Sympathoexcitatory neurotransmission of the chemoreflex in the NTS of awake rats

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1Department of Physiology and Biophysics, Institute of Biological Sciences, Federal University of Minas Gerais, 31270-901, Belo Horizonte, Minas Gerais; and 2Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, 14049-900, Ribeirão Preto, São Paulo, Brazil

Haibara, Andréa S., Leni G. H. Bonagamba, and Benedito H. Machado. Sympathoexcitatory neurotransmission of the chemoreflex in the NTS of awake rats. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R69–R80, 1999.—Cardiovascular responses to chemoreflex activation by potassium cyanide (KCN, 20 µg/rat iv) were analyzed before and after the blockade of ionotropic or metabotropic receptors into the nucleus of the solitary tract (NTS) of awake rats. Microinjection of ionotropic antagonists [6,7-dinitroquinoxaline-2,3-dione or kynurenic acid (Kyn)] into the lateral commissural NTS (NTSlat), the midline commissural NTS (NTS_mid), or into both (NTSlat + mid), produced a significant increase in basal mean arterial pressure, and the pressor response to chemoreflex activation was only partially reduced, whereas microinjection of Kyn into the NTS_mid produced no changes in the pressor response to the chemoreflex. The bradycardic response to chemoreflex activation was abolished by microinjection of Kyn into the NTSlat or into NTSlat + mid but not by Kyn microinjection into the NTS_mid. Microinjection of α-methyl-4-carboxyphenylglycine, a metabotropic receptor antagonist, into the NTSlat or NTS_mid produced no changes in baseline mean arterial pressure or heart rate or in the chemoreflex responses. These results indicate that 1) the processing of the parasympathetic component (bradycardia) of the chemoreflex seems to be restricted to the NTSlat and was blocked by ionotropic antagonists and 2) the pressor response of the chemoreflex was only partially reduced by microinjection of ionotropic antagonists and not affected by injection of metabotropic antagonists into the NTSlat or NTS_mid or into NTSlat + mid in awake rats.

nucleus of the solitary tract; arterial chemoreceptors; cardiovascular regulation; N-methyl-D-aspartate receptors; non-N-methyl-D-aspartate receptors; metabotropic receptors; kynurenic acid; 6,7-dinitroquinoxaline-2,3-dione; α-methyl-4-carboxyphenylglycine

The peripheral chemoreceptors are important in the regulation of respiratory and cardiovascular functions (8, 25), and the neurotransmission of the chemoreceptors afferent in the NTS as well as in other areas of the ventral medulla has been extensively studied (1, 20, 21, 27, 28, 39). Pharmacological studies have indicated that excitatory amino acid (EAA) receptors in the commissural NTS play an important role in the neurotransmission of the peripheral chemoreflex (39–41). However, most of these experiments were performed while subjects were under anesthesia, which may have a distorting effect on the cardiovascular and ventilatory responses to chemoreflex activation (10). In addition, microinjection of L-glutamate into the NTS of conscious rats elicited opposite cardiovascular responses when compared with the responses obtained in the same animals under anesthesia (23), further confirming that neurotransmission in the NTS is deeply affected by the anesthesia.

In a previous study, we showed that bradycardic response induced by chemoreflex activation was mediated by N-methyl-D-aspartate (NMDA) receptors in the commissural NTS because bilateral microinjection of DL-2-amino-5-phosphonovaleric acid (AP-5), a selective NMDA receptor antagonist, into the lateral portion of the commissural NTS (NTSlat) blocked the bradycardic response in a dose-dependent manner. However, AP-5 produced no effect on the pressor response to the chemoreflex, suggesting that the sympathoexcitatory component of the chemoreflex may be mediated by non-NMDA receptors (16). On the basis of such findings, the present study aimed to evaluate the role of non-NMDA receptors in the neurotransmission of the pressor response to chemoreflex activation (sympathoexcitatory component) in the lateral and medial aspects of the commissural NTS.

Anatomic studies by Finley and Katz (9) have shown that the projections of the carotid body afferents occur mainly in the NTSlat. Electrophysiological studies by Mifflin (27) further confirmed that neurons in this area received input from carotid chemoreceptors. In contrast, recent studies by Chitravanshi and colleagues (2, 3) reported that the carotid chemoreceptor afferents seem to project mainly to the midline portion of the commissural subnucleus of the NTS (NTS_mid), at the calamus scriptorium level. In addition, the blockade of EAA receptors in the NTS_mid of anesthetized rats abolished the pressor and ventilatory responses to chemoreflex activation (39). Therefore, we also evaluated the role of EAA receptors (ionotropic and metabotropic) in the different subregions of the commissural NTS (lateral and midline portions) in the neurotrans-
mission of the sympathoexcitatory component of the chemoreflex (pressor response) in conscious rats. A preliminary report of our findings has been published as an abstract (15).

