GABA-mediated neurotransmission in the ventrolateral NTS plays a role in respiratory regulation in the rat

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GABA-mediated neurotransmission in the ventrolateral nucleus of the solitary tract (vNTS) plays a role in respiratory regulation in the rat. We recently reported that neurons in the region of the vNTS promote the transition from inspiration to expiration and function as part of the inspiratory off-switch (IOS) for instituting artificial respiration when necessary. Cervical apneusis; apnea; inspiratory off-switch

WE RECENTLY REPORTED that neurons in the region of the ventrolateral nucleus of the solitary tract (vNTS) of the rat have an important role in the control of respiratory function (50). Our hypothesis was that these neurons act as part of the “inspiratory off-switch” (IOS) and terminate the inspiratory phase of the respiratory cycle. This was tested using drugs that hyperpolarize (GABA), disfacilitate (kynurenic acid), and block both axonal conduction and action potential generation (tetrodotoxin). These drugs were used as tools to ascertain the function of the vNTS and were not used to delineate the neurotransmitter systems involved. Bilateral microinjection of each of these agents prolonged inspiration duration (TI) and evoked an apneustic pattern of breathing. These results supported our hypothesis that neurons in the region of the vNTS promote the transition from inspiration to expiration and function as part of the IOS.

As vNTS neurons appear to function as an integral part of the IOS in the rat, we next focused our attention on the question of which central nervous system neurotransmitter systems control the level of activity of these neurons. Recent data of Ellenberger (11) suggest a widespread role for GABAergic regulation of respiratory function within respiratory-related portions of the NTS (e.g., ventrolateral, interstitial, and commissural subnuclei). This was demonstrated for glutamic acid decarboxylase (GAD) immunohistochemistry. Relatively high GAD immunoreactivity was found associated with each of these subnuclei. Terai and colleagues (45) found positive staining of presumptive terminals in the vNTS using a polyclonal antibody to GABA. These investigators also examined the distribution of GABA\textsubscript{A} receptors in the NTS and reported that this subunit was moderately to highly expressed within the vNTS. They used a monoclonal antibody to the β2/3 subunit of the GABA\textsubscript{A} receptor. Finally, we and others reported that TI increases after intravenous administration of baclofen to selectively activate GABA\textsubscript{B} receptors (20, 44).

Thus the primary aim of the present study was to evaluate the role of GABAergic neurotransmission in the vNTS of the rat in the control of respiratory function with a specific focus on control of the inspiratory phase of the respiratory cycle.

MATERIALS AND METHODS

General Experimental Methods

Microinjection experiments were performed on 115 adult male Sprague-Dawley rats (Taconic, Germantown, NY) weighing 250–450 g. Anesthesia was instituted with an intraperitoneal injection of a cocktail containing 800 mg/kg urethane and 60 mg/kg a-chloralose dissolved in 0.9% saline. This cocktail was also used to supplement anesthesia as necessary to maintain the animal areflexic to toe pinch. The trachea was cannulated to provide access to the airway and for instituting artificial respiration when necessary. Cervical apneusis; apnea; inspiratory off-switch

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vagus nerves were exposed bilaterally and sectioned in all animals to prevent slowly adapting pulmonary stretch receptor afferents from influencing central respiratory rhythm. The superior laryngeal nerve has been demonstrated to project to the vNTS region in the cat (25) and, to exclude this input as well, two animals were studied in which both superior laryngeal nerves were sectioned along with bilateral cervical vagotomy. No obvious alteration in breathing or response to microinjected drugs was observed (data not shown). The left carotid artery was isolated, and PE-50 tubing was inserted to obtain blood pressure, which was recorded using a bridge amplifier connected to a MacLab (ADInstruments, Milford, MA) data-acquisition system. Temperature was maintained at 37 ± 2°C with an infrared heating lamp. Changes in respiration were assessed by monitoring diaphragmatic electromyogram (dEMG) activity. The dEMG was obtained by making an incision below the chest cavity and inserting a hooked bipolar platinum-iridium electrode into the right half of the costal diaphragm. The electrode was coupled to a Tektronix AM 502 differential AC preamplifier (Tektronix, Wilsonville, OR). The output signal of the amplifier was fed into an audiometer and displayed on a storage oscilloscope and computer monitor. The dEMG signal was continuously recorded using a sampling rate of 5 kHz and integrated by the MacLab software using a 100-ms time window. Heart rate was obtained from the blood pressure signal and was averaged over eight-beat bins to minimize beat-to-beat fluctuations. All data were stored on computer (Apple Macintosh PowerPC connected to MacLab) for later viewing and analysis. We measured arterial blood gases and pH in three animals during the baseline period after surgery and before drug administration. The values for pH, PO$_2$, and PCO$_2$ were normal and stable (based on previously reported values for the rat; see Ref. 38). Arterial blood gases and pH were only assessed during the control period. We did not monitor these values during the response to microinjected drugs, as respiratory changes occurred immediately or within seconds after microinjection into the vNTS and thus it was felt that these respiratory alterations could not be due to secondary changes caused by disturbances in blood gases or pH.

Stereotaxic Surgery and Microinjection Procedure

Animals were placed in a prone position in a small animal stereotaxic frame (Kopf, Tujunga, CA). The dorsal medulla was exposed via a limited occipital craniotomy with retraction of the dura and retraction of the cerebellum in an analogous fashion as described in the cat (33). Our experience from previous microinjection studies (50) using the calamus scriptorius (i.e., caudal tip of the area postrema) as our reference point guided us in determining microinjection coordinates for the specific subnuclei of the NTS. Briefly, Fast green FCF dye (5%) was microinjected into the dorsal medulla at a series of coordinates, and the resulting dye locations were compared with known locations of various subnuclei according to the studies of Kalia and Sullivan (18). These experiments provided the coordinates used for our experiments. Coordinates used for vNTS microinjections were as follows: 0.5 mm rostral to calamus scriptorius; 0.9–1.1 mm lateral to midline; 0.5–0.8 mm below the dorsal surface of the brain stem. Coordinates used for medial NTS (mNTS) injections were 0.5 mm rostral to calamus scriptorius, 0.4–0.6 mm lateral to midline, and 0.4 mm below the dorsal surface of the brain stem. Lateral medullary microinjection coordinates (corresponding to the dorsomedial reticular nucleus, DMRN) used for control microinjections outside the NTS subnuclei were 0.5 mm rostral to calamus scriptorius, 1.4–1.6 mm lateral to midline, and 0.7 mm below the dorsal surface of the brain stem. Double-barreled (ID 0.23 mm; FHC, New Brunswick, ME) glass micropipettes were pulled and the tips cut to 20–40 μm outside diameter. Micropipettes were placed in a bracket fixed to the arm of an electrode carrier, and PE-90 tubing was connected to the barrels. Drugs were loaded or ejected using negative or positive pressure, respectively, generated by a syringe connected to the tubing. Calibration tape (Formaline 9006B; Wheeling, IL), consisting of alternating bands of clear or black bands, was affixed to the side of the pipette barrel. As the distance between bands as well as the interior diameter of the pipette barrel was known, we could determine the volume of drug solution ejected by observing the distance of travel of the meniscus during injection. With the use of this technique, drug volume ejected was 45 nl (±5 nl). The effective spread of drug volume, estimated according to the theoretical model of Nicholson (31) would be <173–292 μM². All drug solutions were dissolved in 0.9% saline, and pH was adjusted to 7.30–7.40. Microinjected drugs used in these experiments were the following: nipecotic acid (0.1 and 0.5 M; Sigma, St. Louis, MO), muscimol hydrochloride (2.2 mM; RBI, Natick, MA), baclofen hydrochloride (1.7 mM; Tocris Neuramin, Ballwin, MO), bicuculline methiodide (0.56 mM; RBI), CGP-35348 (0.11 M; RBI), SR-95531 (Gabazine, 0.56 mM; RBI), and kynurenic acid (22 mM; RBI). All drug doses were within dose ranges reported in previously published studies (4, 10, 13, 17, 47, 48).

