Glycine at hypoglossal motor nucleus: genioglossus activity, CO₂ responses, and the additive effects of GABA

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Morrison, Janna L., Sandeep Sood, Xia Liu, Hattie Liu, Eileen Park, Philip Nolan, and Richard L. Horner. Glycine at hypoglossal motor nucleus: genioglossus activity, CO₂ responses, and the additive effects of GABA. J Appl Physiol 93: 1786–1796, 2002. First published August 2, 2002; 10.1152/japplphysiol.00464.2002.—There is evidence for glycinergic inhibition of hypoglossal motoneurons. Altered mechanisms with integrative reflex respiratory control have also not been determined. This study tests the hypotheses that glycine and GABA are likely to be released together within the hypoglossal motor nucleus and indicates that glycine-mediated inhibition of hypoglossal motoneurons with effects antagonized by strychnine (5, 6, 24, 25). There is also evidence in vivo for strychnine-sensitive inhibitory postsynaptic potentials recorded from hypoglossal motoneurons after electrical stimulation of the lingual nerve in cats (40). In addition, application of glycine by iontophoresis to the hypoglossal motor nucleus suppresses the antidromic field potential elicited in the hypoglossal motor nucleus by electrical stimulation of the whole hypoglossal nerve, although it has not been established whether these phenomena were recorded from retractor or protruder motoneurons (41). Accordingly, few studies have determined the effects of glycine at the hypoglossal motor nucleus on motor outflow to the GG muscle in vivo, and, to our knowledge, no studies have determined the interaction of such glycine mechanisms with integrative reflex respiratory control, such as the GG muscle responses to systemic hypercapnia.

Glycine-immunoreactive fibers in the hypoglossal motor nucleus are also intermingled with fibers containing GABA, another major inhibitory neurotransmitter (18). GABA inhibits hypoglossal motoneurons via the GABA<sub>A</sub>-receptor agonist (6, 24) and decreases motor outflow to GG muscle in vivo (19, 20, 41). Because glycine and GABA are likely to be released together within the hypoglossal motor nucleus and individual hypoglossal motoneurons contain receptors for both neurotransmitters (24), it is important to determine whether the recruitment of such glycine and GABA mechanisms explains the periods of major GG suppression in behaviors such as rapid eye movement sleep.

hypercapnia; pharyngeal muscles; obstructive sleep apnea; control of breathing

GLYCINE IS A MAJOR INHIBITORY neurotransmitter in the central nervous system (e.g., for review, see Ref. 30). The role of glycine in the motor control of upper airway dilator muscles, such as the genioglossus (GG) muscle of the tongue, remains incompletely understood. There is immunohistological (31, 43) and autoradiographic (29, 47) evidence for the presence of both glycine and its receptor in the hypoglossal motor nucleus, the source of motor outflow to the GG. From in vitro studies of neonatal rat brain stem, there is also evidence for glycine-mediated inhibition of hypoglossal motoneurons with effects antagonized by strychnine (5, 6, 24, 25). There is also evidence in vivo for strychnine-sensitive inhibitory postsynaptic potentials recorded from hypoglossal motoneurons after electrical stimulation of the lingual nerve in cats (40). In addition, application of glycine by iontophoresis to the hypoglossal motor nucleus suppresses the antidromic field potential elicited in the hypoglossal motor nucleus by electrical stimulation of the whole hypoglossal nerve, although it has not been established whether these phenomena were recorded from retractor or protruder motoneurons (41). Accordingly, few studies have determined the effects of glycine at the hypoglossal motor nucleus on motor outflow to the GG muscle in vivo, and, to our knowledge, no studies have determined the interaction of such glycine mechanisms with integrative reflex respiratory control, such as the GG muscle responses to systemic hypercapnia.