**METHODS**

Guide cannula implantation in direction of NTS. Male Wistar rats weighing 230–270 g were used in the present study. Four days before the experiments, the rats were deeply anesthetized with tribromoethanol (250 mg/kg ip; Aldrich, Milwaukee, WI) and placed in a stereotaxic frame (Kopf, Tujunga, CA) for guide cannula implantation. When the rat reacted to frequent toe pinching during stereotaxic surgery, additional tribromoethanol was injected. The technique described by Michelini and Bonagamba (26) was adapted to implant guide cannulas in the following three experimental protocols: 1) bilateral guide cannula in the direction of the NTSlat (0.5 mm lateral to midline and ~0.5 mm rostral to calamus scriptorium); 2) one guide cannula in direction of the NTSmed (at midline at level of calamus scriptorium); and 3) three guide cannulas, with two implanted in the direction of the NTSlat and one implanted in the direction of the NTSmed. The implants of all guide cannulas were performed in accordance with the coordinates of Paxinos and Watson (29). To implant each guide cannula, we made a small window in the skull caudal to the lambda and a 15-mm-long stainless steel guide cannula (22 gauge; Small Parts) was introduced perpendicularly through the window at the following coordinates: 0.5 (NTSlat) or 0.0 mm (NTSmed) lateral to the bregma, 14.00 (NTSlat) or 14.5 mm (NTSmed) caudal to the bregma, and 7.9 mm below the skull surface at the bregma (NTSlat and NTSmed). The tip of the guide cannula was positioned ~1.0 mm above the dorsal surface of the brain stem. The guide cannula was fixed to the skull with methacrylate and watch screws and then closed with an occluder until time of experimentation.

Arterial and venous cannulation. One day before the experiments, while rats were under tribromoethanol anesthesia, a catheter (PE-10 connected to PE-50; Clay Adams, Parsippany, NJ) was inserted into the abdominal aorta through the femoral artery for measurement of pulsatile arterial pressure (PAP), mean arterial pressure (MAP), and heart rate (HR). A second catheter was inserted into the femoral vein for systemic administration of potassium cyanide (KCN). Both catheters were tunneled subcutaneously and exteriorized through the back of the neck to be connected to the pressure transducer on the subsequent day. PAP and MAP were measured in conscious, freely moving rats with a pressure transducer (model CDX I; Cobe, Lakewood, CO) connected to a Narcorace 80 physiological recorder (Narco Bio-Systems, Austin, TX). HR was measured with a Narco Biotechnomach Coupler (model 7302).

Microinjections into NTS. For microinjections into the NTS, a 33-gauge needle (Small Parts) 1.5 mm longer than the guide cannula was connected by PE-10 tubing to a 1-µl syringe (Hamilton, Reno, NV). After removal of the occluder, the needle for microinjection was carefully inserted into the guide cannula and manual injection was started 30 s later. For bilateral microinjection, the microinjection was initially performed on one side, the needle was withdrawn and repositioned in the contralateral side, and the second injection was performed. The same procedure was used in the protocols with three guide cannulas. Therefore, the time interval of microinjections into the NTS in the different protocols was ~1 min. The volume for each microinjection was 100 nl in all experimental protocols.

Activation of chemoreflex. The chemoreflex was activated by intravenous injection of KCN (20 µg/rat; Merck, Darmstadt, Germany) in accordance with the procedures described by Franchini and Krieger (10) and was previously validated for our experimental conditions (16). These previous studies demonstrated that the cardiovascular responses to KCN injection were reproducible, and no habituation was observed when the same dose of KCN was systematically injected at intervals of 10 min (16).

Drugs. The drugs microinjected into the NTS were diluted in artificial cerebrospinal fluid (aCSF) containing (in mM) 3 KCl, 0.6 MgCl₂, 2 CaCl₂, 132 NaCl, 24 NaHCO₃, and 4 dextrose or 0.9% saline. The solutions were freshly dissolved in aCSF, except 6,7-dinitroquinoxaline-2,3-dione (DNQX), which was dissolved in 2.5% DMSO (Sigma, St. Louis, MO). Sodium bicarbonate was added to the solutions to adjust the pH to a range of 7.0–7.4.

