Glutamatergic Antagonism in the NTS Decreases Post-Inspiratory Drive and Changes Phrenic and Sympathetic Coupling During Chemoreflex Activation

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Costa-Silva JH, Zoccal DB, Machado BH. Glutamatergic antagonism in the NTS decreases post-inspiratory drive and changes phrenic and sympathetic coupling during chemoreflex activation. J Neurophysiol 103: 2095–2106, 2010. First published February 17, 2010; doi:10.1152/jn.00802.2009. For a better understanding of the processing at the nucleus tractus solitarius (NTS) level of the autonomic and respiratory responses to peripheral chemoreceptor activation, herein we evaluated the role of glutamatergic neurotransmission in the intermediate (iNTS) and caudal NTS (cNTS) on baseline respiratory parameters and on chemoreflex-evoked responses using the in situ working heart-brain stem preparation (WHBP). The activities of phrenic (PND), cervical vagus (cVNA), and thoracic sympathetic (tSNA) nerves were recorded before and after bilateral microinjections of kynurenic acid (Kyn, 5 nmol/20 nl) into iNTS, cNTS, or both simultaneously. In WHBP, baseline sympathetic discharge markedly correlated with phrenic bursts (inspiration). However, most of sympathoexcitation elicited by chemoreflex activation occurred during expiration. Kyn microinjected into iNTS or into cNTS decreased the postinspiratory component of cVNA and increased the duration and frequency of PND. Kyn into iNTS produced no changes in sympathoexcitatory and tachypneic responses to peripheral chemoreflex activation, whereas into cNTS, a reduction of the sympathoexcitation, but not of the tachypnea, was observed. The pattern of phrenic and sympathetic coupling during the chemoreflex activation was an inspiratory-related rather than an expiratory-related sympathoexcitation. Kyn simultaneously into iNTS and cNTS produced a greater decrease in postinspiratory component of cVNA and increase in frequency and duration of PND and abolished the respiratory and autonomic responses to chemoreflex activation. The data show that glutamatergic neurotransmission in the iNTS and cNTS plays a tonic role on the baseline respiratory rhythm, contributes to the postinspiratory activity, and is essential to expiratory-related sympathoexcitation observed during chemoreflex activation.

INTRODUCTION

The nucleus tractus solitarius (NTS) is the site of first synapses of peripheral afferents arising from the respiratory and cardiovascular systems, such as pulmonary, baro-, and chemoreceptors (Loewy 1990; Miffin 1992). Besides, intermingled with interneurons and neurons of different orders in the NTS, it is also found groups of inspiratory and expiratory neurons, namely dorsal respiratory group (DRG) (Berger 1977; de Castro et al. 1994; Jiang and Shen 1991; Lipski et al. 1983; Richerson and Getting 1992; Saether et al. 1987; Subramanian et al. 2007). Although its involvement in the resting respiratory pattern generation is not well understood, there is evidence showing that the DRG is involved in the processing of somatic reflexes, modulating the respiratory and sympathetic activities (Bonham and McRimmon 1990; Bonham et al. 1993; Dobbins and Feldman 1994; Otake et al. 1989; Subramanian et al. 2007). Previous studies have demonstrated that the antagonism of glutamatergic receptors in the NTS leads to important changes in respiratory and cardiovascular baseline parameters (Bonham et al. 1993; Braccialli et al. 2008; Machado and Bonagamba 2005), revealing that γ-glutamate is released tonically at this level, and it is important to the control of respiratory and sympathetic activities. In addition, other studies demonstrated the involvement of γ-glutamate and its receptors in the neurotransmission of cardiorespiratory reflexes, such as the peripheral chemoreflex (Almado and Machado 2005; Bonham et al. 1993; Braga et al. 2007; Vardhan et al. 1993; Zhang and Mifflin 1993).

The activation of peripheral chemoreceptors produces increases in sympathetic, parasympathetic, and respiratory activities (Braga et al. 2007; Haïbara et al. 1995). Previous studies demonstrated that the respiratory and sympathetic responses to peripheral chemoreflex activation are tightly entrained, and the increase in sympathetic activity occurs in bursts mainly during the expiratory phase (Dick et al. 2004; Mandel and Schrehofer 2009). This pattern of respiratory-sympathetic coupling differs from that observed during eupnea in which the sympathetic activity exhibits peaks of discharges during the end of inspiration (Gilbey et al. 1986; Malpas 1998; Zoccal et al. 2008).