Accordingly, the present study tests the hypothesis that increasing glycine at the hypoglossal motor nucleus will suppress GG muscle activity, even in the presence of reflex respiratory stimulation by systemic hypercapnia, and that the suppressant effects of glycine will be antagonized by strychnine. We also hypothesize that a combination of glycine and the GABA<sub>A</sub>-receptor agonist will be antagonized by strychnine. We also determined whether coapplication of glycine and muscimol (GABA<sub>A</sub>-receptor agonist) to the HMN is additive in suppressing GG activity. Twenty-four urethane-anesthetized, tracheotomized, and vagotomized rats were studied. Diaphragm and GG activities, the electroencephalogram, and blood pressure were recorded. Microdialysis probes were implanted into the HMN for delivery of artificial cerebrospinal fluid (control), glycine (0.0001–10 mM), or muscimol (0.1 μM). Increasing glycine at the HMN produced graded suppression of GG activity (P < 0.001), although the GG still responded to stimulation with 7% inspired CO₂ (P = 0.002). Strychnine (0.1 mM) reversed the glycine-mediated suppression of GG activity, whereas combined glycine and muscimol were additive in GG muscle suppression. It remains to be determined whether the recruitment of such glycine and GABA mechanisms explains the periods of major GG suppression in behaviors such as rapid eye movement sleep.

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receptor agonist muscimol at the hypoglossal motor nucleus will produce additive suppression of GG activity. Understanding the potential for inhibitory neurotransmitters such as glycine and GABA to suppress GG activity and modulate integrative reflex respiratory responses, such as those produced by CO₂ stimulation, is important because major suppression of GG activity occurs in certain behaviors, such as rapid eye movement (REM) sleep, both in animals (11, 22, 27) and humans, especially during phasic REM sleep events (36, 45). REM sleep also effectively abolishes GG responses to CO₂ (11). However, although inhibitory postsynaptic potentials have been recorded at hypoglossal motoneurons during the pharmacological model of REM sleep induced by pontine carbachol (9, 46), the potential role of glycine and GABA in the suppression of GG activity remains open to question (17). However, before determination of whether such inhibitory mechanisms are recruited or not in REM sleep, it is important to determine the presence (or absence) of such mechanisms and to characterize their interaction with integrative respiratory control mechanisms. This study has direct relevance to the neural control of GG activity as well as clinical disorders, where suppression of GG muscle activity can lead to airway occlusion and obstructive sleep apnea (33).

METHODS

Animal Preparation and Surgical Procedures

Twenty-four male Wistar rats (Charles River, mean body weight, 293 ± 7 g; range, 242–388 g) were studied. All procedures conformed to the recommendations of the Canadian Council on Animal Care, and the University of Toronto Animal Care Committee approved the experimental protocols. The rats were anesthetized with urethane (0.5 g/ml, 1 g/kg ip), and surgical levels of anesthesia were then maintained, as necessary, by inhalation of halothane (typically 0.2–2%). Halothane was administered via an anesthetic mask and then through a tracheal tube after tracheotomy (see below). The rats spontaneously breathed a mixture of 50% room air and 50% oxygen throughout all procedures. The rats were also given atropine sulfate (0.5 g/ml, 1 mg/kg ip) to minimize airway secretions. Body temperature was monitored with a rectal probe and maintained between 36 and 38°C with a water pump and heating pad (T/Pump-Heat Therapy System, Gaymar, NY).

After the onset of effective surgical anesthesia, as judged by abolition of hindlimb withdrawal and corneal blink reflexes, the rats were tracheotomized and bilaterally vagotomized. Vagotomy was performed to prevent reflex inhibition of GG muscle originating in vagal afferents (1, 37) and to eliminate the confounding effect of vagal reflex responses to the changes in breathing rate and depth elicited by hypercapnic stimuli (see RESULTS). A polyethylene catheter was inserted in the femoral vein for continuous infusion of a solution containing 7.5 ml of 0.9% saline, 2.0 ml of 5% dextrose, and 0.4 ml of 1 M NaHCO₃ at a flow rate of 0.4 ml/h. A bolus dose of dexamethasone (0.1 ml at 2 mg/ml) was also given to minimize brain edema. A catheter inserted into the femoral artery was used to record arterial blood pressure. To record the diaphragm electromyogram (EMG), multistranded stainless steel wires (AS636, Cooner Wire, Chatsworth, CA) were sutured into the costal diaphragm with an abdominal approach.

