Behavioral/Systems/Cognitive

Neurokininergic Mechanism within the Lateral Crescent Nucleus of the Parabrachial Complex Participates in the Heart-Rate Response to Nociception

Pedro Boscan,[1] Mathias Dutschmann,[2] Horst Herbert,[3] and Julian F. R. Paton[1]

[1]Department of Physiology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, United Kingdom, [2]Department of Physiology, University of Göttingen, 37073 Göttingen, Germany, and [3]Department of Animal Physiology, University of Tübingen, 72076 Tübingen, Germany

We wanted to ascertain whether the lateral parabrachial nucleus was involved in mediating the heart-rate response evoked during stimulation of somatic nociceptors. Reversible inactivation of the lateral parabrachial nucleus, using a $GABA_A$ agonist, reduced the reflex tachycardia evoked during noxious (mechanical) stimulation of the forelimb by ~50%. The same effect was observed after blockade of neurokinin 1 receptors within the lateral parabrachial nucleus, indicating a possible involvement for substance P as a neurotransmitter. Immunocytochemistry revealed a strong expression of substance P-immunoreactive fibers and boutons in all lateral subnuclei, but they were particularly dense in the lateral crescent subnucleus. Histological verification showed that the most effective injection sites for attenuating the noxious-evoked tachycardia were all placed in or near to the lateral crescent nucleus of the lateral parabrachial complex. Many single units recorded from this region were activated by high-intensity brachial nerve stimulation. The brachial nerve evoked firing responses of some of these neurons was reversibly reduced after local delivery of a neurokinin 1 receptor antagonist. However, only a minority of these neurons followed a paired-pulse stimulation protocol applied to the spinal cord, suggesting a predominance of indirect projections from the spinal cord to the parabrachial nucleus. We conclude that the cardiac component of the response to somatic nociception involves indirect spinal pathways that most likely excite neurons located in the lateral crescent nucleus of the parabrachial complex via activation of neurokinin 1 receptors.

Key words: **pain; NK_1; parabrachial complex; dorsal horn; heart rate; respiratory**

Introduction

The parabrachial complex (PB) has extensive afferent and efferent connections with multiple areas throughout the central neuraxis that regulate autonomic function (Saper and Loewy, 1980; Fulwiler and Saper, 1984). In particular, the lateral parabrachial nucleus (LPB) represents an integral part of the central autonomic network controlling the cardiovascular system. Its stimulation (electrical or chemical) evokes a sympathoexcitatory effect that is manifested as tachycardia and hypertension (Mraovitch et al., 1982; Ward, 1988; Paton et al., 1990; Chamberlin and Saper, 1992; Korte et al., 1992; Lara et al. 1994).

The LPB has also been implicated in the processing of nociceptive afferent information: LPB neurons respond to noxious stimuli, as demonstrated by either single-unit recording (Bernard and Besson, 1990; Bernard et al., 1994; Bester et al., 1995; Menendez et al., 1996) or staining for c-fos expression (Lanteri-Minet et al., 1993, 1994; Bellavance and Beitz, 1996; Hermanson and Blomqvist, 1996; Bester et al., 1997). Thus, a potential role for the LPB could be in mediating the cardiovascular reflex response during nociception (Saper, 1995).

The LPB receives direct inputs from dorsal horn neurons of the spinal cord (Kitamura et al., 1989, 1993; Menetrey and De Pommery, 1991; Bernard et al., 1995; Feil and Herbert, 1995). Immunocytochemical staining revealed that lateral subnuclei of the LPB, which are innervated by ascending spinal afferents, exhibit axonal terminals positive for substance P (SP) (Milner et al., 1984; Milner and Pickel, 1986; Standeart et el., 1986; Block and Hoffman, 1987). Interestingly, this neuropeptide was also found within neurons lying within the dorsal horn of the spinal cord (Leah et al., 1988). Thus, these spinal neurons might be a possible source of SP within different subnuclei of the LPB.

Using microinjection, electrophysiological, and immunocytochemical techniques, the present study was designed to challenge the hypothesis that the LPB mediates the cardiac response to nociceptive stimulation and that this, in part, is mediated by SP. Initially, we assessed the role of the LPB in mediating the reflex cardiovascular response evoked during somatic nociception. Subsequently, we tested whether this was mediated via a spino-parabrachial pathway that released SP acting on neurokinin type 1 (NK_1) receptors on LPB neurons.

Parts of this work have been published previously (Boscan and Paton, 2001a).

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Correspondence should be addressed to Prof. Julian F. R. Paton, Department of Physiology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK. E-mail: julian.f.r.paton@bristol.ac.uk.

P. Boscan's present address: Veterinary Medical Teaching Hospital, University of California Davis, Davis, CA 95616-8747.

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Materials and Methods

Three sets of studies were performed: (1) microinjection of drugs to test the role of the LPB in mediating the tachycardia evoked after stimulation of forelimb nociceptors; (2) extracellular recording from single LPB neurons responding to somatic afferent stimulation before and during local ejection of pharmacological agents; and (3) SP immunocytochemistry within the LPB.

The working heart–brainstem preparation. The microinjection and extracellular recording experiments were performed in a decerebrate, unanesthetized, and intra-arterially perfused working heart–brainstem preparation (WHBP) (for details, see Paton, 1996) of Sprague Dawley rats (75–150 g; either sex). We chose to use the WHBP because the pattern of cardiorespiratory reflex responses evoked by stimulating noxious afferents (Boscan and Paton, 2001b, 2002a; Boscan et al., 2002a) is consistent and comparable with that reported in lightly anesthetized or conscious animals and humans (Roizen et al., 1981; Abram et al., 1983; Ness and Gebhart, 1990; Bang and Lund, 1991; Barr, 1998; al'Absi et al., 1999; Oberlander et al., 1999).

Briefly, rats were anesthetized deeply with halothane until there was no sign of withdrawal reflexes after a pinch to the tail and a forepaw. The rat was decerebrated precollicularly and cerebellectomized, sparing the pons and leaving the medulla intact. All structures rostral to the level of the decerebration were aspirated. Because this procedure made the animal insentient, anesthesia could be withdrawn. The preparation was bisected subdiaphragmatically, and the descending aorta was isolated and cannulated with a double-lumen catheter to perfuse the upper body. The perfusate was made from a Ringer's solution that contained 1.25% Ficoll and was gassed with 95% oxygen and 5% carbon dioxide at 31°C. Peripheral temperature taken from the forelimb was 27°C. Perfusion was at constant flow using a roller pump (28–32 ml/min; model 505S; Watson Marlow, Falmouth, UK). The second lumen of the catheter was used to monitor aortic perfusion pressure (PP) via a transducer. A phrenic nerve was isolated at the level of the thorax, and its activity was recorded via a suction electrode. The respiratory motor pattern consisted of an incrementing discharge indicative of an eupnoeic-like pattern (St-John and Paton, 2003), which was used to gauge the adequacy of oxygenation of the brainstem and hence viability of the preparation. The electrocardiogram (ECG) was recorded simultaneously with the activity of the phrenic nerve as the suction electrode was within close vicinity of the left ventricle. The R wave of the ECG was discriminated using a window discriminator to trigger transistor–transistor logic pulses used to determine instantaneous heart rate (HR). Phrenic nerve activity (PNA) and ECG signals were amplified and filtered (8 Hz to 3 kHz; Neurolog modules 104 and 125; Digitimer, Hertfordshire, UK) and displayed on an oscilloscope.

