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Critical Role of B3 Serotonergic Cells in Baroreflex Inhibition during the Defense Reaction Triggered by Dorsal Periaqueductal Gray Stimulation

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ABSTRACT

The present study was designed to identify the serotonergic pathway causing baroreflex inhibition associated with the defense reaction in rats. Under conditions that produce physiological responses typical of the defense reaction, electrical stimulation of the dorsal periaqueductal gray (dPAG) was found to double c-Fos immunoreactive serotonergic neurons within the mid-rostrocaudal extent of the B3 group (which comprises the raphe magnus and the lateral paragigantocellular reticular nuclei) in anesthetized rats. Local blockade of neuronal activity by microinjection of muscimol (a $GABA_A$ receptor agonist) directly into the B3 region prevented the inhibitory effect of dPAG activation on the cardiac baroreflex. Conversely, neuron activation by local application of D,L-homocysteic acid into B3 region caused baroreflex inhibition that was suppressed by microinjection of granisetron (a $5\text{-}HT_3$ antagonist) into the nucleus tractus solitarius. These results show that activation of serotonergic cells in the mid-portion of B3 group is critical to trigger baroreflex inhibition occurring during the defense reaction evoked by dPAG stimulation. J. Comp. Neurol. 506:108–121, 2008.

Indexing terms: heart rate; serotonin; c-Fos; tractus solitarius; periaqueductal; ventromedial medulla

The baroreflex is a regulatory mechanism devoted to maintain constant blood pressure (Dampney et al., 2002). However, to permit an adequate response of the animal during acute stressful conditions, like those at the origin of the defense reaction (Hilton, 1963), the blood pressure and the heart rate must be increased in order to enhance the muscular blood flow. In agreement with this physiological need (Nosaka, 1996), several studies showed that the cardiac (parasympathetic) response of the baroreflex is suppressed during the defense reaction (Inui and Nosaka, 1993; DiMicco et al., 2002; Sevoz-Couche et al., 2003).

Serotonin (5-HT) plays an important role in the central control of cardiovascular parameters (Laguzzi et al., 1984; Wolf et al., 1985). Recently, we found that 5-HT mediates the inhibition of the cardiac baroreflex elicited by stimulation of the dorsolateral part of the periaqueductal gray (dPAG) (Comet et al., 2004), a key area within the circuits underlying the defense reaction (Hockman and Talesnik, 1971; Hilton and Redfern, 1986; Yardley and Hilton, 1986; Bandler et al., 2000).

The 5-HT effect appeared to be mediated through activation of $5\text{-}HT_3$ receptors located in the nucleus tractus solitarius (NTS) (Sevoz-Couche et al., 2003), where arterial baroreceptor afferents project (Reis et al., 1981). However, the source of 5-HT released within the NTS during dPAG activation to induce inhibition of the baroreflex is still unknown.

To address this question we performed a first set of experiments, aimed at determining the location of central

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serotonergic cells activated during the defense reaction induced by dPAG electrical stimulation. Using double immunolabeling in urethane-anesthetized rats, we analyzed, within 5-HT-immunoreactive cells, the increase of c-Fos protein evoked by electrical stimulation of dPAG. In a second set of experiments, we directly examined the implication of the serotonergic region identified previously (the B3 group) in the inhibition of the baroreflex cardiac response triggered by dPAG stimulation. We first analyzed the effect of inactivation of the B3 region on the suppression of the aortic baroreflex cardiac response (ACR) induced by dPAG activation (either by electrical stimulation or by local microinjection of D,L-homocysteic acid [DLH]). Inactivation of the B3 region was achieved with local microinjections of muscimol (a specific $GABA_A$ receptor agonist, which induces a local blockade of synaptic transmission). Finally, we analyzed the capacity of B3 activation (by local microinjection of DLH) to inhibit ACR, before and after NTS 5-HT_3 receptor blockade by microinjection of granisetron into the NTS.

MATERIALS AND METHODS

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. 75-116 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 pressure 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 second). Rectal temperature was maintained at 37°C with a thermostatically controlled heating blanket.

Experiment 1. Immunohistochemical labeling: c-Fos evoked in serotonergic cells by dPAG stimulation

Electrical stimulation of the dPAG. Urethane-anesthetized rats were placed in a stereotaxic frame with the head fixed in horizontal position. A craniotomy was performed and a bipolar stimulating electrode was lowered into the left dPAG using predetermined (Sevoz-Couche et al., 2003) stereotaxic coordinates (P −6.7; L 0.5; V −4.5, in mm from bregma) from Paxinos and Watson's atlas (2005). This region was identified by monitoring cardiovascular responses: an increase in both mean blood pressure and heart rate, caused by local electrical stimulation (50 Hz, 1 ms pulse duration, 150 μA, 20 minutes with 10 s-on/10 s-off). In the sham group the electrode was lowered into dPAG but no current was passed through and the baseline cardiovascular parameters were not changed.

Perfusion. Two hours after the end of the stimulation, anesthetized animals were perfused through the heart with: 1) warm (37°C) heparinized saline (0.9% NaCl) solution (over 3 minutes), 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 (over 20 minutes), and finally by 3) 20% sucrose solution (over 10 minutes). 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 four containers filled with PBS, allowing their parallel processing as four serial groups of floating sections.

Immunohistochemistry. To label c-Fos protein (c-Fos), two series of sections were preincubated for 2 hours 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 c-Fos antibody (1/8,000, polyclonal IgG made in rabbit against amino acids

Abbreviations

3, 7, and 10,	Nuclei of 3rd, 7th, and 10th nerves
4v,	4th ventricle
5-HT,	Serotonin, 5-hydroxytryptamine
6n, 7n, 8n,	6th, 7th, 8th nerves
ACR,	Aortic cardiac response
acs7,	Accessory nucleus of 7th nerve
AS,	Aortic depressor nerve
B3,	3rd serotonergic group (including the RMg and the LPGi)
c-Fos,	C-Fos protein
cRPa,	Caudal RPa
Cu,	Cuneate nucleus
DAB,	3,3′-diaminobenzidine
DCDp,	Dorsal cochlear nucleus deep core
DLH,	D,L-Homocysteic acid
dPAG,	Dorsolateral PAG
ECG,	Electrocardiogram
Gi,	Gigantocellular reticular nucleus
Gr,	Gracile nucleus
HR,	Heart rate
icp,	Inferior cerebellar peduncle
lPAG,	Lateral PAG
LPGi,	Lateral paragigantocellular reticular nucleus
LSO,	Lateral superior olive
ml,	Medial lemniscus
mlf,	Medial longitudinal fasciculus
MSO,	Medial superior olive
NGS,	Normal goat serum
NTS,	Nucleus tractus solitarius
PAG,	Periaqueductal gray matter
PBS,	Phosphate-buffered saline
PCRt,	Parvicellular reticular nucleus
Pn,	Pontine nuclei
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
rRPa,	Rostral RPa
RVLM,	Rostroventrolateral medulla
sol,	Tractus solitarius
sp5,	Spinal trigeminal tract
Sp5,	Trigeminal nucleus
SPO,	Superior paraolivary nucleus
VC,	Ventral cochlear nucleus
Ve,	Vestibular nuclei

115–165 of human c-Fos; Santa Cruz Biotechnology, 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 c-Fos antibody has the expected molecular weight of ≈62 kDa (pers. commun.). We confirmed in our laboratory that after noxious stimuli the c-Fos labeling markedly increased in the dorsal horn, in agreement with consensus data (Hunt et al., 1987; Menetrey et al., 1989; Bullit, 1990). After incubation with c-Fos antibody, sections were rinsed for 30 minutes with PBS, then incubated for 1 hour with the secondary antibody (biotinylated goat antirabbit, 1/200, Vector, Burlingame, CA) in PBS containing 0.4% Triton X-100 and 1% NGS. After a second rinse for 30 minutes with PBS, the sections were finally incubated for 1 hour 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 minutes with PBS, then 15 minutes 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 minutes 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 was mounted on gelatin-coated slides and coverslipped. This first series was thus labeled only for c-Fos.

For the second series, sections were first processed as above and then underwent a second procedure to label 5-HT. This procedure was similar to the first one except that: 1) the primary antibody was anti-5-HT (1/160, 000; polyclonal antibody made in rabbit against serotonin conjugated to bovine serum albumin [BSA] by glutaraldehyde, Calbiochem, La Jolla, CA; refs PC 228L, batch D21789-1; the specificity of the 5-HT antibody was controlled after preadsorption against BSA and the distribution of 5-HT immunoreactive cells found with this antibody matched that described in the literature [Steinbusch, 1981]); the primary antibody solution also contained 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 c-Fos and 5-HT.