The rats were then placed in a stereotaxic apparatus (model 962, Kopf, Tujunga, CA) in the flat skull position by using an alignment tool (Kopf model 944). Stainless steel wire electrodes were inserted bilaterally, under direct vision, into the GG muscle. In two additional rats, tongue EMG recordings were made before and after bilateral section of the medial and then lateral branches of the hypoglossal nerve. Two stainless steel screws (1.5 mm diameter) attached to insulated wire (30 gauge) were implanted in the skull over the frontal-parietal cortex to record the electroencephalogram (EEG). The EEG electrodes were placed ~2 mm anterior and 2 mm to the right of bregma, and 3 mm posterior and 2 mm to the left of bregma (12).

Recording Procedures

All electrical signals were amplified and filtered (Super-Z head-stage amplifiers and BMA-400 amplifiers/filters, CWE, Ardmore, PA). The EEG was amplified by 1,000 and filtered between 1 and 100 Hz, whereas the GG and diaphragm EMGs were amplified by 2,000 and filtered between 100 and 1,000 Hz. The electrocardiogram was removed from the diaphragm EMG by using an oscilloscope and an electronic blanker (model SB-1, CWE). The moving-time averages (time constant = 200 ms) of the GG and diaphragm EMGs were also obtained (S76-01, Coulbourn, Lehigh Valley, PA). The EEG and EMG signals were calibrated by using the built-in microvolt calibrator (20 μV to 1 mV) on the head-stage amplifiers. Blood pressure was measured with a transducer (DT-XX, Ohmeda, Madison, WI) and an appropriate amplifier (PM-1000, CWE). Inspired CO₂ concentration was measured with a CO₂ analyzer (CAPStar-100, CWE). All raw signals, along with the moving-time averages of the GG and diaphragm EMGs, were recorded on chart paper (TA11, Gould, Valley View, OH) and computer (1401 interface, Spike 2 software, CED, Cambridge, UK).

Microdialysis

The microdialysis probes were 240 μm in diameter with a 1-mm cuprophane membrane and a 6,000-Da cutoff (CMA/11 14/01, CSC, St. Laurent, Quebec). The probes were lowered slowly through a small hole drilled at the junction of the intraparietal and occipital bones and aimed at the hypoglossal motor nucleus by using the following coordinates: 13.86 ± 0.16 (SE) mm posterior to bregma (range, 12.90–16.85 mm), 0.31 ± 0.02 mm lateral to the midline (range, 0.15–0.55 mm), and 9.46 ± 0.15 mm ventral to bregma (range, 7.00–10.50 mm). When the microdialysis probe initially penetrated the hypoglossal motor nucleus, a transient burst of GG EMG activity was observed, and the probe was then lowered a further 0.5 mm to these final coordinates. This burst of GG activity during insertion of the probe was transient (lasting an average of 3 min and 40 ± 35 s in the group of rats) and was useful as a preliminary indication of probe placement at the beginning of the experiment (e.g., Ref. 14). The insertion of the probe did not affect the diaphragm EMG, respiratory rate, or blood pressure.

The microdialysis probes were connected to FEP Teflon tubing (inside diameter = 0.12 mm) that in turn was connected to 1.0-ml plastic syringes via a zero-dead space switch (Uniswitch, BAS, West Lafayette, IN). The lag time for fluid to travel to the tip of the probe from the switch was 3 min and 38 s. The probes were perfused with artificial cerebrospinal fluid (aCSF) at a flow rate of 2.1 μl/min. The aCSF was made fresh on the day of each experiment with a composition (in
mM) of 125 NaCl, 3 KCl, 1 KH2PO4, 2 CaCl2, 1 MgSO4, 25 NaHCO3, and 30 d-glucose. The aCSF was warmed to 37°C before CaCl2 was added (23). The aCSF was then bubbled with 100% CO2 to a physiological pH of 7.35–7.45. The rats stabilized for at least 30 min after insertion of probes before any interventions.

**Experimental Protocol**

All interventions were typically performed between 1300 to 1900 during steady-state periods with stable breathing, blood pressure, and high-voltage and low-frequency EEG activity.