Stimulation methods. For the microinjection studies, a forelimb was stimulated at the level of the paw with constant mechanical pressure applied using a custom made forceps fitted with a precalibrated strain gauge that was connected to a differential amplifier, as described previously (Boscan et al., 2002a). This allowed the intensity of the pinch stimulus to be monitored and maintained constant during each experiment. The contact surface area of this pinching device was 5×2 mm. Stimulus intensity and duration were 2 ± 0.4 N/mm^2 and 8 ± 3 s, respectively, but the exact intensity and duration were determined for each preparation. Once determined, the intensity and duration of the stimulus remained constant throughout the experiment and produced consistent cardiorespiratory reflex responses for at least 3 h when a period of >6 min was allowed between consecutive trials.

During extracellular single neuronal recording (see below), the brachial nerve plexus was isolated and electrically stimulated with a silver wire bared at one end and wrapped around the entire plexus. Both the plexus and the exposed tip of the wire were embedded in low-melting-point paraffin wax to provide electrical insulation. A pulse generator was used to deliver electrical stimuli suitable for activating C-fibers to the brachial plexus (0.25 ms, 0.2–0.5 Hz, 10 V; Digitimer). In addition, the cervical spinal cord (C3–C4) was stimulated via a custom-made bipolar electrode (wire diameter, 0.12 mm). This electrode was in direct contact with the dorsolateral edges of the spinal cord and stimulated axonal fibers of passage. LPB neurons were tested for orthodromic inputs using a paired-pulse protocol in which two stimuli separated by 10 ms were delivered (width, 0.1–0.2 ms; frequency, 0.2–0.5 Hz; and amplitude, 3–6 V). This paired-pulse protocol was used for identifying putative relatively direct connections and based on that used previously (Miles, 1986; Scheuer et al., 1996; Zhang and Mifflin, 2000).

Microinjection studies. In the absence of the cerebellum in the WHBP, the surface landmarks of the dorsal medulla and pons were seen clearly. The fourth ventricle, the cerebellar peduncules, the caudal edge of the inferior colliculi, and the lateral edge of the brainstem were used as reference landmarks for positioning micropipettes into the LPB. A three-barreled micropipette (external tip diameter, 10–30 μm) was placed into the dorsolateral pons with a three-dimensional micromanipulator under visual control of a binocular dissecting microscope and using coordinates according to the atlas of Paxinos and Watson (1986). The volume microinjected (40 nl) was assessed by measuring the distance moved by the meniscus through a second binocular microscope fitted with a precalibrated eyepiece reticule. Drugs were microinjected into the LPB bilaterally at a depth of 400–600 μm from the dorsal medullary surface. The nociceptor-mediated reflex tachycardia was retested 1–2 min after microinjection.

Controls. The following drugs were used in the microinjection studies: a $GABA_A$ receptor agonist, isoguvacine (0.2 and 0.4 nmol); and a specific NK_1 receptor antagonist, CP-99,994 (2 pmol). The efficacy of this antagonist was challenged with SP (0.4 nmol). Furthermore, the specificity of action of CP-99,994 was assessed by recording the evoked heart-rate response to glutamate microinjection (4 nmol) before and after delivery of CP-99,994. To control for drug spread and localization of the site of action, isoguvacine (0.2 and 0.4 nmol) was also microinjected into the medial parabrachial (MPB) nucleus. To control against volume-related artifacts, saline (0.9%; 40 nl) was microinjected into the LPB. In all cases, saline failed to modify baseline heart rate, perfusion pressure, phrenic nerve activity, or nociceptor-evoked reflex responses. Finally, microinjection sites were marked with 2% pontamine sky blue dye (40 nl).

At the end of the experiment, the brainstem was removed, fixed (1–2 d; 4% paraformaldehyde containing 20% sucrose), processed for histological analysis (50 μm sections), and counterstained with neutral red. The microinjection sites were analyzed using a light microscope. Injection sites, as marked by pontamine sky blue, were documented on semi-schematic drawings [according to the atlas of Paxinos and Watson (1986)] of transverse sections through the dorsolateral pons.

Extracellular recording. Microelectrodes were aimed into comparable LPB sites as those in the microinjection studies (see above); these were ipsilateral to the stimulated brachial nerve. We used two-barreled microelectrodes (external tip diameter of 2–5 μm) for extracellular recording and drug application. The first barrel was filled with 100 mM glutamate in 1.5 M NaCl and used to record single units (resistance, 15–32 MΩ). The extracellularly recorded action potentials were amplified, filtered (8 Hz to 3 kHz; Neurolog modules 104 and 125), and displayed on an oscilloscope. The second barrel contained CP-99,994 (5 or 10 mM) to block NK_1 receptors. CP-99,994 was ejected with positive pressure applied from a custom-made pressure ejection system fitted with solenoids and valves that permitted control of the applied pressure. The applied pressure was monitored continuously on an analog gauge. Neurons encountered were tested for a synaptic response after brachial nerve stimulation, and the evoked excitatory responses were retested after CP-99,994 application. CP-99,994 was applied for 2–10 min. Washout of the drug effect occurred over a period of 10–30 min. Because high concentrations of CP-99,994 can exert nonspecific effects on sodium channels, action potential spike height was measured during application of glutamate (100 mM) in control and during delivery of CP-99,994.

