min with PBS, then incubated for 1 h with the secondary antibody (biotinylated goat anti-rabbit, 1/200, Vector, Burlingame, CA) in PBS containing 0.4% Triton X-100 and 1% NGS. After a second rinse for 30 min with PBS, the sections were finally incubated for 1 h in the avidin–biotin–horseradish peroxidase solution (one drop of A + one drop of B complex per 10 ml; ABC Vectastain kit Elite, Vector). The sections were then rinsed, and immunolabeling proceeded using two peroxidase histochemical procedures. For the first series, sections were rinsed for 15 min with PBS, then 15 min with TRIS (0.12 M) buffered (pH 7.4) solution, before labeling (black/gray) with 3,3′-diaminobenzidine (DAB)-nickel enhanced technique: they were incubated in TRIS buffered solution containing 0.04% DAB (Sigma–Aldrich, St. Quentin-Fallavier, France) + 0.2% ammonium nickel sulfate (Sigma–Aldrich) supplemented with H_2O_2 every 4 min in order to obtain increasing H_2O_2 concentrations (0.00015%, 0.0003%, 0.0006%, 0.0012%, 0.0024%, 0.0048%).

After an extensive final rinsing, one series, labeled for Fos only, was mounted on gelatin-coated slides and coverslipped. The second series of sections underwent a second procedure to label 5-HT. This procedure was similar to the first one except that: (1) we used primary 5-HT antibody (1/160,000; Calbiochem; Refs. PC 228L, batch D21789-1); this polyclonal antibody was made in rabbit against 5-HT conjugated to bovine serum albumin (BSA) by glutaraldehyde; the specificity of the 5-HT antibody was controlled after preadsorption against BSA: the distribution of 5-HT immunoreactive cells found with this antibody matched that de-

scribed in the literature [68]; the primary antibody solution contained also 0.0048% H_2O_2 to inactivate remaining peroxidases; (2) the amount of avidin–biotin–horseradish-peroxidase was lowered to one drop of A + one drop of B complex per 20 ml; and (3) the sections were finally labeled (brown/yellow) using 0.04% DAB only. This second series was thus double-labeled for both Fos and 5-HT.

The third series was not processed for immunohistochemistry but run in parallel for Nissl staining by thionin. In a few cases, the third series of sections underwent the processing for 5-HT labeling only as technical control of double labeling.

2.3.4. Visualization and counting of Fos- and 5-HT-immunoreactive neurons

Neurons were observed under brightfield illumination in 50 μm thick coronal sections (150 μm apart) in regions containing serotonergic neurons, which project to the NTS: the raphe pallidus [RPa] (B1 serotonergic group), the raphe obscurus [ROb] (B2 group), the LPGi and the RMg (B3 group), and the raphe dorsalis [RDr] (B6 and B7 groups) nuclei [71]. Location of brainstem nuclei containing 5-HT neurons was based on observation of serotonergic groups and adjacent Nissl stained sections. Nomenclature and boundaries of these areas were adopted according to Paxinos and Watson [62]. Plotting and counting were based on the observation of the Fos + 5-HT double-labeled series.

We took into account Fos + 5-HT double-labeled neurons after careful individual examination of each neuron at high magnification (×40). Double-labeled as well as Fos- or 5-HT- single-labeled neurons were plotted and counted with Mercator software (Explora Nova, La Rochelle, France) connected to the microscope with (1) a CCD color video camera which sent tricolor red, green and blue (RGB) output to a computer and (2) XY stage captors which sent the micrometric location of the section to the computer. The outline of the section and the main structures were drawn at low magnification (×4, ×10), and single as well as double-labeled neurons were plotted at higher magnification (×20, ×40), the computer providing continuous synchronization between plotting and section location during moving or magnification changes. Finally, we got a camera lucida drawing-like illustration of labeled cells distribution within the section. In each section, within each structure delimited as indicated previously, we counted the numbers of 5-HT + Fos double-labeled (n_d), 5-HT single-labeled (n_h) and Fos single-labeled (n_f) neurons. Then in each animal, we calculated for each structure extending in n_s sections, the mean numbers per section of 5-HT + Fos double-labeled ($d = \sum[n_d]/n_s$), 5-HT single-labeled ($h = \sum[n_h]/n_s$) and Fos single-labeled ($f = \sum[n_f]/n_s$) neurons. Finally, in each group of n_a animals, we calculated for each structure, the mean numbers per section of 5-HT + Fos double-labeled ($D = \sum[d]/n_a$), 5-HT single-labeled ($H = \sum[h]/n_a$) and Fos single-labeled ($F = \sum[f]/n_a$) neurons.

Digitized photomicrographs were made using a CCD color video camera, connected to a microscope, which sent RGB output to a Macintosh computer. Images at different focal planes were captured and digitized using a 24-bit color-scale Openlab software (Improvision, Coventry, UK). An operator allowed the combination, pixel-by-pixel, of images in different focal planes. These operations resulted in the production of one image by incorporating the darker value of the corresponding pixel in each focal plane for each color plan. Images were exported to Adobe-Photoshop (version 8.0.1) in order to mount adjacent digitized images as a final large field high-resolution image. Then, brightness, contrast and image scale were adjusted. Finally, additional indications and/or anatomical landmarks were incorporated into the figure.

2.4. Experiment 3 – chemical blockade of B3 region or NTS: Effect on noxious stimulus-induced inhibition of the cardiac baroreflex

2.4.1. Bilateral microinjections of fluorescent muscimol into the B3 region

Chemical blockade of the B3 region was made using bilateral microinjections of bodipy TMR-X conjugate muscimol (Ref: M-23400, Invitrogen, Pontoise, France), which permits labeling of the injection site [1].

Urethane-anesthetized and catheterized rats were placed in a stereotaxic frame with the head fixed in ventroflexed position (incisor bar lowered 8 mm below the Paxinos and Watson's atlas standard position [62]). A craniotomy was performed, and a glass micropipette (tip diameter < 50 μm) connected to a Hamilton microsyringe filled with bodipy muscimol or saline was lowered bilaterally at stereotaxic coordinates (11 mm caudal to bregma; lateral 0.8 mm on both sides; depth 9.5 mm from the cerebellum surface, Paxinos and Watson's atlas [62]) which correspond to the B3 region found in experiment 2 to present an increased density of double-labeled (Fos + 5-HT) cells in response to noxious stimulation ⩾48 °C. The bodipy muscimol was injected during 4 min with a pump pushing the Hamilton microsyringe, using the same micropipette for both sides. Muscimol was microinjected at a dose (5 mM, 100 nl) in the range of doses used previously [8,50].