The third series of sections underwent processing for 5-HT labeling only and the fourth series was not processed for immunohistochemistry but run in parallel for Nissl staining by thionin.

Visualization and counting of c-Fos- and 5-HT-immunoreactive neurons. Neurons were observed under brightfield illumination in 50-μm-thick coronal sections (200 μm apart) in regions containing serotonergic neurons (the lateral paragigantocellular reticular [LPGi] nucleus, the raphe magnus [RMg], the raphe pallidus [RPa], the raphe obscurus [ROb], and the raphe dorsalis [RDr] nuclei). Location of brainstem nuclei containing 5-HT neurons was based on observation of adjacent Nissl-stained sections. Nomenclature and boundaries of these areas were adopted according to Paxinos and Watson (2005).

We took into account c-Fos + 5-HT double-labeled neurons after careful individual examination of each neuron at high magnification (40×). Double-labeled as well as c-Fos- or 5-HT-single-labeled neurons were plotted and counted with a camera lucida attachment.

Digitized photomicrographs were made using a CCD color video camera, connected to a microscope, which sent a tricolor (RGB) output to a Macintosh computer. Images at different focal planes were captured and digitized using a 24-bit color-scale Openlab software (Improvision, Conventry, 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 of red, green, and blue color plans. Images were exported to Adobe Photoshop (v. 6.0, San Jose, CA) 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.

Experiment 2. Chemical blockade or activation of the serotonergic region found in experiment 1: effect on the cardiac baroreflex

General procedure for microinjections. Rats underwent the same surgery as that used in Experiment 1. Either a bipolar stimulating electrode or a single-barrel glass micropipette (<100 μm external diameter) connected to a Hamilton microsyringe was lowered unilaterally into the dPAG at the same stereotaxic coordinates as those used in Experiment 1. This region was identified by recording the typical cardiovascular responses of the defense reaction (i.e., increases in mean blood pressure and heart rate) induced by either local electrical stimulation (50 Hz, 1 ms pulse duration, 150 μA, 5 s) or microinjection of D,L-homocysteic acid (DLH, 0.3 M, 100 nL). The microinjection of saline into dPAG produced no cardiovascular effects (Comet et al., 2004).

In addition, a craniotomy was performed to allow the chemical activation (with DLH) or blockade (with muscimol, 5 mM, 100 nL; in the range of doses used previously [Blessing and Nalivaiko, 2001; Morrison, 2003]) of regions found in Experiment 1 to present an increased density of double-labeled (c-Fos+5-HT) cells following dPAG electrical stimulation.

Sites of microinjections were identified in brain coronal sections (70 μm) stained with thionin, either by local deposit of Fast green (2%) at the tip of the micropipette previously used for microinjections of DLH or muscimol into the B3 region or granisetron into NTS, or by the track of the micropipette in case of chemical stimulation of dPAG.

Quantification of the aortic cardiac baroreflex. The left aortic depressor nerve (AS) was dissected from the vagus nerve by a lateral approach and placed on silver bipolar hook electrodes for electrical stimulation. The baroreflex response was triggered by electrical stimulation of the aortic nerve (20 Hz, 1 ms, 100 μA). The aortic cardiac response (ACR) was defined as the ratio of the

maximal decrease in heart rate (ΔHR) over HR baseline value (ACR = ΔHR / HR baseline).

Effects of chemical and electrical activation of dPAG on the cardiac baroreflex. The AS was stimulated for 4 seconds while monitoring cardiovascular changes (ACR "control"). Five minutes later, electrical (50 Hz, 1 ms pulse duration, 150 μA, 10 s) or chemical (DLH 0.3 M, 100 nL) stimulation of dPAG was performed. "Experimental" baroreflex responses were determined ≈5 seconds after the beginning of electrical stimulation or 60 seconds after DLH (chosen as the delays after which the best inhibition of ACR was observed in both cases) or saline microinjection (for control).

Effects of chemical blockade of B3 region on the inhibition of the cardiac baroreflex induced by dPAG chemical and electrical stimulation. Ten minutes after microinjection of muscimol (5 mM, 100 nL) or saline (for control) into the B3 region the effect of dPAG electrical or chemical stimulation upon ACR was analyzed as described above and compared with the effect of dPAG stimulation without B3 microinjections.

Effects of NTS 5-HT$_3$ receptor blockade on the inhibition of the cardiac baroreflex induced by chemical stimulation of the B3 region. Under the same conditions as those used previously for dPAG stimulation (Sévoz-Couche et al., 2003; Comet et al., 2004), we analyzed whether B3 stimulation-evoked effects on ACR could be prevented by prior (10 minutes) bilateral microinjections into the NTS of granisetron (250 pmol in 100 nL of saline), a selective 5-HT$_3$ receptor antagonist (Hoyer et al., 2002).

Statistical analyses

Absolute values are expressed as means ± SEM of n rats. Statistical analyses were performed using a two-way (variable factors: ACR, dPAG stimulation, or B3 microinjections) or a three-way (variable factors: ACR, dPAG stimulation, B3 microinjections, or ACR, B3 microinjections, NTS microinjections) analysis of variance (ANOVA) as stated, followed by Bonferroni's test. Statistical analysis of the c-Fos + 5-HT cell numbers in Experiment 1 and the basal cardiovascular parameters' modifications following local microinjections in Experiment 2 were made using Student's paired *t*-test.

RESULTS

Experiment 1. Effects of dPAG stimulation on c-Fos expression in serotonergic cells

The c-Fos expression, evoked by electrical stimuli applied into dPAG, was examined in brainstem areas containing serotonergic neurons known to project on the NTS. In these areas we analyzed the resulting increase in the number of c-Fos-expressing neurons within two populations, the serotonergic one (increase of c-Fos + 5-HT double-labeled neurons) and the nonserotonergic one (increase of c-Fos single-labeled neurons). These increases were determined by comparing an experimental group of rats (n = 6) subjected to dPAG stimulation with a sham group (n = 5) processed under the very same conditions, but without dPAG stimulation.

Electrical stimulation of the dPAG evoked a strong increase of c-Fos expression around the site of stimulation and bilaterally in most of PAG extent (data not shown). In particular, a high density of c-Fos-labeled neurons was systematically found in the contralateral dPAG, indicating that this area was activated during our repetitive unilateral stimulation procedure. Such bilateral activation of the dPAG might explain the bilateral increase in c-Fos expression depicted below. Among the serotonergic groups projecting to the NTS, dPAG stimulation evoked a strong increase of c-Fos immunolabeling only in the B3 group, which includes the RMg and the LPGi (Fig. 1).

The increase in c-Fos + 5-HT double-labeled neuron number was confined to the mid-rostrocaudal extent of the B3 serotonergic group (Fig. 1B1), from 11.3–10.4 mm behind the bregma (Paxinos and Watson, 2005). At this level a strong increase of c-Fos expression in serotonergic neurons was evoked bilaterally by dPAG stimulation in both the RMg and the LPGi (Fig. 1B2), with a moderate predominance on the side ipsilateral to stimulation. Considering absolute values, i.e., mean number of neurons per rat, it could be determined that the number of double-labeled neurons increased by 108% (from 225 ± 19 to 468 ± 43 neurons per rat; $n_1 = 5$, $n_2 = 6$, $P < 0.001$) in the ipsilateral LPGi, by 104% (from 94 ± 9 to 191 ± 21; $P < 0.01$) in the ipsilateral RMg, by 98% (from 85 ± 9 to 167 ± 27; $P < 0.05$) in the contralateral RMg, and by 79% (from 227 ± 20 to 407 ± 54; $P < 0.01$) in the contralateral LPGi. Among 5-HT immunoreactive neurons the mean proportion of double-labeled 5-HT + c-Fos neurons in the ipsilateral LPGi increased from 53 ± 4% (225 out of 425 serotonergic neurons per rat) in sham rat to 81 ± 4% (468 out of 576) in dPAG-stimulated rats. This proportion increased from 22 ± 3% (sham: 94 out of 425) to 46 ± 2% (dPAG stimulation: 191 out of 414) in the ipsilateral RMg. Corresponding percentages were 20 ± 2% (85 out of 432) and 40 ± 6% (167 out of 419) in the contralateral RMg, and 50 ± 2% (227 out of 454) and 76 ± 3% (407 out of 534) in the contralateral LPGi. Importantly, the absolute number of 5-HT neurons counted did not change after dPAG stimulation in any of the serotonergic groups examined above, except for the ipsilateral LPGi. In this nucleus the number of labeled serotonergic neurons was 425 ± 35 (n = 5) in sham conditions and increased to 576 ± 37 neurons (n = 6) per rat ($P < 0.05$) after dPAG stimulation. This explains why the increases in absolute and relative values were not identical for the ipsilateral LPGi.