It is well described that the respiratory system markedly modulates the sympathetic nerve discharge (Dick et al. 2004; Haselton and Guyenet 1989; Malpas 1998; Zoccal et al. 2008), and two main mechanisms contribute to the respiratory rhythmic oscillations in the sympathetic activity: information arising from peripheral receptors, such as baroreceptors and pulmonary stretch receptors, and central coupling of respiratory and sympathetic neurons located in the pons and medulla (Adrian et al. 1932; Bernardi et al. 2001; Häbler et al. 1996). Considering that the NTS is an important site for the respiratory and autonomic control, it is possible that this region also plays an important role in the entrainment of respiratory and sympathetic activities not only in resting conditions but also during acute hypoxic stimulus. Thus, in the present study we sought to assess the role of glutamatergic neurotransmission in the different subregions of the NTS on the maintenance of baseline respiratory activity as well as on the sympathetic and respiratory coupling in resting and during acute hypoxia. To reach these goals, microinjections of glutamate receptors antagonism were performed in different subregions of NTS using an in situ working heart-brain stem preparation.
(WHBP) in which vagus, phrenic, and thoracic sympathetic nerve activities were simultaneously recorded.

METHODS

Animals

All experiments were performed in juvenile male Wistar rats (65–85 g) obtained from the Animal Care of the University of São Paulo, Ribeirão Preto, Brazil. The animals were maintained in standard environmental conditions (23 ± 2°C, mean ± SD; 12:12 h dark/light cycle) with water and chow ad libitum. All experimental protocols were approved by the Ethical Committee on Animal Experimentation of the School of Medicine of Ribeirão Preto, University of São Paulo (Protocol 80/2007) and are in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

In situ WHBP

The animals were deeply anesthetized with halothane (Astra Zeneca, Cotia, SP, Brazil) such that the withdrawal responses to noxious pinching of the tail and paw were absent. The animals were then transected caudal to the diaphragm, exsanguinated, and submerged in a cooled Ringer solution. They were decerebrated at the precoculic level to make insentient, skinned, and had descending aorta isolated. Preparations were then transferred to a recording chamber, the descending aorta was cannulated and perfused retrogradely with Ringer solution (containing, in mM: 125 NaCl, 24 NaHCO3, 3 KCl, 2.5 CaCl2, 1.25 MgSO4, 1.25 KH2PO4, and 10 glucose) containing 1.25% Ficoll (an oncotic agent; Sigma, St Louis, MO) using a roller pump (Watson-Marlow 502s, Falmouth, Cornwall, UK) via a double-lumen cannula. The perfusion pressure was maintained in the range of 50–70 mmHg by adjusting the rate flow between 21 and 25 ml/min and by adding vasopressin to the perfusate (600–1,200 pM, Sigma) as previously described (Zoccal et al. 2008). The perfusate was gassed continuously with 5% CO2-95% O2, warmed to 31–32°C, and filtered by a window discriminator the R-wave was captured with the phrenic activity giving a continuous physiological index of preparation viability. The electrocardiogram was acquired from phrenic nerve activities were simultaneously recorded.

Data acquisition

Electrode activities from all nerves were obtained using glass suction electrodes held in a micromanipulator (Narishige, Tokyo, Japan). Left phrenic nerve discharges were recorded from its central end using a unipolar electrode, and its rhythmic ramping activity gave a continuous physiological index of preparation viability. The electrocardiogram was acquired from phrenic recording and by a window discriminator the R-wave was captured and the inter-R wave interval displayed as heart rate (HR). The left cervical vagus and left thoracic sympathetic (at the level of T9–T12) nerves were cut distally and their central activities recorded using bipolar glass suction electrodes. All signals were amplified, band-pass filtered (0.05–5 kHz), and acquired in an A/D converter (CED 1401, Cambridge Electronic Design, Cambridge, UK) to a computer using Spike2 software (version 5, CED).

Chemoreflex activation

Peripheral chemoreceptors were stimulated by injections of potassium cyanide (KCN 0.05%, 50 μl) into the descending aorta of the WHBP via the perfusion cannula as previously described (Braga and Machado 2006; Braga et al. 2007). The stimulation of the peripheral chemoreflex receptors by KCN 0.05% produced consistent autonomic and respiratory responses, which present low variability within and among the WHBPs.

Microinjection into the NTS

The coordinates used for microinjections into the NTS were determined by stereotaxic atlas (Paxinos and Watson 1998), using the calamus scriptorius (CS) as reference point. Drugs were applied bilaterally via glass micropipette (tip diameter, 20–30 μm) and the injected volume (~20 nl) was determined by previous calibration of the picopump system (Picospritzer II, Parker Instruments). Microinjections were performed into the intermediate NTS (iNTS; 0.3 mm rostral to the CS; 0.3 mm lateral to midline), into the caudal NTS (cNTS; 0.3 mm caudal to the CS; 0.2 mm lateral to midline), or simultaneously into the iNTS and cNTS. The depth of the microinjections was 0.3–0.4 mm ventral to the dorsal surface. Bilateral microinjections into the iNTS and cNTS were performed sequentially in these sites within a time period no longer than 90 s.