The injection sites of bodipy muscimol were precisely located thanks to the orange emission (572 nm) by the fluorophore linked to muscimol. In addition, 5-HT cells were visualized in brain coronal sections (100 μm) thanks to the green fluorescent emission (519 nm) of alexa 488 anti-rabbit (Ref: A-11034, Invitrogen) used after incubation of sections with rabbit anti-5-HT antibody (1/40,000, see above).

2.4.2. Bilateral microinjections of granisetron into the NTS

Chemical blockade of NTS 5-HT_3 receptors was made using local microinjections of granisetron (a selective 5-HT_3 antagonist; gift of GlaxoSmithKline Labs, Harlow, UK). Urethane-anesthetized and catheterized rats were placed in a stereotaxic frame. We exposed the dorsal surface of the brain stem through a small incision of the atlo-occipital membrane with limited occipital craniotomy. A glass micropipette (tip diameter < 50 μm), connected to a Hamilton microsyringe filled with granisetron or saline, was then lowered 500 μm laterally into the commissural NTS at the level of the calamus scriptorius. Bilateral microinjections of granisetron (250 pmol, 100 nl, injection duration: 2 min) into the NTS were made using the same micropipette.

The sites of granisetron microinjections in NTS were identified in brain coronal sections (100 μm) stained with thionin, by local deposit of pontamine sky blue (2%) at the tip of the micropipette previously used for granisetron microinjections.

2.4.3. Effects of chemical blockade of B3 region on the inhibition of the cardiac baroreflex induced by noxious thermal stimulation

Thirty minutes after bilateral microinjections of muscimol (5 mM, 100 nl) or saline (for control) into the B3 region, the effect of noxious thermal stimulation (48 °C, 52 °C) upon BCR was analyzed as described above, and compared to the effect of noxious thermal stimulation without B3 microinjections.

2.4.4. Effects of NTS 5-HT_3 receptor blockade on the inhibition of the cardiac baroreflex induced by noxious thermal stimulation

Twenty minutes after bilateral microinjections of granisetron (250 pmol, 100 nl) or saline (for control) into the NTS, the effect of noxious thermal stimulation (48 °C, 52 °C) upon BCR was

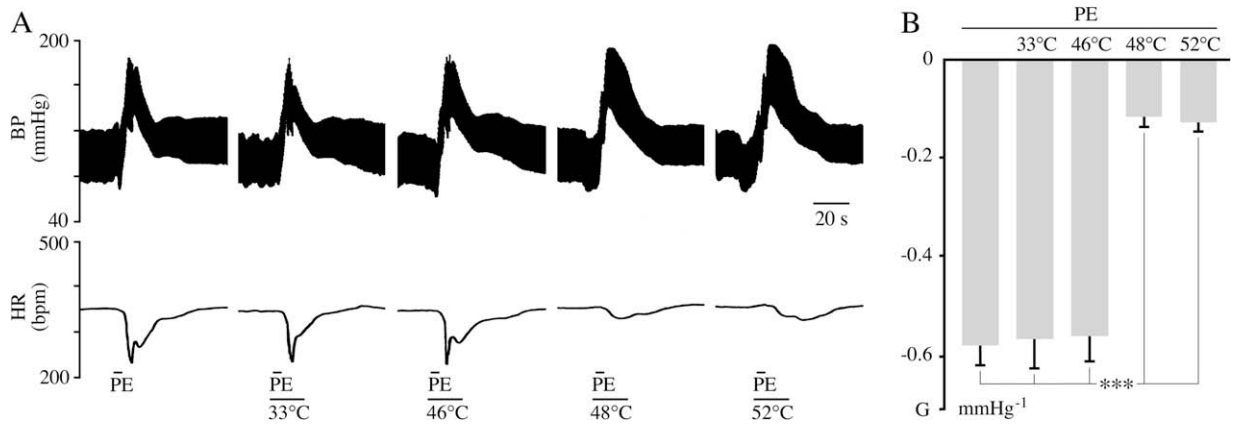

Fig. 1. Effects of innocuous and noxious thermal stimuli on baroreflex cardiac responses. (A) Representative tracings showing the baroreflex bradycardia evoked by intra-arterial phenylephrine (PE) only and by PE during 33, 46, 48, and 52 °C stimulation of the hindpaw. Note that the inhibition of baroreflex cardiac response was elicited only by 48 and 52 °C highly nociceptive temperatures. (B) Histogram of the mean (+S.E.M.) baroreflex gain elicited by PE only ($n = 30$) and by PE during 33 °C ($n = 11$), 46 °C ($n = 12$), 48 °C ($n = 13$), 52 °C ($n = 25$) thermal stimuli. Note that the mean gain (G) of baroreflex was clearly unchanged by 33 and 46 °C whereas it was strongly decreased (absolute value) by 48 and 52 °C. Each of PE only, 33, and 46 °C groups was significantly different from each of 48 and 52 °C groups ($p < 0.001$). BP: blood pressure; G: gain of cardiac baroreflex; HR: heart rate; ***$p < 0.001$.

analyzed as described above, and compared to the effect of noxious thermal stimulation without NTS microinjections.

2.5. Statistical analyses

Absolute values are expressed as means ± S.E.M. of n rats. Statistical analyses were performed using Student's paired t-test. A p value $\leqslant 0.05$ was considered as the limit for statistical significance.

