Role of nucleus tractus solitarius (NTS) 5-HT₃ receptors in the defense reaction-induced inhibition of the aortic baroreflex in rats

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LIST OF ABBREVIATIONS

CBaR: cardiac baroreflex response

CPBG: 1-(m-chlorophenyl)-biguanide

DMH: dorsomedial nucleus of the hypothalamus

dPAG: dorsal part of the periaqueductal gray

ECG: electrocardiogram

HR: heart rate

MBP: mean blood pressure

NTS: nucleus tractus solitarius

SBaR: sympathetic baroreflex response
ABSTRACT

Different stressful conditions elicit a typical behaviour called the defense reaction. Our aim was to determine whether 5-HT3 receptors in the nucleus tractus solitarius (NTS) are involved in (i) the inhibition of the baroreflex bradycardia and (ii) the rise in blood pressure which are known to occur during the defense reaction. In urethane-anesthetized rats, the defense reaction was elicited by electrical stimulation of the dorsomedial nucleus of the hypothalamus (DMH) or the dorsal part of the periaqueductal gray (dPAG). Direct electrical stimulation of the aortic depressor nerve was used to trigger the typical baroreflex responses. Aortic stimulation at high (100-150 µA) and low (50-90 µA) intensity produced a decrease in heart rate of -39 to -44 % (relative to baseline, Group 1 responses, n=113) and -19 % to -24 % (Group 2 responses, n=43), respectively. In spontaneously breathing rats, Group 1 and Group 2 bradycardiac responses were inhibited during DMH (-75 ±4% and -96 ±4%, n=38 and n=11, respectively), as well as dPAG (-81±3 % and -95±4 %, n=36 and n=10, respectively) stimulation. The aortic baroreflex bradycardia was hardly affected by DMH or dPAG stimulation when bicuculline (5 pmol), a specific GABA_A receptor antagonist, had previously been microinjected into the NTS. Likewise, NTS microinjections of granisetron, a specific 5-HT3 receptor antagonist, prevented, in a dose-dependent manner, the baroreflex bradycardia inhibition. In addition, intra-NTS granisetron did not affect the rise in blood pressure induced by either site stimulation. These data show that 5-HT3 receptors in the NTS are involved in the GABAergic inhibition of the aortic baroreflex bradycardia, but not in the rise in blood pressure, occurring during the defense reaction elicited by DMH or dPAG stimulation.
INTRODUCTION

The dorsal part of the periaqueductal gray (dPAG) and several nuclei in the hypothalamus constitute a brain aversive system that coordinates aggressive or defense-type behavioural patterns (for reviews see Mancia and Zancchetti 1981; Depaulis et al. 1994). The defense reaction, elicited by either electrical or chemical stimulation of these sites, is characterized by dynamic vascular changes such as blood flow redistribution among organs (Humphreys et al. 1971; Bandler and Carrive 1988; Smith et al. 1990), and both hypertension and tachycardia in response to sympathetic activation. One can expect that the hypertension elicited during the defense reaction will activate arterial baroreceptors. But the resulting arterial baroreflex responses, i.e. hypotension and bradycardia, would limit the blood flow supply to skeletal muscles, thereby counteracting behavioural performance. Indeed, the cardiovagal component of the arterial baroreflex is suppressed during the defense reaction elicited by stimulation of either dPAG (Hockman and Talesnik 1971; Nosaka et al. 1993) or specific nuclei in the hypothalamic defense area (such as the dorsomedial nucleus of the hypothalamus, DMH) (Gebber and Snyder 1970; Coote et al. 1979). However, the effects of such brain stimulations on the sympathetic component of the baroreflex are still a matter of controversy (Humphreys et al. 1971; Coote et al. 1979; Smith and Barron 1989; Nosaka et al. 1993).

It is suggested that activation of GABA<sub>A</sub> receptors in the nucleus tractus solitarius (NTS) probably underlay baroreflex inhibition during the defense reaction (Jordan et al. 1988; Mifflin et al. 1988). We previously demonstrated that a similar mechanism was involved in the inhibition of the baroreflex bradycardia produced by activation of 5-HT<sub>3</sub> receptors in the NTS (Merahi et al. 1992; Sévoz et al. 1996a). In addition, activation of these serotonergic receptors are known to induce, like the defense reaction, a rise in blood pressure caused by
sympathoexcitation (Nosjean et al. 1995; Sévoz-Couche et al. 1998). Moreover, (i) numerous projections do exist from the dPAG and the hypothalamus to different raphe nuclei where are located serotoninergic neurons (Hosoya 1985; Hosoya et al. 1987; Behzadi et al. 1990; Vertes and Crane 1996; Hermann et al. 1997), and (ii) part of these neurons actually project into the NTS (Steinbusch 1984; Thor and Helke 1987; Schaffar et al. 1988).

All these considerations are compatible with the idea that 5-HT₃ receptors in the NTS may be involved in both a GABAergic inhibition of the baroreflex bradycardia and the increase in blood pressure which occur during the defense reaction elicited by dPAG or DMH stimulation in the rat. In order to test these hypotheses, we have analysed the effects of NTS microinjections of bicuculline, a GABAₐ receptor antagonist (Desarmenien et al. 1984), on the inhibition of aortic baroreflex bradycardia caused by dPAG and DMH stimulations. We have also analysed the effects of microinjections of granisetron, a specific 5-HT₃ receptor antagonist (Sanger and Nelson 1989; Callera et al. 1997), into the NTS, on both the inhibition of the baroreflex bradycardia and the rise in blood pressure caused by the latter stimulations. In addition, in an attempt to solve the pending question of the relationships between the defense reaction and the sympathetic component of the baroreflex, the effects of dPAG and DMH stimulations on the vascular baroreflex response to aortic nerve stimulation were also investigated in rats whose parasympathetic tone had been suppressed by α-methyl-atropine.