As illustrated in Figure 2A, the number of c-Fos + 5-HT double-labeled neurons was already substantial in the mid-rostrocaudal extent of the B3 group (in both the LPGi and the RMg) in sham animals. Nevertheless, this number increased markedly in dPAG-stimulated rats (Fig. 2B). The marked increase of double-labeled neurons in ipsilateral LPGi is illustrated at higher magnification in Figure 3A,B.

In contrast, in the rostral and the caudal portions of the B3 area (Fig. 1A,C, respectively), no increase of c-Fos expression in response to dPAG stimulation was observed in serotonergic neurons (Fig. 1A2,C2). Among 5-HT immunoreactive neurons the mean proportion of double-labeled 5-HT + c-Fos neurons was 18 ± 4% (37 out of 207 serotonergic neurons, per rat) in sham conditions and remained stable at 22 ± 2% (45 out of 205) after dPAG stimulation in the rostral portion of the B3 area. In the caudal portion of the B3 area, this percentage was 5 ± 1% (32 out of 648) and 7 ± 2% (43 out of 612) in sham conditions and after dPAG stimulation, respectively.

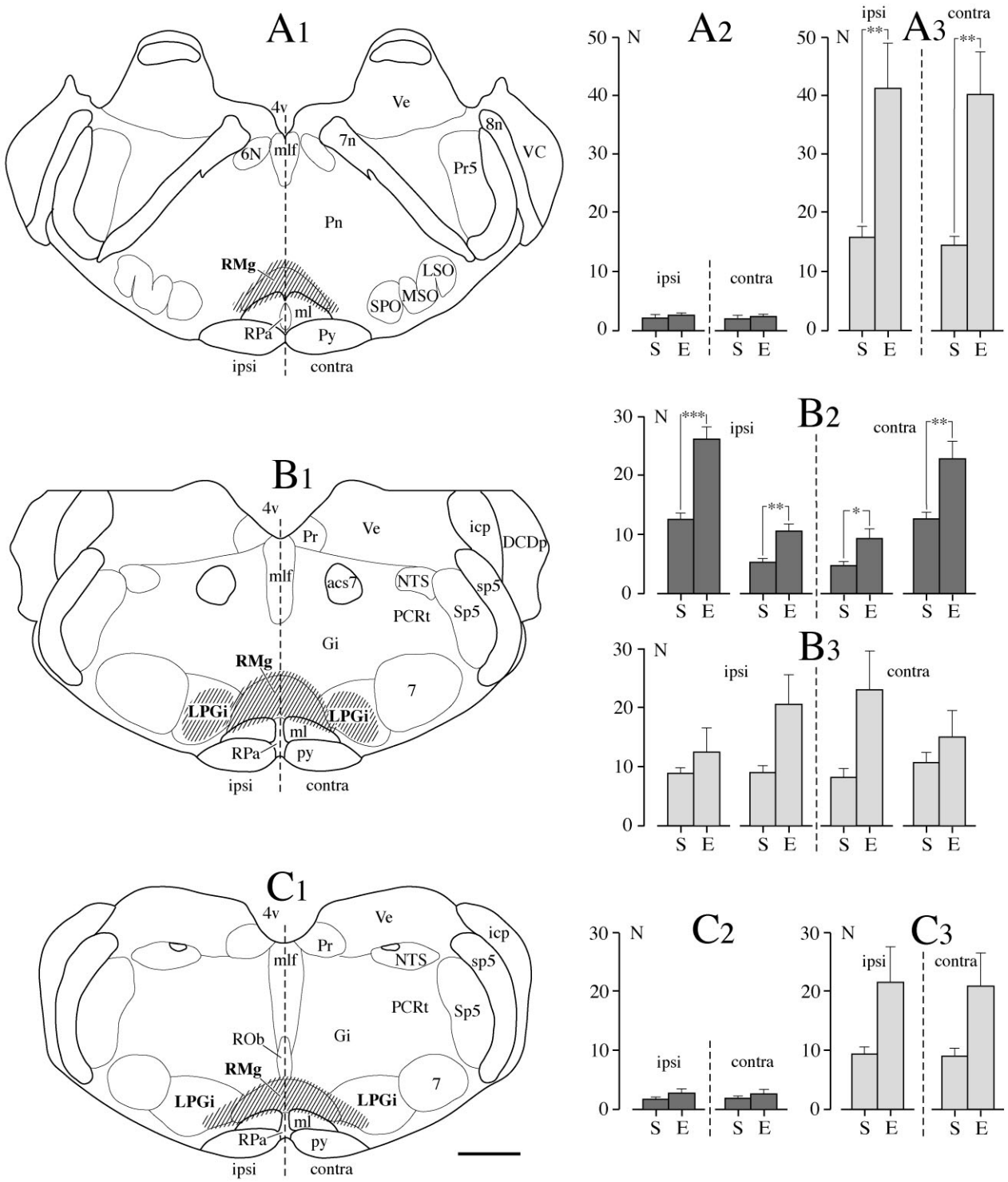

Fig. 1. Location and quantification (mean number of immunolabeled neurons per section) of c-Fos increase within B3 subregions in response to dorsal periaqueductal gray (dPAG) stimulation. **A1,B1,C1:** Sections of the brainstem at the level of the rostral, mid-rostrocaudal, and caudal portions (bregma −10.2, −10.9, and −11.8 mm, respectively; Paxinos and Watson, 2005) of the B3 serotonergic group (hatched area). The histograms of the mean number per section (N ± SEM) of serotonergic cells labeled for c-Fos (filled in dark gray [A2,B2,C2]) and nonserotonergic cells labeled for c-Fos (filled in light gray [A3,B3,C3]) are presented facing the level of the B3 region analyzed. **A2,A3:** Histograms corresponding to the rostral portion of the B3 region (RMg [ipsi] and RMg [contra] were analyzed separately). **B2,B3:** Histograms corresponding to the mid-rostrocaudal extent of the B3 region, with, from left to right, analysis in LPGi (ipsi), RMg (ipsi), RMg (contra), and LPGi (contra). **C2,C3:** Histograms corresponding to the caudal portion of the B3 region (RMg [ipsi] and RMg [contra] were analyzed separately). Ipsi: side ipsilateral to the dPAG stimulus; contra: contralateral side; dashed line: separation between ipsi and contralateral side for both sections and histograms; S: "Sham" group without stimulation of dPAG (n = 5); E: "Experimental" group with dPAG stimulation (n = 6); N: number of neurons per section; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. Abbreviations: see list. Scale bar = 1 mm.