Assessment of Apneusis, Apnea, Analysis of Data

All respiratory values were obtained from a software-derived integration (100-ms time window) of the raw dEMG signal. This signal will be described as the integrated diaphragmatic EMG (iEMG). Postdrug values were taken at the time of maximal change in Ti as well as at various time-points after experimental intervention. Ti and expiratory duration (T_e) were measured in earlier studies “by hand,” which entailed a “click and drag” method on the computer screen, highlighting each portion of the respiratory phase within each respiratory cycle from which the computer determined duration. Later studies used a computer-automated “macro” command that was designed to determine the duration of time the iEMG signal remained 5% above the value of the inter-breath interval (for Ti) or within 5% of the inter-breath value (for T_e). Using this automated procedure, Ti and T_e values could be generated for every breath in the experimental record. This method was employed to generate the figures illustrating the response to microinjected drugs over a 400-breath period. Experiments analyzed both by hand and by the macro were compared and found to be consistent with each other. iEMG amplitude and respiratory rate were averages obtained in all experiments by a click-and-drag method over 10 breaths (for baseline) or 5 breaths (after experimental interventions).

An apneustic breath was defined as a prolongation of inspiration in which the depth of breathing (i.e., iEMG amplitude) was maintained at a level equal or nearly equal to the maximum iEMG amplitude of control breaths for a duration >650 ms and having a Ti of >900 ms. Onset of apneusis was judged to occur at the second apneustic breath. Duration of apneustic breathing was determined as the time from the second apneustic breath (i.e., onset) to the occurrence of the second-to-last apneustic breath. Apneusis was defined as a cessation of inspiratory activity of >5 s duration.
occurring after drug administration. It was observed (see results) that unilateral microinjection of vehicle occasionally produced transient respiratory effects, principally a prolongation of T1 but sometimes included cessation of breathing that reversed with the termination of the microinjection. For this reason, we defined drug-induced apnea as occurring only after administration of the drug had been completed.

If a drug either prolonged T1, evoked apneas, or both, then cardiorespiratory variables were measured at the time of maximal T1 and 5 min after the drug administration. The means of five successive respiratory cycles were used to calculate the values reported in this study. In instances where other than obvious reduction of T1, apnea, or both was produced, postdrug cardiorespiratory values were taken within the first 5 s after microinjection had been completed. This was done to minimize the effects of altered respiration on cardiovascular function. The duration of apnea was determined as the time between cessation of breathing after completion of drug administration and the last point in the experimental record at which respiration could not be maintained unsupported.

**Experimental Protocols**

In all cases, a stable iEMG baseline of >5 min was observed before any experimental intervention. All animals with extreme baseline patterns of respiration (i.e., T1 >700 ms, Tc >1,000 ms, or respiratory rate <35 breaths/min) were excluded from our studies. Micropipettes were inserted either bilaterally or unilaterally as dictated by the needs of the experiment and the animal was allowed to stabilize for at least 5 additional minutes before a baseline recording was obtained. Subsequently, drugs of interest or vehicle (saline) were microinjected over a period of ~30 s per injection. In 10 cases, Fast green FCF dye (5%) contained in the adjacent barrel was microinjected into coordinates of the vlNTS at the conclusion of the experiment to verify the location of our microinjections. In preliminary studies, Fast green dye was observed to produce late-developing alterations in respiration. Thus, in all experiments, dye was not administered with the drug of interest and the microinjection location was noted from microscopic assessment of brain tissue slices in which pipette tracks could be clearly discerned. Studies were designed to maximize the amount of information obtained from each animal. Thus a number of different microinjection protocols were used depending on the drugs used and the expected response observed from preliminary studies. All experiments using GABA receptor agonists or reuptake inhibitors involved bilateral microinjection as it was noted previously (50) that apneas were only produced when the region of the vlNTS was inhibited bilaterally. All antagonist studies involved unilateral microinjections as it was observed in preliminary experiments that this was sufficient to produce significant respiratory alterations. Additionally, GABAergic antagonists were not microinjected before GABAergic agonists/reuptake inhibitors because they produced clear respiratory effects on their own. Thus, unless the antagonist was given to assess antagonist effects per se, antagonists were given unilaterally after bilateral agonist-induced apneas had stabilized; generally, this occurred 20–60 s after the end of agonist administration.

Cardiorespiratory effects of either dose of nipeptastic acid (i.e., 5 nmol or 25 nmol) were brief (<4 min in all cases) and demonstrated complete recovery. Because of this, both doses of drug could be tested in the same animal. Typically this involved either bilateral microinjection of one dose followed by a 10- to 20-min waiting period before bilateral microinjection of the other dose (i.e., dose response) into the same site or the bilateral microinjection of the same dose into a different site (i.e., into the mNTS or DMN for site response). Experiments were conducted such that dose or site order was randomized. The time interval allowed to elapse between injections was chosen to be more than double the longest duration of cardiorespiratory effects observed with the highest dose of nipeptastic acid tested in the vlNTS to avoid the effects of a prior intervention. No animal received more than two sets of microinjections.

As mentioned previously, unilateral microinjection of GABA receptor antagonists could elicit robust respiratory effects, depending on the site of microinjection. Respiratory rhythm frequently returned to approximately baseline values, which allowed us to test the opposite side of the brain after a 15- to 20-min period of recovery. No animal received more than one microinjection on either side of the brain stem. Cardiorespiratory effects of l-glutamate microinjection were of even shorter duration and were made unilaterally (as this was shown to produce cardiorespiratory effects). Thus, because of the short duration of the l-glutamate effect, multiple unilateral microinjections could be made in each animal. In these experiments, the dose was always the same (300 pmol) but the target site was chosen randomly as was side order. No animal received more than four unilateral microinjections. In experiments in which drugs produced apnea, animals were ventilated only if mean arterial pressure fell markedly, typically 30 s after the onset of apnea. Once placed on the ventilator, the animal was tested once or twice per minute to determine if spontaneous respiration would occur with removal from artificial ventilation. The animal was placed back on the respirator until testing indicated the animal could maintain respiratory rhythm unsupported.

**Histology**

Upon completion of the experiment, animals were killed by administration of an overdose of pentobarbital sodium through the arterial line. The brain was rapidly removed and placed in a solution of 6% buffered paraformaldehyde and transferred within 24–48 h into a solution of 20% sucrose in phosphate-buffered saline. Brains remained in this latter solution for at least 24 h before sectioning at 50 µm. Sections were stained with neutral red or cresyl violet to facilitate identification of nuclear groups, and the site of microinjection was determined in the following manner: as the ventral-most extent of identified pipette tracks or the site of highest density of Fast green FCF dye. Experiments without bilateral pipette tracks by microscopic assessment were not included in these studies. Thus the 115 animals used in this study all had identifiable pipette tracks. Although the calamus scriptorius was used as a reference point for microinjection coordinates, our descriptions of the placement of microinjection sites in the rostrocaudal axis use as a reference point the opening of the central canal into the 4th ventricle (see Fig. 1), commonly referred to as “obex” (28, 29).