MATERIALS AND METHODS
General procedures

Experiments were performed on 156 male Sprague-Dawley rats, weighing 330-370 g, which had been kept under controlled environmental conditions (ambient temperature: 21±1°C, 60% relative humidity, food and water ad libitum, alternate 12 h: 12 h light/dark cycles) for at least one week after receipt from the breeding center (CER Janvier, Le Genest-St Isle, France). Procedures involving animals and their care were all conducted in conformity with institutional guidelines, which are in compliance with national and international law policies (Council directive n° 87-848, 19 October 1987, Ministère de l’Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions n° 0299 to M.H. and 0314 to R.L.).

Rats were anaesthetized by an intraperitoneal (i.p.) injection of urethane (1.6 g kg^{-1}) and the depth of anaesthesia was regularly assessed by pinching a hindpaw and monitoring the stability of arterial blood pressure and heart rate. In case of withdrawal reflex and/or significant variations of these cardiovascular parameters, a supplementary dose of urethane was given (0.1-0.2 g kg^{-1} i.v.). A cannula was inserted into the femoral vein for administration of drugs or additional doses of urethane. Arterial pressure was 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, Courtaboeuf, France). The R wave of ECG was discriminated with a window discriminator and used to generate pulses. Arterial blood pressure and ECG pulse signals were relayed to a 1401 interface (1401 Plus, CED, Cambridge, UK) connected to a computer running Spike 2 software (CED, Cambridge, UK). Heart rate (HR) was automatically computed from R wave pulses and displayed as instantaneous rate in beats per
minute (bpm). Rectal temperature was maintained at 37° C with a thermostatically controlled heating blanket.

Surgery

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 on the left side into either the hypothalamus or the periaqueductal gray. DMH and dPAG were identified by using stereotaxic coordinates (P 3.0-3.6, L 0.5, V 8-9 and P 6.3-7.0, L 0.5, V 4.5-5, respectively, from Paxinos and Watson’s atlas 1986, see Fig 1) and observing typical autonomic responses of the defense reaction caused by electrical stimulation at these sites: mydriasis, vibris movements, rise in blood pressure, tachycardia and hyperventilation. Intensities of electrical stimuli (50 Hz, 1 ms pulse duration) delivered to DMH and dPAG were 300 µA and 200 µA, respectively. The left aortic depressor nerve was dissected from the vagus nerve by a lateral approach and placed on silver bipolar hook electrodes for electrical stimulation (20 Hz, 1 ms pulse duration). Stimulation of the aortic depressor nerve induced the typical cardiorespiratory responses of the baroreflex: hypotension, bradycardia and apnea. In order to confirm that DMH and dPAG stimulations were able to inhibit the baroreflex bradycardia, we assessed the effects of such stimulations on (i) the maximal reflex bradycardia (170-200 bpm, Group 1 responses) that could be obtained without producing any significant nerve damage even after repeated aortic depressor nerve stimulation (in a set of experiments, n=10 rats, 15 successive stimulations of the aortic depressor nerve produced baroreflex cardiovagal responses that differed from each other by less than 10 %), and on (ii) bradycardia corresponding to half of the maximal reflex response (Group 2 responses). Group 1 and Group 2 reflex cardiac responses were obtained by adjusting for each rat the aortic depressor nerve stimulation intensity within 100-150 µA and 50-90 µA, respectively.
Different rats were used in experiments aimed at inducing Group 1 and Group 2 responses. Once determined, the appropriate intensity of aortic nerve stimulation was maintained constant in each rat throughout the overall experimental procedure.

For microinjections of neuroactive substances into the NTS, we first exposed the dorsal surface of the brainstem through a limited occipital craniotomy. A single-barrel glass micropipette (<100 µm external diameter), connected to a Hamilton microsyringe filled with drugs or saline, was then lowered bilaterally into the commissural tractus solitarius at the level of the calamus scriptorius (Sévoz et al. 1996 a,b). Bilateral microinjections (100 nl) were made within 1 min using the same micropipette. In experiments aimed at studying 5-HT\textsubscript{3} receptor agonist-5-HT\textsubscript{3} or GABA\textsubscript{A} receptor antagonist interactions, the antagonist chosen was microinjected bilaterally into the NTS and then, 5 min later, another micropipette filled with the 5-HT\textsubscript{3} receptor agonist was lowered for bilateral microinjections into the very same sites. The effects of NTS microinjections were tested on the defense reaction-induced inhibition of the maximal reflex cardiac responses (i.e. Group 1) only.

Quantification of the cardiovagal baroreflex response

The cardiovagal baroreflex response (CBaR) was defined as the maximal decrease in HR observed during stimulation of the aortic depressor nerve, expressed as a percentage of basal HR value. In order to eliminate the influence of defense reaction-induced sympathetic activation on heart rate activity (Inui et al. 1994; Fontes et al. 2001), rats were pretreated with propranolol (0.4 mg/kg i.v.), a β-adrenergic receptor antagonist, in a limited set of experiments (n=16). Systemic administration of propranolol blocked for more than an hour the increase in HR produced by DMH or dPAG stimulation, and induced a slight and non-significant reduction of both Groups of CBaR, as compared to non-pretreated rats. Although
propranolol crosses the blood-brain barrier, it had no significant effect on sympathetic outflow (see Tank et al. 2001).