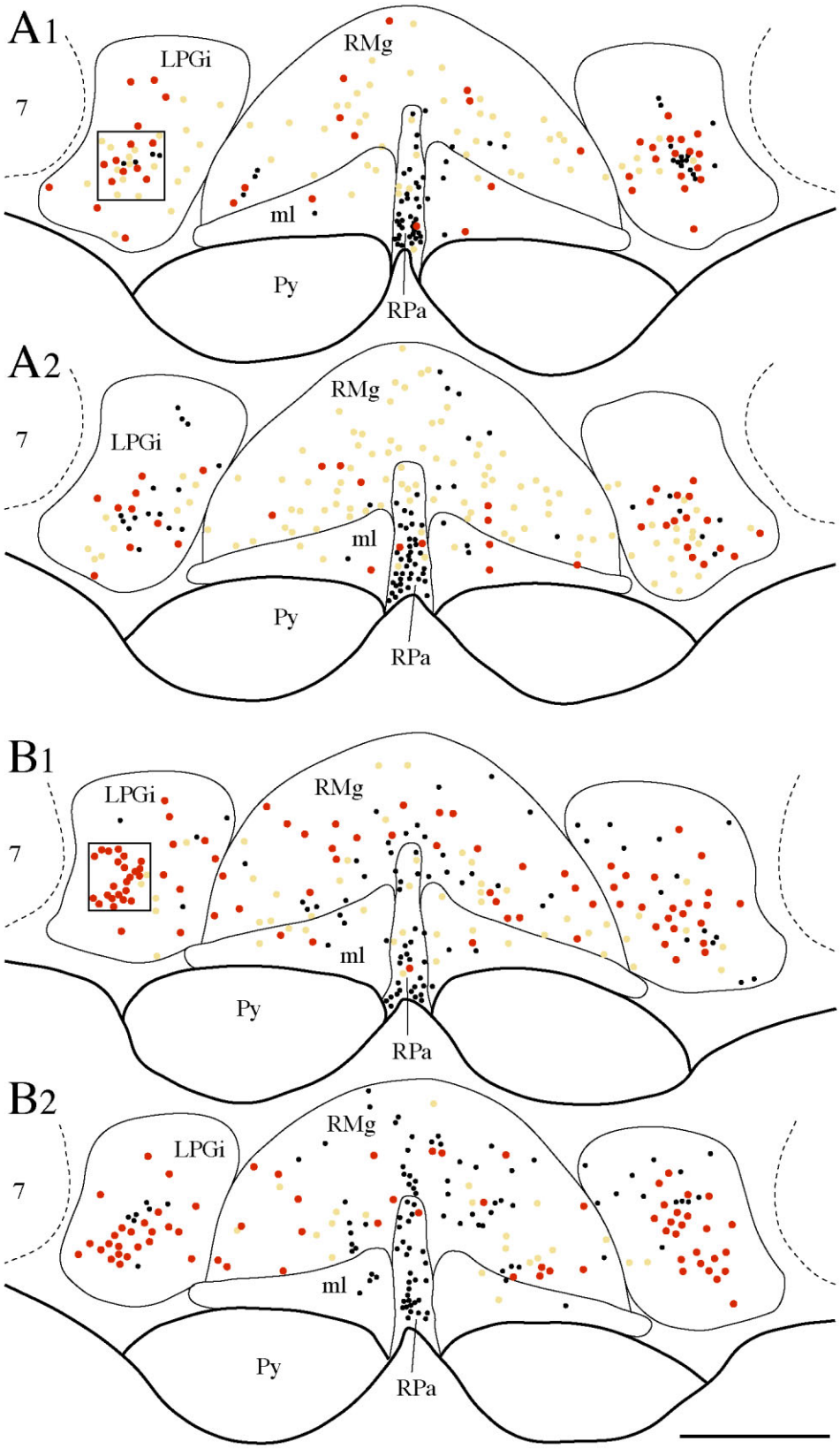

Fig. 2. Camera lucida drawing of neurons labeled for c-Fos and/or 5-HT at two levels of the mid-rostrocaudal extent of the B3 region. The drawings depict neurons expressing 1) both c-Fos and 5-HT (red circle), 2) only 5-HT (yellow point), or 3) only c-Fos (black point). One "Sham" (not stimulated) animal (**A1,2**) is illustrated in coronal sections at levels caudal to bregma approximately −10.9 mm and −10.6 mm, respectively (Paxinos and Watson, 2005). One "Experimental" animal, with dPAG stimulation (**B1,2**), is illustrated at the same levels for comparison. The framed areas in A1 and B1, in the ipsilateral LPGi, indicate the location of the high-magnification microphotographs in Figure 3A,B. Note the increased density of double-labeled neurons and the decreased density of single 5-HT-labeled neurons in B1,2 compared with A1,2. Abbreviations: see list. Scale bar = 500 μm.

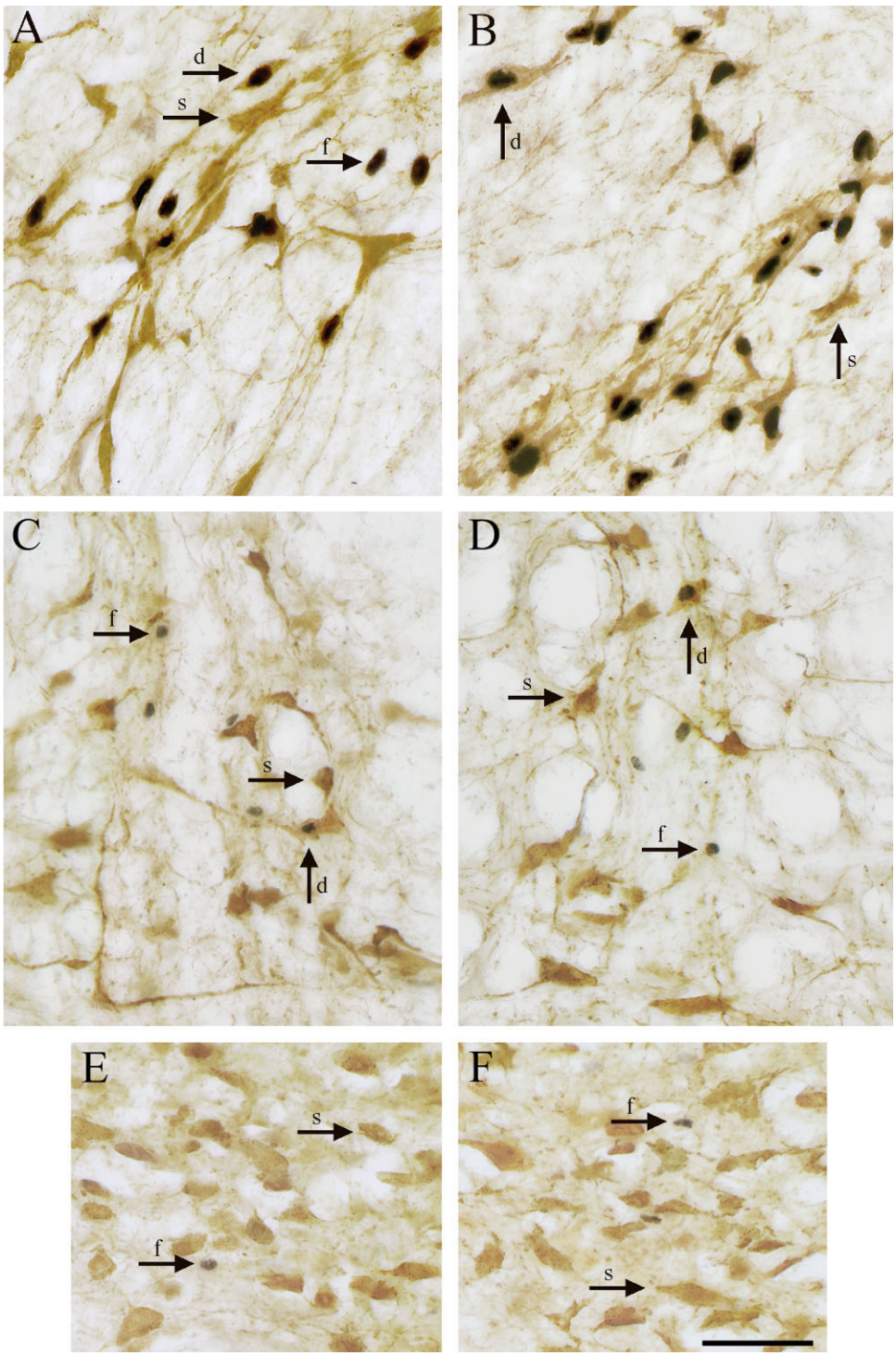

Fig. 3. Color digitized photomicrographs of neurons labeled for c-Fos and/or 5-HT in three serotonergic groups. **A,B:** Photomicrographs of the lateral paragigantocellular (LPGi) regions framed in Figure 2A1,B1, respectively. A: "Sham," not stimulated, animal. B: "Experimental" animal, with dPAG stimulation. Note, 1) the high c-Fos expression and 2) the increased number of double-labeled neurons in B compared to A. **C,D:** Photomicrographs of the raphe obscurus nucleus in "Sham" and "Experimental" rats, respectively. **E,F:** Photomicrographs of the raphe dorsalis nucleus in "Sham" and "Experimental" rats, respectively. Note, 1) the low c-Fos expression in C–F, and 2) the absence of c-Fos change from C to D and from E to F. Arrows with d, s, and f indicate examples of c-Fos + 5-HT double-labeled (black core surrounded by brown staining), 5-HT single-labeled (cytoplasm brown staining), and c-Fos single-labeled (nucleus black staining) neurons, respectively. Scale bar = 50 μm.