**Immunohistochemistry Protocol**

**Tissue preparation.** Five rats that received no prior surgical intervention were deeply anesthetized with pentobarbital sodium (50 mg/kg) and perfused through the aorta with 250 ml of a 0.1 M PBS (pH 7.4) solution. This was followed by 500 ml of 2% paraformaldehyde solution in 0.1 M PBS solution (pH 7.4). The brains were removed and postfixed in this
Effects of nipecotic acid microinjected bilaterally into the NTS on indexes of respiratory and cardiovascular function

<table>
<thead>
<tr>
<th>Site Studied/Experimental Condition</th>
<th>Dose (nmol)</th>
<th>Apneusis Incidence</th>
<th>Apneusis Duration</th>
<th>R-R, breaths/min</th>
<th>iEMG, %change</th>
<th>BP, mmHg</th>
<th>HR, beats/min</th>
<th>Apneusis Onset</th>
<th>Time to Peak</th>
<th>Apneusis Duration</th>
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<tr>
<td>vlNTS (n = 12)</td>
<td>5</td>
<td>3/12</td>
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<td></td>
<td></td>
<td>5 ± 3</td>
<td>10 ± 2</td>
<td>16 ± 6</td>
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<tr>
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<td>459 ± 20</td>
<td>653 ± 59</td>
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<td>86 ± 4</td>
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<td>Peak effect</td>
<td>782 ± 114*</td>
<td>453 ± 84*</td>
<td>48 ± 3</td>
<td>-6 ± 1</td>
<td>97 ± 6*</td>
<td>503 ± 13</td>
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<td>5 min post</td>
<td>458 ± 19</td>
<td>686 ± 67</td>
<td>50 ± 2</td>
<td>-1 ± 2</td>
<td>73 ± 4*</td>
<td>489 ± 14</td>
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<td>-2 ± 5</td>
<td>13 ± 4</td>
<td>157 ± 32</td>
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<tr>
<td>Baseline</td>
<td>516 ± 25</td>
<td>512 ± 63</td>
<td>56 ± 3</td>
<td></td>
<td>74 ± 2</td>
<td>454 ± 14</td>
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<tr>
<td>Peak effect</td>
<td>1,470 ± 239*</td>
<td>335 ± 62*</td>
<td>34 ± 4*</td>
<td>3 ± 7</td>
<td>91 ± 6*</td>
<td>465 ± 14</td>
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<tr>
<td>5 min post</td>
<td>531 ± 37</td>
<td>546 ± 77</td>
<td>55 ± 4</td>
<td>5 ± 3</td>
<td>73 ± 5</td>
<td>474 ± 15</td>
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<tr>
<td>Baseline</td>
<td>436 ± 27</td>
<td>766 ± 83</td>
<td>50 ± 3</td>
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<td>79 ± 5</td>
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<td>480 ± 24</td>
<td>758 ± 85</td>
<td>49 ± 3</td>
<td>-4 ± 2</td>
<td>105 ± 7*</td>
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<td>74 ± 5</td>
<td>482 ± 10</td>
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Values are means ± SE. Onset time is in relation to the end of bilateral drug administration. vlNTS, ventrolateral nucleus of the solitary tract; mNTS, medial nucleus of the solitary tract; T1, inspiratory duration; Te, expiratory duration; iEMG, integrated EMG amplitude; BP, blood pressure; HR, heart rate. *P < 0.05 vs. baseline. †P < 0.05 vs. vlNTS (5 nmol).

Fixative solution for at least 24 h at 4°C. Brains were then transferred to a 30% sucrose solution in PBS, pH 7.3, and allowed to equilibrate for at least 48 h at 4°C. Brains were removed, cut in 30 μm sections and mounted on gelatin-coated Superfrost+ glass slides (Fisher Scientific, Pittsburgh, PA). Brain slices from the level of the caudal brain stem to 1 mm rostral to the opening of the 4th ventricle were stored at -80°C until processed for immunohistochemistry. These slices usually included cerebellar tissue in addition to medullary tissue. As the distribution of GABA receptor subtypes in the cerebellum has been previously described (14, 27), the cerebellar tissue was used as a positive control for our antisera.

Antisera. To label GABA<sub>A</sub> receptors, we used a commercially available (Upstate Biotechnology, Lake Placid, NY), affinity purified polyclonal rabbit antibody formed against the synthetic peptide QPSQDELDUNTTUFT, which corresponds to amino acids 1–15 of the subunit of this receptor. Similarly, to label GABA<sub>B</sub> receptors, we used a polyclonal guinea-pig antibody (Chemicon, Temecula, CA) against the synthetic peptide PSEPPDRILSCDGSRVHLHYK that is common to both R1β and R1β isoforms.

Protocol. Each coronal section was circled with a PAP pen (Research Products International, Mount Prospect, IL) to form incubation wells. The sections were then rehydrated for 20 min with PBS solution, blocked for 20 min with a 5% reconstituted milk solution containing 0.3% Triton X-100 along with 0.2% sodium azide in PBS, and incubated with one or more primary antibodies in this blocker solution. Primary antibody incubation with either the rabbit anti-GABA<sub>A</sub> receptor (1:300 dilution), guinea pig anti-GABA<sub>B</sub> receptor (1:500), or a combination of these primary antibodies was allowed to proceed for 1 h at room temperature followed by 18–24 h of incubation at 4°C. Primary solution was washed off and sections were then washed in PBS and incubated for 2 h in either PVC-conjugated donkey anti-rabbit IgG, TRITC-conjugated donkey anti-guinea pig IgG diluted 1:200 in PBS (Jackson ImmunoResearch Laboratory, West Grove, PA), or both solutions. Sections were then placed under a coverslip with anti-fading and fluorescence mounting medium ( Vectashield, Vector, Burlingame, CA).

Immunofluorescence assessment. Immunofluorescence was examined using a Nikon fluorescence microscope (model Eclipse E600, Tokyo, Japan). Images were captured using a charge-coupled device camera (Optronics Engineering model DEI-750, Goleta, CA) and Bioquant TCW95 v.250.4 software (Bioquant-R&M Biometrics, Nashville, TN). Images were then processed using Adobe Photoshop 4.0 (Adobe, Mountain View, CA). Image processing consisted of reduction of brightness, increase of contrast, and sharpening to enhance fluorescence. Control sections stained with either one or none of the primary antibodies in the presence of both secondary antibodies produced staining selective only for the primary antibody (i.e., no “cross-over” or “bleed-through”) staining was observed upon switching from FITC to Texas red visualization or vice versa; data not shown).