SP immunocytochemistry. Six rats were anesthetized deeply with an overdose of chloral hydrate (60 mg/100 body weight, i.p.) and perfused through the aorta with PBS (0.01 M, pH 7.4, 0.9% NaCl), followed by 500 ml of ice-cold fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. The brains were then removed and postfixed at 6°C overnight in 4% paraformaldehyde containing 5% sucrose. Frozen sections of 50 μm were cut in the transverse plane through the pons. The sections through the pons were divided into three series and mounted on gelatin-coated slides. One series was Nissl stained

with thionin and coverslipped. The remaining series were immunocytochemically processed for SP. Sections were washed in Tris-buffered saline (50 mM, pH 7.6) and incubated in a blocking solution containing 10% normal goat serum, 2% bovine serum albumin, and 0.3% Triton X-100 in Tris-buffered saline. After 45 min, the sections were transferred into the primary antisera, i.e., rabbit anti-SP (Incstar, Stillwater, MN), diluted 1:500 in a carrier containing 1% goat serum, 1% bovine serum albumin, and 0.3% Triton X-100 in Tris-buffered saline. The incubation was for 40–48 h at 6°C. Thereafter, the sections were rinsed for 30 min and transferred to an unlabeled goat anti-rabbit IgG antiserum (Dako, High Wycombe, UK; Diagnostika, Hamburg, Germany), diluted 1:50 in carrier, and incubated for 1.5 h at room temperature. After repeated rinsing in Tris-buffered saline, they were incubated in a rabbit peroxidase–antiperoxidase complex (Dako), diluted 1:200 in the carrier, again for 1.5 h at room temperature, followed by several rinses in Tris-buffered saline. The final visualization of SP-IR was accomplished by processing the sections in Tris-buffered saline, containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2, for 20 min. The incubation was terminated by transferring the sections into Tris-buffered saline with repeated rinsing. Thereafter, the sections were mounted on gelatin-coated slides, air dried, dehydrated in alcohol, cleared in xylene, and coverslipped with Entellan.

Sections through the PB were analyzed with a light microscope equipped with a CCD camera (CF 20 DXC; Kappa, Göttingen, Germany). Digital images were taken from the dorsolateral parts of the PB in which SP-IR was visible.

Data analysis. All data collected during the electrophysiological studies were relayed via a 1401 CED interface to a computer running Spike 2 software (Cambridge Electronics Design, Cambridge, UK) with custom-written scripts for data acquisition and on-line and off-line analysis. The peak reflex responses in heart rate, phrenic nerve activity cycle length, and perfusion pressure were measured and compared with baseline values. For single unitary activity, the firing frequency was measured before, during, and after single pulse electrical stimulation of the brachial nerve. The electrically evoked peak firing frequency was compared with the frequency of ongoing discharge. The criterion used to establish an increased firing rate response was when evoked firing frequency increased significantly (i.e., by >25%) relative to basal discharge. To determine LPB neuronal sensitivity to NK_1 receptor antagonism, peristimulus time histograms (10 ms bin width) were plotted to show brachial nerve-evoked activity before, during, and after CP-99,994 application.

Significance of data were assessed using a two-way ANOVA and paired two-tailed Student's *t* test to the raw data. All values quoted are the mean ± SEM, and *n* is the number of preparations for the microinjection experiments and the number of cells in the extracellular single-unit recording study. Differences were taken as significant at the 95% confidence limit.

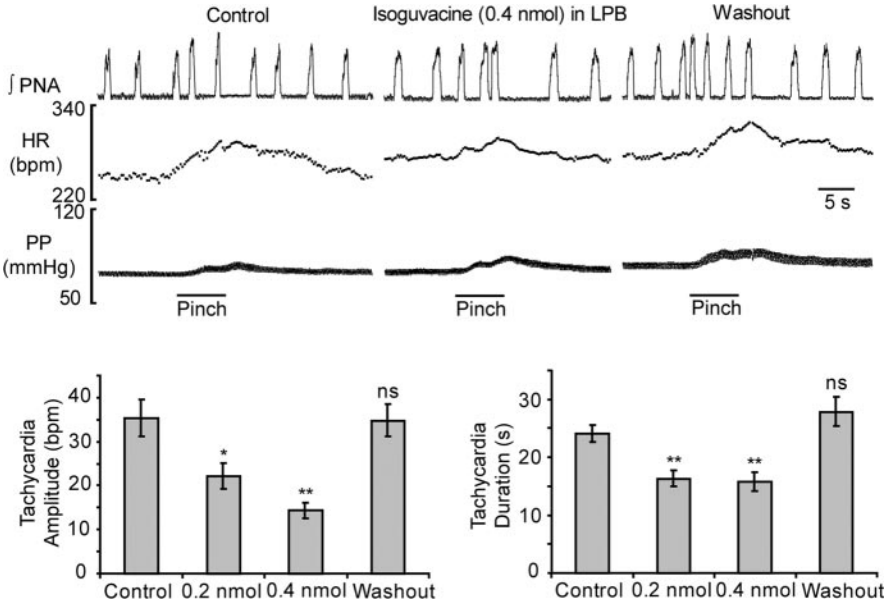

Figure 1. Bilateral microinjection of isoguvacine (a $GABA_A$ receptor agonist) into the LPB attenuates the reflex tachycardic response evoked by stimulation of forelimb nociceptors. The attenuation was reversible after a 10 min washout period. In contrast, the reflex changes in respiratory activity and peripheral vascular resistance remained unaffected. $*p < 0.05$; $**p < 0.01$; two-way ANOVA and paired two-tailed Student's *t* test. ns, Not significant.

Results

Microinjection studies

Role of LPB in mediating the nociceptive-evoked cardiorespiratory reflex response

In nine WHBPs, basal HR [294 ± 12 beats per minute (bpm)], PP (68.3 ± 1.7 mmHg), and cycle length of PNA (5.4 ± 0.6 s) were measured. Mechanical noxious pinch of the forelimb evoked a tachycardia (+36 ± 4.2 bpm), hypertension (+4.3 + 0.5 mmHg), and decrease in PNA cycle length (−2.4 + 0.3 s; $p < 0.01$ for all values). Bilateral microinjection of isoguvacine (a $GABA_A$ receptor agonist) into the LPB, to reversibly inactivate neurons, did not modify baseline cardiorespiratory parameters but did attenuate the noxious-induced reflex tachycardia. The tachycardia amplitude was attenuated in a dose-dependent manner from either 36 ± 4.2 to 22.2 ± 3 or 14.4 ± 1.8 bpm using 0.2 and 0.4 nmol of isoguvacine, respectively ($n = 9$; $p < 0.01$) (Fig. 1; Table 1). In addition, the duration of the tachycardia was attenuated equally at both doses (from 24.1 ± 1.5 to 16.3 ± 1.5 or 15.8 ± 1.6 s; $n = 9$; $p < 0.01$). After 15 min, the noxious-evoked tachycardia recovered fully (Fig. 1). In contrast, isoguvacine neither attenuated the noxious-evoked hypertension nor the decrease in PNA cycle length.

Histological analysis showed that the effective microinjections sites were all placed in the LPB (see Fig. 3, capital letters). Microinjections placed into or nearby the lateral crescent nucleus were most effective (≥50% attenuation of cardiac response) (see Fig. 3, bold *A–C*, *F*, *M*). In contrast, in a different group of WHBPs ($n = 8$), isoguvacine (0.2 or 0.4 nmol) microinjected outside the LPB area, such as into the MPB or Kölliker-Fuse area (KF), failed to affect the noxious-evoked tachycardia (i.e., 31.8 ± 3 or 29.4 ± 3 bpm; $p = 0.3$ or 0.2, respectively). These injection sites are summarized in Figure 3 (see *D*, *E*, *H–L*, *N–Q*). It was noted that isoguvacine microinjection into the MPB–KF changed PNA pattern and frequency (data not analyzed), whereas ongoing basal HR was unaltered (e.g., 273.6 ± 6.6 vs 281 ± 6.6 bpm). From these studies, we concluded that the lateral crescent nucleus of the LPB, but not its medial counterpart or the KF, plays a role in mediating the nociceptive-evoked reflex tachycardia.