Quantification of the sympathetic baroreflex response
Stimulation of the aortic depressor nerve produced a decrease in blood pressure due to the combination of baroreflex sympathetic inhibition (vasodilation) and parasympathetic excitation (decrease in HR) (see Nosaka et al. 1993). In order to investigate the effect of DMH or dPAG stimulation on the baroreflex sympathetic component only, the parasympathetic tone was suppressed by a pretreatment with α-methyl-atropine (30 µg/kg i.v., Guyenet et al. 1987).

The sympathetic component of the baroreflex (SBaR) was defined as the maximal decrease in blood pressure observed during stimulation of the aortic depressor nerve, expressed as a percentage of basal mean blood pressure (MBP) value in α-methyl-atropine-pretreated rats.

Experimental procedure
After adjusting the intensity of stimulation of the aortic depressor nerve in order to obtain Group 1 or Group 2 CBaR responses, and testing the cardiovascular effects of either DMH or dPAG stimulation, the aortic depressor nerve was stimulated for 4 s while monitoring cardiovascular changes (“control”). Five min later, when MBP and HR had returned to baseline levels, DMH or dPAG stimulation was turned on to trigger the defense reaction. The aortic nerve was then stimulated again for 4 s when the cardiovascular changes associated with the evoked defense reaction were maximal (i.e. usually 3 s after the beginning of DMH or dPAG stimulation, “experimental”). DMH or dPAG stimulation was turned off 3 s after the end of aortic nerve stimulation. The effect of the defense reaction on the baroreflex responses was calculated as the percent change in “experimental” versus “control” conditions.
In another set of experiments (n=20), the same procedures were carried out in rats with a tracheal cannula for ventilation with room air. End-tidal CO₂ was maintained close to 4 % by adjusting the ventilation rate. These experiments were meant to eliminate the changes in respiration elicited by DMH or dPAG stimulation and evaluate their possible influence on the inhibition of the baroreflex bradycardia caused by either site stimulation.

In experiments aimed at analysing the effects of intra-NTS microinjections of granisetron or bicuculline on the defense reaction-induced inhibition of the baroreflex bradycardia, 3 determinations of Group 1 CBaR inhibition were performed prior to microinjections and considered stable if they differed from each other by less than 10 %. Then, microinjections were performed and another Group 1 CBaR inhibition was determined 15 min later, to be compared with the 3rd determination of the preceding series. Only one experimental procedure was carried out in each rat.

**Histology**

At the end of experiments, electrolytic lesions (50 Hz, 4 mA, 20 s) were made at DMH or dPAG stimulation sites, and methylene blue was microinjected into the injection sites within the NTS. Rats were then perfused intracardially with saline and a solution of 4 % paraformaldehyde in 0.1 M sodium phosphate, pH 7.4. After perfusion, the brain was removed from the skull and coronal sections (60 µm) were cut and stained with Nissl substance.

**Statistics**

Absolute values are expressed as means ±S.E.M of n rats. One-way analysis of variance (ANOVA) followed by multiple comparison Scheffé’s test were used to compare the effects
of NTS microinjections of different doses of granisetron on CBaR inhibition during DMH or dPAG stimulation. Other comparisons were made with Student’s paired or unpaired t-test. Differences were considered significant at P<0.05.

Drugs

α-Methyl-atropine (Sigma-Aldrich, St Quentin-Fallavier, France), bicuculline methiodide (Sigma-Aldrich), CPBG (Research Biochemical Inc, Natick, MA, USA), DL-propranolol (Sigma-Aldrich) and granisetron (Smith Kline-Beecham, Harlow, UK) were dissolved in saline. The pH of all solutions microinjected into the NTS was adjusted to 7.4.

RESULTS

In all rats used in these studies (n=156), the baseline values of HR and MBP were 401±5 bpm and 93±2 mmHg, respectively. Electrical stimulation of DMH or dPAG (Fig 1) produced marked increases in HR and MBP (Table 1). On the other hand, electrical stimulation of the aortic depressor nerve produced typical baroreflex cardiovascular changes: decreases in HR and MBP (Table 1). The ranges of cardiovagal baroreflex responses (CBaR) were -39 % to -44 % in Group 1 (n=113), and -19 % to -24 % in Group 2 (n=43). HR and MBP returned to normal levels within 3 min after cessation of the aortic stimulation.

Effects of DMH and dPAG stimulations on the sympathetic baroreflex response (SBar) in α-methyl-atropine-pretreated rats

As explained in Materials and Methods, SBar was determined in rats pretreated with α-methyl-atropine. On its own, α-methyl-atropine (30 µg/kg i.v., n=22) produced an increase in HR that lasted for more than 30 min but no significant change in basal MBP (Table 1).
aortic baroreflex bradycardia was totally blocked when α-methyl-atropine was given 10 min before electrical stimulation of the aortic depressor nerve (Fig 2). In addition, this compound slightly, but significantly, reduced Group 1 (-35±1 % and -22±1 % before and after α-methyl-atropine, respectively, n=11, p<0.05) and Group 2 (-28±1 % and -20±2 % before and after α-methyl-atropine, respectively, n=11, p<0.05) SBaR. In α-methyl-atropine-pretreated rats, the remaining Group 1 SBaR was not affected by electrical stimulation of DMH (-22±2 % and -23±3 % before and during DMH stimulation, respectively, n=6, Fig 2) or dPAG (-23±2 % and -22±2 % before and during dPAG stimulation, respectively, n=5) site. Likewise, Group 2 SBaR was unaffected by either site stimulation: -20±3 % and -19±3 %, before and during DMH stimulation, respectively (n=6), and -19±3 % and -20±2 %, before and during dPAG stimulation, respectively (n=5).

These effects of α-methyl-atropine lasted for at least one hour.

Effects of DMH and dPAG stimulations on the cardiac baroreflex response (CBaR).