Experimental protocols. All studies were performed in conscious, freely moving animals. The experimental protocol for the study of neurotransmission into the NTS consisted of the activation of the carotid chemoreflex before and after microinjection of EAA antagonists into the NTSlat or NTSmed or into both sites (NTSlat + NTSmed) by using three guide cannulas. The NTS was functionally identified by previous microinjection of glutamate (1 nmol/100 nl), which, in accordance with our previous studies (5, 6, 23), produces pressor and bradycardic responses of short duration. The peak changes in MAP and HR in response to chemoreflex activation were evaluated before and 10 min after the microinjections of EAA receptor antagonists into the NTS. A third injection of KCN was performed 40 min after antagonist microinjection to evaluate the reversibility of the blockade. The EAA receptor antagonists microinjected into the NTS were DNQX (Research Biochemicals International, 400 lg/ml), a selective non-NMDA receptor antagonist, in three different doses (0.1, 0.5, or 2.0 nmol/100 nl), kynurenic acid (Kyn; Sigma), a nonselective ionotropic antagonist, in one dose (10 nmol/100 nl), and α-methyl-4-carboxyphenylglycine (MCPG; Research Biochemicals International), a selective metabotropic antagonist, in two different doses (2.5 and 5.0 nmol/100 nl). Each rat used in each of the three experimental protocols (DNQX, Kyn, or MCPG) received only one dose of the antagonist. The groups of rats that received microinjection of EAA receptor antagonists into the NTS also received a control microinjection of saline with DMSO (DNQX group) or aCSF (Kyn and MCPG groups) in each individual experiment at least 30 min previous to the microinjection of the respective antagonist.

The selectivity of DNQX for non-NMDA receptors was evaluated in a specific protocol in which NMDA, a selective NMDA receptor agonist (10 pmol/100 nl), was microinjected into the NTS before and after different doses of DNQX, and the effectiveness of MCPG in blocking metabotropic receptors was tested in a specific protocol in which trans-(-)-1-amino-1,3-cyclopentanedicarboxylic acid (trans-ACPD; Research Biochemicals International), a selective metabotropic receptor agonist (250 pmol/100 nl), was microinjected into the NTS before and after microinjection of MCPG (2.5 and 5.0 nmol/100 nl, respectively).

Histological examination. At the end of the experiments, 100 nl of Evans blue dye (2%) were microinjected for histological identification of the sites of microinjection, and later the animals were submitted to intracardiac perfusion with saline followed by 10% buffered Formalin while they were under ether anesthesia. The brains were removed and stored in buffered Formalin for 2 days. Serial coronal sections (10–15 µm thick) were obtained and stained by the Nissl method. Only the rats in which the microinjection sites were located in...
the lateral or midline or in both portions of the commissural NTS, in accordance with the protocol, were considered for data analysis.

Data analysis. All data are expressed as means ± SE. The results were analyzed by one-way ANOVA, and the differences between individual means were determined by Student's t-test, with the level of significance set at 0.05 in all analyses.

RESULTS

Effect of bilateral microinjection of DNQX into NTSlat on cardiovascular responses to chemoreflex activation. Figure 1 illustrates the effects of bilateral microinjection of increasing doses of DNQX (0.1, 0.5, and 2.0 nmol/100 nl) into the NTSlat on the cardiovascular responses to chemoreflex activation with KCN (20 µg iv) of three different rats, representative of their respective groups. Figure 1A shows that DNQX (0.1 nmol/100 nl) produced no changes in the pressor or bradycardic responses to chemoreflex activation, whereas the dose of 0.5 nmol/100 nl (Fig. 1B) attenuated the pressor response but produced no change in the bradycardic response induced by chemoreflex activation. Figure 1C shows that the dose of 2.0 nmol/100 nl also attenuated the pressor response to the chemoreflex in the same way as observed with the dose of 0.5 nmol/100 nl and blocked the bradycardic response. Bilateral microinjection of DNQX into the NTS produced a significant increase in basal MAP. Table 1 shows the absolute values of MAP and HR before and 10 min after bilateral microinjection of DNQX into the NTSlat and indicates that DNQX produced a significant increase in basal MAP at all doses, without any significant changes in basal HR.

The data related to the effects of DNQX on the chemoreflex responses are summarized in Fig. 2 and show that increasing doses of DNQX reduced but did not abolish the pressor response to the chemoreflex activation when compared with the control response.

<table>
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<tr>
<th>DNQX, nmol/100 nl</th>
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<th>Before</th>
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<th>After</th>
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<tr>
<td>0.1</td>
<td>7</td>
<td>99 ± 4</td>
<td>111 ± 2*</td>
<td>339 ± 7</td>
<td>331 ± 10</td>
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<tr>
<td>0.5</td>
<td>5</td>
<td>114 ± 6</td>
<td>139 ± 7*</td>
<td>373 ± 15</td>
<td>390 ± 6</td>
</tr>
<tr>
<td>2.0</td>
<td>6</td>
<td>100 ± 5</td>
<td>126 ± 5*</td>
<td>338 ± 12</td>
<td>342 ± 12</td>
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Values are means ± SE. MAP, mean arterial pressure; HR, heart rate; NTSlat, lateral portion of the commissural nucleus of the solitary tract. *Statistically different in relation to values before microinjection of 6,7-dinitroquinoxaline-2,3-dione (DNQX) (P < 0.05, paired t-test).
Changes in the bradycardic response (doses of 0.1 and 0.5 nmol/100 nl induced no significant effects on the pressor or bradycardic responses to the chemoreflex, the microinjection of DNQX at the doses of 0.1 and 0.5 nmol/100 nl, respectively) in the three groups of rats studied. In relation to the parasympathetic component of the chemoreflex, the microinjection of DNQX at the doses of 0.1 and 0.5 nmol/100 nl in comparison with control. Bottom: changes in HR were significantly reduced only after DNQX at 2.0 nmol/100 nl, a dose that is not selective for non-NMDA receptors (Table 4). One-way ANOVA and differences between individual means were determined by Student’s modified t-test with Bonferroni correction for multiple comparisons.