Experimental protocols

To antagonize the ionotropic glutamatergic receptors in the NTS, bilateral microinjections of an effective dose of kynurenic acid (Kyn, 250 mM, 5 nmol/20 nl) (Braga et al. 2006) were performed into iNTS (Fig. 1, A1), into cNTS (Fig. 1, A3) or into iNTS and cNTS simultaneously (Fig. 1B, I and 3). In all experimental groups, chemoreflex was activated before (2 activations to obtain consistent control responses) and 2, 10, 30, 45, and 60 min after Kyn microinjections.

Data analysis

All the analyses were performed off-line in rectified and integrated (τ = 100 ms) signals using Spike 2 software with custom-written scripts. Vagus and thoracic sympathetic nerve recordings were subtracted from the electrical noise obtained after the death of WHBP.

FIG. 1. A: schematic coronal sections of the brain stem showing the sites of the center of microinjections of kynurenic acid (black circles) into the intermediate nucleus tractus solitarius (iNTS, A1) or caudal NTS (cNTS, A3). B: schematic coronal sections of the brain stem showing the sites of the center of microinjections of kynurenic acid (black circles) into the iNTS and cNTS simultaneously (B, I and J). C: photomicrographs of coronal sections of the brain stem of rats representative of the group that received microinjections into the cNTS (J) and iNTS (J) showing the tracts and the center of the microinjections (white arrows). A2–C2 correspond to the calamus scriptorius (CS), which was used as an anatomical reference. The iNTS was located 0.3 mm rostral and the cNTS 0,3 mm caudal to CS. Schematic drawings modified from Paxinos and Watson (1998). AP, area postrema; XII, hypoglossal nucleus. Scale bar: 500 μm.
**Baseline measurements**

Phrenic nerve activity was assessed by its frequency (Hz) and duration (s) of discharges. In relation to central vagus activity, phrenic-triggered averages were obtained from 10 respiratory cycles. Area under the curve of these averages was calculated and divided into two components: inspiratory (coincident with phrenic burst) and postsipnatory (coincident with expiratory phase). Thus the perceptual proportion between inspiratory or postsipnatory components and the total area (inspiratory plus postsipnatory areas) were obtained to express the vagus activity.

**Responses to chemoreflex activation**

Phrenic nerve response to the chemoreflex activation was assessed by the difference between baseline phrenic burst frequency and the peak of response observed after the stimulus (Δ PND; expressed in Hz). Likewise, bradycardic response was also evaluated by difference of heart rate before and after the stimulus (Δ HR; expressed in bpm) and the peak values were observed in a time window ≤10 s after the stimulus. The sympathetic response was assessed by the measurement of area under the curve, in a time window ≤10 s after the stimulus, and expressed as percentage values (Δ thoracic sympathetic nerve activity, tSNA; in %) in relation to the activity before the stimulus.

**Analysis of respiratory-sympathetic coupling**

Phrenic-triggered averages of tSNA (10 respiratory cycles) were obtained, and the proportion of inspiratory (coincident with phrenic discharge) and expiratory components of tSNA (between phrenic discharges) were measured by the area under the curve and expressed in percentage in relation to the total area (inspiratory plus expiratory activities). This analysis was performed at rest and during peripheral chemoreflex activation.

**Histology**

At the end of each experiment, the brain stem was removed and fixed by immersion for 5 days in 10% buffered formalin. Histological procedures were performed to verify the micropipette track and the site of microinjections in the NTS. Serial transverse sections (18 μm thickness) were cut and stained with cresyl violet using the Nissl method. The rats in which the center of microinjection in the NTS was identified in only one side, whereas in the other side, the center was dorsal and out of the NTS were used as part of an unilateral misplaced group and compared with the group that received bilateral microinjections into the NTS.

**Drugs**

Kyn, a glutamate ionotropic receptor antagonist, was purchased from Sigma Chemical. The dose of Kyn used (Kyn, 250 mM, 5 nmol/20 nl) was determined in a previous study from our laboratory (Table 1). Potassium cyanide (KCN 0.05%, 50 μl) was purchased from Merck (Darmstadt, Germany). The drugs were diluted in NaCl 0.9% sterile solution (Samtec Biotecnology, Ribeirão Preto, Brazil), and their pHs were adjusted to 7.4 using sodium bicarbonate (Reagen, Rio de Janeiro, Brazil).

**Statistic analysis**

The results were expressed as means ± SE and analyzed by repeated measures analysis of variances followed by post hoc Newman-Keuls test. Differences were considered significant when $P < 0.05$. The comparisons were carried out on GraphPad Prism software (GraphPad Software, version 4).