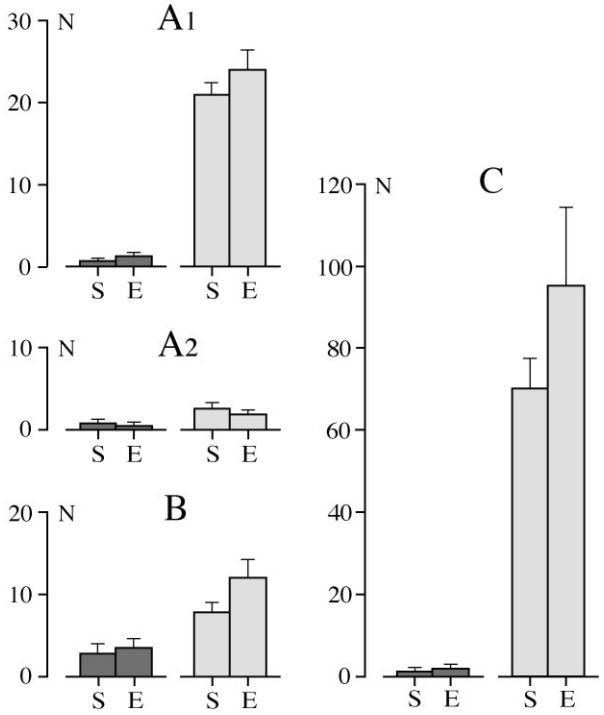

Fig. 4. Histograms of the mean number per section (N ± SEM) of serotonergic cells labeled for c-Fos (dark gray) and nonserotonergic cells labeled for c-Fos (light gray) in serotonergic brainstem regions outside the B3 group. **A1:** Rostral portion of the raphe pallidus nucleus; A2: caudal portion of the raphe pallidus nucleus. **B:** Raphe obscurus nucleus. **C:** Raphe dorsalis nucleus. S: "Sham", not stimulated group, (n = 5); E: "Experimental" group with dPAG stimulation (n = 6); N: number of neurons per section.

The pattern of c-Fos increase in nonserotonergic neurons within the B3 area was different from the one observed in serotonergic neurons (Fig. 1A3). In the rostral portion of RMg the absolute number (mean number of neurons per rat) of c-Fos single-labeled neurons increased by 160% and 175% (from 285 ± 32 to 738 ± 136 and from 261 ± 25 to 720 ± 129 neurons per rat; $n_1 = 5$, $n_2 = 6$, $P < 0.01$ in each case) on the ipsilateral and contralateral sides, respectively. In the middle and caudal portion of the B3 region we observed only a tendency to an increase of c-Fos single-labeled neurons in the proper RMg, excluding the LPGi nuclei (Fig. 1B3,C3).

In other 5-HT neuron-containing regions such as the rostral RPa (rRPa) (surrounded by the RMg, see Fig. 2), the caudal RPa (cRPa) (caudal to RMg), the ROb, and the RDr the stimulation of dPAG did not evoke any significant increase of c-Fos expression in serotonergic as well as nonserotonergic neurons (see absolute numbers in Figs. 3C–F and 4). Interestingly, the medial portion of RDr contained only a very few c-Fos single-labeled neurons (Fig. 3E,F). The substantial number of c-Fos single-labeled neurons indicated in the histogram in Figure 4C was confined to the external portion of RDr, which spreads in the ventrolateral PAG. Among 5-HT-immunoreactive neurons in the rRPa the mean proportion of double-labeled 5-HT + c-Fos neurons was low in sham rats: 11 ± 3% (16 out of 143 serotonergic neurons, per rat), and did not significantly change at 18 ± 4% (25 out of 138) after dPAG stimulation. In the cRPa it was 6 ± 3% (22 out of 371) under sham conditions and 3 ± 1% (12 out of 397) after dPAG stimulation. Corresponding percentages were 9 ± 4% (79 out of 877) and 11 ± 3% (96 out of 872) in the ROb, and 0.8 ± 0.6% (29 out of 3,635) and 1.3 ± 0.3% (47 out of 3,592) in the RDr.

Experiment 2. Effects of chemical blockade or activation of the serotonergic region found in Experiment 1 on the cardiac baroreflex

In the second set of experiments we used local microinjections of inhibitory substances into the regions (at −11.0 mm caudal to bregma) identified in Experiment 1: the middle caudorostral extent of RMg and adjacent LPGi, as those where dPAG stimulation produced a marked increase in the density of double-labeled (c-Fos +5-HT) cells. The aim of these experiments was to examine the effects of RMg and LPGi inactivation on the inhibition of the baroreflex bradycardia occurring during dPAG stimulation that evoked a defense-like reaction. The effects of chemical activation of RMg and LPGi by local microinjection of DLH was also investigated in the same series of experiments.

In urethane-anesthetized animals, baseline values of mean blood pressure and heart rate were 97 ± 4 mmHg and 399 ± 14 bpm, respectively.

Cardiovascular responses to electrical or chemical activation of dPAG. Electrical stimulation of the dPAG reduced the bradycardia elicited by aortic nerve stimulation by 72% (ACR: −0.29 ± 0.01 [−115 ± 8 bpm from a baseline of 400 ± 8 bpm] before stimulation; −0.08 ± 0.01 during dPAG stimulation; n = 5, $P < 0.005$, 2-way ANOVA) (Fig. 5A). Microinjection of DLH into the dPAG produced similar effects (68% reduction; ACR reduced from −0.33 ± 0.02 to −0.10 ± 0.01; n = 15, $P < 0.005$) (Fig. 5B).

In addition, electrical as well as chemical dPAG activation produced significant increases in heart rate and mean blood pressure (Table 1).

Effects of muscimol microinjection into the B3 region on the cardiovascular responses to electrical or chemical activation of dPAG. After microinjections of muscimol (5 mM), a specific $GABA_A$ receptor agonist, into the intermediate B3 area (Fig. 6B), ACR was only reduced by −17% (ACR "control" and ACR "experimental": −0.27 ± 0.01 and −0.22 ± 0.02, respectively, n = 5) during dPAG electrical stimulation (Fig. 5A), and by −12% (ACR "control" and ACR "experimental": −0.29 ± 0.02 and −0.26 ± 0.02, respectively, n = 15) during DLH-evoked chemical activation of dPAG (Fig. 5B).

In addition, microinjections of muscimol into the B3 area produced a transient (5-minutes) decrease in both heart rate and mean blood pressure (Table 1). However, these microinjections did not modify (2-way ANOVA, $P > 0.05$) the increases in heart rate (+73 ± 6 bpm and +70 ± 5 bpm, after muscimol) and mean blood pressure (+42 ± 7 mmHg and 48 ± 5 mmHg, after muscimol) normally induced by electrical or chemical activation of dPAG, respectively (see Table 1).

As controls, we verified that 1) electrical and chemical dPAG activation-induced inhibition of ACR after saline microinjections into the B3 region at the same sites (−69% and 70%, respectively, n = 4 in both cases) was not sig-