3. Results

3.1. Experiment 1. Effect of thermal noxious stimuli on the cardiac baroreflex

In this first series of experiments, we tested the effects of hindpaw stimulation with increasing noxious thermal stimuli (46, 48 and 52 °C) and the neutral thermal stimulus (33 °C) upon the

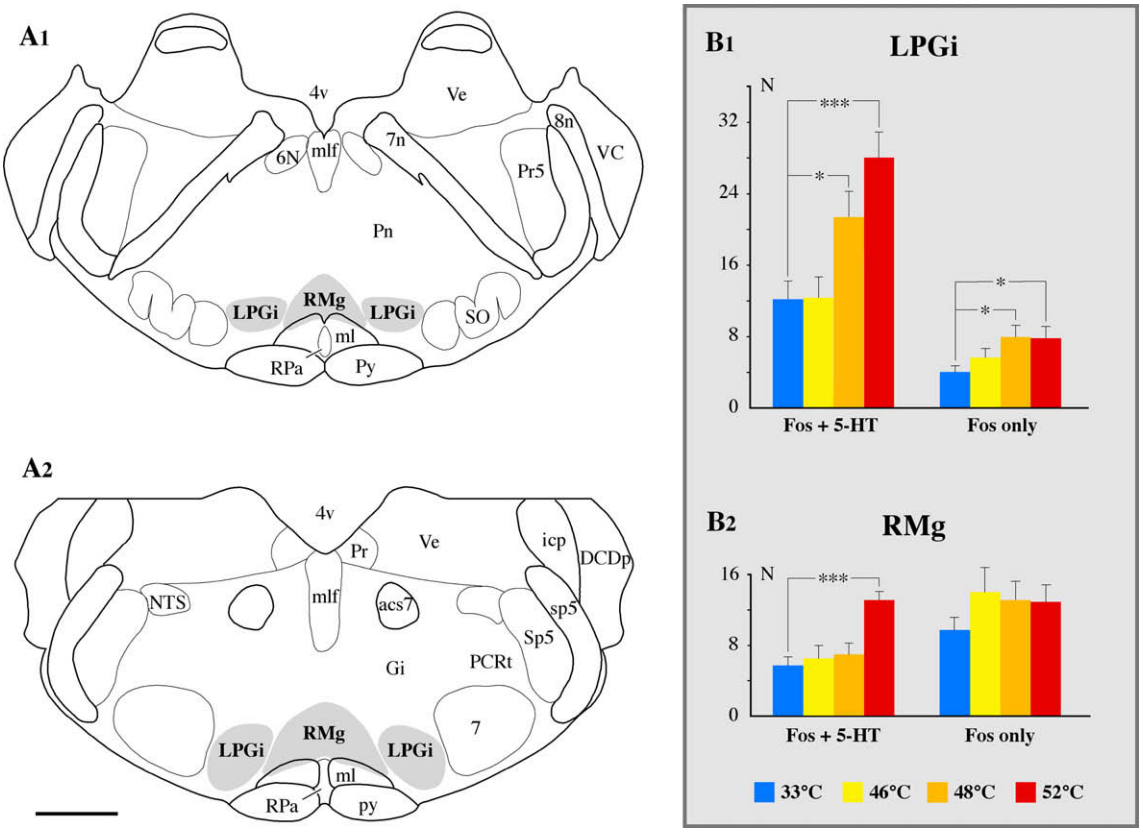

Fig. 2. Location and quantification (mean number of immunolabeled neurons per section) of Fos expression within B3 region in response to thermal noxious stimuli. (A1–A2) Coronal sections of the brainstem at the level of the rostral and the mid portions (bregma −10.2 and −10.9 mm, according to Ref. [62]) of B3 serotonergic group (gray area). (B) Histograms of the number of serotonergic and non-serotonergic Fos positive cells in both the ipsilateral and contralateral LPGi (B1) and the RMg (B2), within the rostral + mid portion of the B3 region (gray areas in A). Blue, yellow, orange, and red bars indicate the mean (+ S.E.M.) number (N) of Fos expressing cells per section after 33 °C ($n = 9$), 46 °C ($n = 5$), 48 °C ($n = 5$), and 52 °C ($n = 10$) thermal stimuli, respectively. Fos + 5-HT: serotonergic Fos positive cells; Fos only: non-serotonergic Fos positive cells. LPGi: lateral paragigantocellular nucleus; RMg: raphe magnus nucleus; ***$p < 0.001$, *$p < 0.05$. Abbreviations in A1, A2: see list. Scale bar = 1 mm.

BCR elicited by PE intra-arterial injection. Importantly, the sympathetic tachycardia response to noxious stimuli was suppressed by pretreatment with atenolol to unmask the bradycardia component of the baroreflex (see Section 2).

In urethane-anesthetized and atenolol-treated animals, baseline values of mean blood pressure and heart rate were 86 ± 3 mmHg and 309 ± 12 bpm, respectively. Application of 33 and 46 °C stimuli did not change significantly the blood pressure and the heart rate. In contrast, 48 and 52 °C stimuli produced a strong increase in blood pressure (+44 ± 5% and +47 ± 6% over the baseline, respectively; $n = 6$, $P < 0.001$) and a slight increase in heart rate (+7.0 ± 1.5% over the baseline in both cases; $n = 6$, $P < 0.01$).

The 33 °C neutral and the 46 °C mid-nociceptive stimuli did not change the BCR induced by PE (bradycardia ≈ −120 bpm) whereas the 48 and 52 °C strong nociceptive stimuli depressed markedly this response (bradycardia ≈ −25 bpm) (Fig. 1A). The histogram in Fig. 1B indicates that the mean gain of baroreflex elicited by PE ($G = -0.58 \pm 0.04$ mmHg^{-1}, $n = 30$) was unchanged during 33 °C ($G = -0.57 \pm 0.06$, $n = 11$) and 46 °C stimulation ($G = -0.56 \pm 0.05$, $n = 12$) whereas it was strongly decreased ($P < 0.001$) during 48 °C ($G = -0.11 \pm 0.02$, −81%, $n = 13$) and 52 °C ($G = -0.13 \pm 0.02$, −78%, $n = 25$) stimulation.

Considering the hypothesis that the baroreflex inhibition is driven by serotonergic neurons (see Section 1), the aim of experiment 2 was to identify which of these neurons are activated by noxious stimuli that inhibit the cardiac baroreflex. Thus, in experiment 2, we used serotonergic labeling coupled with visualization of Fos expression evoked by noxious temperature identified in experiment 1.