Role of NK_1 receptors in the LPB

As shown above, the LPB mediates ~50% of the reflex tachycardia evoked during mechanical stimulation of forelimb nociceptive afferents. We considered SP as a putative neurotransmitter released in the LPB after somatic nociceptor stimulation. In seven WHBPs, basal HR was 262 ± 6 bpm, and stimulation of forelimb mechanical nociceptors evoked a tachycardia of 34.2 ± 1.8 bpm, with a duration of 30.4 ± 1.6 s. Microinjection of the NK_1 recep-

Table 1. Baseline and reflex cardiorespiratory values during control and after bilateral microinjection of drugs into the LPB

	Basal HR (bpm)	Tachycardia (bpm)	Basal PP (mmHg)	Hypertension (mmHg)	Basal PNA cycle length (s)	Tachypnea (s)
Control	294 ± 12	36 ± 4.2	68.3 ± 1.7	4.3 ± 0.5	5.4 ± 0.6	−2.4 ± 0.3
Isoguvacine (0.2 nmol)	300 ± 11	22.2 ± 3	70.5 ± 2.3	4.6 ± 0.9	4.8 ± 0.5	−1.7 ± 0.2
Isoguvacine (0.4 nmol)	306 ± 14	14.4 ± 1.8	73.8 ± 1.8	3.5 ± 0.4	5.1 ± 0.7	−2.2 ± 0.5
CP-99,994 (2 pmol)	264 ± 7	18.6 ± 1.7	73.7 ± 2.6	3.1 ± 0.3	6 ± 0.6	−3 ± 0.4
SP (0.4 nmol)	270 ± 11	12 ± 1.2	74.2 ± 2.5	0.37 ± 0.2	5.9 ± 1.2	0.6 ± 1.4
SP + CP-99,994 (0.4 nmol + 2 pmol)	268 ± 16	4.8 ± 1	75.9 ± 3.5	0.04 ± 0.2	5 ± 1	0.8 ± 1.5
Glutamate (4 nmol)	276 ± 19	15 ± 1.3	73 ± 3.4	1.3 ± 0.4	4.6 ± 0.4	−1.8 ± 0.3
Glutamate + CP-99,994 (4 nmol + 2 pmol)	299 ± 12	15.6 ± 3	80.3 ± 2.6	1.1 ± 0.6	5.5 ± 0.9	−1.5 ± 1.1

Drugs included isoguvacine (a $GABA_A$ agonist), CP-99,994 (an NK_1 receptor antagonist), SP, glutamate, and respective combinations.

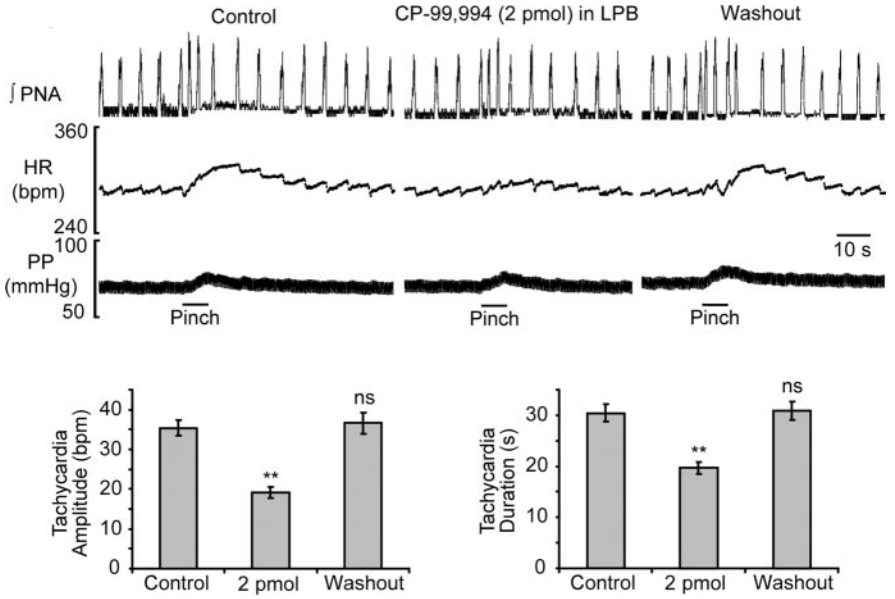

Figure 2. Bilateral microinjection of CP-99,994 (an NK_1 receptor antagonist) into the LPB reversibly attenuates the amplitude and duration of the tachycardia evoked after somatic nociception. Although the tachycardia was attenuated, the changes in respiratory activity and peripheral vascular resistance remained unaffected. $^{**}p < 0.01$; two-way ANOVA and paired two-tailed Student's *t* test. ns, Not significant.

tor antagonist (CP-99,994; 2 pmol) in LPB did not modify basal HR (264 ± 7 bpm) but attenuated reversibly both the magnitude and duration of the nociceptive-evoked tachycardia (18.6 ± 1.7 bpm; 19.6 ± 1.2 s; $p < 0.01$) (Fig. 2, Table 1). The reflex tachycardia recovered to 33 ± 2.2 bpm after 10–15 min.

To assess the efficacy of antagonism of CP-99,994 in these experiments, SP (0.4 nmol) was microinjected unilaterally into the LPB and evoked a tachycardia of 12 ± 1.2 bpm ($n = 4$). After microinjection of CP-99,994 (0.2 nmol), this tachycardia was reduced to 4.8 ± 1 bpm ($p < 0.01$) (Table 1). In comparison, the tachycardia evoked by a unilateral microinjection of glutamate (4 nmol) was unaffected by CP-99,994 (15 ± 1.3 vs 15.6 ± 3 bpm; $p = 0.9$; $n = 7$) (Table 1). Histological analysis (Fig. 3, 1–7) showed that the injection sites were all in the LPB, and injections into or nearby the lateral crescent nucleus were again most effective (≥50% attenuation of cardiac response) (Fig. 3, bold 1, 3, 5–7). These studies are consistent with the notion that SP acting on NK_1 receptors particularly in the lateral crescent nucleus of the LPB are important in mediating the nociceptive-evoked reflex tachycardia.