**Results**

**Sympathetic and phrenic coupling before and during chemoreflex activation**

During eupneic breathing, sympathetic nerve discharge markedly correlates with phrenic bursts (Fig. 2A) with 63 ± 3% of activity during the inspiration (Fig. 2, B1 and C1). During chemoreflex activation, we observed two different patterns of coupling (phase 1 and 2, Fig. 2A). In the first phase, 44 ± 3% of the sympathetic excitation occurred during inspiration and 56 ± 3% during expiration. In the second phase, 24 ± 3% of the sympathetic excitation occurred during inspiration and 76 ± 3% during expiration (Fig. 2, B2 and C2).

**Antagonism of glutamatergic transmission in the iNTS**

Bilateral microinjections of Kyn (250 mM, 5 nmol/20 nl) into the iNTS produced significant changes in the basal respiratory pattern. As shown in Table 1, it was observed a significant increase in frequency and in duration of phrenic discharge. In addition, as illustrated in Fig. 3B, microinjection of Kyn into the iNTS produced important changes in the pattern of vagus nerve when compared with the control condition (Fig. 3A), with decrease in the postsipnatory component (77.3 ± 1.6 vs. 33.0 ± 3.2%, n = 8) and increase in the inspiratory component (22.7 ± 1.6 vs. 67.0 ± 3.2%, n = 8; Fig. 3, C and D).

In relation to the peripheral chemoreflex responses, Kyn into the iNTS did not change the tachypneic and sympathetically-activated responses but significantly reduced the bradycardic response at 2 and 10 min, which was back to control values 60 min later (Figs. 4A and 5A). Figure 6 (A1–C1) shows that the pattern of basal coupling between sympathetic and phrenic activity was not altered by the antagonism of glutamate receptors in the iNTS, as 62 ± 12% of sympathetic activity remained during inspiration. However, the normal pattern of the coupling during chemoreflex activation was affected by Kyn in the iNTS (Fig. 6A2). It was also observed that 78 ± 8% of the sympathetic excitation in response to chemoreflex was correlated with inspiratory phase and 22 ± 8% with expiratory phase (Fig. 6, B2 and C2).

**Antagonism of glutamatergic transmission in the cNTS**

Bilateral microinjections of Kyn into the cNTS produced a significant increase in frequency and duration of phrenic discharge (Table 1). In addition, microinjections of Kyn into the cNTS produced important changes in the pattern of vagus nerve activity when compared with control (Fig. 7, A and B) with a decrease in its postsipnatory component (23.9 ± 1.3 vs. 38.9 ± 2.3%, n = 8) and an increase in its inspiratory component (23.9 ± 1.3 vs. 61.1 ± 2.3%, n = 8) as illustrated in Fig. 7, C and D.

In relation to the peripheral chemoreflex responses, Kyn into the cNTS did not affect the tachypneic response. On the other hand, the sympathoexcitatory response was significantly reduced 2 min after Kyn (113 ± 10 vs. 67 ± 17%, n = 8).
Likewise, bradycardic response was also reduced at 2, 10, and 30 min after Kyn. Those responses returned to control values 60 min later (Figs. 4B and 5B). Similar to iNTS, the antagonism of glutamate receptors in the cNTS did not alter the basal pattern of coupling between sympathetic and phrenic activity (Fig. 8, A1–C1). However, the coupling during chemoreflex activation was altered as the sympathetic excitation was correlated mainly with inspiration (61 ± 6%, Fig. 8, A2–C2).

Simultaneous antagonism of glutamate transmission in the iNTS and cNTS

Kyn microinjected bilaterally into the iNTS and cNTS produced large increases in both frequency and duration of phrenic discharge, as shown in Table 1. In addition, Kyn into the iNTS and cNTS changed vagus activity (Fig. 9B) when compared with control (A). Postinspiratory component of the vagus nerve activity decreased (81 ± 1 vs. 27 ± 8%, n = 9) and inspiratory

| TABLE 1. Frequency and duration of phrenic discharge before and after bilateral microinjection of kynurenic acid into the intermediate NTS, caudal NTS, or simultaneously in both intermediate and caudal NTS of rats |
|-----------------|-----------------|-----------------|-----------------|
|                 | iNTS            | cNTS            | iNTS and cNTS   |
|                 | control         | Kyn             | control         | Kyn             | control         | Kyn             |
| PND, Hz         | 0.31 ± 0.03     | 0.63 ± 0.07*    | 0.39 ± 0.02     | 0.88 ± 0.11*    | 0.37 ± 0.04     | 0.76 ± 0.09*    |
| DI, s           | 0.52 ± 0.25     | 1.1 ± 0.2*      | 0.57 ± 0.04     | 0.84 ± 0.13*    | 0.55 ± 0.04     | 1.2 ± 0.2*      |

Values are means ± SD. iNTS and cNTS, intermediate and caudal nucleus tractus solitarius, respectively; PND, phrenic nerve discharge; DI, duration of phrenic discharge (seconds); Kyn, kynurenic acid. *P < 0.05 in relation to the control.
component increased (19 ± 1 vs. 73 ± 8%, n = 9) after simultaneous microinjections of Kyn into iNTS and cNTS, as illustrated in Fig. 9, C and D.