Fig. 1. Response to bilateral microinjection of nipecotic acid into the ventrolateral nucleus of the solitary tract (NTS) in the anesthetized, vagotomized, and spontaneously breathing rat. A: photomicrograph of ventrolateral NTS (vlNTS) microinjection sites, denoted by arrows, which are observed at 0.1 mm caudal to obex. Bar = 0.25 mm. B: baseline cardiorespiratory activity. Time bar = 1 s. C: microinjection of 5 nmol/45 nl nipecotic acid into the left vlNTS (right vlNTS microinjection accomplished within the preceding 45 s). Black arrow denotes the end of microinjection. D: 2 min postbilateral microinjection of 5 nmol nipecotic acid, note return to baseline cardiorespiratory activity. E: 400 breath time-course depicting the effect of nipecotic acid on inspiratory duration (Ti). Bilateral nipecotic acid administration occurs during the inset bar and denoted “nip.” F: baseline cardiorespiratory activity 21 min after D. Gray arrow denotes the start of microinjection of 25 nmol/45 nl nipecotic acid into the left vlNTS region (right vlNTS microinjection accomplished within the preceding 30 s) and ends at black arrow in G. H: 2 min postbilateral microinjection of 25 nmol nipecotic acid. Note apneusis is still evident. I: 400 breath time course depicting the effect of 25 nmol nipecotic acid on T<sub>i</sub>, BP, blood pressure; dEMG, raw diaphragmatic electromyogram signal; iEMG, integrated dEMG signal; HR, heart rate; XII, hypoglossal nucleus; DMV, dorsal motor nucleus of the vagus; TS, solitary tract.
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VLNTS GABA AND RESPIRATORY REGULATION

Data Analysis

Data presented are the means ± SE. Statistical analysis was performed using a one-way repeated measures ANOVA test (to detect significant differences between baseline and various postdrug values) and a Student-Newman-Keuls test (for specific group comparisons). When comparing the incidence of apneustic breathing between treatment groups and between treatment groups and vehicle controls, a Fisher’s exact test was used. In all cases, \( P < 0.05 \) was the criterion used for statistical significance.

Research described in this paper fully conforms with the American Physiological Society’s “Guiding Principles for Research Involving Animals and Human Beings.”

RESULTS

Effects of Bilateral Microinjection of Nipecotic Acid Into the vlNTS Region

First, we ascertained whether endogenous release of GABA in the vlNTS affects respiration. We recorded raw (dEMG) and integrated (iEMG) diaphragmatic EMG activity, arterial blood pressure, and heart rate before, during, and after bilateral microinjections of either a low (5 nmol, \( n = 12 \)) or a high (25 nmol, \( n = 8 \)) dose of nipecotic acid, a GABA reuptake inhibitor (Table 1, Fig. 1). Low doses of nipecotic acid increased \( T_I \) (170 ± 25% of baseline) and decreased \( T_E \) (69 ± 10% of baseline). In 3 of 12 animals, the breathing pattern became apneustic after completing the bilateral microinjection (Fig. 1, B-E). Although mild prolongation of \( T_I \) and shortening of \( T_E \) were observed occasionally with unilateral injection, large changes in \( T_I \), \( T_E \), or apneustic breaths were never observed with unilateral microinjection of nipecotic acid. Large alterations in respiratory rhythm, apneusis, or both developed either during the time of the microinjection of nipecotic acid into the second vlNTS region (Fig. 1) or within seconds after completion of bilateral microinjection (see time course for apneusis in Table 1). Effects on respiration were brief and at 5 min postmicroinjection, values obtained for \( T_I \) and \( T_E \) were equal to the control values (Table 1). Indeed, using \( T_I \) as the endpoint of a drug effect, it appeared that the effect of nipecotic acid was a threshold dose for inducing apneusis.

Six animals received bilateral microinjections of 5 nmol nipecotic acid into the mNTS. The data are shown in Table 1, and a representative experiment appears as Fig. 3. Nipecotic acid exerted no respiratory effect when microinjected into the mNTS; however, the drug did produce an increase in mean arterial blood pressure. No effect on heart rate was noted. The time course of the pressor response was similar to the time course of the cardiorespiratory effects of nipecotic acid noted in the vlNTS (Table 1, 5 min postinjection data; Fig. 3, B-D). Microinjection sites for all of the experiments performed in the mNTS are depicted in Fig. 2.
Effects of Bilateral Microinjection of Muscimol and Baclofen Into the vlNTS Region

Bilateral microinjections of the GABA_A receptor agonist muscimol (100 pmol) produced a significant increase in T_i, a significant decrease in T_e, and apneusis in 9 of 10 animals. Although T_i was often prolonged and T_e reduced after unilateral microinjection, apneusis was never observed after unilateral microinjection of muscimol. Onset of apneusis occurred during (7/9) or within 40 s (2/9) after the contralateral microinjection of muscimol into the vlNTS. Respiratory rate decreased due to the increased T_i. iEMG amplitude was significantly elevated (Table 2) at the time of peak respiratory effects; however, iEMG amplitude was not significantly higher than baseline when assessed 10–20 s after entering apneusis (+7 ± 15%). Although respiratory effects (i.e., apneusis) were observed immediately upon bilateral microinjection of muscimol, these effects continued to increase in severity and peaked 62 ± 18 s after microinjection. This contrasts with nipecotic acid in which the peak effects were seen almost immediately. The duration of apneusis was also longer with muscimol than with nipecotic acid (compare data of Table 2 with data of Table 1). Cardiovascular changes were similar to that seen with nipecotic acid, i.e., blood pressure in-

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Fig. 2. Summary of microinjection sites. Camera lucida reconstruction detailing microinjection sites of nipecotic acid of two different doses: 5 (A) and 25 nmol (B). Microinjection sites that were associated with apneusis are indicated by black circles; those that were not associated with apneusis are indicated with gray circles. Note that microinjections of 5 nmol nipecotic acid into the medial NTS described in RESULTS are also included in this figure. Coordinates listed describe location relative to obex; see METHODS for description. AP, area postrema.
creased significantly but heart rate was not significantly affected.

Bilateral microinjection of the GABA_B receptor agonist baclofen (75 pmol) produced a very similar profile of cardiorespiratory effects as observed with muscimol. T_i significantly increased, T_e significantly decreased, and apneusis was produced in 10 of 11 animals. Respiratory rate was decreased due to the increase in T_i, and the iEMG amplitude was elevated significantly at the time of the peak respiratory effects (Table 2). Although T_i was often prolonged and T_e reduced after unilateral microinjection, apneusis was never observed after unilateral microinjection of baclofen. However, apneusis developed nearly immediately (14 ± 4 s) after completion of bilateral microinjection of baclofen. Peak respiratory effects (i.e., apneusis) were observed 56 ± 12 s after bilateral microinjection of baclofen and generally appeared to have the same time course of action as muscimol. As in the case of muscimol, if iEMG amplitude was assessed 10–20 s after developing baclofen-induced apneusis (rather than during the peak effect approximately a minute after drug administration) it

Table 2. Effects of vLNTS microinjection of GABAergic agonists (or vehicle) on indexes of respiratory and cardiovascular function