Substance P in the lateral parabrachial nucleus

Figure 4 shows that the LPB exhibits strong SP-IR at all rostrocaudal levels, whereas other nuclei of the parabrachial complex, such as the Kölliker-Fuse or medial nucleus, are essentially devoid of SP-IR. SP-IR in the LPB was generally restricted to fibers and punctuated button-like IR (Fig. 4, left column, arrows), whereas somatic labeling was hardly detected. In accordance with the most effective injection sites for the NK-1 receptor antagonist CP-99,994, SP-IR was most prominent in the lateral crescent nucleus of the intermediate section of the parabrachial complex, whereas other subnuclei of the LPB, such as the external lateral or central nuclei, were less intensively labeled (Fig. 4*B*,*C*, right column). In the rostral regions of the LPB, the internal lateral and extreme lateral nuclei also showed prominent SP-IR (Fig. 4*A*). At the caudal level, there was still clear SP-IR in the LPB but at lesser intensity as for the intermediate sections. Overall, the SP-IR suggests that the lateral PB receives a large terminal field of SP containing afferent inputs, which is apparently accentuated in the lateral crescent nucleus.

Extracellular recordings from the LPB

Role of LPB NK_1 receptors in mediating inputs from the brachial nerve

In 52 neurons recorded in LPB, electrical stimulation of the brachial nerve increased the firing rate. The ongoing activity of these cells was 2.2 ± 0.4 Hz, which increased to 8.6 ± 1.4 Hz ($p < 0.01$) during single-pulse stimulation of the brachial nerve. The brachial nerve-evoked response was sensitive to the NK_1 receptor antagonist in a subpopulation of neurons (13 of 52, or 25%) (Fig. 5). When CP-99,994 (5 or 10 mM) was applied to these 13 neurons through the second barrel of the microelectrode, the ongoing activity did not change (i.e., 1.9 ± 0.5 vs 1.7 ± 0.4 Hz), but the brachial nerve-evoked response was reduced from 8.4 ± 1.4 to 4.4 ± 0.9 Hz. After ~30 min, recovery from CP-99,994 occurred (i.e., firing response of 7.9 ± 1.8 Hz), but this was limited to 5 of the 13 neurons tested (Fig. 5). Despite a lack of recovery from CP-99,994 in these neurons, the evoked response after application of glutamate (100 mM) was unchanged ($n = 4$). Glutamate-

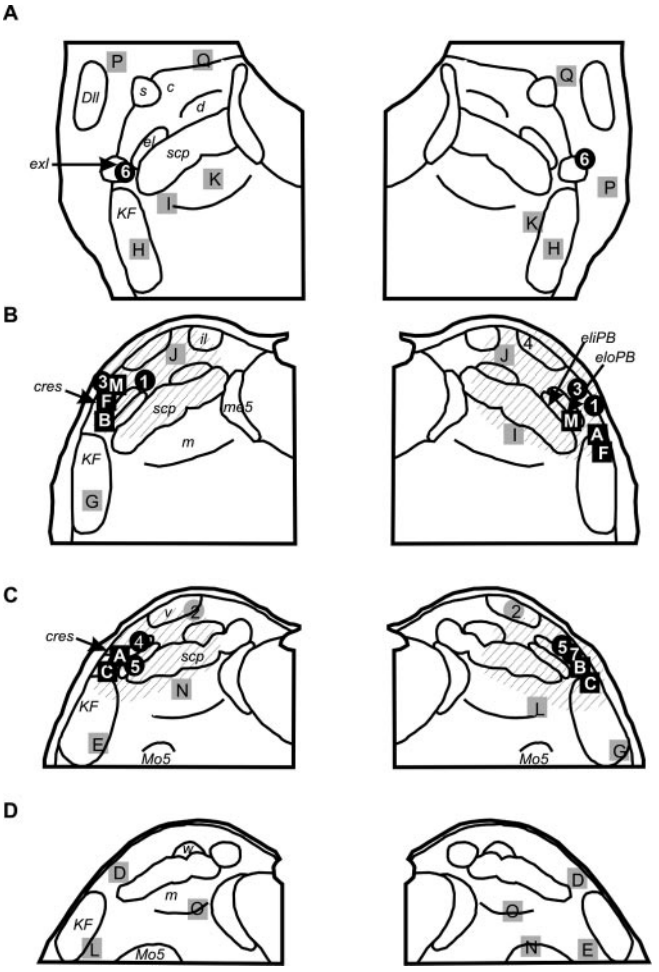

Figure 3. Semi-schematic drawings of coronal sections through the parabrachial complex (*A–D*, rostral to caudal) illustrating the injection sites for isoguvacine (squares) and for the NK_1 receptor antagonist CP-99,994 (circles). To identify the individual pairs of bilateral isoguvacine injection sites, compare the letters *A–Q*. The pairs of bilateral CP-99,994 injection sites are labeled with numbers 1–7, and extracellular recording sites are indicated by the shaded area. The circles and squares shown in black represent the most effective injection sites in relation to the attenuation of the nociceptive tachycardia (≥50% attenuation). Symbols shown in gray were ineffective or only showed weak attenuation of the cardiac response. Dll, Dorsal nucleus of the lateral lemniscus; me5, mesencephalic tract of the trigeminus; Mo5, motor nucleus of the trigeminus; scp, superior cerebellar peduncle, nuclei of the parabrachial complex; c, central nucleus; cres, lateral crescent nucleus; d, dorsal nucleus; el, external lateral nucleus (eli, inner section; elo, outer section); exl, extreme lateral nucleus; il, internal lateral nucleus; v, ventral nucleus; s, superior nucleus; w, waste nucleus.