3.2. Experiment 2. Effects of thermal noxious stimuli on Fos expression in serotonergic cells

The increase of Fos expression evoked within serotonergic brainstem nuclei (RPa, ROb, B3 region and RDr, see Section 2) by thermal noxious stimuli applied to the right hindpaw (46 °C, $n = 5$; 48 °C, $n = 5$; 52 °C, $n = 10$) was analyzed in comparison with a sham group stimulated with neutral temperature (33 °C, $n = 9$). We observed that strong (52 °C, 48 °C) thermal noxious stimuli evoked significant Fos increase only in two of the structures analyzed: primarily in the rostral two thirds of the B3 region and to a lesser extent in the ROb. In contrast, moderate noxious stimulus (46 °C) did not evoke any Fos expression in these nuclei.

Throughout the rostral two thirds of the B3 region (Fig. 2A1,2), between bregma −11.8 and −10.4 mm [62], the 52 °C noxious stimulus evoked a marked ($P < 0.001$, $n_1 = 9$, $n_2 = 10$) increase of Fos expression in serotonergic neurons of the LPGi on both sides (with a moderate contralateral predominance) and the RMg (Fig. 2B). The number of Fos + 5-HT double-labeled neurons was increased by 131% (from 12.1 ± 2.1 to 28.0 ± 2.8 neurons per section) in LPGi on both sides and by 128% (from 5.7 ± 0.9 to 13.0 ± 1.0) in the RMg. The 48 °C noxious stimulus also increased, but by 75% only (from 12.2 ± 2.1 to 21.4 ± 2.9), the Fos expression in serotonergic neurons of LPGi ($P < 0.05$, $n_1 = 9$, $n_2 = 10$) (Fig. 2B1) whereas no increase was observed in the RMg (Fig. 2B2). Importantly, the total number of serotonergic neurons did not change with noxious stimuli.

The Fig. 3 shows that the increase of Fos expression at 48 and 52 °C affected mainly the serotonergic neurons, which were primarily grouped, close to the nuclei of 7th nerves, in a very lateral and well individualized portion of the B3 group, delineated here as the LPGi. Fig. 4 is a typical higher magnification photomicrograph of Fos increase within the contralateral LPGi when the stimulus temperature raised from 33 to 52 °C.

In the rostral B3 region, the Fos expression is also evoked in non-serotonergic neurons by 48 and 52 °C noxious stimuli. The resulting increase in Fos single-labeled neurons was significant ($P < 0.05$) in the LPGi (Fig. 2B1), whereas only a tendency was observed in the RMg (Fig. 2B2).

In the ROb, only the 52 °C noxious stimulus evoked a moderate but significant increase of Fos expression restricted to serotonergic neurons (from 2.7 ± 0.4 to 4.4 ± 0.4 neurons per section, $n_1 = 9$, $n_2 = 10$, $P < 0.01$). In these conditions, 30 ± 13 serotonergic neurons only were activated per animal in the ROb.

3.3. Experiment 3. Effects of chemical blockade of the B3 region and the NTS upon the cardiac baroreflex inhibition by noxious stimuli

3.3.1. Effect of muscimol microinjection into the B3 region

The aim of this experiment was to examine the effect of inactivation of the region identified in experiment 2, i.e., the rostral two thirds of B3 region, where a marked increase of Fos + 5-HT double-labeled cells was noted in response to noxious stimuli ⩾52 °C. For this purpose, we used bilateral microinjections of muscimol

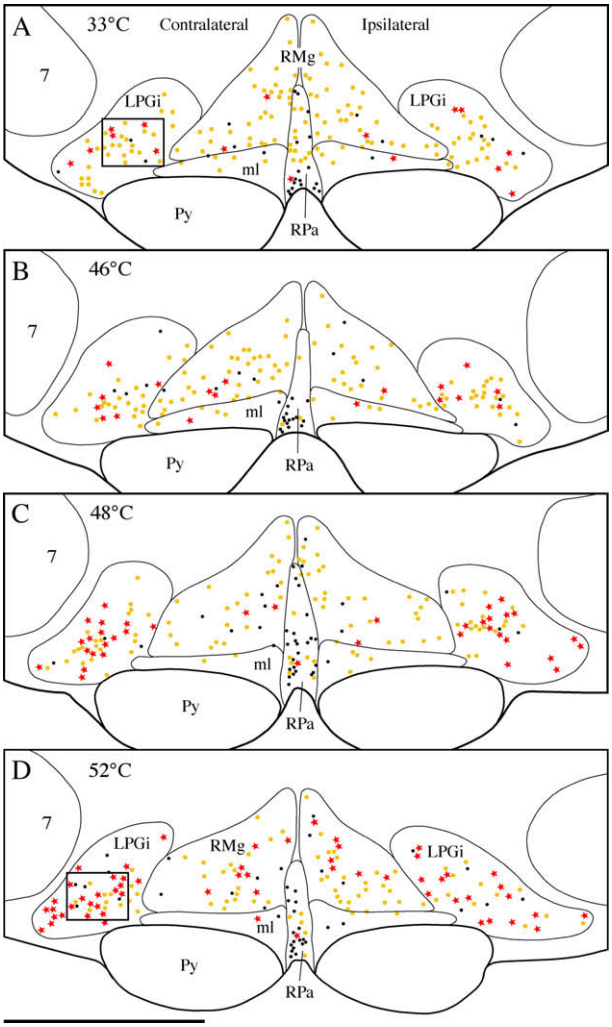

Fig. 3. Distribution of neurons labeled for Fos and/or 5-HT in the mid-rostrocaudal extent of the B3 region (A–D) Camera lucida drawings of coronal sections at bregma level = −10.9 mm [62] for rats whose right hindpaw was dipped into waterbath at 33, 46, 48, and 52 °C, respectively. Each drawing depicts neurons expressing (1) both Fos and 5-HT (red star), (2) only 5-HT (brown/yellow circle) or (3) only Fos (small black point). The framed areas in (A) and (D), in the contralateral LPGi, indicate the location of the high magnification microphotographs of Fig. 4A and B. Note the increased density of double-labeled neurons and the decreased density of single 5-HT labeled neurons in (D) compared to (A). Ipsilateral, Contralateral: side ipsilateral, contralateral to stimulated paw. Abbreviations: see list. Scale bar = 1 mm.