In relation to chemoreflex responses, Kyn into the iNTS and cNTS produced: a significant reduction in the tachypneic response at 2 (0.44 ± 0.06 vs. 0.02 ± 0.06 Hz), 10 (0.44 ± 0.06 vs. 0.02 ± 0.08 Hz), and 30 min after (0.44 ± 0.06 vs. 0.21 ± 0.07 Hz, n = 9) as illustrated in Fig. 4C and summarized in Fig. 5C2; a significant reduction in the sympathoexcitatory response at 2 (102 ± 12 vs. 24 ± 6%), 10 (102 ± 12 vs. 34 ± 5%), and 30 min after (102 ± 12 vs. 84 ± 8%, n = 9), as illustrated in Fig. 4C and summarized in Fig. 5C3; and a significant reduction in the bradycardic response until 45 min as illustrated in Fig. 4C and summarized in Fig. 5C1. All responses to chemoreflex activation were back to control 60 min after Kyn microinjections, as illustrated in Figs. 4C and 5C.

It is important to note that simultaneous Kyn microinjections into the iNTS and cNTS abolished the correlation between phrenic and sympathetic nerve activity in the basal as well as during chemoreflex activation as clearly shown in Fig. 4C2.

**Unilateral microinjection of Kyn into the NTS**

Considering that in the WHBP the approach to place micropipettes in the NTS is performed using a stereoscopic microscope, the index of misplaced microinjections is quite low. In this study, we considered in the misplaced group those animals in which the microinjection into the NTS was performed unilaterally and in the contralateral side it was dorsal and out of the NTS (Supplemental Fig. S1,1 A and B). In this misplaced group (n = 7), unilateral microinjection of Kyn produced a statistically significant increase in the duration of the inspiration (0.51 ± 0.03 vs. 0.67 ± 0.06 s, Supplemental Fig. S2A) as well as in the respiratory frequency (0.37 ± 0.02 vs. 0.58 ± 0.06 Hz, Supplemental Fig. S2C). However, the effect of the unilateral microinjection on the respiratory frequency was significantly smaller than that observed in the group with bilateral microinjections of Kyn into the cNTS (0.58 ± 0.06 vs. 0.88 ± 0.11 Hz, n = 8, Supplemental Fig. S2D). In relation to the effects of unilateral microinjection of Kyn into the NTS on the autonomic and respiratory responses to chemoreflex activation, the data show that no significant changes were observed in the tachypneic (0.47 ± 0.06 vs. 0.51 ± 0.05 Hz, Supplemental Fig. S3C) and in the sympathoexcitatory responses (99 ± 9 vs. 83 ± 11%, E), whereas in the animals that received bilateral microinjections into the cNTS, the sympathoexcitatory response was significantly reduced (113 ± 10 vs. 67 ± 17%, n = 8, F). With respect the bradycardic responses, it was significantly reduced in the animals with unilateral microinjection (−265 ± 16 vs. −180 ± 27 bpm), whereas in the group that received bilateral microinjections, it was almost abolished (−235 ± 26 vs. −8 ± 3 bpm, n = 8, Supplemental Fig. S3, A and B). Therefore these data point out that in spite of an effect of unilateral microinjection of Kyn into the cNTS on baseline respiratory parameters and on the autonomic and respiratory responses to chemoreflex activation, it was significantly smaller than in the group receiving bilateral microinjections of Kyn into the cNTS. Altogether, these data show that unilateral microinjection of Kyn into the cNTS is enough to alter the baseline respiratory parameters and indicate that the effects of this antagonist are specific for the NTS because the magnitude of changes in

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1 The online version of this article contains supplemental data.
DISCUSSION

The main findings of the present study were: L-glutamate in the NTS plays a tonic role controlling the duration of inspiration, postinspiratory output of vagus nerve is dependent on glutamatergic transmission in the NTS, and the antagonism of ionotropic glutamate receptors in the iNTS or cNTS did not alter the coupling between phrenic and sympathetic activities at rest while it changed the pattern of coupling during chemoreflex activation, inhibiting the expiratory-related sympathoexcitation. These results represent new insights about the role of NTS neurons in the modulation of respiratory pattern and its interaction with the sympathetic nervous system, especially during chemoreflex activation.