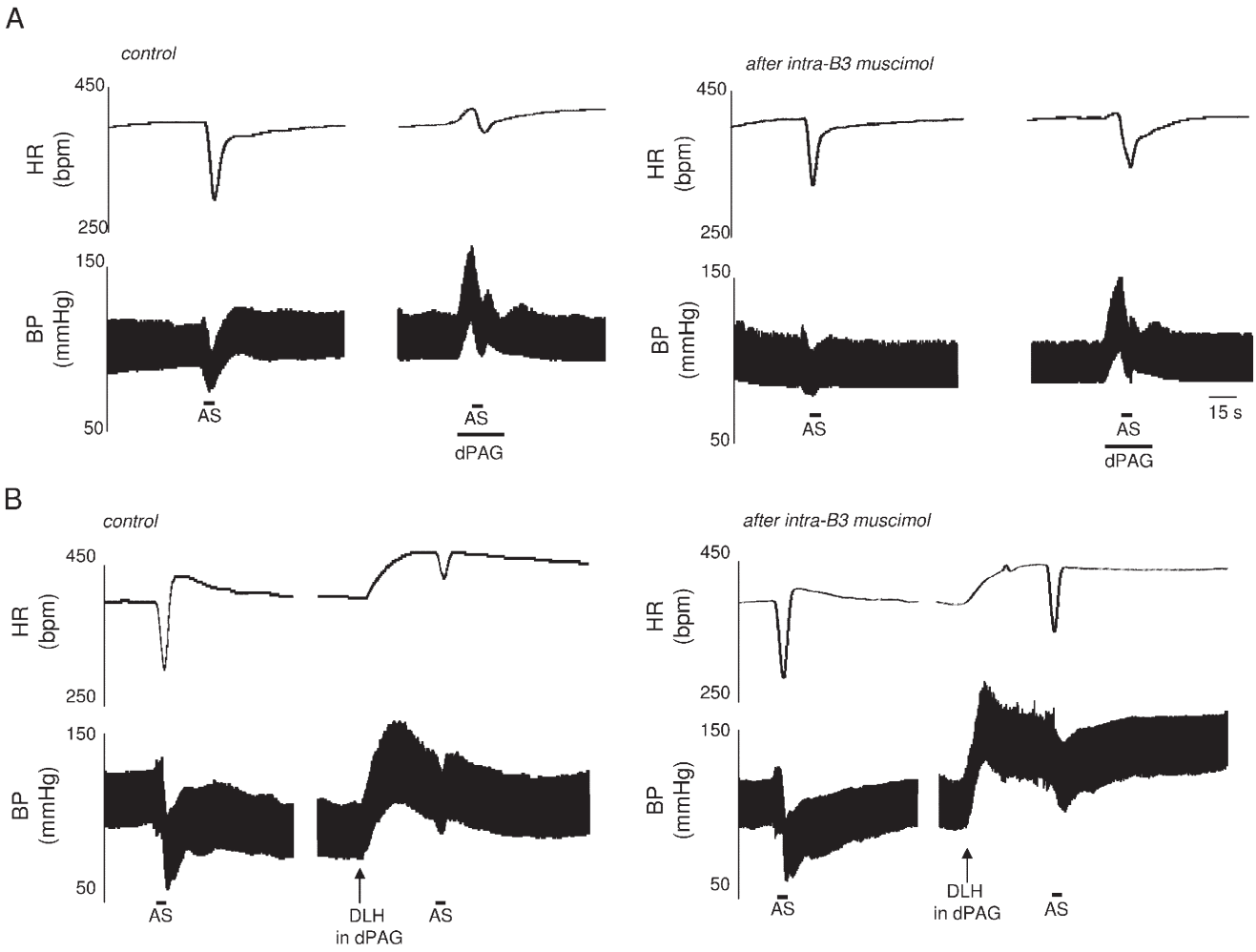

Fig. 5. Inhibitory effects of electrical and chemical stimulation of dPAG on baroreflex cardiac responses before (control) and after muscimol microinjections into the B3 region. Representative tracings showing that the baroreflex bradycardia evoked by AS was reduced (control, left) by electrical (**A**) and chemical (DLH, 0.3 M) (**B**) activation of dPAG. Pretreatment with muscimol (5 mM) into the B3 region prevented (right) the inhibitory effects of both types of dPAG activation on the baroreflex bradycardia.

TABLE 1. Changes in Basal Mean Blood Pressure (ΔMBP) and Heart Rate (ΔHR) Induced by Electrical Stimulation of the Dorsolateral Quadrant of the Periaqueductal Gray (dPAG-elec), and Microinjections of D,L-Homocysteic Acid (DLH) into dPAG (dPAG-DLH), Muscimol, or DLH into B3 Region (B3-muscimol or B3-DLH, Respectively), and Granisetron into the Nucleus of the Tractus Solitarius (NTS-granisetron)

Treatment	*n*	ΔMBP (mmHg)	ΔHR (bpm)
dPAG-elec	5	+45 ± 5* (101 ± 4)	+70 ± 10* (405 ± 10)
dPAG-DLH (0.3 M, 0.1 μL)	15	+43 ± 4* (97 ± 5)	+74 ± 9* (386 ± 8)
B3-muscimol (5 mM, 0.1 μL)	16	−27 ± 4* (99 ± 6)	−44 ± 6* (397 ± 13)
B3-DLH (0.3 M, 0.1 μL)	20	+22 ± 2* (98 ± 3)	+40 ± 5* (405 ± 11)
NTS-granisetron (2.5 mM, 0.1 μL)	7	+3 ± 1 (100 ± 4)	+4 ± 3 (398 ± 7)

Negative or positive numbers indicate a decrease or an increase, respectively, in MBP and HR from the baseline values indicated in parenthesis. Values are the means ± SEM of independent determinations in *n* rats.
*$P < 0.05$ (Student's *t*-test).

nificantly different from that obtained in nonpretreated rats (see above), and 2) ACR was not significantly affected (2-way ANOVA, $P > 0.05$) by microinjections into the B3 region of saline (+10%, n = 4) or muscimol (−11%, n = 4) in rats not subjected to dPAG activation.

Cardiovascular responses to chemical activation of B3 region before and after injection of granisetron into the NTS. ACR was reduced (−55%) after microinjection of DLH (n = 16) into the intermediate B3 area (Fig. 7). Indeed, ACR "control" was −0.28 ± 0.01 and ACR "experimental" was −0.13 ± 0.01 (n = 15, 2-way ANOVA, $P < 0.005$). This effect of DLH was prevented in rats that had been pretreated with granisetron (250 pmol, bilateral microinjections), a 5-HT_3 antagonist, into the commissural NTS (Fig. 6C) (ACR "control" and "experimental": 0.26 ± 0.02 and 0.25 ± 0.02, respectively, n = 7, Fig. 8).

In addition, an increase in heart rate and mean blood pressure was observed after microinjection of DLH into B3 area, but granisetron produced, on its own, no cardiovascular changes (Table 1).

DISCUSSION

The data presented herein show that the B3 area (RMg + LPGi) is the primary serotonergic source respon-

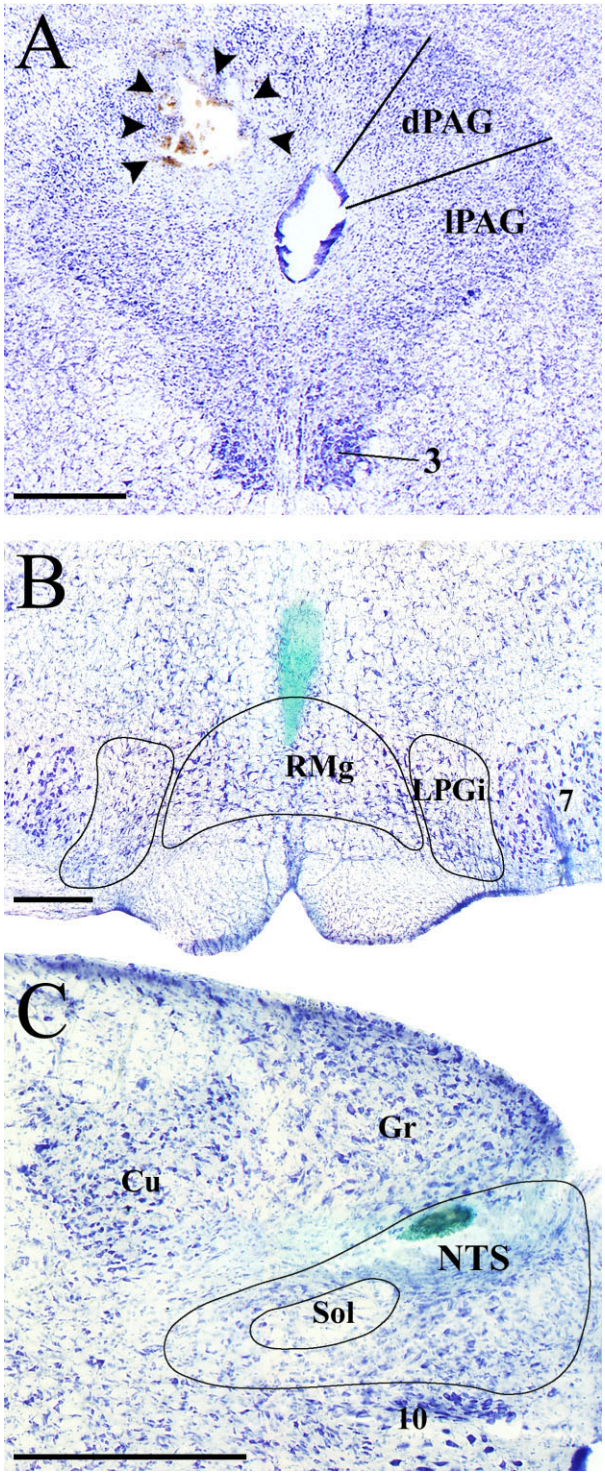

Fig. 6. Localization of chemical stimulation in dPAG (**A**) and microinjections into the B3 group (**B**) and NTS (**C**). **A:** Photomicrograph showing a representative micropipette track (arrows) for chemical stimulation (microinjection of DLH, 0.3 M) in the dPAG that produced cardiovascular responses typical of the defense reaction (coronal section, bregma level −6.8 mm; Paxinos and Watson, 2005). **B:** Photomicrograph showing Fast green deposit around the tip of the micropipette used for muscimol microinjection into the B3 region (comprising the RMg and the LPGi) (bregma level −10.7 mm). **C:** Photomicrograph showing Fast green deposit around the tip of the micropipette used for granisetron microinjection into the NTS (bregma level −13.5 mm). Abbreviations: see list. Scale bars = 500 μm (A–C).