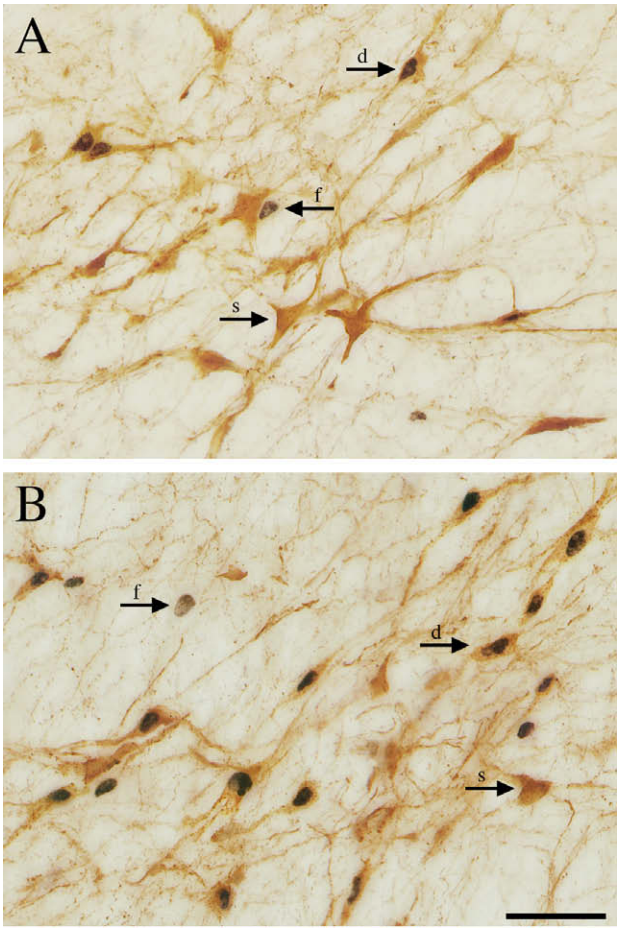

Fig. 4. Color digitized photomicrographs of neurons labeled for Fos and/or 5-HT in the B3 region (A) and (B): photomicrographs of the lateral paragigantocellular (LPGi) regions framed in Fig. 3A and D, respectively. (A) 33 °C Stimulated animal. (B) 52 °C Stimulated animal. Note the strong increase of Fos + 5-HT double-labeled neurons in (B) compared to (A). *Arrows* with *d*, *s* and *f* indicate examples of Fos + 5-HT double-labeled (black core surrounded by brown staining), 5-HT single-labeled (cytoplasm brown staining) and Fos single-labeled (nucleus black staining) neurons, respectively. Scale bar = 50 μm.

(a specific agonist of $GABA_A$ receptors that induces strong inhibition of neuronal firing [20]) centered on the rostral two thirds of B3 (at bregma −11 mm).

After bilateral microinjections (see for the rat No. 98, Fig. 5A2) of fluorescent muscimol (5 mM in 0.1 μl, on each side), that spread within the rostral B3 region, the inhibition of BCR elicited by 52 °C noxious stimulation was prevented (Fig. 6A). The histogram in Fig. 6B indicates that the mean gain of baroreflex elicited by PE ($G = -0.59 \pm 0.06$, $n = 12$) was unchanged after injection of muscimol into rostral B3 ($G = -0.58 \pm 0.07$, $n = 15$), whereas the gain decrease of PE-evoked baroreflex during 52 °C stimulation ($G = -0.16 \pm 0.03$, $n = 12$) was significantly prevented ($P < 0.001$) by this local pharmacological treatment ($G = -0.46 \pm 0.06$, $n = 15$). Other microinjections of fluorescent muscimol illustrated in Fig. 5A1, as well as microinjections of pure muscimol within B3 region, produced similar prevention of baroreflex inhibition by noxious stimulation, without changes of cardiovascular baseline values. In contrast, the few microinjections of muscimol ($n = 7$) that were made outside the B3 group did not prevent the inhibition of baroreflex elicited by 52 °C noxious stimulation.

3.3.2. Effect of granisetron microinjection into the NTS

The aim of this last series of experiments was to examine the hypothesis that baroreflex inhibition by noxious stimulation is mainly mediated by 5-HT acting at $5\text{-}HT_3$ receptors within the NTS. Thus we tested the effect of bilateral microinjections of granisetron, a specific $5\text{-}HT_3$ receptor antagonist, into the NTS.

After bilateral microinjections of granisetron into the caudal NTS, at a level close to the calamus scriptorius (Fig. 5B), the inhibition of BCR elicited by 52 °C noxious stimulation was prevented (Fig. 7A, compare A2 with A1). Importantly, granisetron did not produce any changes of cardiovascular baseline values. The histogram in Fig. 7B indicates that the mean gain of baroreflex induced by PE after intra-NTS microinjections of granisetron ($G = -0.41 \pm 0.04$, $n = 15$)