Role of NTS in the maintenance of basal respiratory rhythm

Different subtypes of inspiratory and expiratory neurons were identified along the NTS (de Castro et al. 1994; Saether et al. 1987; Subramanian et al. 2007), such as pump cells, a group of neurons located in the ventrolateral and medial subnucleus of the NTS that exhibited an augmenting pattern of activity during inspiration (Bonham and McCrimmon 1990; Bonham et al. 1993; Moreira et al. 2007; Saether et al. 1987; Takakura et al. 2007). Besides, groups of inspiratory-decrementing and postinspiratory cells are also observed in the ventrolateral and medial subnucleus of the NTS (Subramanian et al. 2007). Studies by Subramanian et al. (2007) showed that the activation of inspiratory cells in the parameters evaluated were greater when both sides of the cNTS were reached by the microinjections of Kyn.
the medial subnucleus of the NTS is associated to a reduction in the respiratory frequency, suggesting that those cells play an inhibitory modulation on the respiratory rhythm. Our data are in agreement with these observations because we demonstrated that glutamate in the iNTS and cNTS, acting on its ionotropic receptors, play a tonic inhibitory role on the respiratory rhythm. In this scenario, we hypothesize that this inhibitory role of glutamate in the NTS may involve the activation of inhibitory pathways to or within the ventral respiratory column (Moreira et al. 2007; Paton 1997; Takakura et al. 2007), which may contribute to generation of postinspiratory activity required for inspiratory switch-off (Smith et al. 2007). This hypothesis is supported by our findings demonstrating that the antagonism of glutamate receptors in the NTS significantly reduced the postinspiratory activity of central vagus activity. In addition, the data showing that unilateral microinjection of the Kyn into the cNTS is enough to produce significant changes on the respiratory pattern support the concept that glutamatergic neurotransmission in the NTS play a key role in the control of the respiratory rhythm.

According to the site of microinjection into the NTS, Kyn induced different changes in the baseline respiratory pattern. Microinjections of Kyn into the iNTS produced increases mainly in duration of inspiration, whereas Kyn in the cNTS induced changes in respiratory frequency, suggesting that glutamatergic mechanisms in the iNTS are important for regulation of the duration of inspiration and that respiratory neurons in this NTS subregion may modulate the inspiratory off-switch. In this way, it was described that along the NTS, in the ventrolateral subnucleus of the NTS, it is located a group of second order neurons, named pump cells, which receive afferents from pulmonary stretch receptors and play a role in inspiratory off-switch (Bonham and McCrimmon 1990; Bonham et al. 1993; Marchenko and Sapru 2000; Saether et al. 1987). Besides, it was shown that excitatory inputs to these cells were mediated by l-glutamate in the iNTS (Almado and Machado 2005; Miyazaki et al. 1999). Considering that the volume (20 nl) used in the our microinjections can spread to a radius as large as 200 μm (Nicholson 1985), it is possible that the Kyn had reached other subnucleus of the iNTS and cNTS, such as ventrolateral. Thus the changes in respiratory pattern following microinjection of Kyn into the NTS may be due to, at least in part, the spread of the drug solution to those subnucleus.

**Fig. 5.** A: average changes in HR (Δ HR, A1), in tSNA (Δ tSNA, A2), and in PND (Δ PND, A3) induced by chemoreflex activation (KCN 0.05%) before (C, ■) and 2, 10, 30, 45, and 60 min after the microinjections of Kyn (●) into the iNTS (n = 8). B: average changes in HR (Δ HR, B1), in tSNA (Δ tSNA, B2), and in PND (Δ PND, B3) induced by chemoreflex activation (KCN 0.05%) before (C, ■) and 2, 10, 30, 45, and 60 min after the microinjections of Kyn (●) into the cNTS (n = 8). C: average changes in HR (Δ HR, C1), in tSNA (Δ tSNA, C2), and in PND (Δ PND, C3) induced by chemoreflex activation (KCN 0.05%) before (C, ■) and 2, 10, 30, 45, and 60 min after the microinjections of Kyn (●) into the iNTS and cNTS (n = 9). *P < 0.05; **P < 0.001 in relation to the control response (C).
As discussed in the preceding text, ventrolateral subnucleus of the NTS receives afferents inputs from pulmonary stretch receptors and is involved in the inspiratory off-switch. However, in WHBP the pulmonary afferents are absent (as the chest is open and the lungs are static), showing that mechanisms other than pulmonary stretch re-

![Diagram](image)

**Fig. 6.** A: tracings of 1 WHBP representative of the group, showing raw and integrated (∫) tSNA and PND during baseline condition 2 min after Kyn microinjections into the iNTS (A1) and during chemoreflex activation (KCN 0.05%, A2) of 1 WHBP after Kyn microinjections into the iNTS. B: phrenic-triggered averages of tSNA during rest (control, B1) and during the chemoreflex activation (B2) of 1 WHBP representative of the group, showing the inspiratory (I) and expiratory-related areas (E) of the tSNA. Phrenic-triggered averages of tSNA during rest (top) and chemoreflex activation (bottom). C: average proportion of the group of inspiratory (Insp, coincident with phrenic discharge) and expiratory components of tSNA (Exp, between phrenic discharges) at rest (control, C1) and during the chemoreflex activation 2 min after Kyn into the intermediate NTS (C2, n = 8).