**Study 1: Glycine at the hypoglossal motor nucleus and responses to CO2.** In seven rats, all signals were recorded during microdialysis perfusion of aCSF (i.e., control) and glycine (glycine hydrochloride, FW: 111.5, Sigma Chemical, St. Louis, MO) dissolved in aCSF at doses of 0.0001, 0.001, 0.01, 0.1, 1.0, and 10 mM. Doses were applied in ascending order for a total of ~40 min at each dose. After 20 min after an increment in dose, signals were recorded before, during, and after steady-state (6 min) application of 7% inspired CO2. For aCSF and each level of glycine delivered to the hypoglossal motor nucleus, analyses were performed over 1-min periods immediately before CO2 application, in the 6th min of CO2 application, and 6 min after removal of CO2. To determine whether prior exposure of the hypoglossal motor nucleus to glycine affected subsequent responses to the higher doses, a further study was performed in four additional rats in which only the three highest doses of glycine (i.e., 0.1, 1.0, and 10 mM) were applied by using the same protocol described above.

**Study 2: Antagonism of glycine with strychnine at the hypoglossal motor nucleus.** A second study in six rats was performed to determine whether strychnine, a glycine-receptor antagonist, could reverse the effects of glycine at the hypoglossal motor nucleus. The protocol involved perfusion of the hypoglossal motor nucleus for 25 min with each of the following agents in the following order: aCSF, glycine (1 mM), strychnine (0.1 mM; strychnine hydrochloride, FW: 370.9, Sigma Chemical), a return to glycine, and, finally, a switch back to aCSF. Data were analyzed 20 min after a switch between drugs. The focus of the study was the change in GG activity from the first glycine-to-strychnine condition compared with the second glycine-to-aCSF condition.

**Study 3: Combined glycine and GABA A-receptor stimulation at the hypoglossal motor nucleus.** A third study was performed to determine the effects on GG muscle activity of microdialysis perfusion of glycine and the GABA A-receptor agonist muscimol (muscimol hydrobromide, FW: 195.0, Sigma Chemical) into the hypoglossal motor nucleus, first alone and then together. For each experiment, aCSF was first perfused into the hypoglossal motor nucleus followed by either glycine (four rats, 0.01 mM) or muscimol (three rats, 0.1 μM). After this initial response to the first agent applied alone, a switch was made to perfuse both agents together. As such, a total of seven rats ended the protocol with glycine and muscimol applied together. Each drug was perfused for 35 min. For aCSF, or when glycine and muscimol were applied together, data were also recorded before, during application of steady-state (>6 min) CO2, followed by a 10-min recovery period. Preliminary studies showed that the doses of glycine (see results) and muscimol (19, 20) used were sufficient to clearly decrease GG muscle activity when applied individually to the hypoglossal motor nucleus. Data were analyzed 30 min after a switch between drugs.

**Data Analysis**

Breath-by-breath measurements of GG and diaphragm activities were calculated and averaged in consecutive 5-s epochs. The EMGs were analyzed from the moving-average signal (above electrical zero) and were quantified in arbitrary units. Electrical zero was the voltage recorded with the amplifier inputs grounded. GG activity was quantified as mean tonic activity (i.e., difference between basal activity at end expiration and electrical zero), peak inspiratory activity, and phasic respiratory-related activity (the difference between peak inspiratory and tonic end-expiratory activity). In practice, there was no tonic GG activity in this anesthetized preparation; therefore, only phasic respiratory activity is presented. The amplitude of the diaphragm EMG was quantified as the difference between the peak inspiratory and prior end-expiratory values’ moving-time-averaged signal, and the mean values of diaphragm amplitude, respiratory rate, and blood pressure were also calculated for each 5-s epoch. Diaphragm minute activity was calculated as the product of diaphragm amplitude and respiratory rate. The EEG was sampled by computer at 500 Hz and analyzed on overlapping segments of 1,024 samples, windowed using a raised cosine (Hamming) function, and subjected to a fast-Fourier transform to yield the power spectrum. The window was advanced in steps of 512 samples, and the mean power spectrum of the EEG signal over each 5-s analysis epoch was calculated. The power contained within six frequency bands was recorded both as absolute power and as the percentage of the total power of the EEG signal. The band limits were δ1 (0.5–2 Hz), δ2 (2–4 Hz), θ (4–7.5 Hz), α (7.5–13.5 Hz), β1 (13.5–20 Hz), and β2 (20–30 Hz). All respiratory, blood pressure, and EEG values were written to a spreadsheet and matched to the corresponding intervention at the hypoglossal motor nucleus, and the respective level of CO2, to provide a grand mean for each variable, for each intervention, in each rat.