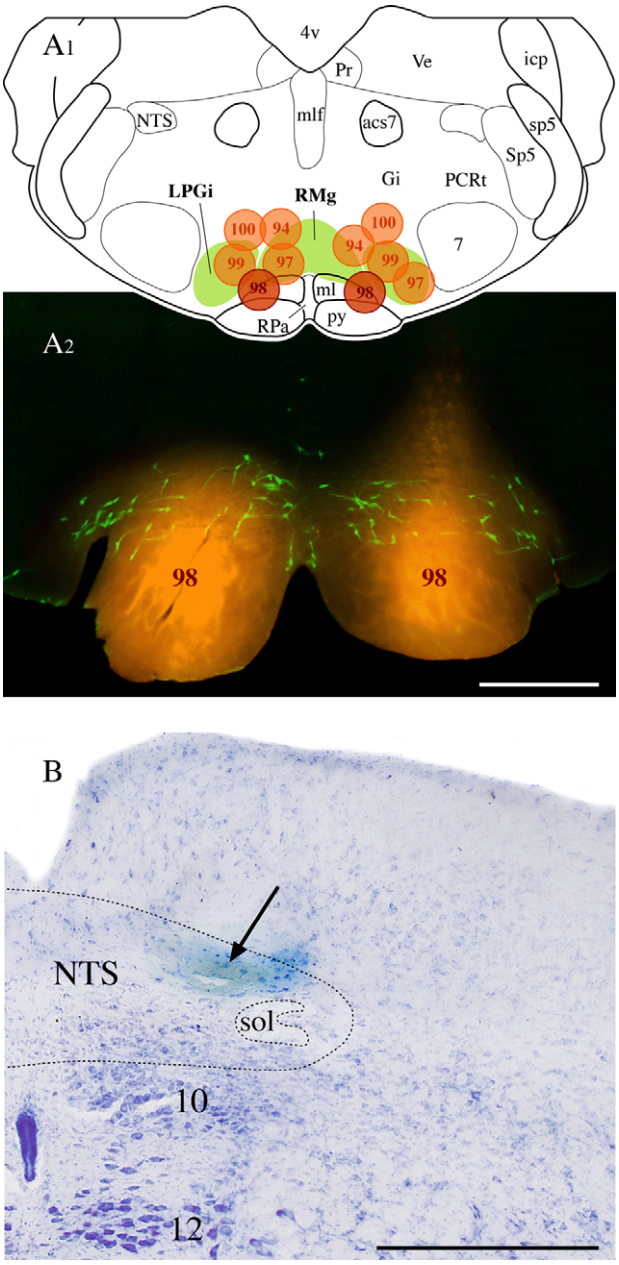

Fig. 5. Microinjection sites of fluorescent bodypi muscimol into the B3 group (A) and pontamine blue dye into the NTS (B). (A1) Coronal section at the level of B3 group (green) showing bilateral bodipy muscimol microinjections. Each couple of bilateral microinjections, in a given animal, was identified by the same number in two orange circles. (A2) Photomicrograph showing typical case (No. 98) of bilateral microinjections of bodipy muscimol (orange fluorescence) within the serotonergic neuron area (green) of the B3 group (comprising the RMg and the LPGi; bregma ≈ −11 mm). (B) Photomicrograph showing pontamine blue deposit (arrow) around the tip of the micropipette used to inject granisetron into the NTS (bregma ≈ −13.5 mm). Abbreviations: see list. Scale bars = 500 μm (A2 and B).

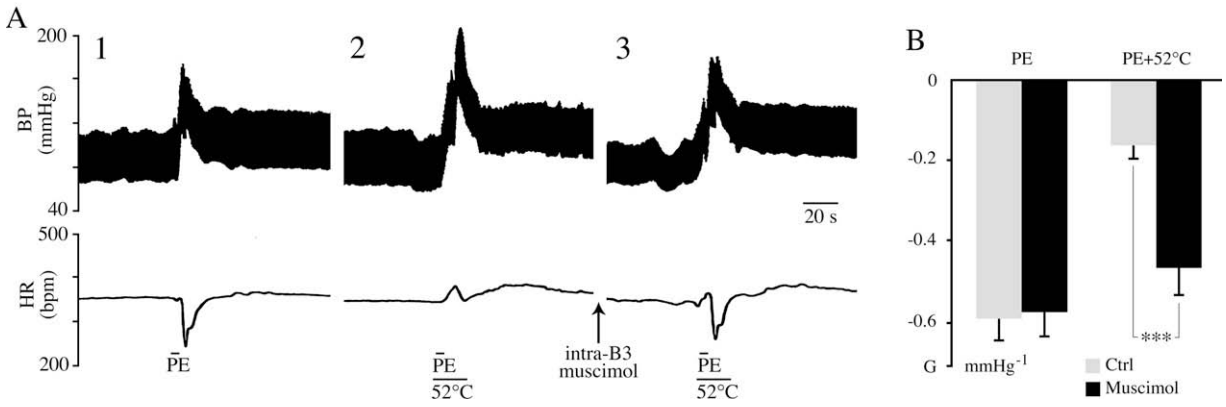

Fig. 6. Effects of muscimol microinjection into the B3 region upon the baroreflex inhibition by noxious thermal stimuli. (A) Representative tracings showing, in the same animal, (1) the baroreflex bradycardia evoked by PE, (2) the inhibition of the baroreflex by 52 °C noxious stimulus, and (3) the full prevention of the latter inhibition by prior bilateral microinjections of fluorescent muscimol (5 mM) into the B3 region (injection No. 98 shown in Fig. 5). (B) Histogram of the mean (+S.E.M.) baroreflex gain elicited by PE only (PE) and by PE during 52 °C noxious stimulation (PE + 52 °C), before (gray) and after (black) muscimol microinjection into the B3 region (each bar: $n = 15$). Note that the mean baroreflex gain elicited by PE only was clearly unchanged by muscimol, whereas the strong decrease in baroreflex gain elicited by 52 °C noxious stimulation was markedly reduced by intra-B3 muscimol. BP, blood pressure; G, gain of cardiac baroreflex; HR, heart rate; $^{***}p < 0.001$.

did not significantly differ from that measured after local micoinjections of saline ($G = -0.47 \pm 0.10$, $n = 5$). On the other hand, the mean decrease of the baroreflex gain triggered by 52 °C stimulation was not changed after microinjections of saline into the NTS ($G = -0.07 \pm 0.02$, $n = 5$), but was completely prevented ($P < 0.001$) after local microinjections of granisetron ($G = -0.42 \pm 0.05$, $n = 7$).

4. Discussion

This study showed that noxious thermal stimuli ⩾48 °C inhibited strongly the cardiac component of the baroreflex, whereas noxious stimuli ⩽46 °C had no effect. It demonstrated, in parallel, that stimuli ⩾48 °C evoked primarily a strong Fos expression within serotonergic neurons of B3 group (RMg + LPGi), whereas stimuli ⩽46 °C did not evoke Fos expression in serotonergic neurons. These data suggest a key role for B3 serotonergic neurons in the inhibition of baroreflex bradycardia by intense noxious stimuli.

The prevention of baroreflex inhibition by muscimol-induced B3 local inactivation supported the involvement of the B3 region in the inhibitory mechanisms triggered by noxious stimuli. Finally, the involvement of 5-HT was demonstrated using intra-NTS granisetron (a specific 5-HT_3 antagonist), which prevented noxious stimuli-induced inhibition of baroreflex bradycardia.