<table>
<thead>
<tr>
<th>Drug Studied/Experimental Condition</th>
<th>Dose (nmol)</th>
<th>Apneusis Incidence</th>
<th>T_i, ms</th>
<th>T_e, ms</th>
<th>R-R, breaths/min</th>
<th>iEMG, %change</th>
<th>BP, mmHg</th>
<th>HR, beats/min</th>
<th>Apneusis Onset, s</th>
<th>Time to Peak, s</th>
<th>Apneusis Duration, s</th>
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<tbody>
<tr>
<td>Muscimol</td>
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<tr>
<td>(n = 10)</td>
<td>100</td>
<td>9/10</td>
<td>455 ± 17</td>
<td>703 ± 43</td>
<td>51 ± 2</td>
<td>—</td>
<td>76 ± 4</td>
<td>455 ± 10</td>
<td>2 ± 4</td>
<td>62 ± 8</td>
<td>1,074 ± 444</td>
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<td>Baseline</td>
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<tr>
<td>Peak effect</td>
<td></td>
<td></td>
<td>1,174 ± 94*</td>
<td>526 ± 46*</td>
<td>36 ± 3*</td>
<td>12 ± 2*</td>
<td>100 ± 4*</td>
<td>481 ± 8</td>
<td>1,524 ± 235*</td>
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<tr>
<td>Baclofen</td>
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<tr>
<td>(n = 11)</td>
<td>75</td>
<td>10/11</td>
<td>519 ± 37</td>
<td>606 ± 47</td>
<td>54 ± 3</td>
<td>—</td>
<td>90 ± 4</td>
<td>482 ± 12</td>
<td>14 ± 4</td>
<td>56 ± 12</td>
<td>1,524 ± 240</td>
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<tr>
<td>Peak effect</td>
<td></td>
<td></td>
<td>1,293 ± 139*</td>
<td>521 ± 41*</td>
<td>34 ± 2*</td>
<td>14 ± 5*</td>
<td>110 ± 7*</td>
<td>496 ± 10</td>
<td>1,293 ± 235*</td>
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<tr>
<td>Saline</td>
<td>(n = 7)</td>
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<tr>
<td>+10 s</td>
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<td></td>
<td>402 ± 30</td>
<td>658 ± 70</td>
<td>52 ± 4</td>
<td>—</td>
<td>82 ± 6</td>
<td>468 ± 19</td>
<td>76 ± 19</td>
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<tr>
<td>+60 s</td>
<td></td>
<td></td>
<td>376 ± 8</td>
<td>769 ± 71</td>
<td>51 ± 4</td>
<td>1 ± 22</td>
<td>79 ± 8</td>
<td>461 ± 20</td>
<td>79 ± 7</td>
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</table>

Values are means ± SE. Onset time is in relation to the end of bilateral drug administration. *P < 0.05 vs. baseline.
was not significantly different from baseline (+18 ± 17%). Cardiovascular alterations also were similar to that of muscimol: mean arterial pressure was elevated and heart rate remained unchanged with the cardiovascular time course mirroring that of the respiratory effects (Table 2).

To assure that the doses of drugs tested were effective and selective in stimulating each type of GABA receptor, studies were performed wherein apneustic breathing was produced by bilateral microinjection of each GABA receptor agonist into the vlNTS region followed by a unilateral microinjection of the respective GABA receptor antagonist into the same site. As can be seen in Figs. 4A and 5A, apneustic breathing produced by 100 pmol muscimol was readily reversed by subsequent administration of the GABA<sub>A</sub> receptor antagonist bicuculline methiodide, 25 pmol (n = 5). Additionally, pressure increases observed with bilateral muscimol administration into the vlNTS also were reversed by bicuculline (Fig. 4A). The specific GABA<sub>B</sub> receptor antagonist CGP-35348 was similarly able to reverse apneusis produced by 75 pmol baclofen (Figs. 4B and 5B) and also reduced the baclofen-induced pressor response (Fig. 4B). Although apneusis was produced only after bilateral microinjection of the agonist, reversal of apneusis required only unilateral administration of the antagonist. Additionally, reversal of apneustic breathing always preceded the reversal of GABA receptor agonist-induced pressor response, usually by 15 s to 1 min.

Cross-blocking studies in which the GABA<sub>B</sub> receptor antagonist was given after GABA<sub>A</sub> receptor agonist administration and the GABA<sub>A</sub> receptor antagonist given after GABA<sub>B</sub> receptor agonist demonstrated the specificity of these drugs for the receptor subtypes. CGP-35348 was unable to reduce the apneusis breathing produced by muscimol (Fig. 5C), and bicuculline was ineffective in reversing baclofen-induced apneustic breathing (Fig. 5D). Furthermore, CGP-35348 was unable to reverse the increased blood pressure produced by muscimol and bicuculline was unable to counteract the increase in pressure after microinjection of baclofen (data not shown).

**Effects of Bilateral Microinjection of Drug Vehicle Into the vlNTS Region**

Seven animals received bilateral microinjections of drug vehicle (saline, 0.9%; pH 7.4) and data obtained at 10 and 60 s after the second microinjection was completed are summarized in Table 2. Cardiorespiratory function was not significantly affected at either of these two time points. Acute respiratory effects could occasionally be observed during vehicle injection and consisted of a reduction in respiratory rate due to prolongation of Te. These respiratory effects returned toward normal within seconds of the end of microinjection. In 2 of 14 sides (as these were bilateral microinjections in 7 animals) administration of vehicle produced a transient apnea (8 and 11 s duration) that abated immedi-

![Fig. 4. Reversal of muscimol and baclofen-induced apneusis with bicuculline and CGP-35348, respectively. A: left depicts apneusis produced by bilateral microinjection of muscimol 100 pmol into the vlNTS. Middle indicates the effect of unilateral microinjection of bicuculline 25 pmol into the vlNTS (black arrow denotes end of microinjection). Reversal of apneusis occurs during administration. Right obtained 1 min after bicuculline microinjection. B: left depicts apneusis produced by 75 pmol baclofen. Gray arrow at the end of the left denotes beginning of microinjection of CGP-35348. Middle indicates completion of CGP-35348 microinjection 40 s later (black arrow) with clear reversal of apneusis during drug administration. Right obtained 1 min after CGP 35348 injection. Time bars = 1 s.](http://ajpregu.physiology.org/)
ately upon cessation of vehicle injection. Blood pressure was not observed to change. In no case did vehicle administration mimic the apneustic response produced by nipecotic acid and GABAergic receptor agonists.

Effects of Unilateral Microinjection of GABA Receptor Antagonists Into the vlNTS Region

Our intent, initially, was to microinject antagonists bilaterally as we had done with nipecotic acid and the GABA receptor agonists. However, we observed that unilateral microinjection of bicuculline methiodide (25 pmol) produced a transient apnea (Fig. 6A). Seven animals received a total of 10 unilateral microinjections of bicuculline methiodide. Seven of these bicuculline microinjections produced apnea, all but one of which occurred immediately. Four of these apnea episodes were so severe as to require artificial respiration. Apnea duration lasted 73 ± 21 s (range 10–152 s) and recovery was observed, although in one case the animal died when artificial respiration was not instituted soon enough. The onset of apnea was so abrupt (Fig. 6A) that in six of these instances there was no period of time where accurate measures of Ti, Te, and iEMG amplitudes could be obtained. Mean blood pressure and heart rate changes were followed but not analyzed in these animals because we felt that any changes that occurred would most likely be secondary to the apnea and/or to placing the animals on artificial respiration. However, in one animal, apnea developed slowly, occurring 80 s after microinjection. In this animal, a reduction in Ti and prolongation of Te was observed immediately after microinjection of drug and progressed slowly until a brief apnea developed. Neither blood pressure nor heart rate changed appreciably during this interval before the onset of apnea.