Values are means ± SE. No statistical difference in change in \( \Delta \) MAP or \( \Delta \) HR was observed when values before and after DMSO microinjection were compared (paired t-test). Injections were made in same group of rats that received microinjection of DNQX (0.1, 0.5, and 2.0 nmol/100 nl).

**(Table 2).** Changes in MAP and HR in response to chemoreflex activation before and 10 min after bilateral microinjection of 2.5% DMSO into NTSlat in 3 groups of rats. Top: significant differences in pressor response were observed after doses of 0.5 and 2.0 nmol/100 nl in comparison with control. Bottom: changes in HR were significantly reduced only after DNQX at 2.0 nmol/100 nl, a dose that is not selective for non-NMDA receptors (Table 4). One-way ANOVA and differences between individual means were determined by Student’s modified t-test with Bonferroni correction for multiple comparisons.

**Table 3.** Changes in MAP and HR in response to chemoreflex activation before and 10 min after misplaced microinjections of DNQX or Kyn into areas adjacent to NTS

Values are means ± SE. No statistical difference in \( \Delta \) MAP or \( \Delta \) HR was observed when values before and after misplaced microinjections were compared (paired t-test).
significantly reduced the cardiovascular responses to chemoreflex activation. In these cases, the pressor responses were significantly reduced (NTSlat, +50 ± 2 vs. +30 ± 3 mmHg; NTSlat+mid, +48 ± 3 vs. +22 ± 8 mmHg) and the bradycardic responses were almost abolished (NTSlat, −225 ± 28 vs. −18 ± 7 beats/min; NTSlat+mid, −213 ± 28 vs. −11 ± 5 beats/min). In contrast, microinjection of Kyn into the NTSmid did not affect the pressor (+57 ± 5 vs. 39 ± 8 mmHg) or bradycardic response (−191 ± 37 vs. −163 ± 64 beats/min) to chemoreflex activation. Microinjection of Kyn into the NTSlat, NTSmid, or NTSlat+mid induced a significant increase in baseline MAP but not in baseline HR (Table 5).

The effects of Kyn microinjection into the NTSlat or NTSlat+mid on the chemoreflex were reversible, considering that 60 min after microinjection of the antagonist, the cardiovascular responses to chemoreflex activation were similar to those observed before microinjection. In addition, bilateral microinjection of the vehicle (aCSF) into the NTSlat (n = 6) did not modify the pressor (+48 ± 3 vs. +51 ± 1 mmHg) or bradycardic (−214 ± 26 vs. −216 ± 27 beats/min) responses to chemoreflex activation.

The effects of Kyn on the cardiovascular responses to chemoreflex activation were restricted to the NTS, because misplaced microinjections into areas adjacent to the NTS did not modify the pressor or bradycardic responses to chemoreflex activation (Table 3).

Effect of microinjection of MCPG into NTSlat or NTSmid on cardiovascular responses to chemoreflex activation. Neither dose of MCPG (2.5 and 5.0 nmol/100 nl) modified the cardiovascular responses to chemoreflex activation. Therefore, the data for the dose of 2.5
Figure 5 shows the tracings of one rat in which microinjection of MCPG into the NTSmid also did not affect the responses to chemoreflex activation, and Fig. 5B shows the tracings of one rat in which bilateral microinjection of MCPG into the NTSlat induced no changes in the pressor or bradycardic response to chemoreflex activation after microinjection of Kyn (10 nmol/100 nl) into NTSlat (n = 6), NTSmid (n = 6), or NTSlat-mid (n = 6) of 3 different groups of rats. *Statistically different in relation to control response (P < 0.05, paired t-test).