In spontaneously breathing non-pretreated rats, both Groups of evoked CBaR were inhibited during DMH and dPAG stimulation (Table 2). This inhibition was transient as it was no longer observed when CBaR was determined a few seconds only after cessation of DMH or dPAG stimulation. Similar results were obtained in artificially-ventilated rats (Group 1 and Group 2 CBaR inhibition during DMH stimulation: -74±1 %, n=4, and -95±2 %, n=6, respectively; Group 1 and Group 2 CBaR inhibition during dPAG stimulation: -81±5 %, n=5, and -95±5 %, n=5, respectively).

Intravenous administration of saline affected neither basal HR and MBP (Table 1), nor Group 1 CBaR (40±2 % and 42±2 %, before and after saline i.v., n=4, respectively). In some experiments, propranolol (0.4 mg/kg i.v) was given in order to eliminate the DMH- and dPAG-induced increase in HR due to sympathetic activation (see Materials and Methods).
Propranolol did not affect basal MBP and HR values (Table 1). In addition, like saline, propranolol pretreatment did not interfere with the decrease in Group 1 and Group 2 baroreflex bradycardia caused by DMH or dPAG stimulation 10 min later (Table 2).

**Effects of NTS bilateral microinjections of bicuculline and granisetron on the defense reaction-induced inhibition of CBaR.**

Microinjections of saline (n=8) at the level of the calamus scriptorius affected neither HR and MBP (Table 1) nor Group 1 CBaR (-41±1 % and -40±2 %, before and after NTS saline, n=8, respectively). In addition, saline did not affect the defense reaction-induced inhibition of Group 1 CBaR triggered by DMH (-76±3 % and -76±3 %, before and after NTS saline, respectively, n=4) or dPAG (-82±4 % and -81±4 %, before and after NTS saline, respectively, n=4) stimulation.

Intra-NTS microinjections of bicuculline (5 pmol, Fig 3), a specific GABA<sub>A</sub> receptor antagonist, elicited a decrease in both HR and MBP (Table 1) but no changes in Group 1 CBaR (Fig 4 A). However, bicuculline microinjected 15 min before the experimental procedure markedly reduced (for more than 30 min) the inhibition of Group 1 CBaR normally induced by DMH stimulation (n=10, Fig 4 A and Fig 5). Likewise, bicuculline (n=8) prevented the inhibition of Group 1 CBaR normally observed during dPAG stimulation (Fig 5).

Intra-NTS microinjections of granisetron (Fig. 3), a specific 5-HT<sub>3</sub> receptor antagonist, 15 min before the experimental procedure, reduced in a dose-dependent manner the inhibition of Group 1 CBaR by DMH (n=20) or dPAG (n=20) stimulation (Fig 6). The maximal effect was obtained with the dose of 250 pmol and lasted for more than 30 min (Fig 4 B). At this dose, granisetron produced no changes in HR, MBP (Table 1), and Group 1 CBaR (Fig 4 B).
Effects of NTS bilateral microinjections of bicuculline and granisetron on the CPBG-induced inhibition of CBaR.

Control experiments confirmed that bilateral NTS microinjection of CPBG (600 pmol, n=7) induced an increase in blood pressure without affecting HR (see Sévoz et al., 1996a). In addition, this treatment prevented Group 1 CBaR (Table 1 and Fig 7 A) normally evoked by aortic stimulation. The effects of CPBG lasted for at least 45 min. Intra-NTS microinjections of granisetron (250 pmol, n=6) or bicuculline (5 pmol, n=6) 5 min prior to local application of CPBG (600 pmol) completely prevented the inhibitory effect of the latter drug on Group 1 CBaR (Fig 7 B and 7 C).

Effects of NTS bilateral microinjections of granisetron on the defense reaction-induced rise in blood pressure.

Granisetron (250 pmol) did not affect the hypertensive response to electrical stimulation of the DMH (ΔMBP: +50±4 mmHg from a baseline of 95±5 mmHg and +46±3 mmHg from a baseline of 92±3 mmHg, before and after granisetron, respectively, n=6) or the dPAG (ΔMBP: +58±5 mmHg from a baseline of 105±6 mmHg and +56±6 mmHg from a baseline of 98±4 mmHg, before and after granisetron, respectively, n=6). In addition, granisetron (250 pmol) did not affect the increase in heart rate caused by DMH (ΔHR: +40±4 bpm vs +42±7 bpm, before and after granisetron, respectively, n=6) or dPAG (ΔHR: +70±8 bpm vs +71±9 bpm, before and after granisetron, respectively) stimulation.

DISCUSSION
The principal results of the present studies clearly indicate that DMH and dPAG stimulations block the aortic baroreflex bradycardia through the activation of 5-HT\textsubscript{3} and GABA\textsubscript{A} receptors in the NTS.

Electrical stimulation of dPAG in anaesthetized animals is well known to induce an increase in basal cardiorespiratory parameters, which is characteristic of the defense reaction (Inui and Nosaka 1993; Lovick 1993; Bandler and Shipley 1994). In the same manner, electrical stimulation of several regions in the hypothalamus known as the hypothalamic defense area, such as the anterior (Simon et al. 1985) and the posterior (Smith and Barron 1989) areas, and in particular the dorsomedial nucleus (DMH, DiMicco et al. 1996; Bernardis and Bellinger 1998), which has been shown to be the most appropriate site for evoking cardiovascular changes typical of this behavioural reaction (Duan et al. 1996; Nosaka 1996; Fontes et al. 2001), produces similar cardiovascular and respiratory changes. These data were confirmed in the present study by using the same conditions as those previously described for the electrical stimulation of DMH in anaesthetized rats (Barron and Heesch 1990), which have also been applied to dPAG stimulation.