In contrast to the intense respiratory effect observed with bicuculline methiodide, unilateral microinjection of CGP-35348 (5 nmol/45 nl) only rarely produced apnea. In the 10 experiments performed in seven animals, only one animal exhibited apnea, and this event lasted just 13 s. The primary effects of CGP-35348 were to decrease Ti and to increase Te. Thus, at 30 s after unilateral microinjection of CGP-35348, Ti was reduced from 451 ± 19 to 423 ± 13 ms (P < 0.05) and Te was increased from 604 ± 48 to 742 ± 53 ms (P < 0.05) (see Fig. 6B). Recovery from these effects of CGP-35348 occurred within 1 min. Blood pressure was observed to be significantly decreased 30 s after unilateral microinjection of CGP-35348 (79 ± 4 to 67 ± 5 mmHg); however, heart rate remained unchanged.

Three animals received a combination of the two antagonists (bicuculline methiodide, 25 pmol, and CGP-35348, 5 pmol, in a total volume of 45 nl), and this cocktail was microinjected unilaterally into the vlNTS in each animal with the procedure repeated in two animals on the contralateral side for a total of five microinjections. In all instances, apnea occurred immediately, and in three cases required artificial ventilation of the animal (average duration of apnea in each animal tested was 154 ± 91 s; range 17–329 s). Measurements of Ti, Te, iEMG amplitude, mean blood pressure, and heart rate were not obtained because of the quick onset of apnea and the influence of respiratory changes on cardiovascular function. A representa-

Fig. 5. GABA receptor subtype antagonists act specifically to reverse only corresponding agonist-induced apneusis. Agonists were microinjected bilaterally into the vlNTS (muscimol 100 pmol, baclofen 75 pmol) while antagonists were microinjected unilaterally (bicuculline 25 pmol, CGP-35348 5 nmol). Bar groups denoted A and B depict appropriate agonist/antagonist competition while bar groups C and D depict the results of cross-blocking experiments. Agonist data obtained at maximal response. Postantagonist data obtained 1 min after antagonist administration. *P < 0.05 vs. baseline.
tive experiment depicting the effects of the cocktail appears as Fig. 6C.

Quaternary salts of bicuculline are also capable of blocking calcium-dependent potassium channels (37). This is not the case for the GABAA receptor antagonist SR-95531 (Gabazine) (37). Hence, we performed three experiments in three rats wherein unilateral microinjections of 25 pmol of SR-95531 were made into the vlNTS region. In each instance, an immediate apnea occurred, and this response was indistinguishable from that produced by bicuculline methiodide. A representative experiment appears as Fig. 7.

**Effects of GABA Receptor Antagonists vs. Nipecotic Acid-Induced Changes in Cardiorespiratory Function**

Next, either bicuculline, CGP-35348, or a combination of both drugs was administered unilaterally into the vlNTS after nipecotic acid-induced apneusis was produced to determine if blockade of either receptor subtype would reverse this respiratory response or if blockade of both receptor subtypes would be required. Neither 25 pmol bicuculline ($n = 5$; representative experiment, Fig. 8A; group means, Fig. 8B) nor 5 nmol CGP-35348 ($n = 4$; representative experiment, Fig. 8C; group means, Fig. 8D) alone was able to reverse nipecotic acid-induced alterations in respiratory rhythm. However, a unilateral microinjection of a combination of bicuculline with CGP-35348 (containing 25 pmol and 5 nmol, respectively) into the region of the vlNTS clearly reversed bilateral nipecotic acid-induced apneustic breathing within 30 s of administration in eight animals tested ($n = 8$; representative experiment, Fig. 8E; group means, Fig. 8F). In experiments in which nipecotic acid was followed by either bicuculline or the combination of bicuculline and CGP-35348, a late-developing apnea was observed that was likely due to the wearing off of nipecotic acid and the continued activity of bicuculline. This apnea could itself be effectively reversed by unilateral microinjection of nipecotic acid.
into the same site that received the antagonist (Fig. 8, A and E).

**Effects of Unilateral Microinjection of Kynurenic Acid Into the vlNTS Region on Bicuculline-Induced Apnea**

The effects seen with bicuculline in some central nervous system systems appear to be due to unopposed influence of the major excitatory neurotransmitter L-glutamate (49). Therefore, preliminary studies were undertaken that tested the hypothesis that bicuculline-induced apnea was caused by unopposed L-glutamate exciting neurons in the vlNTS. This was done by determining whether unilateral microinjection of the nonselective L-glutamate antagonist, kynurenic acid, would prevent the apnea elicited with bicuculline from the same site. Bicuculline initially produced apnea in all three animals studied, severe enough in two cases to require artificial respiration. A period of 20 min was allowed to elapse to permit recovery of respiratory function. Kynurenic acid was then microinjected unilaterally into the same site as bicuculline and 1 min later a second microinjection of bicuculline was administered. Kynurenic acid itself had no significant effects on cardiorespiratory activity 30 s after microinjection. In the presence of kynurenic acid, bicuculline did not produce apnea; however, respiratory rate did decrease slightly (52 ± 1 vs. 49 ± 1 breaths/min, \( P < 0.05 \)) due to an effect on \( T_i \) (598 ± 16 vs. 681 ± 12 ms, \( P < 0.05 \)).

\( T_i \) was unaffected (473 ± 15 vs. 472 ± 28 ms) as was iEMG amplitude. No significant cardiovascular effects were seen, although blood pressure tended to decrease (88 ± 8 vs. 78 ± 11 mmHg). Microinjection of bicuculline either 20 or 40 min later produced a much stronger effect on respiration, especially on \( T_e \) (20 min: 679 vs. 1,132 ms; 40 min: 698 vs. 1,157 ms), although apnea could still not be elicited at this time point. Previous studies by Gordon and colleagues (21, 34) demonstrated that a 2 nmol dose of kynurenic acid was able to attenuate the hypotensive effects of aortic depressor nerve stimulation for up to 30 min, the longest time point tested. An experiment illustrating the antagonism between kynurenic acid and bicuculline appears as Fig. 9.

**Effects of Microinjection of Glutamate Into the Dorsomedial Medulla**

As a further test of our hypothesis that bicuculline-induced respiratory effects were due to unopposed L-glutamate, we microinjected L-glutamate into the vlNTS region to determine whether its effects on respiration mimicked those effects observed with bicuculline. With the use of a dose of 300 pmol L-glutamate, a total of 13 unilateral microinjections were performed in three rats into the vlNTS and adjacent regions. The vlNTS was the site of six microinjections of L-glutamate and resulted in apnea in five of six instances...
Apnea duration was brief and breathing resumed within seconds after the event. No significant alterations of cardiovascular function were observed. Microinjection of L-glutamate into the mNTS unilaterally also produced apnea in two of three instances studied (Table 3, Fig. 10B). Mean arterial blood pressure, however, significantly decreased in this group (Table 3, Fig. 10B). The cardiorespiratory re-
Responses obtained from the mNTS have been reported by other investigators (2, 39, 43) and thus the few microinjections reported here agree with data reported in these studies. As an additional control, four unilateral microinjections were performed at a site 0.4–0.6 mm lateral to the vlNTS, the histology of which appears to place these injections in the dorsomedial reticular nucleus. Under these conditions, L-glutamate did not exhibit an effect on either respiration or mean blood pressure (Table 3, Fig. 10C).