Several lines of experimental evidence support the concept that EAA receptors play a major role in the processing of cardiovascular afferents in the NTS (4, 13, 14, 16, 38) and particularly in the neurotransmission of the chemoreflex in the NTS (2, 3, 20, 27, 39, 40). In a recent study on unanesthetized rats, we showed that the bradycardic response to chemoreflex activation was blocked in a dose-dependent manner by AP-5, an NMDA receptor antagonist, whereas the pressor response was not affected (16). However, previous studies performed on anesthetized rats have reported that administration of EAA antagonists into the NTS blocked the pressor response (sympathoexcitatory component) of the chemoreflex activation (39, 40); such blockade was not observed in the present study in the absence of anesthesia. Bilateral microinjection of DNQX (a selective non-NMDA receptor antagonist) at the dose of 0.5 nmol/100 nl, which does not affect NMDA receptors, significantly reduced but did not abolish the pressor response to chemoreflex activation (+64 ± 4 vs. +34 ± 8 mmHg).

Figure 6 summarizes the data related to the blockade of metabotropic receptors and shows that MCPG (2.5 nmol/100 nl) microinjected into the NTSlat or NTSmid did not modify the pressor response (NTSlat, +56 ± 4 vs. +53 ± 6 mmHg; NTSmid, +45 ± 8 vs. +45 ± 9 mmHg) or in bradycardic response (NTSlat, −241 ± 18 vs. −226 ± 16 beats/min; NTSmid, −210 ± 45 vs. −212 ± 45 beats/min) to chemoreflex activation. Microinjection of MCPG into these two subregions of the commissural NTS did not elicit significant changes in baseline MAP or in baseline HR (Table 6).

The dose of MCPG microinjected into the NTSlat was effective in blocking the metabotropic receptors, because previous microinjection of MCPG (2.5 nmol/100 nl) into the NTSlat of a specific group of rats (n = 8) significantly reduced the depressor (−66 ± 8 vs. −24 ± 9 mmHg) as well as the bradycardic (−267 ± 26 vs. −80 ± 3 beats/min) responses induced by trans-ACPD (250 pmol/100 nl) microinjected into the same site. Similar blockade was achieved with the dose of 5.0 nmol/100 nl of MCPG (data not shown).

Histological analyses of microinjection sites. Figure 7 is a photomicrograph of a transverse section of the brain stem of the rat, showing the site of microinjections in the NTS. Figure 7A is a photomicrograph of a coronal section of the brain stem of one rat representative of the group in which the microinjections were performed bilaterally into the NTSlat. Figure 7B is a photomicrograph of a coronal section of the brain stem of one rat representative of the group in which the microinjections were performed into the NTSmid.

Figure 8 is a schematic representation of the brain stem at the calamus scriptorium level and shows the overlapping sites of Evans blue dye microinjections into the NTSlat (Fig. 8A) or NTSmid (Fig. 8B) of rats used in the protocols of Kyn microinjection into the NTS. The average of the anterior-posterior extension of the area stained by Evans blue dye was 626 ± 42 µm (NTSlat) and 648 ± 89 µm (NTSmid).

**DISCUSSION**

Table 5. Baseline MAP and HR before and 10 min after microinjection of Kyn (10 nmol/100 nl) into NTSlat, NTSmid, or NTSlat-mid.

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<th>Baseline MAP, mmHg</th>
<th>HR, beats/min</th>
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<td></td>
<td>Before</td>
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</tr>
<tr>
<td>NTSlat</td>
<td>6 99 ± 3</td>
<td>121 ± 2*</td>
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<tr>
<td>NTSmid</td>
<td>6 111 ± 4</td>
<td>125 ± 6*</td>
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<tr>
<td>NTSlat-mid</td>
<td>6 102 ± 4</td>
<td>136 ± 4*</td>
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Values are means ± SE. NTSmid, midline portion of the commissural NTS. *Statistically different in relation to values before microinjection (P < 0.05, paired t-test).
17 mmHg), whereas the highest dose of DNQX used (2.0 nmol/100 nl), which is not selective for non-NMDA receptors, did not elicit any additional reduction in the pressor response to chemoreflex activation (+59 ± 4 vs. +33 ± 10 mmHg).

The partial reduction of the pressor response to chemoreflex activation observed after DNQX may be related, at least in part, to the involvement of non-NMDA receptors in the neurotransmission of the sympathoexcitatory component of the chemoreflex in the NTSlat. However, these results require careful interpretation because another possibility to explain why the pressor response to chemoreflex activation was reduced by DNQX is the significant increase in the baseline MAP observed after the bilateral microinjection of DNQX into the NTSlat. With respect to the DNQX protocol, it is important to note that the additional increase in MAP in response to chemoreflex activation was smaller than in the control, probably due to the increase in the baseline sympathetic activity and consequently in MAP. It is also important to emphasize that the pressor response to chemoreflex activation is essentially due to sympathetic activation and not to vasopressin or any other circulating neurohormone, because in previous studies we verified that the treatment with prazosin (intravenously), an a1-adrenoceptor antagonist, almost abolished the pressor response of the chemoreflex (16).