*Effects of the defense reaction on the baroreflex responses*

Controversial results have been reported regarding the effects of the defense reaction on the hypotensive component of the aortic baroreflex. Indeed, electrical stimulation of different areas within the hypothalamic defense area was found to increase (Duan et al. 1996), to decrease (Coote et al. 1979; Nosaka et al. 1993) or to exert no effect (Simon et al. 1985; Barron and Heesch 1990) on the reflex vascular component. In our hands, we noted that the baroreflex hypotensive response remaining after administration of α-methyl-atropine was affected by neither DMH nor dPAG stimulation. These results indicate that the defense
reaction triggered by electrical stimulation of two different brain structures, such as the DMH and the dPAG, did not interfere with the vascular aortic baroreflex response.

However, confirming and extending results obtained in previous studies (Silveira and Graeff 1992; Inui and Nosaka 1993; Fontes et al. 2001), we found that DMH and dPAG stimulations markedly attenuated Groups 1 and 2 bradycardiac responses of the baroreflex triggered by aortic depressor nerve stimulation. Interestingly, similar results were found in rats whose sympathetic cardiac activity was blocked by administration of propranolol, showing that, on its own, the cardiac sympathetic increase associated with the defense reaction did not influence aortic bradycardia. Furthermore, our study showed that the same percentage inhibition of the baroreflex bradycardia during the defense reaction induced by both DMH and dPAG stimulations could be obtained in artificially ventilated rats. Consequently, this inhibition could not be caused by the dramatic changes in frequency and volume respiration that occur normally during the defense reaction in spontaneously breathing animals, even if these data do not eliminate the possibility of some central respiratory influence on the aforementioned inhibition. Finally, the inhibitory influence of the defense reaction on baroreflex bradycardia appeared to involve supraspinal mechanisms because it persisted completely after spinal cord transection (Inui and Nosaka 1993; Nosaka et al. 1993). All these data converge to indicate that dPAG and DMH stimulations exert a centrally mediated inhibitory effect on aortic bradycardia.

NTS microinjections of bicuculline and granisetron: effects on the defense reaction-induced inhibition of the baroreflex bradycardia.

Weiss and Crill (1969) proposed that the NTS, i.e. the first central structure reached by baroreceptor afferents, may be involved, via a presynaptic mechanism, in the defense
reaction-induced inhibition of the baroreflex bradycardia. Interestingly, electrical stimulation of the hypothalamus was found to produce a GABA-mediated inhibition of baroreceptor-sensitive neurons in the NTS (Jordan et al. 1988; Mifflin et al. 1988), and we observed here that NTS bilateral microinjections of a small dose of bicuculline, a selective GABA_A receptor antagonist, prevented the inhibitory effect of the defense reaction on the baroreflex bradycardia. These data were similar to those obtained in our previous studies showing that NTS GABA_A receptors mediate the inhibitory influence of local 5-HT_3 receptor activation on the baroreflex bradycardiac response to systemic administration of phenylephrine (Merahi et al. 1992; Sévoz et al. 1996a). These previous findings led us to suggest that NTS 5-HT_3 receptors could be involved in the defense reaction-induced GABAergic inhibition of the baroreflex bradycardia. The data obtained in the present study provide strong support to this hypothesis because they showed that microinjections of granisetron, a selective 5-HT_3 receptor antagonist, into the NTS, prevented the inhibition of aortic baroreflex bradycardia induced by both DMH and dPAG stimulations. Since NTS 5-HT_3 receptor activation is well known to produce no change in respiration (Sévoz et al. 1996a), a possible central respiratory influence of the defense reaction on the inhibition of the aortic baroreflex bradycardia that would involve these receptors, is unlikely. Analysis of the dose-response curve of granisetron (Figure 6) shows that the minimal dose, 250 pmol, to (almost) completely prevent the inhibition of the baroreflex bradycardia by dPAG and DMH stimulation was i) 50-fold higher than the dose of bicuculline (5 pmol) which produced the same effect and ii) only slightly higher than the threshold dose (175 pmol) of this 5-HT_3 receptor antagonist to significantly reduce the aforementioned inhibition. Interestingly, the dose of intra-NTS bicuculline necessary to block the cardiovascular effects of NTS 5-HT_3 receptor stimulation was also found to be between 10- and 50-fold lower than the effective doses of 5-HT_3 receptor antagonists (Sévoz et al. 1996a; Bonagamba et al. 2000).
One possible explanation of the differences between the relative potencies of bicuculline and granisetron is that the diffusion of the GABA$_A$ receptor antagonist in the NTS is larger than that of the 5-HT$_3$ receptor antagonist. Accordingly, higher doses of granisetron would be necessary for the blockade of 5-HT$_3$ receptors involved in the inhibition of baroreflex bradycardia throughout the NTS. However, further experiments are needed to directly assess this hypothesis.

In our previous studies on 5-HT-mediated inhibition of the cardiac component of the baroreflex, NTS 5-HT$_3$ receptors were activated by local microinjections of nanomolar doses of serotonin or 5-HT$_3$ receptor agonists, and the baroreflex was triggered by phenylephrine-induced increase in blood pressure (Merahi et al. 1992; Sévoz et al. 1996a). As a control, we herein verified that activation of NTS 5-HT$_3$ receptors by local microinjections of CPBG (600 pmol) was also able to block the baroreflex bradycardia evoked by electrical stimulation of the aortic depressor nerve. As expected from an effect specifically mediated by 5-HT$_3$ receptors, the blockade by CPBG could be prevented by prior local microinjections of granisetron. In addition, like that observed in previous studies (Merahi et al. 1992; Sévoz et al. 1996a), prior microinjections of bicuculline were also found to prevent the CPBG-induced inhibition of the aortic baroreflex bradycardia, confirming the involvement of a GABA$_A$-link in this inhibitory effect. The GABA$_A$-mediated inhibitory effect of NTS 5-HT$_3$ receptor activation upon the baroreflex bradycardia was probably the consequence of the activation of a local GABAergic system via a 5-HT$_3$ receptor-mediated release of glutamate from vagal afferents. In line with such a hypothetical mechanism, (i) NTS 5-HT$_3$ receptors are located presynaptically on vagal afferents (Merahi et al. 1992), and (ii) extracellular levels of endogenous glutamate increased markedly within the NTS in response to local 5-HT$_3$ receptor stimulation (Ashworth-Preece et al. 1995). Our previous findings and the present data lead us
to conclude that DMH or dPAG stimulation inhibits the aortic baroreflex bradycardia via the sequential activation of 5-HT$_3$ then GABA$_A$ receptors in the NTS (see Fig 8).