**Distribution of GABA<sub>A</sub> and GABA<sub>B</sub> Receptors in the vlNTS Region**

Analysis of single-labeled as well as double-labeled tissues in five animals presented a consistent picture of GABA<sub>A</sub> receptor subtype distribution. Staining for the α1-subunit of the GABA<sub>A</sub> receptor demonstrated moderate labeling in the vlNTS, most densely localized to processes with less immunoreactive labeling of cell bodies (Fig. 11A). In contrast, immunoreactivity to the
GABAB receptor in the vlNTS was principally found on cell bodies with virtually no staining of neuronal processes (Fig. 11B). Double staining for both receptors demonstrates moderate colocalization of GABA A and GABAB receptors to neuronal cell bodies of the vlNTS but neuronal processes are only labeled with the GABA A receptor antibody (Fig. 11C). Cerebellar tissue, used as a positive control for the antisera used in our experiments, demonstrated a pattern of immunostaining that corroborates previous descriptions of GABA receptor distribution (14, 27): the granular layer is found to be GABA A-immunoreactive positive (Fig. 11D), whereas GABAB immunoreactivity is found primarily on Purkinje neurons (Fig. 11E).

DISCUSSION

Our data suggest that GABA plays a role at the vlNTS in the central control of breathing. This was established using three pharmacologic approaches consisting of microinjection of 1) specific antagonists of GABA A and GABA B receptors, 2) a GABA reuptake inhibitor, and 3) specific agonists of GABA A and GABA B receptors into the vlNTS of the rat. Data obtained with antagonists of GABA A or GABA B receptors, bicuculline methiodide, or CGP-35348, respectively, indicate a reduced duration of TI and an increased duration of TE. Bicuculline, in contrast to CGP-35348, nearly always produced apnea. The production of apnea with GABA A receptor blockade suggests the strongest synaptic GABAergic input to the vlNTS is mediated by GABA A receptors. However, endogenous GABA also activates GABA B receptors at the vlNTS as reflected by the changes in cycle duration after CGP-35348 microinjection. Activation of GABA receptors with muscimol and baclofen and, indirectly, with nipecotic acid increased the duration of TI and decreased the duration of TE. Whereas the responses obtained with muscimol and baclofen were through activation of GABA A and GABA B receptors, respectively, the responses obtained with nipecotic acid were mediated through both GABA A and GABA B receptors.

Finally, both the GABA receptor agonists and the GABA reuptake inhibitor affected TI so strongly that apneustic breathing nearly always occurred. Alterations in GABAergic transmission using the above drugs had little effect on the intensity of inspiratory drive as reflected by the lack of significant effects on the amplitude of the iEMG.

Other investigators have studied GABAergic neurotransmission in the NTS in the rat but not specifically...
in the vlNTS (e.g., Ref. 41). We are aware of only one other study relating to drugs affecting GABAergic neurotransmission in the vlNTS to respiratory function and that is by Miyazaki and colleagues (29). These investigators, using extracellular recordings from respiratory neurons in the vlNTS combined with iontophoretic application of pharmacologic agents, describe a type of respiratory neuron that responds to both bicuculline and the GABAB weak antagonist/partial agonist saclofen. This respiratory neuron received monosynaptic input from slowly adapting pulmonary stretch receptors (SARs; Refs. 28, 29) as well as rhythmic inputs from the central respiratory system. One of these inputs resulted in facilitation of firing at around the period from late inspiration to early expiration (IE facilitation; Fig. 12). IE facilitation was reduced by iontophoretic application of the NMDA receptor channel blocker MK-801, indicating that a glutamatergic synapse on the respiratory neuron was responsible for this excitation during the time of transition from inspiration to expiration (29). Thus blockade of this phasic drive (Fig. 12) would be expected to prolong Ti and could potentially cause apneustic breathing.

These investigators found that iontophoresic application of bicuculline markedly enhances IE facilitation. This, according to their model adapted in Fig. 12, could theoretically shorten Ti or, if intense enough, produce apnea. Indeed, both responses were observed in our study. A presynaptic location on glutamatergic terminals for at least a portion of GABA_A receptors was suggested based on the demonstration that after bicuculline, the firing of the neuron during the IE period (IE facilitation) was greater relative to baseline firing than that seen before bicuculline iontophoresis. According to the model in Fig. 12, excessive glutamate stimulation of these neurons would be expected to shorten Ti or, if the stimulation were strong enough, produce apnea. In support of this idea and schematically depicted in Fig. 12 are our findings that 1) microinjection of glutamate into the vlNTS produces apnea (Fig. 11A; also see Ref. 25) and 2) preliminary studies suggesting that pretreatment with the nonselective

Fig. 11. Distribution of GABA_A and GABA_B receptors in the vlNTS and cerebellum of the rat. Coronal sections of the medulla at the level of the AP and cerebellum were double-labeled using antibodies against the α1-subunit of the GABA_A receptor and the GABA_B receptor. A-C are taken from the vlNTS region. A depicts GABA_A receptor immunoreactivity (GABA_A-IR) within the vlNTS while B depicts GABA_B-IR. Colocalization of receptors is shown in C. D-F are taken from a double-labeling experiment in the cerebellum demonstrating the specificity of the antibodies used. As previously described (14, 28) the granular layer is found to be GABA_A-IR positive (D) while GABA_B-IR is found primarily on Purkinje neurons (E). Little colocalization of receptors is found in this region (F). Scale bar in A is 50 μm and indicates dorsal and medial orientation within the section.
glutamate antagonist kynurenic acid, which blocks both NMDA and non-NMDA receptors, prevents bicuculline-induced apnea (Fig. 10B). Although kynurenic acid will not distinguish between glutamate receptor subtypes, taken together, these data suggest that one role of GABA acting at GABA_A receptors at the vlNTS is to oppose or balance glutamatergic drive at this site and allow an adequate period for completion of the inspiratory phase of respiration.

Miyazaki and colleagues (29) reported that bicuculline increased baseline firing of the respiratory neuron and this is the rationale for our model depicting tonic GABA input synapsing directly onto the respiratory neuron (Fig. 12). Saclofen also increased the baseline firing of the respiratory neuron but had no effect on IE facilitation. We assume therefore that GABA exerts a tonic inhibitory effect on both GABA_A and GABA_B receptors located postsynaptically on the respiratory neuron (Fig. 12) and speculate that blockade of these receptors is largely responsible for shortening T1. We also noted that blockade of GABA_A and GABA_B receptors at the vlNTS also prolonged TE. The reason for this is unclear but would not be unexpected because the respiratory neuron depicted in Fig. 12 receives monosynaptic input from SAR afferents (28, 29) and may function as a neuron mediating the Breuer-Hering reflex-induced prolongation of expiration (15).

Most importantly, the schema shown in Fig. 12 predicts that local administration of GABA, GABA_A, and GABA_B receptor agonists or the GABA reuptake inhibitor nipecotic acid should all produce apneustic breathing. GABA_A receptor agonists would act both directly (postsynaptic) and indirectly (presynaptic) to silence the respiratory neuron, whereas the GABA_B receptor agonist would act directly to evoke the same response. Nipecotic acid would lead to an increase in the concentration of GABA at the synapse and could result in activation of both GABA_A and GABA_B receptors. In fact, in our study we found that nipecotic acid-induced apneusis could only be reversed by a combination of bicuculline and CGP-35348.