The effects observed after bilateral microinjection of DNQX into the NTSlat on baseline MAP were probably due to the blockade of the sympathoinhibitory pathway of the baroreflex and suggest that non-NMDA receptors located on neurons of this subregion of the NTS are

Table 6. Baseline MAP and HR before and 10 min after microinjection of MCPG (2.5 nmol/100 nl) into NTSlat or NTSmid

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<th>MAP, mmHg</th>
<th>HR, beats/min</th>
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<td></td>
<td>Before</td>
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<tr>
<td>NTSlat</td>
<td>8</td>
<td>106 ± 3</td>
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<tr>
<td>NTSmid</td>
<td>5</td>
<td>102 ± 2</td>
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Values are means ± SE. No statistical difference was observed when MAP and HR were compared before and after \(\alpha\)-methyl-4-carboxyphenylglycine (MCPG) microinjection.
involved in the neurotransmission of this component of the baroreflex. This possibility is supported by a previous study by Gordon and Leone (12) showing that non-NMDA receptors in the NTS play a predominant role in mediating the hypotensive response produced by aortic baroreceptor stimulation. In addition, we verified in a previous study that the blockade of the NMDA receptors in the NTS produced no changes in baseline MAP (16).

Another important aspect of the present study relates to the blockade of the bradycardic response to chemoreflex activation observed after the dose of 2.0 nmol/100 nl of DNQX microinjected bilaterally into the NTS. In a previous study, we showed that the cardio-vagal component of this reflex is mediated by NMDA receptors (16). Therefore, the bradycardic response should not be affected by the non-NMDA receptor antagonist unless the dose used is not selective for this receptor subtype. In this respect, a specific protocol used in the present study showed that DNQX at the dose of 2.0 nmol/100 nl is not selective for non-NMDA receptors because it also blocked the cardiovascular responses to NMDA microinjection into the NTS. The protocol using 0.5 nmol/100 nl of DNQX shows that this dose is in the range of selectivity for non-NMDA receptors, because the cardiovascular responses to NMDA receptors and the bradycardic response to chemoreflex activation were not affected, whereas baseline MAP was increased. Therefore, this evidence supports the concept that the cardiovagal component of the chemoreflex is effectively mediated by NMDA receptors in the NTS.

The present data show that DNQX microinjected into the NTS did not abolish the pressor response to chemoreflex activation. To explain these findings, at least two possibilities should be considered. First, the EAA receptors in this subregion of the commissural NTS, particularly the non-NMDA receptors, may not be

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**Fig. 7.** Photomicrographs of transverse sections of brain stem showing bilateral microinjection sites in NTS at level of rostral edge of area postrema (A, NTS protocol) and site of microinjection into NTS at level of calamus scriptorium (B, NTS protocol). Arrows indicate sites of microinjections in NTS marked with Evans blue dye.

**Fig. 8.** Line drawing of transverse section of brain stem from 1 mm rostral to 0.3 mm caudal to calamus scriptorium [adapted from Paxinos and Watson (29)]. Filled areas represent overlapping areas of Evans blue dye distribution in NTS (A) or NTS (B) of all rats used in protocols of Kyn microinjection into NTS or NTS. AP, area postrema; Gr, gracile nucleus; Sol M, medial NTS; Sol C, commissural NTS; CC, central canal; X, dorsal motor nucleus of vagus; XII, hypoglossal nucleus; NA, ambiguus nucleus; CVL, caudal ventrolateral medulla; Cu, cuneate nucleus.
involves the sympathoexcitatory neurotransmission of the chemoreflex. Second, the blockade of non-NMDA receptors only in the NTSlat may not be sufficient to block all the receptors located in different sites of the NTS probably involved in the processing of the afferent information for the chemoreflex. A study by Zhang and Mifflin (40) showed that the pressor response of the chemoreflex was blocked when Kyn, a broad-spectrum EAA receptor antagonist, was microinjected into the NTSlat. In a study by Vardhan et al. (39), the pressor response to chemoreflex activation was also blocked only after the blockade of NMDA and non-NMDA receptors in the NTSmid. Therefore, in these studies (39, 40), which were performed in anesthetized animals, the pressor response to chemoreflex activation was abolished after a nonselective blockade of ionotropic receptors in the NTSlat or NTSmid. Considering these previous studies and also that isolated blockade of NMDA (16) or non-NMDA receptors (present study) in the NTSlat of unanesthetized rats was not able to block the pressor response to chemoreflex activation, we also evaluated the effects of the Kyn microinjection into the NTSlat or NTSmid on the pressor response to chemoreflex activation in unanesthetized rats.