It is noteworthy that, as observed in previous studies (Callera et al. 1997), most histological controls revealed that methylene blue microinjected into the NTS did not reach the area postrema but spread to the dorsal vagal nucleus (data not shown). The dorsal vagal nucleus contains preganglionic vagal motoneurons involved in the control of cardiac activity (Loewy, 1990), and both 5-HT$_3$ and GABA$_A$ receptors are also expressed in this nucleus (Wang et al. 1998; Feng et al. 1990). Therefore, it can be proposed that the defense reaction-induced inhibition of the baroreflex bradycardia might imply, at least in part, a GABA$_A$-mediated inhibition of dorsal vagal motoneurons via the local activation of 5-HT$_3$ receptors. However, this hypothesis is unlikely because (i) it has been shown that microiontophoretic administration of 5-HT$_3$ receptor agonists in the dorsal vagal nucleus excites the local motoneurons (Wang et al. 1998), and (ii) the preganglionic motoneurons involved in the reflex control of heart rate are mostly located in the nucleus ambiguus (Loewy 1990). Thus, the most probable explanation of our data is that activation of NTS 5-HT$_3$ receptors during the defense reaction inhibits, via GABA$_A$-dependent mechanisms, second order NTS neurons at the origin of the baroreflex bradycardia through their projections to the vagal motoneurons in the nucleus ambiguus (Figure 8).

The source of serotonin involved in 5-HT$_3$-mediated inhibition of aortic baroreflex bradycardia is unknown but it is reasonable to assume that it may come from one or several medullary raphe nuclei. Indeed, the NTS receives serotonergic fibers coming from the nuclei raphe obscurus, pallidus and magnus (Thor and Helke 1987). Further studies involving selective lesions of the various bulbar raphe nuclei should help to identify which nucleus
(nuclei) is (are) at the origin of the activation of NTS 5-HT$_3$ receptors to block the baroreflex bradycardia during the defense reaction.

It is important to note that cardiovascular changes, similar to those associated with the defense reaction, can be triggered by other stressful stimuli, including noxious stimuli. In addition to 5-HT$_3$ receptors (this study), NK$_1$ receptors in the NTS have recently been found to be involved in the GABA$_A$-mediated inhibition of the baroreflex bradycardia caused by a somatic noxious stimulus (Boscan et al. 2002). Therefore, it seems reasonable to assume that different neurotransmitters in this structure can contribute to the blockade of baroreflex bradycardia which occurs in response to stressful stimuli. Further experiments are needed to assess whether 5-HT$_3$ receptors in the NTS may also be involved in the baroreflex bradycardia inhibition observed during electrical and/or chemical stimulation of other brain sites than those studied herein (such as parabrachial or amygdala nuclei, see Nosaka 1996), or electrical and/or mechanical noxious stimulation.

*NTS microinjections of granisetron: effects on the defense reaction-induced increase in blood pressure.*

The defense reaction is associated with cardiovascular changes which consist of an increase in cardiac output and a redistribution of blood flow from the skin and viscera (vasoconstriction) to skeletal muscles where occurs a vasodilation to allow activity (see Nosaka 1996). Contradictory results have been reported concerning the participation of the NTS in the tachycardiac response to DMH stimulation (Kunos and Varga 1995; Fontes et al. 2001). In any case, the participation of NTS 5-HT$_3$ receptors in the pressor response to the latter stimulation can be eliminated because (i) NTS 5-HT$_3$ receptor activation does not affect heart rate (Merahi et al. 1992, Sévoz et al 1996a) and (ii) the present data showed that intra-NTS bilateral microinjections of granisetron did not affect dPAG- nor DMH-induced tachycardia.
Like that observed during the electrical stimulation of the dPAG and the DMH, NTS 5-HT\textsubscript{3} receptor activation elicits a significant increase in blood pressure through the excitation of neurons in the rostroventrolateral part of the medulla (Sévoz-Couche et al. 1998; Fontes et al. 2001). Accordingly, it could be hypothesized that NTS 5-HT\textsubscript{3} receptors mediate the defense reaction-induced rise in blood pressure. Indeed, this possibility can be ruled out because we found here that microinjections of granisetron into the NTS did not affect the increase in blood pressure produced by the stimulation of both DMH and dPAG. These data are in line with the idea that the defense reaction-induced sympathoexcitation may be the consequence of a direct activation of the rostroventrolateral part of the medulla (Fontes et al. 2001).

**Conclusion**

It is well known that during stressful conditions such as the defense reaction, the baroreceptor bradycardiac reflex response is inhibited, thereby allowing appropriate behavioral performance. The results reported herein show that 5-HT\textsubscript{3} receptors within the NTS play a key role in this physiological process, but are not involved in the concomitant defense reaction-induced increase in blood pressure. In addition, they confirm that local GABA\textsubscript{A} receptors contribute downstream to the blockade of baroreceptor reflex bradycardia during the defense reaction.
ACKNOWLEDGEMENTS

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aspartate into the NTS are inhibited by local activation of 5-HT(3) receptors.