![Diagram](image)

**Fig. 7.** Tracings of 1 WHBP representative of the group, showing raw and integrated (∫) cVNA before (control, A) and 2 min after bilateral microinjections of Kyn into the cNTS (B). Phrenic-triggered averages of cVNA before (control) and 2 min after bilateral microinjections of Kyn into the cNTS (C). Average proportion of inspiratory (I) and postinspiratory (PI) components of cVNA measured by the area under the curve before (control) and 2 min after bilateral microinjections of Kyn into the caudal NTS (D, n = 8). +P < 0.001 in relation to the control.
Receptors also mediate the control of duration of inspiration. In this way, experiments performed in vagotomized rats showed a normal control of duration of the inspiration (Wasserman et al. 2000), highlighting the hypothesis that central mechanisms may mediate the inspiratory off-switch in absence of afferents from pulmonary stretch receptors.

FIG. 8. A: tracings of 1 WHBP representative of the group, showing raw and integrated (∫) tSNA and PND during rest 2 min after Kyn microinjections into the caudal NTS (A1). A2: the sympathetic and phrenic responses elicited by chemoreflex activation (KCN 0.05%, †) of 1 WHBP after Kyn microinjections into the caudal NTS. B: phrenic-triggered averages of tSNA during rest (control, B1) and during the chemoreflex activation (B2) of 1 WHBP representative of the group, showing the inspiratory (I) and expiratory-related areas (E) of the tSNA. Phrenic-triggered averages of thoracic sympathetic activity (tSNA) during rest (top) and chemoreflex activation (bottom). C: average proportion of the group of inspiratory (Insp, coincident with phrenic discharge) and expiratory components of tSNA (Exp, between phrenic discharges) at rest (control, C1) and during the chemoreflex activation 2 min after Kyn into the cNTS (C2; n = 8).

FIG. 9. Tracings of 1 WHBP representative of the group, showing raw and integrated (∫) cVNA before (control, A) and 2 min after bilateral microinjections of Kyn into iNTS and cNTS (B). Phrenic-triggered averages of cVNA before (control) and 2 min after bilateral microinjections of Kyn into the iNTS and cNTS (C). Average proportion of inspiratory (I) and postinspiratory (PI) components of cVNA measured by the area under the curve before (control) and 2 min after bilateral microinjections of Kyn into the cNTS into the iNTS and cNTS (D, n = 9). +P < 0.001 in relation to the control.
Studies of Miyazaki et al. (1998) demonstrated that the pump cells, located mainly in the ventrolateral subnucleus of the NTS also received inputs from the central respiratory generator and might contribute to the control of duration of inspiration in absence of pulmonary stretch receptors, as is the case in the WHBP.

Reduced postinspiratory drive after antagonism of glutamate transmission in the NTS

The recordings of vagus nerve activity provide us with a central index of inspiratory and postinspiratory activity. The vagus nerve motor output is generated by inspiratory neurons from the rostral ventral respiratory group (rVRG) and postinspiratory neurons from the ventral respiratory column, mainly from the Bötzing complex (Smith et al. 2007; Tian et al. 1997). In the present study, the antagonism of glutamate ionotropic receptors in the iNTS, cNTS, or simultaneously in both subnucleus reduced significantly the postinspiratory and enhanced the inspiratory component of the vagus nerve, suggesting an increase in excitatory drive to rVRG and/or a decrease to Bötzing complex (Núñez-Abades et al. 1993; Song and Poon 2004). This pattern of breathing was similar to two-phase respiratory rhythm produced by removal of pontine circuits (Smith et al. 2007) that is characterized by absence of postinspiratory activity. Likewise, lesion or pharmacological manipulation of parabrachial and/or Kölliker-Fuse pontine nucleus (dorsolateral pons) abolished the postinspiratory output and enhanced the duration of inspiration (Dutschmann and Herbert 2006; Wang et al. 1993), indicating that these pontine nuclei may play a role in postinspiratory activity output as well in inspyatory off-switch. The reduced postinspiratory activity produced by Kyn into the NTS led to increases in frequency discharge and duration of phrenic burst, indicating that glutamate neurotransmission in the NTS contribute to the postinspiratory output and postinspiratory activity contributes to the modulation of the length of expiration and the inspiratory off-switch. Our data suggest that the NTS is part of this complex neuronal network in charge of generating excitatory drive to postinspiratory neurons in the ventral respiratory column or pontine nucleus, which contribute to the eupneic respiratory rhythm (Smith et al. 2007).