The reduction in magnitude of the pressor response to chemoreflex activation induced by microinjection of Kyn into the NTSlat was similar to that induced by microinjection of DNQX into the same area. In another group of rats, microinjection of Kyn into the NTSmid did not affect the pressor response to chemoreflex activation. In both cases (NTSlat and NTSmid), the microinjection of Kyn produced a significant increase in baseline MAP, similar to the effect of DNQX microinjection into the NTSlat. One possibility that cannot be ruled out to explain the reduction in the pressor response to chemoreflex activation after microinjection of Kyn into NTSlat may be related to the increase in baseline MAP, similar to that observed after microinjection of DNQX into the NTSlat. On the other hand, microinjection of Kyn into NTSmid, despite increasing the baseline MAP, produced no attenuation in the pressor response to chemoreflex activation. Therefore, this observation indicates that the reduction in the pressor response to chemoreflex activation by Kyn microinjected into the NTS probably is not associated with the increase in the baseline MAP. However, it is important to note that the baseline increases in MAP produced by Kyn into the NTSlat or NTSmid were different (+22 vs. +13%, respectively). This difference may explain why the increase in MAP in response to chemoreflex activation after Kyn into the NTSlat was smaller than the increase observed after Kyn into NTSmid. Similar findings were obtained with microinjections of DNQX into NTSlat. The dose of 0.1 nmol/100 nl into the NTSlat increased baseline MAP by 12% and produced no effect on the pressor response to chemoreflex activation, whereas the dose of 0.5 nmol/100 nl increased the baseline MAP by 22% and induced a significant reduction in the pressor response to chemoreflex activation. Alternatively, we might also consider the possibility that the increase in baseline MAP, secondary to the basal increase in sympathetic activity after Kyn or DNQX into the NTSlat, reduces the magnitude of the pressor response to chemoreflex activation because the maximal increase of the sympathethic activity remained the same. The experimental protocol in which we performed blockade of EAA receptors in the NTSlat and NTSmid (NTSlat-mid) with Kyn (10 nmol/100 nl) shows that the pressor response to chemoreflex activation was also only partially reduced, suggesting that the processing of the excitatory component of the chemoreflex in the NTS involves neurotransmitters and receptors other than EAAs and their receptors.

In relation to the parasympathetic component of the chemoreflex, our data show that Kyn microinjected into the NTSlat abolished the bradycardic response, probably due to the blockade of NMDA receptors, as we demonstrated previously with microinjections of AP-5 into the same subregion of the NTS (16). However, Kyn microinjected into the NTSmid produced no change in the bradycardic response to chemoreflex activation, suggesting that this subregion of the commissural NTS is not directly involved in the processing of the parasympathetic component of this reflex. A study by Colombari et al. (7) shows that the integrity of NTSmid is essential for the pressor and bradycardic responses to chemoreflex activation, but their findings do not necessarily imply that the neurotransmission of the parasympathetic component of the chemoreflex occurs in this subregion of the NTS. Studies by Finley and Katz (9) have shown dense labeling in the NTSlat after injection of wheat germ agglutinin-conjugated horseradish peroxidase into the vasculally isolated carotid body, whereas a functional study by Vardhan et al. (39) has shown that the NTSmid plays a key role in the neurotransmission of the sympathoexcitatory component of the chemoreflex in the NTS. No study has evaluated the neurotransmission of the parasympathetic component of the chemoreflex, as we did in the present as well as in a previous investigation from our laboratory (16). Because of this controversy related to the site of termination of the chemoreflex afferents and the processing of the sympathetic and parasympathetic components of the chemoreflex in different subregions of the NTS, in the present study we avoided this problem by performing microinjection into the lateral commissural NTS (NTSlat) and into the midline of commissural NTS (NTSmid), or in both (NTSlat-mid), i.e., in all sites in which the chemoreflex neurotransmission seems to be processed. In this case, we observed that the processing of the parasympathetic component of the chemoreflex seems to be restricted to the NTSlat.

Considering that the antagonism of ionotopic EAA receptors in the NTSlat or NTSmid with DNQX or Kyn (NTSlat-mid) was not able to block the pressor response to chemoreflex activation, and also considering previous evidence that metabotropic receptors mediate excitatory transmission in the NTS (11–13), we also evaluated the possible involvement of this class of EAA receptors in the processing of the neurotransmission of the sympathoexcitatory component of the chemoreflex. The microinjection of MCPG, a metabotropic antagonist, into the NTSlat or NTSmid also did not modify the
basal MAP and HR or the pressor and bradycardic responses to chemoreflex activation, indicating that the metabotropic receptors in the NTS play no major role in the neurotransmission of the parasympathetic and sympathetic components of the chemoreflex.