**Histology**

On completion of the experiments, the rats were overdosed with urethane and perfused intracardially with 40 ml of 0.9% saline followed by 20 ml of 10% formalin. Brains were then removed and fixed in 10% formalin. The medullary regions were blocked, transferred to 30% sucrose, and cut in 50-μm coronal sections with a cryostat (Leica, CM 1850, Nussloch, Germany). Each section containing the hypoglossal motor nucleus was mounted and stained with neutral red. Microdialysis sites were localized from the neutral red-stained sections and marked on standard brain maps (28).

**Statistical Analyses**

For all comparisons, differences were considered significant if the null hypothesis was rejected at P < 0.05 by using a two-tailed test. Data were analyzed by using either repeated-measures ANOVA (RM-ANOVA) or paired t-tests, as indicated in the text. Except where noted, the factors were CO2 level and dose of drug for two-way RM-ANOVA and dose of drug for one-way RM-ANOVA. For post hoc t-tests, Dunnett’s test for comparisons with a single control (i.e., aCSF) was used. Analyses were performed by using Sigmastat (SPSS, Chicago, IL). All data are expressed as means ± SE.

**RESULTS**

**Tongue EMG Recordings**

Figure 1 shows an example of the tongue EMG recordings before and after bilateral section of the medial
and then lateral branches of the hypoglossal nerve. Note that the tongue EMG activity was markedly decreased, but not completely abolished, after section of the medial branches of the hypoglossal nerve, showing that recordings were predominantly from the GG muscle. Tongue muscle activity was fully abolished after additional section of the lateral branches of the hypoglossal nerve, indicating that the retractor muscles also contributed a small component to the whole signal. These effects of hypoglossal nerve section were selective for reducing tongue muscle activity, as diaphragm activity was unaffected by the interventions.

**Histology**

Figure 2A shows an example of the lesion site made by the microdialysis probe in the hypoglossal motor nucleus. The locations of all lesion sites from all rats were within the hypoglossal motor nucleus, as shown in Fig. 2B.

**Study 1: Glycine at the Hypoglossal Motor Nucleus and Responses to CO₂**

**GG responses to glycine.** Figure 3A shows an example of the responses of GG muscle to perfusion of glycine into the hypoglossal motor nucleus. This trace shows that increasing glycine caused progressive reductions in GG muscle activity. In this example, there were no changes in diaphragm or EEG activity, but blood pressure decreased at the higher doses of glycine. The group data in Fig. 3B also show that increasing glycine at the hypoglossal motor nucleus produces graded suppression of GG muscle activity. Analysis confirmed that there was a significant effect of glycine on GG muscle activity \( (F_{6,36} = 33.40, P < 0.0001, \text{one-way RM-ANOVA}) \) with a mean decrease of \( \sim 80\% \) at 10 mM glycine. Compared with aCSF, a significant decrease in GG activity with glycine occurred at 0.001 mM \( (P < 0.05, \text{Dunnett’s test}) \).

Effects of glycine on GG responses to CO₂. Figure 4A shows the effects of glycine at the hypoglossal motor nucleus on the GG muscle responses to CO₂. Analyses showed that, despite the progressive decline in GG activity with glycine at the hypoglossal motor nucleus \( (F_{6,36} = 13.84, P < 0.001, \text{two-way RM-ANOVA}) \), there remained a significant stimulating effect of CO₂ \( (F_{2,12} = 11.58, P = 0.002) \) that occurred independently of glycine dose \( (F_{12,72} = 1.44, P = 0.170) \). Although this result showed that the hypoglossal motor nucleus could still respond to excitatory inputs, despite the presence of glycine, further analyses were performed to determine whether glycine altered the percent increase in GG activity with CO₂ stimulation. Analyses of the data shown in Fig. 4B confirmed that there was no effect of glycine on the percent increase in GG activity in response to CO₂ \( (F_{6,36} = 1.93, P = 0.102, \text{one-way RM-ANOVA}) \). For these data, it should be noted that one rat had relatively low-baseline GG activity but large responses to CO₂, which explains why the calculated percent increase in GG activity for the group appears larger than would be anticipated from the group mean values for the raw data (Fig. 4A vs. 3B).