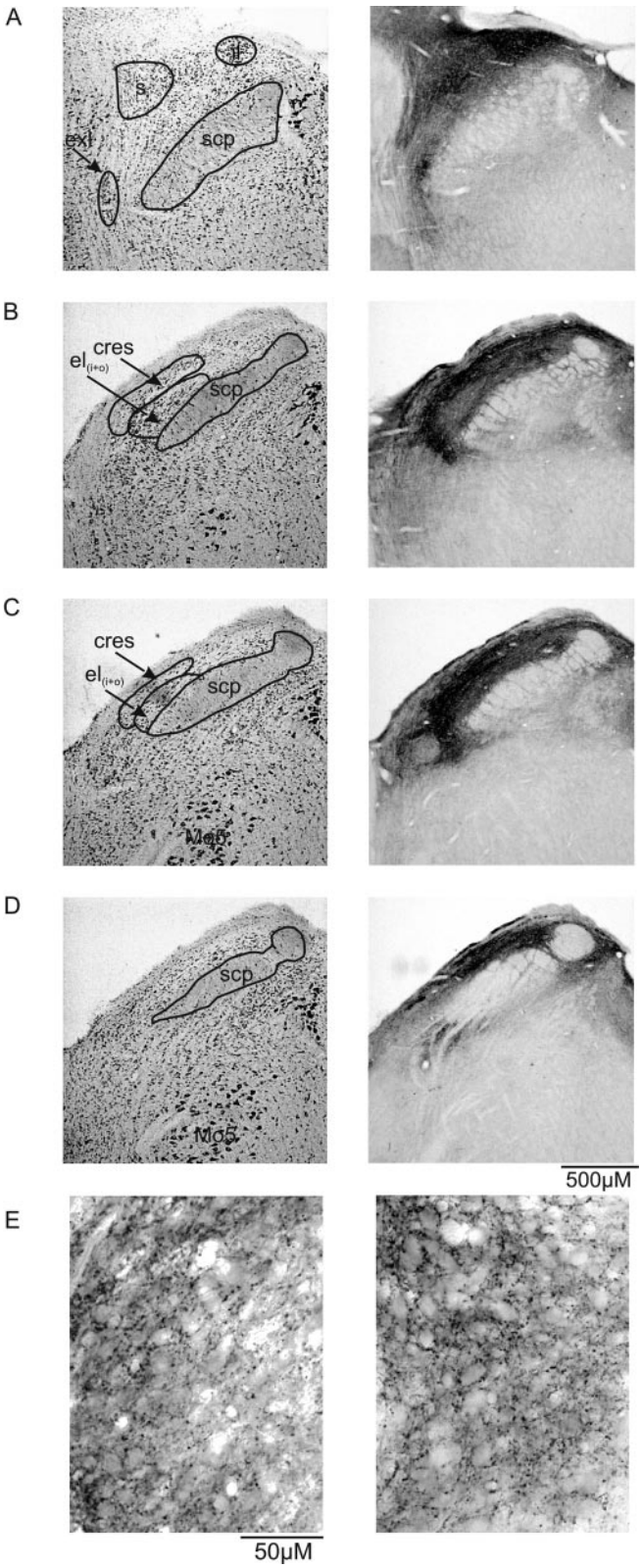

Figure 4. Series of photomicrographs of coronal sections illustrating the cytoarchitecture of the parabrachial complex with thionin staining (left column) and adjacent sections showing immunoreactivity for substance P (right column). *A–D*, From rostral to caudal; compare the dense immunoreactivity of the lateral crescent nucleus with the injection sites presented in Figure 3. *E* is a higher magnification of a substance P-immunoreactive terminal field within the lateral parabrachial nucleus. Note the punctate button-like immunoreactivity surrounding somata (in particular, see top left corner of the right photograph) of the lateral parabrachial complex, suggesting potential SPergeric synaptic contacts in this area. For abbreviations, see Figure 3.

evoked response before CP-99,994 was 10.4 ± 1.3 Hz and, during CP-99,994 administration, was 11.6 ± 2 Hz ($p = 0.58$) (Fig. 5), suggesting a relatively specific effect of the antagonism.

Characteristics of the brachial-evoked firing response in the LPB

The onset latency of the brachial nerve-evoked response from the 13 neurons sensitive to NK_1 receptor antagonism was 45.1 ± 6.7 ms (range of 7–100 ms). This was not different from when CP-99,994 was applied (55.6 ± 8.7 ms; $p = 0.35$) or the latency of neurons insensitive to NK_1 receptor blockade (52.5 ± 6.9 ms; $n = 39$; $p = 0.46$).

The possibility of a relatively direct spinal projection to LPB neurons was tested using a paired-pulse stimulation protocol in which stimuli were separated by 10 ms and applied to the cervical spinal cord. From 10 neurons tested that were sensitive to NK_1 receptor antagonism, only three were capable of following the

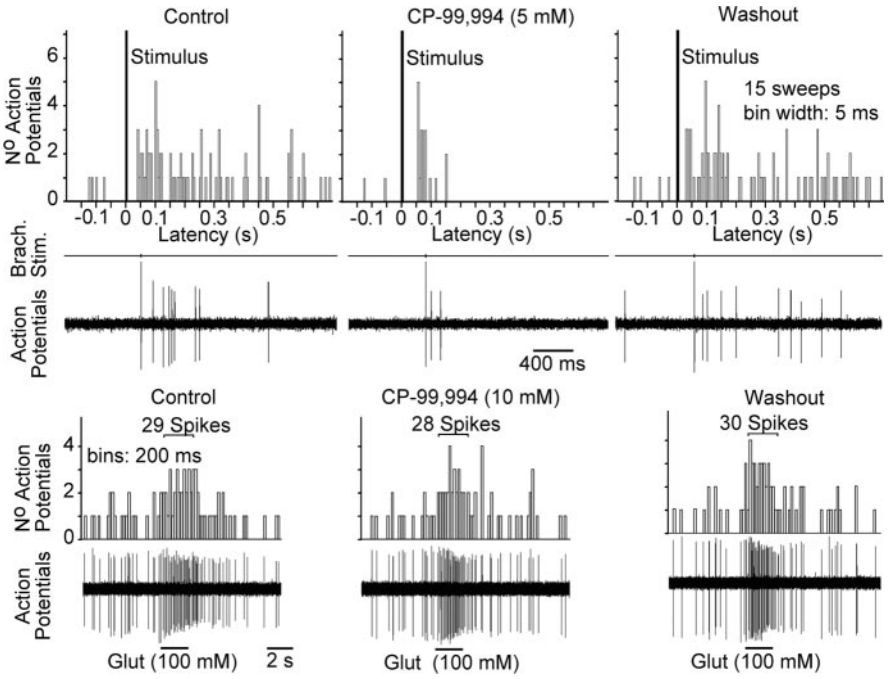

Figure 5. Top, Peristimulus time histogram and raw data from a single LPB neuron demonstrating that the NK_1 receptor antagonist CP-99,994 attenuated the evoked response during electrical stimulation of the ipsilateral brachial nerve (Brach. Stim.). The effect of CP-99,994 was reversible after a 20 min washout period. Bottom, Glutamate (Glut) applied directly to neurons increased firing, which was unaffected with or without NK_1 receptor blockade, indicating integrity of the neurons and an absence of any nonspecific effect of CP-99,994.