The activation of the chemoreflex with KCN in unanesthetized rats produces, in addition to cardiovascular and ventilatory responses, an important behavioral response (10) which seems to be a consequence of the stimulation of the hypothalamic areas involved with the defense reaction (25). Considering that chemical or electrical stimulation of these hypothalamic defense areas increases sympathetic activity (17, 30–33), we may suggest that, in contrast to studies performed under anesthesia, the pressor response induced by chemoreflex activation in the present study depends at least in part on the hypothalamic defense areas. It is important to note that the behavioral responses to KCN, such as exploration of the cage and alertness, were also eliminated by bilateral ligature of the carotid body artery (16) or by deafferentation of the carotid sinus nerve bifurcation (10). Under anesthesia, the alertness and the defense reaction component of the chemoreflex responses are abolished, a fact that may explain why in the studies by Vardhan et al. (39) and Zhang and Miffin (40) working with anesthetized rats, the pressor response of the chemoreflex was blocked by EAA receptor antagonists into the NTS. In addition, it is important to note that in the experiments by Vardhan et al. (39) and Zhang and Miffin (40), no changes in HR were observed in response to chemoreflex activation, whereas in the present study as well as in a previous study from our laboratory (16) we observed an intense bradycardic response. Therefore, when animals are under anesthesia, the behavioral and cardiovascular responses to chemoreflex activation are different, as demonstrated by Franchini and Krieger (10), and the data obtained in conscious and anesthetized animals cannot be easily compared. The degree of involvement of hypothalamic areas in the generation of the pressor response of the chemoreflex in the present study was not experimentally addressed, and therefore further studies on unanesthetized rats are required to explore the possible involvement of hypothalamic projections from and to the NTS on the processing of the sympathoexcitatory component (pressor response) of the chemoreflex.

The involvement of other neurotransmitters in the processing of the chemoreflex afferents in the NTS has been considered. Studies using microdialysis demonstrated that the release of substance P (22, 35) into the NTS was increased during hypoxia. Adenosine may also play a role in the processing of the chemoreflex in the NTS, especially in the projection from the hypothalamic defense areas to the NTS (36, 37). However, the role of these putative neurotransmitters and their different subtypes of receptors in the processing of the pressor response to the chemoreflex in the lateral and medial commissural NTS of unanesthetized rats and particularly in the neurotransmission of the sympathoexcitatory component of the chemoreflex requires additional studies.

The data of the present study indicate that the neurotransmission of the sympathoexcitatory component of the chemoreflex is more complex than the cardiovagal component and suggest that neurotransmitters other than EAAs may play a key role in the processing of the sympathoexcitatory component (pressor response) of the chemoreflex in the lateral and medial commissural NTS.

Perspectives

In a previous study (16), we verified that the bradycardic response to the chemoreflex activation was blocked in a dose-dependent manner by AP-5, a selective NMDA receptor antagonist, whereas the pressor response was not affected. In accordance with those results, we suggested that the sympathoexcitatory component (pressor response) of the chemoreflex could be mediated by non-NMDA receptors at the NTS level, and the experiments presented in the current study were performed to verify this hypothesis. However, the data of this study indicated that different ionotropic (Kyn and DNQX) or metabotropic (MCPG) receptor antagonists were not able to block the pressor response of the chemoreflex, even when Kyn was microinjected simultaneously in three different sites of the NTS. Considering that in a previous study an NMDA receptor antagonist (AP-5) also produced no blockade of the pressor response of the chemoreflex, we may suggest that EAA receptors may not be involved in the processing of the neurotransmission of the sympathoexcitatory component of the chemoreflex in the NTS. Because of this unexpected finding, new and interesting perspectives are open to studies involving the neurotransmission of the sympathoexcitatory component of the chemoreflex in the NTS. One important aspect that must be considered in the present study is related to the fact that the activation of the chemoreflex in unanesthetized rats, in addition to the cardiovascular and respiratory changes, produces behavioral responses, which may contribute to the pressor response. Therefore, studies involving the possible role of the hypothalamic defense area, for example, are required to verify if projections from this area to the NTS participate in the generation of the pressor response to chemoreflex activation. Studies are also required on the possible involvement of other areas, including the parabrachial nucleus and periaqueductal gray in the complex physiological responses to the activation of the chemoreflex in unanesthetized rats, including cardiovascular, respiratory, and behavioral aspects. In addition to the involvement of other areas of the brain in this pressor response, another important aspect to be studied, as a consequence of the present findings, is related to the possibility that neurotransmitters/neuromodulators other than EAAs may play a key role in this neurotransmission. Among these potential neurotransmitters/neuromodulators of the pressor response of the chemoreflex in the NTS, evidence in the literature indicates the involvement of substance P and adenosine in this neurotransmission.
mission. Therefore, experiments using the antagonist of the different subtypes of tachycinergic and purinergic receptors in the NTS should be also performed, preferentially in unanesthetized rats, to verify their possible involvement in the neurotransmission of the pressor response to chemoreflex activation.

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