Specificity of responses to glycine and CO₂. Figure 5 shows that there was no effect of glycine at the hypoglossal motor nucleus on phasic diaphragm activity (Fig. 5A; \( F_{6,36} = 0.914, P = 0.496, \text{two-way RM-ANOVA} \)). However, there was a significant effect of glycine on respiratory rate (Fig. 5B; \( F_{6,36} = 5.52, P < 0.001 \)), although this only became statistically significant compared with aCSF at 1 mM glycine \( (P < 0.05, \text{Dunnett’s test}) \), i.e., a glycine level that was much higher than that which caused suppression of GG activity \( (0.001 \text{ mM}; \text{Fig. 3B}) \). Despite the slight decline in respiratory rate at the higher glycine doses, overall diaphragm minute activity was unaffected by glycine at the hypoglossal motor nucleus (Fig. 5C; \( F_{6,36} = 1.13, P = 0.365 \)). Similarly, although there was a significant

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effect of glycine on blood pressure \( (F_{6,36} = 6.48, P < 0.001), \) this became significant compared with aCSF at 0.1 mM glycine \((P = 0.05, \) Dunnett’s test), i.e., again at a dose higher than the effects on GG activity. There was no effect of glycine at the hypoglossal motor nucleus on the distribution of frequencies in the EEG signal \((all \ F_{6,36} < 1.92, all \ P > 0.100), \) although total EEG power declined with glycine \( (F_{6,36} = 7.45, P < 0.0001) \) with significant changes at 0.001 mM \((P < 0.05, \) Dunnett’s test).

As expected, there was a significant effect of CO₂ on diaphragm activity, with breathing becoming deeper \( (F_{2,12} = 14.13, P < 0.001 from two-way RM-ANOVA) \) and slower \( (F_{2,12} = 5.55, P = 0.02) \) with CO₂ stimulation. However, unlike the significant effects of CO₂ on GG activity \( ( Fig. 4), \) the effect of CO₂ on overall diaphragm minute activity was not statistically significant \( (F_{2,12} = 3.04, P = 0.085). \) Total EEG power was not affected by CO₂ stimulation \( (F_{2,12} = 1.04, P = 0.385), \) although there were slight decreases in blood pressure with CO₂ \( (Fig. 5D; F_{2,12} = 5.26, P = 0.02). \)

**Effect of prior exposure to glycine.** The decreases in GG muscle activity observed with 0.1, 1.0, and 10 mM glycine at the hypoglossal motor nucleus in study 1 \( (i.e., \) after exposure to the preceding lower doses) were similar to the decreases in GG activity measured when 0.1, 1.0, and 10 mM glycine were applied directly without such previous exposure \( (F_{1,2} = 8.17, P = 0.104, \) two-way RM-ANOVA). The application of these three highest doses of glycine alone to the hypoglossal motor nucleus was not associated with any changes in phasic diaphragm activity, respiratory rate, diaphragm minute activity, blood pressure, or total EEG power \((all F_{3,9} < 0.90, all P > 0.480, one-way RM-ANOVA). \)