Taken together, these results demonstrated for the first time that B3, and more specifically LPGi serotonergic neurons, play a key role in the inhibition of baroreflex by noxious stimuli ⩾48 °C.

4.1. Inhibition of baroreflex bradycardia by noxious thermal stimuli

Our data are in agreement with previous reports on baroreflex inhibition by noxious stimuli such as high temperature (55 °C), pinch, visceral distension and nerve stimulation [11,35,56,57,59]. Here we showed for the first time that the baroreflex inhibition occurs only with intense noxious stimuli (48–52 °C). Because strong noxious stimuli induced marked increase in heart rate resulting from sympathetic activation, it was difficult to differentiate the parasympathetic (bradycardia baroreflex) from the sympathetic response (tachycardia). The beta-blocker, atenolol, which suppressed the sympathetic tachycardia, permitted us to unmask the cardiac baroreflex (see Figs. 1, 6 and 7) and to observe clear inhibition of cardiac component (parasympathetic) of baroreflex by noxious stimuli ⩾48 °C. Thenceforth, the sympathetic component of baroreflex was not examined in this study.

4.2. Fos labeling of serotonergic neurons activation in the LPGi

The Fos + 5-HT double labeling precisely identified serotonergic neurons which could contribute to baroreflex inhibition, because they were activated by thermal nociceptive stimulation. Within the population of 5-HT neurons projecting to the NTS [71], 48 °C evoked a significant expression of Fos by those in the LPGi only. Thus, LPGi 5-HT neurons appeared as the most likely serotonergic origin of baroreflex inhibition by 48 °C. This conclusion is further supported by previous data showing that the LPGi provides especially dense serotonergic projection to the NTS [66,71].

The 52 °C Fos experiment supported essentially the same conclusion, since this noxious temperature also evoked the activation of 5-HT neurons in the LPGi, but to a larger extent than 48 °C. In addition, we noted that 52 °C stimulation also triggered moderate activation of 5-HT neurons in the RMg and the ROb. However, because 52 °C did not increase the inhibition of baroreflex more than 48 °C, it is probable that the moderate activation of RMg and ROb neurons by 52 °C is not the main cause of the baroreflex inhibition.

Our finding that neurons in both the RMg and the LPGi were activated by strong noxious stimuli is in general agreement with previous Fos [36,60,72] and 2-deoxyglucose [42,64] studies. Furthermore, Chen et al. [14] also found that noxious stimuli activate 5-HT neurons in the ventromedial medulla.

The increased expression of the nuclear Fos protein is classically used as a marker of neuronal activation [12,28,46]. However, since this marker provides only an indirect view of the neuronal firing, it is interesting to confront Fos with electrophysiological data. Thus, our finding that 52 °C noxious stimulation evoked Fos in moderate number of 5-HT neurons in the RMg (Fig. 2B2) is in good agreement with Gao and Mason [21] who showed that 56 °C noxious stimuli increased the firing in only 17% of RMg 5-HT neurons. Unfortunately, to our knowledge, no electrophysiological study of the response of 5-HT neurons has been focused to LPGi 5-HT neurons.

Interestingly, Gao and Mason [22] also showed that some 5-HT neurons in the RMg can be weakly and phasically activated by strong blood pressure increase (≈60 mmHg). However, it seems improbable that such neuronal response [22] may trigger, on its own, the Fos expression in 5-HT neurons. In support of this inference, we observed that Fos expression increased considerably in 5-HT neurons from 48 °C to 52 °C while the blood pressure enhancement (≈40 mmHg) did not change within this temperature range.