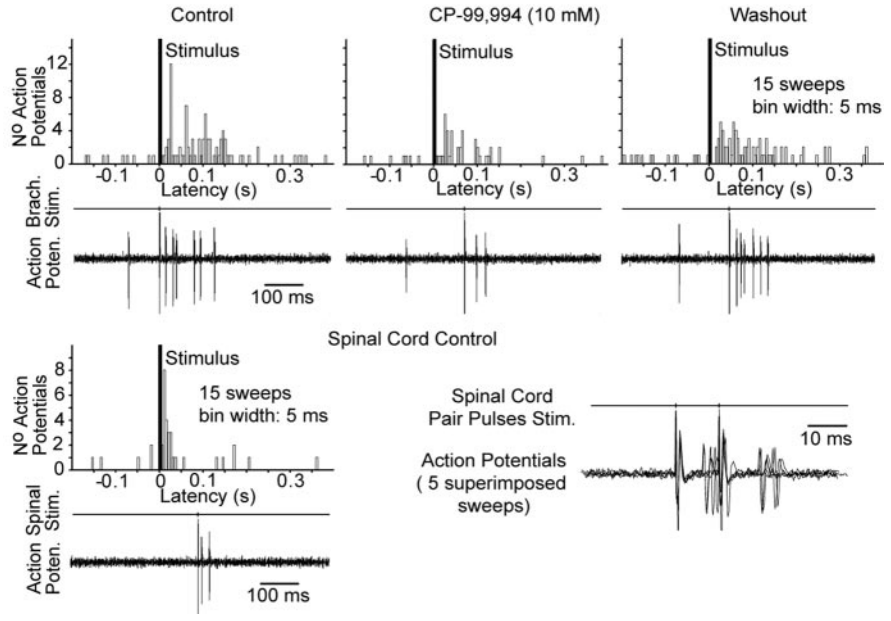

Figure 6. Peristimulus time histogram and raw data from an LPB neuron during electrical stimulation of the ipsilateral brachial nerve (Brach. Stim.) and cervical spinal cord (Spinal Stim.). The synaptic evoked response was sensitive to CP-99,994 in a reversible manner. The spinal input to this neuron was comparable with that evoked from the brachial nerve, albeit at a shorter onset latency. Paired-pulse stimulation with a 10 ms interval of the spinal cord evoked reliable synaptic responses to both stimuli, suggesting a relatively direct synaptic connection from ascending spinal afferents.

paired-pulse protocol (Fig. 6). In these three neurons, the mean onset latency after stimulation of the brachial nerve and spinal cord was 21.2 ± 5 and 14.1 ± 3.8 ms, respectively ($n = 3$). If we subtract the spinal cord onset latency from the brachial nerve onset latency, the mean delay for these three neurons is 7.1 ms. This 7.1 ms is the time delay for the action potentials to travel from the brachial nerve to the cervical spinal cord ascending tract. Considering that the nociceptive afferents in the brachial nerve are C-fibers, the action potential delay in the spinal cord itself is short, suggesting limited time for spinal integration of information before its transfer to the LPB.

Discussion

Our results indicate that the LPB, including the lateral crescent region, is functionally involved in mediating the reflex tachycardia evoked by stimulating forelimb somatic nociceptors. Furthermore, pharmacological blockade of NK_1 receptors in this region was most effective in attenuating the cardiac response to nociceptive stimulation. Electrical stimulation of brachial nerve afferents can trigger firing responses in LPB neurons, with some being sensitive to NK_1 receptor antagonism. This is consistent with our anatomical data showing SP-immunoreactive fibers and terminal fields in the LPB being most pronounced in the dorsal aspects of the LPB, including the lateral crescent, dorsal, and internal lateral subnuclei of the PB.

Nociceptive afferent pathways to the lateral parabrachial nuclei

Although we used a paired-pulse protocol (10 ms separation between pulses) to assess the directness of spinal-LPB inputs, this technique is open to criticism. We acknowledge that it does not prove monosynaptic connectivity, but it does not disprove it either. A false-negative result could occur if both excitatory and inhibitory pathways are coactivated; then, the response to the second stimulus may be shunted by inhibition. Thus, we may have underestimated the number of relatively direct spinal projections to the LPB. We also accept that our stimulation methods may have activated multiple spinal and supraspinal excitatory/inhibitory pathways, making these data very difficult to interpret.

There are both direct and indirect pathways that could mediate the brachial nerve inputs to the LPB described in this study. The first are spino-parabrachial routes. LPB receives multiple projections from the spinal cord. It is well established that the LPB, especially the central and dorsolateral subnuclei, receive abundant and direct projections from spinal nociceptive laminas I and II (Cechetto et al., 1985; Hylden et al., 1985; McMahon and Wall, 1985; Panneton and Burton, 1985; Light et al., 1987; Blomqvist et al., 1989; Menetrey and De Pommery, 1991; Slugg and Light, 1994; Bernard et al., 1995; Craig, 1995; Feil and Her-

bert, 1995; Bester et al., 2000). Spinal cord neurons located in the superficial dorsal horn of thoracic and lumbar spinal segments project mainly to the dorsal lateral and the central lateral PB (Slugg and Light, 1994; Bernard et al., 1995; Craig, 1995; Feil and Herbert, 1995; Bester et al., 2000). In contrast, neurons in the superficial dorsal horn (laminas I–II) of upper cervical segments, the region in which brachial nerve axons terminate, project specifically to the ventral portion of the external lateral PB (Feil and Herbert, 1995). Finally, it should be noted that spinal afferents also terminate within the rostral ventrolateral medulla (Slugg and Light, 1994) and may affect sympathetic activity via this route. Indeed, such a projection would explain why only ~60% of the tachycardia could be abolished after inactivation of the LPB and why the latter also failed to affect the arterial pressure response.

The second are indirect routes. In addition to direct routes, there are also numerous indirect projections between the dorsal horn and LPB. One relay that receives spinal afferents and projects to the LPB is the nucleus tractus solitarius (NTS) (Loewy and Burton, 1978; Ricardo and Koh, 1978; Beckstead et al., 1980; Granata and Kitai, 1989; Herbert et al., 1990). Moreover, the NTS receives various visceral and spinal sensory afferents that are involved in integrating nociceptive afferent inputs (Toney and Mifflin 1995, 2000; Boscan and Paton, 2001b, 2002b; Boscan et al., 2002a,b). In the context of the present findings, it is of interest that the NTS mediates ~50% of the tachycardia evoked by activating forelimb nociceptors (Boscan and Paton, 2001b). This is very similar to the reduction after inactivating the LPB (~60%). Thus, it appears that the NTS and LPB play a major role in the heart-rate response to somatic nociception. Based on the connectivity between these regions, it is conceivable that the NTS relays this ascending information to the LPB. Despite the caveats discussed above, this is upheld by the finding that most LPB neurons did not follow the paired-pulse protocol applied to the spinal cord, suggesting indirect ascending inputs.

The LPB also receives abundant inputs from neurons located in other brainstem regions that could potentially relay nociceptive information. These include the lateral reticular formation, periaqueductal gray matter, and spinal trigeminal nucleus. All are innervated by spinal afferents, activated by noxious stimulation, and project to LPB (Cechetto et al., 1985; McMahon and Wall, 1985; Milner and Pickel, 1986; Herbert et al., 1990; Lanteri-Minet et al., 1994; Bernard et al., 1995; Craig, 1995; Feil and Herbert, 1995; Krout et al., 1998; Imbe et al., 1999; Keay and Bandler, 2002). However, it is not clear whether these projections contain substance P.