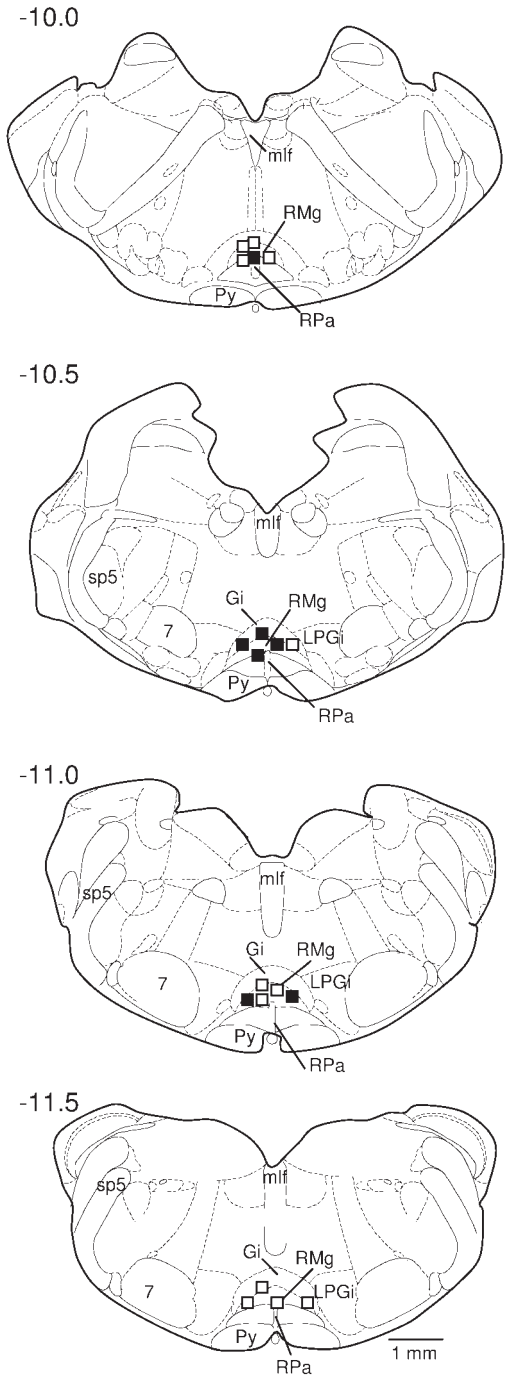

Fig. 7. Coronal brain sections showing the localization of sites where chemical (DLH, 0.3 M) stimulation was performed in the raphe magnus and the lateral paragigantocellular reticularis nuclei. Camera lucida drawings of serial coronal sections (levels from bregma are indicated on left) displaying sites where DLH microinjections produced baroreflex inhibition (filled symbols). Open symbols show locations where DLH microinjections produced no significant effect on the cardiac reflex response. Abbreviations: see list.

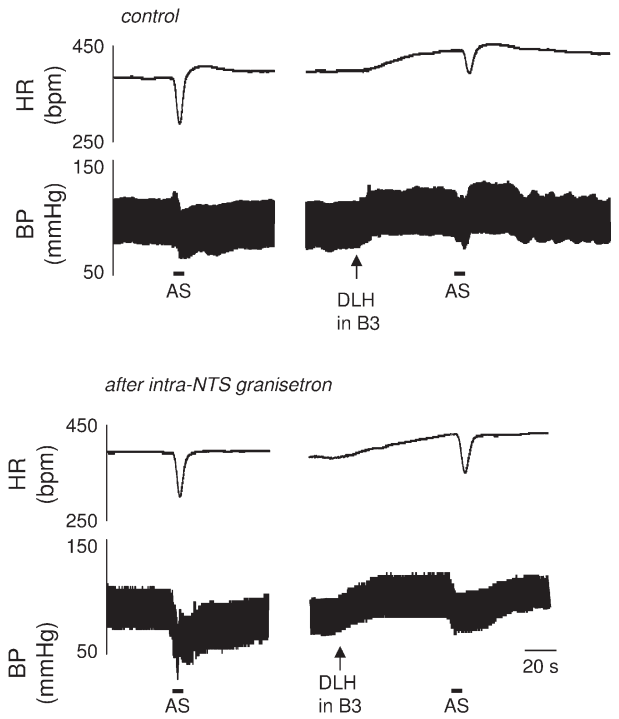

Fig. 8. Effects of microinjection of granisetron into the NTS on the inhibitory effect of B3 stimulation on baroreflex bradycardia. Representative tracings showing that the reflex bradycardia evoked by electrical stimulation of the AS was inhibited by prior microinjection of DLH (30 mM) into the intermediate B3 region (control). This inhibitory effect of DLH was prevented by microinjection of granisetron (250 pmol) into the NTS.

sible for the 5-HT-mediated inhibition of baroreflex bradycardia that occurs during the defense-like reaction evoked by dPAG stimulation in rats.

The inhibition of baroreflex bradycardia occurring during the defense-like reaction induced by electrical as well as chemical stimulation of dPAG is primarily a 5-HT-mediated phenomenon (see Introduction), acting through NTS presynaptic 5-HT_3 receptors (Sevoz-Couche et al., 2003; Comet et al., 2004). Indeed, 5-HT neuron activation is a prerequisite for the occurrence of baroreflex bradycardia inhibition evoked by dPAG stimulation. The ROb, RPa, RDr, RMg, and LPGi nuclei are at the origin of 5-HT projections to the NTS (Thor and Helke, 1987; Schaffar et al., 1988). However, up to this study, we did not know the portion of this serotonergic system that actually acts on the NTS to mediate baroreflex bradycardia inhibition evoked by dPAG stimulation.

The c-Fos immunolabeling experiments revealed that dPAG electrical stimulation evoked a strong increase of c-Fos expression in 5-HT neurons within a restricted portion of the B3 group, whereas no change was observed in other portions of the serotonergic system. In particular, the c-Fos immunolabeling technique seemed to dismiss the small group of 5-HT neurons contained in rostral RPa (surrounded by RMg 5-HT neurons) from a role in baroreflex inhibition. It is probable that the strong increase of c-Fos immunolabeling in B3 serotonergic cells was mostly due to a direct effect of dPAG stimulation rather than a possible consequence of an indirect feedback evoked from the resulting increases in blood pressure and heart rate. Indeed, no increase of c-Fos expression was described in the B3 region in response to blood pressure changes (Dampney and Horiuchi, 2003). Furthermore, the B3 region receives most of its afferent projections from the PAG (Fardin et al., 1984), whereas it receives none or only sparse projection from the NTS (the primary afferent relay for blood pressure messages) and the ventrolateral medulla (the main sympathetic center) (Hermann et al., 1997).

Although our discussion is chiefly focused on serotonergic neurons because we demonstrated that 5-HT plays a key role in baroreflex inhibition (see above), it must be noted that in the rostral RMg, c-Fos was also strongly evoked by dPAG stimulation in non-5-HT neurons. Thus, it is possible that non-5-HT neurons also contributed to some extent to the inhibition of baroreflex evoked by dPAG stimulation.

The expression of the nuclear protein c-Fos is classically considered a marker of neuron excited by synaptic activation (Hunt et al., 1987; Menetrey et al., 1989; Bullit, 1990; Morgan and Curran, 1991; Luckman et al., 1994; Hughes and Dragunow, 1995). Accordingly, our c-Fos data strongly suggest that, among 5-HT neurons, only those located within the serotonergic B3 group were excited by both the dPAG stimulation and the associated defense reaction. However, it has to be emphasized that the c-Fos technique can provide only indirect evidence in support of neuron excitation. Consequently, c-Fos data always need further confirmation using other approaches.

In this context, it was especially relevant that muscimol-induced inactivation of the mid-rostrocaudal portion of the B3 region suppressed almost completely the inhibitory effect of both electrical and chemical stimulations of dPAG on baroreflex bradycardia. These data confirmed that the B3 neurons, but not necessarily those with serotonergic phenotype, were responsible for at least 80% of the inhibition of the baroreflex bradycardia caused by the dPAG stimulation inducing defense reaction. The observation that chemical stimulation restricted to the mid-rostrocaudal portion of the B3 region inhibited baroreflex bradycardia provided additional direct support to the idea that neurons in this B3 subregion play a key role in such cardiovascular regulation. However, none of the data recalled above specifically demonstrated the involvement of serotonergic neurons in the B3 area. Importantly, the local microinjection of a 5-HT_3 antagonist (granisetron) into the NTS was found to depress the effect of B3 stimulation. This result together with the c-Fos and previous data in our laboratory (see Introduction) provide strong support for the conclusion that the serotonergic neurons within the mid-portion of the B3 group play a critical role in the baroreflex inhibition evoked by dPAG stimulation.