Ventilatory and autonomic responses to peripheral chemoreflex activation after antagonism of glutamate receptors in the NTS

Peripheral chemoreflex activation in the WHBP induces bradycardia and tachypnea and an increase in sympathetic nerve activity (see Fig. 4). In present study, we verified that antagonism of glutamate receptor in the iNTS, in the cNTS, or in both simultaneously, reduced the bradycardic response to peripheral chemoreflex activation. Studies by Haibara et al. (1995) performed in awake rats demonstrated that the antagonism of N-methyl-d-aspartate (NMDA) receptors in the NTS abolished the bradycardic response to chemoreflex activation, indicating that activation of the cardio-vagal component of this reflex in the NTS is mediated by NMDA receptors. Herein we showed that glutamatergic neurotransmission in the iNTS and cNTS contributes to bradycardia of this reflex. In relation to tachypneic response, we showed that Kyn microinjected into iNTS or cNTS did not alter the tachypneic response to chemoreflex activation. However, after microinjection of Kyn into iNTS and cNTS simultaneously, the activation of chemoreflex induced an apneustic pattern of breathing, suggesting an abolishment of excitatory drive to postinspiratory neurons, which are important to inspiratory off-switch (Baeky et al. 2008). It is well established that peripheral chemoreceptors activation provides a powerful excitatory drive to the respiratory network and involves a synchronized activation of both inspiratory and expiratory neurons from different areas, which will provide the source for coordinated responses of inspiratory and expiratory activity. Here we hypothesize that the respiratory disruption produced by glutamatergic antagonism in the NTS may lead to several changes in the processing of chemoreflex at brain stem level, including alterations in sympathetic activity output during chemoreflex.

We observed that sympathetic nerve activity in normal conditions is well correlated with phrenic discharges, reaching the peak at the end of inspiration. In addition, we also observed that during chemoreflex activation, the pattern of coupling was different. The analyses performed in the present study provide evidences about a differential recruitment of neurons after chemoreflex activation. We observed two distinct phases during chemoreflex activation. First, the sympathetic excitation occurs at both inspiratory and expiratory periods of respiratory cycle, and second, sympathetic excitation occurs mainly during expiration. Studies in cats suggested that the sympathetic response elicited by peripheral chemoreflex activation (by hypoxic or cytotoxic hypoxia) consists of two components: one dependent and another independent on phrenic nerve activity (Huang et al. 1988). Further, it was observed that the sympathetic excitation to chemoreflex activation occurs mainly at expiratory phase of the breathing cycle in anesthetized and vagotomized rats (Dick et al. 2004; Koshiya et al. 1993; Mandel and Schreihofer 2009). These findings provided evidence that during peripheral chemoreceptors activation, there is a different recruitment of neurons responsible for generation of respiratory and sympathetic coupling. The present study shows that the antagonism of glutamate ionotropic receptor in the iNTS or cNTS produced no effects on baseline respiratory and sympathetic coupling at rest. Nevertheless the respiratory and sympathetic coupling during chemoreflex activation was deeply affected by the antagonism in the iNTS or cNTS. It was observed that the increase in sympathetic nerve activity elicited by activation of chemoreflex was correlated mainly with inspiratory phase. These data show that glutamatergic neurotransmission in the iNTS or cNTS is essential for generating the excitatory drive to expiratory neurons during chemoreflex activation, which would be responsible for generating a large increase in sympathetic activity during expiratory phase. In addition, we observed that in absence of that excitatory drive to expiratory neurons, a distinct recruitment of inspiratory-related neurons provides the excitatory drive to sympathetic neurons to preserve the large increase in thoracic sympathetic nerve activity in response to chemoreflex activation. Thus our data suggest that glutamatergic inputs to NTS participate of neural network to generate respiratory and sympathetic coupling at rest and during chemoreflex activation.
Conclusions

Glutamatergic inputs to NTSs that are independent of pulmonary stretch receptors play a key role in the maintenance of the euvneic respiratory rhythm, modulating the neuronal network involved generation of inspiratory activity. In relation to peripheral chemoreflex, we concluded that the expiratory-related sympathoexcitatory during hypoxia is dependent on intact postinspiratory activity of three-phase respiratory rhythm and that the glutamatergic neurotransmission in the NTS is essential to maintain this pattern of breathing and consequently normal sympathetic and phrenic coupling during chemoreflex activation.

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