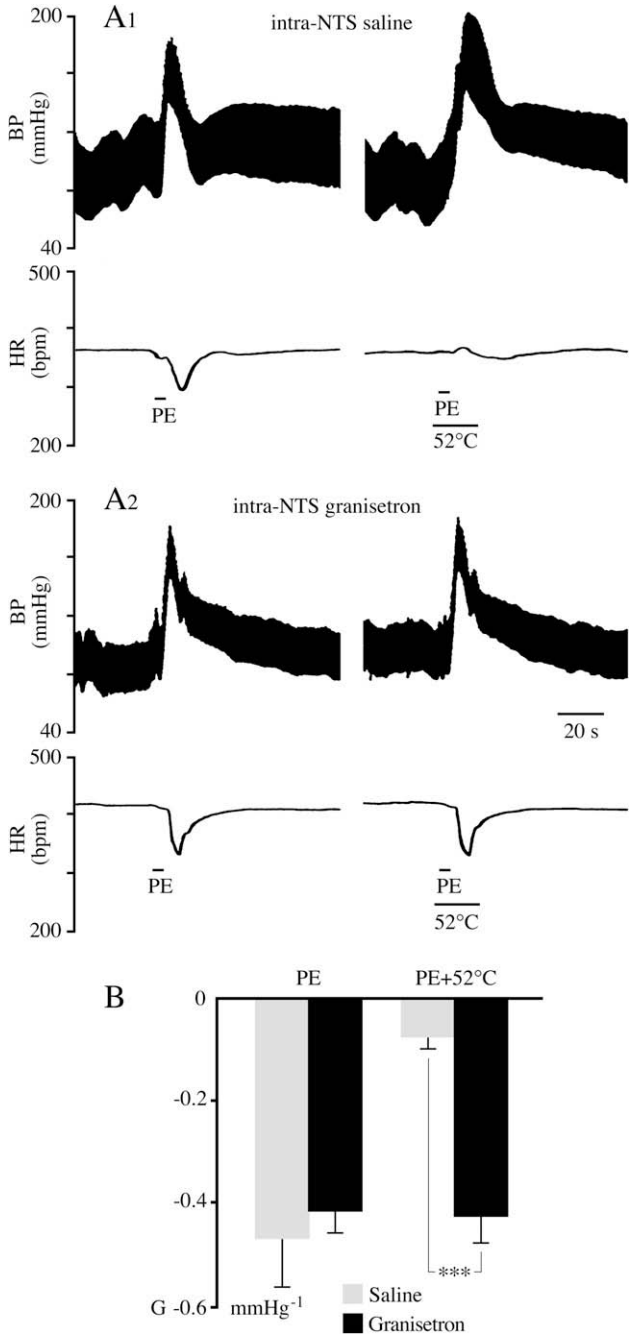

Fig. 7. Effects of granisetron microinjection into the NTS upon the baroreflex inhibition by noxious thermal stimuli. (A1) In control conditions, the tracing shows that PE-evoked baroreflex bradycardia and that concomitant 52 °C thermal noxious stimulation inhibited this response. (A2) After granisetron microinjections into the NTS, the tracing shows that PE-evoked baroreflex bradycardia persisted during 52 °C noxious stimulation. (B) Histogram of the mean (+S.E.M.) baroreflex gain elicited by PE only (PE) and by PE during 52 °C noxious stimulation (PE + 52 °C), after saline (gray, each bar: $n = 5$) or granisetron (black, each bar: $n = 7$) microinjection into the NTS. Note that the mean baroreflex gain by PE only was clearly unchanged by granisetron, whereas the strong decrease of baroreflex gain elicited by 52 °C noxious stimulus was markedly prevented by intra-NTS granisetron. BP: blood pressure; G: gain of cardiac baroreflex; HR: heart rate; ***$p < 0.001$.

4.3. Inactivation of the B3 region and blockade of 5-HT3 receptors in the NTS

The use of fluorescent muscimol allowed the visualization of bilateral microinjection sites with its main spreading in the B3 region [1]. When the prevention of baroreflex inhibition was regularly observed (i.e. 30 min after the microinjections), the muscimol spreading at inhibitory concentration within the B3 region could be estimated to extend 1 mm from the injection site [20,43] i.e. ≈0.5 mm beyond the halo of muscimol spreading visible in Fig. 5A2; therefore, most neurons within the B3 region were certainly inactivated. This further supports the idea that the B3 region plays a cardinal role in baroreflex control, in line with previous data showing that local excitation of B3 neurons elicited an inhibition of baroreflex [6]. However, because muscimol caused non-specific inactivation of B3 neurons, further investigations were needed to really demonstrate the specific involvement of 5-HT in noxious stimuli-evoked baroreflex inhibition.

In this context, our data showing that bilateral microinjections of granisetron (a specific 5-HT_3 receptor antagonist [27]) into the caudal NTS prevented the inhibition of baroreflex by noxious stimuli are especially relevant. Indeed, the NTS is the primary center of the baroreflex that receives aortic and cardiac barosensitive afferents [13,61,65]. Furthermore, the NTS contains abundant 5-HT innervation with high density of 5-HT_3 receptors, contrasting with adjacent regions almost completely devoid of 5-HT [19,48]. Thus, it can be inferred that the effect of granisetron within the NTS can be exerted through the local blockade of 5-HT-mediated inhibition of baroreflex bradycardia. Supporting the specificity of this effect, it must be emphasized that intra-NTS saline microinjection did not affect the baroreflex whereas intra-NTS granisetron prevented the modulation of baroreflex by noxious stimuli without affecting the baroreflex itself.

4.4. Involvement of LPGi serotonergic neurons in the inhibition of baroreflex by noxious stimuli

Because baroreflex inhibition appears to be mediated by 5-HT, the serotonergic neurons of the LPGi, which are primarily activated by noxious stimuli, play certainly a critical role in this inhibition. Thus, under noxious stimulation, 5-HT released from LPGi afferents should activate vagal glutamatergic afferents via presynaptic 5-HT_3 receptors within the NTS [19,31,48]. The resulting facilitation would activate GABAergic interneurons, which, in turn, would inhibit NTS neurons involved in baroreflex bradycardia [16,47,67] (see Fig. 9 in [6]).

4.5. Transmission of nociceptive messages to the LPGi

The way through which nociceptive messages activate the LPGi neurons remains unknown. Because, (i) the main afferent projection to the LPGi originates in the dPAG [51] and (ii) the dPAG–LPGi–NTS circuit has been involved in baroreflex inhibition during the defense reaction evoked by dPAG stimulation [6], it can be hypothesized that the dPAG conveys nociceptive messages to the LPGi–NTS circuit which inhibits the baroreflex. Nociceptive messages could be transmitted to the dPAG via the major spino-parabrachio-hypothalamic-dPAG nociceptive circuit [5,23]. In support of this hypothesis, it must be emphasized that structures of the latter circuit, i.e. the dPAG, the parabrachial area and the medial hypothalamus, are all involved in both nociception and modulation of baroreflex [5,6,9,10,58,67]. However, it has to be recalled that the LPGi also receives direct but more diffuse afferent projections from the parabrachial area, several reticular nuclei (deep mesencephalic, gigantocellular, parvocellular, lateral reticular nuclei) and the deep laminae of the dorsal horn of the spinal cord [39,54], which all process, at least in part, nociceptive messages.

4.6. A physiological role for serotonergic neurons in the LPGi

Unbearable noxious stimuli (i.e. stimuli ⩾48 °C) induce an inhibition of baroreflex bradycardia, which allows the full blood pres-

sure increase necessary to carry out the behavior (withdrawal, flight, fight) required by the painful situation. We demonstrated here that the LPGi serotonergic neurons are key actors of this inhibition of baroreflex by noxious stimuli.

We showed previously that the LPGi serotonergic neurons participate also in inhibition of baroreflex bradycardia by dPAG stimulation [6]. Because in both dPAG stimulation and nociception, the baroreflex inhibition is as a noteworthy component of the defense reaction, it can be speculated that LPGi serotonergic neurons probably have a specific role in defense reaction. Indeed, the LPGi projects not only to the NTS but also to the spinal cord, the ambiguous nuclei, the locus cœruleus, and numerous brainstem reticular areas [2,24,32]. Furthermore, convergent data established that the LPGi participates in the control of main physiological functions such as nociception [4,7,45,69], sympathetic tone [15], motricity [49], awakeness/sleep [17,38] and sexuality [18,52,70]. Thus, during painful stimulation, LPGi serotonergic neurons might trigger autonomic and motivational defensive behavior, including cardiovascular, motor tone, and vigilance enhancement, and nociception and sexual inhibition, to cope with vital emergency.

Conflicts of interest

In the present work there has been no conflict of interest.

Acknowledgements

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