Our results are also congruent with anatomical data showing that the dPAG massively projects to the B3 region (Beitz et al., 1983; Fardin et al., 1984; Li and Lovick, 1985; Cameron et al., 1995; Hudson and Lumb, 1996; Hermann et al., 1997), which, in turn, provides a significant set of serotonergic projection to the NTS (Thor and Helke, 1987; Schaffar et al., 1988). Accordingly, it can be reasonably inferred that a dPAG-B3-NTS circuit is involved in baroreflex inhibition associated with the defense-like reaction evoked by dPAG stimulation.

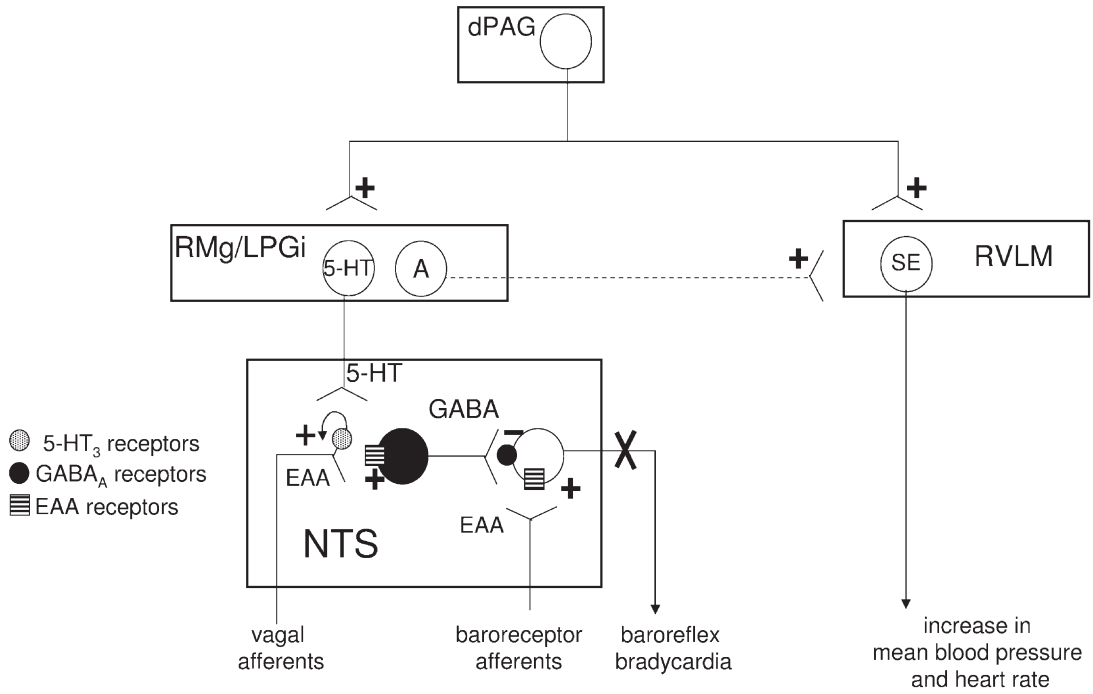

Fig. 9. Schematic representation of putative medullary pathways producing baroreflex inhibition during the defense reaction. Arterial baroreceptor afferents are known to excite barosensitive NTS cells through the activation of excitatory amino acid (EAA) receptors (small striped square) to induce vagal baroreceptor reflex bradycardia. Our previous (Sevoz-Couche et al., 2003; Comet et al., 2004) and present findings show that, during the defense-like reaction triggered by dPAG stimulation, 5-HT released from neurons originating in the RMg and the LPGi nuclei activates 5-HT_3 receptors (small dotted circle) localized presynaptically on vagal glutamatergic afferents in the NTS. The resulting facilitation of glutamate release ends with the excitation of GABAergic interneurons, which, in turn, inhibit baroreflex bradycardia through the stimulation of $GABA_A$ receptors (small black circle) on barosensitive NTS cells. In addition, the defense reaction also elicits an increase in blood pressure and heart rate through dPAG projections to sympathoexcitatory (SE) cells in the RVLM. Finally, tonic activation of nonserotonergic (A) cells in LPGi and RMg also raises blood pressure and heart rate, probably via excitation of SE neurons in RVLM (dotted line), but independently of the defense reaction. + = excitatory effect; − = inhibitory effect.

This B3 serotonergic area might mediate baroreflex inhibition not only from the dPAG but also from other central regions involved in the defense reaction such as the mediodorsal hypothalamus (Sevoz-Couche et al., 2003). Interestingly, when DLH was microinjected into the caudal portion of the B3 area, no significant effect was observed on the baroreflex (see Fig. 7), in agreement with the results of c-Fos experiments. In fact, the very caudal portion of the RMg (like the ventrolateral periaqueductal gray [vPAG]) has been found to exert a facilitatory influence on baroreflex bradycardia (Inui et al., 1994). Baroreflex bradycardia therefore appears to be controlled by both a facilitatory vPAG-caudal B3 circuit and an inhibitory dPAG-middle B3(-NTS) circuit.

The dichotomy observed between the middle B3 area (baroreflex depression) and the caudal B3 area (baroreflex facilitation) might also be extended to blood pressure changes. Indeed, we observed in the present study that chemical stimulation of middle B3, as that of dPAG, produced an increase in blood pressure, whereas Schenberg and Lovick (1994) previously reported that chemical stimulation of caudal B3 prevents, at least partly, a blood pressure increase due to dPAG stimulation. In the same line, it can be recalled that Nakamura et al. (2004, 2005) found that the caudal B3 (including caudal RMg and local portion of RPa), but not the middle B3, participated in cardiovascular changes associated with thermoregulation.

In agreement with previous studies (Nosaka et al., 1993; Comet et al., 2004), we observed that chemical stimulation of dPAG triggered typical cardiovascular responses, i.e., increases in blood pressure and heart rate, normally associated with the defense reaction. On the other hand, activation of sympathetic premotor neurons located in the rostroventrolateral region of the medulla (RVLM) (Sun and Guyenet, 1987; Guyenet et al., 1989; Cravo et al., 2003) is known to evoke such cardiovascular responses (Lovick, 1985, 1992; Verberne and Struyker Boudier, 1991) but through a nonserotonergic mechanism (Comet et al., 2004). Interestingly, we found here that chemical activation of both RMg and LPGi also produced an increase in heart rate and mean blood pressure, thereby suggesting that dPAG sympathetic responses could be due to activation of nonserotonergic neurons in RMg and LPGi. Furthermore, microinjections of muscimol into these areas did not prevent the cardiovascular modifications induced by dPAG stimulation, in agreement with previous data showing that this stimulation in fact activates directly sympathoexcitatory neurons in the RVLM (Bandler et al., 2000). Figure 9 depicts how dPAG, on the one hand, and RMg and LPGi, on the other, can send independent projections into RVLM to raise basal blood pressure and heart rate. This hypothetical view is supported by data from Hudson and Lumb (1996), which

show that dPAG sends collateral projections to both RMg and RVLM.

In conclusion, during the defense-like reaction evoked by dPAG stimulation, the inhibition of baroreflex bradycardia appears to be due to the activation of only a restricted group of serotonergic neurons in the mid-rostrocaudal extent of the B3 group that comprises the RMg and LPGi nuclei. However, the increase in basal heart rate and mean blood pressure caused by dPAG stimulation is not mediated through B3 activation, as depicted in the hypothetical model of Figure 9.

Numerous studies have also shown that serotonergic (as well as nonserotonergic) neurons in the B3 region can produce either antinociception or facilitation of nociceptive processing (Basbaum and Fields, 1984; Besson and Chaouch, 1987; Suzuki et al., 2005; but see Mason, 2005). Furthermore, the PAG and the ventromedial medulla (which comprises the B3 region) have been considered the "efferent channel" of descending pain control system from the brain to the dorsal horn in the spinal cord (Besson et al., 1991; Vanegas and Schaible, 2004). Thus, it can be suggested that, during the defense reaction, serotonergic neurons in the B3 group are responsible for several physiological responses, including antinociception and cardiovascular adjustments, which are necessary to cope with a vital emergency. However, in the case of a depressive state induced by repetitive stress, they may also be responsible for baroreflex dysregulations that contribute to cardiovascular diseases (Watkins and Grossman, 1999; Pitzalis et al., 2001; Grippo et al., 2002; Grippo and Johnson, 2002).

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