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LEGENDS to FIGURES

Figure 1: **Camera lucida drawings of frontal brain sections showing the localization of sites in DMH and dPAG where electrical stimulation produced the inhibition of aortic baroreflex bradycardia.**

Sections showing representative sites of electrical stimulation (50 Hz, 1 ms) in DMH (A, ■) and dPAG (B, ●). Numbers represent the distance (in mm) from bregma, according to Paxinos and Watson (1986).

A. cc: corpus callosum; CPu: caudate putamen; Ce: central amygdaloid nucleus; D3V: dorsal third ventricle; DMH: dorsomedial hypothalamic nucleus; VMH: ventromedial hypothalamic nucleus; f: fornix; mt: mamillothalamic tract.

B. dPAG: dorsal part of the periaqueductal gray; mPAG: medial part of the periaqueductal gray; DG: dentate gyrus; tfp: transverse fibers pons.

Figure 2: **The vascular component of the baroreflex induced by aortic depressor nerve stimulation was not affected by DMH stimulation.**

Representative tracings showing that the decrease in HR in response to aortic depressor nerve stimulation (AS, Group 1) in naive rats (left) was blocked by systemic administration of α-methyl-atropine (30 µg/kg i.v., middle). In addition, the vascular response of the aortic baroreflex remaining in α-methyl-atropine-pretreated rats was unchanged when AS occurred during the rise in blood pressure elicited by DMH stimulation (right).

All tracings were obtained from the same rat.
Figure 3: **Camera lucida drawings of frontal brainstem sections showing the localization of microinjection sites in the NTS.**

Sections showing the anatomical distribution of CPBG (A, 600 pmol, ♦, n=4), granisetron (B, 250 pmol, ◊, n=4) and bicuculline (C, 5 pmol, ⊛, n=4) bilateral microinjections at the level of the calamus scriptorius (- 14.1 mm from bregma, according to Paxinos and Watson, 1986).


Figure 4. **NTS microinjections of bicuculline (5 pmol) and granisetron (250 pmol) prevented the inhibition of cardiac baroreflex response (CBaR) by DMH stimulation.**

A and B, upper panels: Representative tracings showing that Group 1 aortic baroreflex bradycardia in response to aortic depressor nerve stimulation (AS, control) was inhibited during DMH stimulation (experimental).

A and B, lower panels: 10 min after NTS bilateral microinjections of bicuculline (5 pmol, A) or granisetron (250 pmol, B), the aortic baroreflex bradycardia (control) was unchanged compared to before NTS microinjections. However, this cardiac response in response to AS was no longer inhibited during DMH stimulation (experimental, left) 15 min after bicuculline (A) or granisetron (B). Inhibition of the aortic baroreflex bradycardia caused by DMH stimulation tended to recover 25 min after NTS microinjection (experimental, middle) and was similar to the control inhibition 10 min later (experimental, right).

All tracings in A were from the same rat, and tracings in B were from another rat.
Figure 5. **NTS microinjections of bicuculline (bic) prevented the inhibition of CBaR during DMH and dPAG stimulation.**

The inhibition of Group 1 CBaR by DMH (n=10) or dPAG (n=8) stimulation (empty bars) was greatly reduced 15 min after NTS bilateral microinjections of bicuculline (5 pmol, black bars).

Values are means ± S.E.M.

*p<0.05 compared to CBaR inhibition prior to bicuculline microinjections.

Figure 6. **Dose-dependent preventive effect of NTS microinjections of granisetron on the inhibition of CBaR by DMH or dPAG stimulation.**

Dose-dependent prevention by NTS bilateral microinjections of granisetron of the inhibition of Group 1 CBaR by DMH ( ■ ) and dPAG ( ◊ ) stimulation, compared to before granisetron (control on abscissa). Inhibition of CBaR is expressed as percentage of basal values determined in the absence of DMH or dPAG stimulation.

Values are means ± S.E.M of n rats.

*p<0.05 compared to before granisetron (control).

§p<0.05 compared to lower dose.

Figure 7: **The inhibitory effect of NTS microinjections of CPBG on aortic baroreflex bradycardia was prevented by prior microinjections of granisetron into the same sites.**

A. Tracings showing that, in naive rats, AS (Group 1) elicited a decrease in HR (left) which was blocked by prior bilateral microinjections of CPBG (600 pmol) into the NTS (right).
The aortic baroreflex bradycardia (left) was not reduced by NTS microinjections of CPBG (right) when bicuculline (5 pmol, B) or granisetron (250 pmol, C) was microinjected 15 min before CPBG.

Figure 8. **Medullary pathways possibly involved in the cardiovascular changes associated with the defense reaction.**

It is known that: 1/ vagal baroreceptor afferents (black) excite NTS cells (“A” and “B”), 2/ NTS “A” cells activate preganglionary (PG) neurons within the nucleus ambiguous (NAmb), to induce the cardiovagal bradycardia of the baroreflex, 3/ NTS “B” cells are responsible for the baroreflex vasodilation. Our previous findings (Sévoz et al. 1996 a, b) showed that activation of 5-HT₃ receptors (small circles, 5-HT₃ R), localized presynaptically on non-cardiovascular (non-CV) vagal afferents in the NTS (in grey), triggers the glutamatergic activation of local GABAergic interneurons thereby inhibiting (grey cross) the baroreflex bradycardia via GABAₐ receptors (GABAₐR). Data reported herein showed that of NTS 5-HT₃ receptors are activated (by 5-HT released from NTS terminals of serotonergic neurons in raphe nuclei?) during the defense reaction triggered by DMH or dPAG stimulation, thereby producing the same GABA-mediated inhibition of baroreflex bradycardia.