Role of LPB as a major integrator for somatic nociceptive information

Previous studies manifested an important role for the LPB in mediating somatic nociceptive information arising form the dorsal horn using both electrophysiological techniques (Bernard and Besson, 1990; Bernard et al., 1994; Bester et al., 1995; Menendez et al., 1996) and c-Fos expression profiles (Lanteri-Minet et al., 1993, 1994; Bellavance and Beitz, 1996; Hermanson and Blomqvist, 1996; Bester et al., 1997). Thus, the functional role of LPB is currently seen as a major relay of nociceptive afferent inputs to higher brain areas. The major efferent projection targets include the thalamus, hypothalamus, amygdala, and cortex (Saper and Loewy, 1980; Fulwiler and Saper, 1984; Bernard and Besson, 1990; Moga et al., 1990; Hurley et al., 1991; Allen and Cechetto, 1992; Bernard et al., 1996; Jasmin et al., 1997; Bester et al., 1995, 1997; Krout and Loewy, 2000; Gauriau and Bernard, 2002). In addition, the LPB also has descending connections to medullary sites involved in autonomic function; these are described below.

Role of LPB as an integrator of the cardiovascular response to nociception

In addition to playing a role in nociception, the LPB is an integral part of the central autonomic network controlling cardiovascular function (Mraovitch et al., 1982; Ward, 1988; Paton et al., 1990; Chamberlin and Saper, 1992; Korte et al., 1992; Lara et al., 1994). Electrical or chemical stimulation of the LPB inhibits baroreceptor-evoked excitatory synaptic responses of NTS neurons (Felder and Mifflin, 1988; Paton et al., 1990; Len and Chan, 2001). Interestingly, we have shown that nociceptive afferents can also inhibit the cardiac component of the baroreceptor (and peripheral chemoreceptor) reflex, as well as the firing responses of physiologically characterized baroreceptive NTS neurons. These effects appear to be mediated via an intrinsic $GABA_A$ergic system (Boscan and Paton, 2002a; Boscan et al., 2002a). Thus, LPB may play an important role in inhibiting the baroreceptor reflex during noxious stimulation. This may be one of the mechanisms producing the reflex tachycardia observed during painful stimuli, especially with the simultaneous presence of increased arterial pressure. However, with projections from the LPB to the ventrolateral medulla (see below) (Fulwiler and Saper, 1984; Herbert et al., 1990), this route cannot be excluded for mediating sympathoexcitatory responses.

The precise LPB subregions contributing to the regulation of cardiovascular function is not well defined. In our study, pharmacological blockade of the lateral crescent subnucleus was most effective in attenuating the nociceptor-evoked tachycardia. Indeed, the lateral crescent nucleus provides a main source for descending medullary projections targeting both the rostral and caudal ventrolateral medulla and NTS (Fulwiler and Saper, 1984; Herbert et al., 1990). This region was also characterized previously as the LPB subnucleus that produced pronounced cardiovascular responses after electrical and chemical stimulation (Chamberlin and Saper, 1992). Nevertheless, previous studies concerned with afferent connectivity of the lateral crescent nucleus with the cervical spinal cord yielded contrasting results. Bernard et al. (1995) demonstrated direct and dense projections from the cervical spinal cord to the lateral crescent nucleus. Other tract tracing studies revealed predominant afferent termination fields within the external lateral nucleus (Slugg and Light, 1994; Feil and Herbert, 1995), whereas labeling in other lateral parabrachial nuclei, including the lateral crescent, was sparse. However, a direct participation of the external lateral nucleus in mediating the cardiovascular effects can be primarily excluded. First, the external lateral nucleus lacks descending pathways (Fulwiler and Saper, 1984; Herbert et al., 1990). Second, because of decerebration of the animal model used, a potential involvement of ascending connections with the hypothalamus (Mifflin et al., 1988) or amygdala are invalid for the present study. A possibility is that intranuclear connections that exist between the external lateral and lateral crescent nuclei could contribute to the tachycardic response to brachial stimulation. In support of this, some neurons of the ventral portion of the external lateral nucleus show dendritic domains that cross nuclear boundaries (Herbert and Bellintani-Guardia, 1995), suggesting potential dendritic synaptic contacts with cells of the lateral crescent nucleus. Thus, direct cervical spinal inputs to external lateral nucleus could be processed via dendro-dentritic contacts between neighboring LPB subnuclei.

Precise conclusions on the involvement of direct spinal pro-

jections to the lateral crescent nucleus in mediating the nociceptive tachycardia cannot be drawn from the results of the present study. On one hand, our microinjection studies clearly point to the lateral crescent, but, on the other, we failed to prove monosynaptic inputs to putative nociceptive neurons of the lateral crescent. Nevertheless, the lateral crescent nucleus contains a dense SPergic innervation (Fig. 4) that could potentially originate from the spinal cord (Bernard et al., 1995); this needs to be investigated in more detail in future studies.

Role of substance P and NK_1 receptors in the LPB

In the present study, SP-IR was abundantly expressed in fibers and button-like structures in the LPB. However, densities of SP-IR for the specific subnuclei differed (Fig. 4). SP-IR was pronounced in the lateral crescent, dorsal, and internal lateral nuclei, whereas in the external lateral and central subdivisions, SP-IR was less intense. These findings provide the anatomical substrate for the electrophysiological recordings after blocking NK_1 receptors.

The central and dorsolateral subnuclei receive strong and direct projections from spinal nociceptive laminas I and II (see above). The projection to central nucleus could account for the LPB neurons that were primarily insensitive to the NK_1 receptor antagonist but responded to the orthodromic paired-pulse stimulation, indicating a relatively direct non-SP projection. However, most of the neurons sensitive to the NK_1 receptor antagonist failed to follow the orthodromic paired-pulse stimulation, suggesting nondirect spinal inputs. It is reported that some NTS projections to LPB (including the lateral crescent subnucleus) contain SP-IR (Milner et al., 1984; Milner and Pickel, 1986; Riche et al., 1990). Furthermore, SP-IR in LPB can be elevated by activation of the NTS during electrical stimulation of the vagus nerve (Saleh and Cechetto, 1996). These findings demonstrate that NTS projections to LPB can release SP after stimulation of nociceptors, although we have not anatomically analyzed these projections in the present study.

In conclusion, we suggest that the majority of the tachycardic response to brachial nociceptive stimulation might be mediated via an indirect route from the cervical spinal cord (perhaps relaying in the NTS) to the lateral crescent nucleus. This pathway is dependent to some extent on SP release within the lateral crescent nucleus. The slight discrepancy in the strength of the attenuation of the tachycardia after isoguvacine (~60%) and NK_1 receptor antagonist (~45%; see Results) might be explained by additional inputs from the cervical spinal cord to the external lateral nucleus. This input is then potentially processed via dendritic synapses between external lateral and lateral crescent nuclei. Finally, this input is likely to be SP independent because we could not detect SP-immunoreactive cells in the external lateral nucleus.

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