We used an immunohistochemical approach to assess whether GABA receptors are present in the vlNTS. Our data demonstrate that both GABA_A and GABA_B receptors are localized primarily to neuronal processes and cell bodies of neurons located in the vlNTS, respectively. Terai and colleagues (45) used a monoclonal antibody directed against a subunit of the GABA_A receptor (β2/3), which is commonly expressed along with the α3-subunit in native GABA_A receptors (26). They reported that GABA_A receptors were moderately to highly expressed within the vlNTS and ventral NTS, with moderate immunoreactivity to the β2/3 subunit in the interstitial subnucleus. Immunoreactivity to the β2/3 subunit decreased rapidly medially, and was almost absent within the mNTS itself (45). Additionally, Terai and colleagues (45) used a polyclonal antibody to GABA and found GABA-immunoreactive puncta (which they state are probably nerve terminals) in the vlNTS. According to these investigators, immunoelectron microscopic studies of others have indicated that such puncta are GABA-containing presynaptic terminals, and this supports the tonic GABA input to IE neurons depicted in Fig. 12. To our knowledge, no studies have been published in which the distribution of GABA_B receptors within the subnuclei of the rat NTS has been assessed.

We do not directly record from neurons in the vlNTS in our experiments and demonstrate that the neurons exhibit a respiratory pattern of discharge. Nevertheless, this seems likely as respiratory neurons can be found in this region (7, 28, 42, 46) and the neurons we are affecting appear to have similar inputs to those described by Miyazaki and colleagues (28, 29). Thus our data strongly suggest that GABA plays an important role in controlling the activity of respiratory neurons located in the dorsal respiratory group of rats and that this takes place specifically in the vlNTS. Our evidence for site specificity is based primarily on our nipecotic acid data and on our recently published data on GABA (50). Data obtained with microinjection of 5 nmol of nipecotic acid into the vlNTS or the adjacent mNTS revealed significant respiratory changes elicited from the vlNTS but no significant respiratory changes evoked from the mNTS. Evidence that nipecotic acid microinjections into the mNTS were accurately placed was demonstrated not only anatomically (Figs. 2 and 3) but functionally wherein a pressor effect occurred that was at least as great if not greater than the pressor effect obtained with both 5 and 25 nmol of nipecotic acid microinjected into the vlNTS (Table 1). Furthermore, our previous study (50), which compared the respiratory effects of microinjection of GABA into the vlNTS and the mNTS, revealed identical respiratory changes could be elicited from the vlNTS to those noted with nipecotic acid and muscimol from the vlNTS in
the present study, but no comparable respiratory changes were evoked from the mNTS. On the other hand, GABA elicited robust pressor effects from the mNTS that were at least equal in magnitude (if not greater) than that produced from the vlNTS (50).

vlNTS neurons in other species such as the cat represent a major monosynaptic drive to the phrenic motor nucleus and central pattern generation (1, 8, 32). Functional evidence for this drive was not observed in our studies. Perturbation of GABAergic transmission had relatively little or no effect on iEMG amplitude but, in contrast, affected rhythm generation. This might be expected because the rat showed only a minor monosynaptic connection to the phrenic motor nucleus (7, 9, 12, 36, 51). However, the vlNTS has extensive projections to other brain stem regions (16, 28, 51). Data obtained from both electrophysiologic studies (28) and neuroanatomical studies (28) in the rat indicated neural connectivity to the dorsolateral pons, primarily to the Kölliker-Fuse nucleus with some projections to adjacent parabrachial nuclei (28). Since apneustic breathing as well as apnea could be elicited with some projections to adjacent parabrachial nuclei (28), it is tempting to suggest that the site of action of baclofen into the vlNTS evokes the same pattern of the Ko

Since apneustic breathing as well as apnea can be evoked by changing the activity of neurons in the region of the Kölliker-Fuse nucleus (6, 30), we speculate that the connectivity demonstrated between the vlNTS and the dorsolateral pons might explain a significant portion of the respiratory changes evoked from the vlNTS. Studies performed in the cat have provided evidence of GABAergic inputs to respiratory neurons of the vlNTS from the Bötzing complex, presumably from expiratory neurons (22, 23). This connectivity from the Bötzing complex to the vlNTS, however, seems to be absent in the rat (3). Thus the source of afferent GABAergic tone into the vlNTS of the rat (Fig. 12) is not known. Apneustic breathing required that a bilateral microinjection of direct and indirect GABA receptor agonists be administered, whereas apnea could be elicited by unilateral microinjection of a GABA receptor antagonist. These results suggest that the vlNTS receives bilateral inhibitory (GABA) drive to maintain the integrity of the IOS and that excitatory input to each vlNTS region is capable or sufficient to affect inspiratory termination. Apneas, therefore, would not be expected to occur unless the vlNTS was inhibited bilaterally. Unilateral stimulation of these neurons within the vlNTS, either directly with glutamate microinjection or indirectly with bicuculline, would be expected to produce high rates of firing in these neurons. As one intact vlNTS region is capable of effecting the IOS, hyperstimulation of these neurons would be expected to produce a prolonged activation of the IOS resulting in apnea.

Focusing on the possibility that GABAergic transmission within the vlNTS participates in the control of the IOS, we have published reports from our laboratory and others indicating that systemic administration of baclofen produces apneustic breathing without consistent changes in Tc (19, 35, 44). Since microinjection of baclofen into the vlNTS evokes the same pattern of apneustic breathing without an increase in Tc (Table 2), it is tempting to suggest that the site of action of systemically administered baclofen is the vlNTS.

Our data obtained with L-glutamate (Table 3) and data of Marchenko and Sapru (24) suggest that vlNTS neurons are dedicated for controlling respiratory function. Evidence for this is that microinjection of an excitatory amino acid into the vlNTS in a small volume (present study and see Ref. 24) produces only a change in respiratory function and no change in cardiovascular function. The mNTS, considered a primary site for cardiovascular regulation, has also been implicated in the control of respiration and contains second-order neurons in the Breuer-Hering reflex pathway (2). Indeed, these investigators reported that with microinjection of small volumes of an excitatory amino acid (not exceeding 3 nl), only respiratory effects could be observed. In our study and in the study of Marchenko and Sapru (24) we could not separate respiratory effects from cardiovascular effects upon microinjection of glutamate into the mNTS. However, we did note only cardiovascular effects when 5 nmol of nipecotic acid were microinjected into the mNTS (Table 1). Regarding cardiovascular effects elicited from the mNTS with nipecotic acid, Sved (40) reported that GABA under the conditions of reuptake inhibition with nipecotic acid acts primarily on GABA_A receptors to elicit a cardiovascullar response (i.e., a rise in arterial pressure). On the other hand, both bicuculline and CGP-35348 microinjected into the mNTS exerted effects per se. Both agents given separately decreased arterial pressure, approximately by the same magnitude (41). Thus tonic GABAergic drive to the mNTS acts to raise arterial pressure, presumably by acting equally on GABA_A and GABA_B receptors, whereas the indirect GABAergic effect of nipeptic acid results in a rise in arterial pressure due to excitation of GABA_B receptors.

In summary, evidence from pharmacologic and immunohistochemical approaches performed in the vlNTS of the anesthetized rat reveal that GABAergic transmission is crucially important in controlling the activity of these neurons and therefore in modulating respiratory function. Furthermore, the vlNTS appears to contain a pool of neurons dedicated to respiration and forms a major component of the IOS.

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