⊕: excitatory effect; ⊙: inhibitory effect
Table 1: Changes in heart rate (ΔHR) and mean blood pressure (ΔMBP) during DMH, dPAG and aortic depressor nerve (AS) stimulations, and after systemic administration of saline, propranolol or α-methyl-atropine, or bilateral microinjections of saline, CPBG, granisetron or bicuculline into the NTS, in ventilated and non-ventilated rats (data did not differ between ventilated and non-ventilated rats. Pooled data are presented).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>ΔHR (bpm)</th>
<th>ΔMBP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>brain stimulations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMH (300 µA)</td>
<td>71</td>
<td>+42±5 (397±12)*</td>
<td>+54±3 (84±3)*</td>
</tr>
<tr>
<td>dPAG (200 µA)</td>
<td>66</td>
<td>+72±12 (372±26)*</td>
<td>+57±7 (92±10)*</td>
</tr>
<tr>
<td>aortic stimulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS (100-150 µA, Group 1)</td>
<td>113</td>
<td>-177±6 (409±4)*§</td>
<td>-32±1 (90±1)*§</td>
</tr>
<tr>
<td>AS (50-90 µA, Group 2)</td>
<td>43</td>
<td>-89±6 (416±5)*</td>
<td>-25±1 (89±2)*</td>
</tr>
<tr>
<td>i.v injections</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>saline</td>
<td>4</td>
<td>-5±1 (415±9.)</td>
<td>-2±1 (92±3)</td>
</tr>
<tr>
<td>propranolol (0.4 mg/kg)</td>
<td>8</td>
<td>-47±8 (390±18)*</td>
<td>-6±1 (98±6)</td>
</tr>
<tr>
<td>α-methyl-atropine (30 µg/kg)</td>
<td>22</td>
<td>+46±7 (432±15)*</td>
<td>+5±1 (100±6)</td>
</tr>
<tr>
<td>intra-NTS injections</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>saline</td>
<td>4</td>
<td>-4±1 (403±11)</td>
<td>-4±1 (91±3)</td>
</tr>
<tr>
<td>bicuculline (5 pmol)</td>
<td>24</td>
<td>-67±4 (404±9)*</td>
<td>-24±2 (94±3)*</td>
</tr>
<tr>
<td>granisetron (250 pmol)</td>
<td>18</td>
<td>-6±2 (415±7)</td>
<td>+7±1 (91±4)</td>
</tr>
<tr>
<td>CPBG (600 pmol)</td>
<td>7</td>
<td>+5±1 (420±8)</td>
<td>+26±2 (82±4)*</td>
</tr>
</tbody>
</table>

Positive numbers indicate an increase and negative numbers a decrease from the baselines indicated in parentheses. Each value is the mean ±S.E.M. of n rats. * p<0.05 compared to before treatment. § p<0.05 compared to aortic nerve stimulation at lower intensity.
Table 2: Percentage inhibition of Group 1 and Group 2 cardiovagal baroreflex responses (CBaR) during DMH or dPAG stimulation, in non-pretreated and i.v. saline- or propranolol (0.4 mg/kg)-pretreated spontaneously breathing rats.

<table>
<thead>
<tr>
<th>Brain stimulation</th>
<th>pretreatment</th>
<th>Group 1 CBaR inhibition (%)</th>
<th>Group 2 CBaR inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMH (300 µA)</td>
<td>none</td>
<td>-75±4 (n=38)</td>
<td>-96±3 (n=11) §</td>
</tr>
<tr>
<td></td>
<td>saline</td>
<td>-72±3 (n=4)</td>
<td>-91±3 (n=4) §</td>
</tr>
<tr>
<td></td>
<td>propranolol</td>
<td>-74±4 (n=4)</td>
<td>-92±3 (n=4) §</td>
</tr>
<tr>
<td>dPAG (200 µA)</td>
<td>none</td>
<td>-81±3 (n=36)</td>
<td>-96±4 (n=10) §</td>
</tr>
<tr>
<td></td>
<td>saline</td>
<td>-79±2 (n=4)</td>
<td>-93±2 (n=4) §</td>
</tr>
<tr>
<td></td>
<td>propranolol</td>
<td>-81±2 (n=4)</td>
<td>-96±4 (n=4) §</td>
</tr>
</tbody>
</table>

n= number of rats.

§ p<0.05 compared to inhibition of Group 1 CBaR
A

control

before bicuculline

HR (bpm)

AS

DMH

after bicuculline

10 min

300

500

5 s

AS

DMH

AS

DMH

AS

DMH

B

control

before granisetron

HR (bpm)

AS

DMH

10 min

300

500

15 min

25 min

35 min

after granisetron

DMH

10 min

300

500

15 min

25 min

35 min

5 s

AS

DMH

AS

DMH

AS

DMH

control experimental

control experimental
Inhibition of Group 1 CGR (%)

DMH dPAG

before bic after bic before bic after bic

*
Inhibition of Group 1 CBR (%)

granisetron (pmol/100 nl)
A control
HR (bpm)
AS
CPBG
naive
300
500
300
500

B bicuculline
HR (bpm)
AS
control
CPBG
300
500
300
500

C granisetron
HR (bpm)
AS
control
CPBG
5 s
300
500
300
500
baroreceptor vagal afferents

non-CV vagal afferents

baroreceptor vagal afferents

DMH/dPAG

PG

NAmb

baroreflex bradycardia

baroreflex vasodilation

NTS

GABA

GABA\(_A\) R

5-HT\(_3\) R

5-HT

S-HT

Raphe?

B

A

44