**Study 2: Antagonism of Glycine Responses With Strychnine at the Hypoglossal Motor Nucleus**

Figure 6A shows an example of the typical suppression of GG muscle activity after a switch from aCSF to glycine at the hypoglossal motor nucleus and then reversal of this suppression with application of strychnine. In contrast, a switch back to aCSF from glycine does not reverse the suppression when measured over the same time frame, showing that the responses with strychnine were not a time effect due to washout of glycine. Data for the group of six rats are shown in Fig. 6B. Analysis showed that there was a significant effect of drug treatment on GG activity \( (F_{4,16} = 5.55, P = 0.005, one-way RM-ANOVA). \) Further post hoc analyses showed that a switch from aCSF to glycine caused a significant decrease in GG activity \( (t(5) = 5.50, P = 0.003, paired t-test), \) whereas application of strychnine returned GG activity to levels indistinguishable from that of the aCSF controls \( (t(5) = 1.25, P = 0.268). \) Reapplication of glycine returned GG activity to values...
significantly below aCSF \( [t(5) = 3.63, P = 0.015] \), and activity remained significantly below this control after a subsequent switch back to aCSF \( [t(5) = 5.36, P = 0.003] \), confirming that the strychnine effect was due to drug rather than washout of glycine.

**Study 3: Combined Glycine and GABA\(_A\)-receptor Stimulation at the Hypoglossal Motor Nucleus**

Figure 7A shows examples of the combined administration of glycine and muscimol to the hypoglossal motor nucleus in two of the seven rats. In the first rat (Fig. 7A, left), GG muscle activity is shown for aCSF, glycine, and then combined glycine and muscimol at the hypoglossal motor nucleus, whereas responses to aCSF, muscimol, and then combined muscimol and glycine are shown for the second rat (Fig. 7A, right). Group data from all rats (Fig. 7B) showed that coapplication of muscimol and glycine to the hypoglossal motor nucleus caused a significant decrease in GG activity [mean decrease = 59.2 ± 7.7% from aCSF, 95% confidence interval for the change = 40.3–78.0%, \( t(6) = 7.69, P < 0.001 \), paired \( t \)-test]. Individual application of muscimol or glycine caused suppression of GG activity by 42.5 ± 8.3 and 28.4 ± 15.1%, respectively, from aCSF, such that the algebraic sum of their individual effects (i.e., 70.9%) was within the 95% confidence interval for the actual effect observed when they were applied together.

In the presence of combined administration of glycine and muscimol to the hypoglossal motor nucleus, GG activity still increased with CO\(_2\) stimulation (Fig. 8). Analyses showed that the increase in GG activity with CO\(_2\) was statistically significant \( (F_{2,12} = 3.94, P = 0.048, \text{two-way RM-ANOVA}) \), with this stimulating effect occurring independently of whether aCSF or combined glycine and muscimol were present at the hypoglossal motor nucleus \( (F_{2,12} = 0.62, P = 0.556) \). Furthermore, the percent increase in GG activity with CO\(_2\) was similar whether aCSF or combined glycine and muscimol was applied to the hypoglossal motor nucleus \( [24.6 ± 6.8 \text{ vs. } 24.8 ± 8.6\%], \text{respectively}, \ t(6) = 0.03, P = 0.976 \), paired \( t \)-test], although there was a clear suppression of overall GG activity with glycine and muscimol (Fig. 8; \( F_{1,6} = 17.19, P = 0.006 \), two-way RM-ANOVA).

**DISCUSSION**

This study shows that increasing glycine at the hypoglossal motor nucleus in vivo produces graded suppression of GG muscle activity, with application of...
strychnine reversing this suppression. The glycine-mediated suppression of GG activity occurred both in the absence of, and in the presence of, reflex respiratory stimulation with systemic hypercapnia. Nonetheless, at each level of glycine, the GG muscle was still able to respond to CO₂ stimuli, and the proportional increase in GG activity was not affected by glycine at the hypoglossal motor nucleus. Overall, these results confirm previous in vitro studies (5, 6, 24) and extend them to the in vivo preparation where the interaction with integrative reflex respiratory control could also be determined. In the present study, we also showed that the combined effect of glycine and muscimol, a GABA_A-receptor agonist, at the hypoglossal motor nucleus was additive in the suppression of GG activity. Moreover, with combined administration of glycine and muscimol, the GG muscle was also able to respond to CO₂ stimuli, although overall activity was decreased compared with aCSF. These results are relevant to the neural control of hypoglossal motor outflow, because recent data show that glycine and GABA are released together onto hypoglossal motoneurons, with individ-

Fig. 4. A: group data (n = 7) showing phasic respiratory GG activity during control microdialysis of aCSF into the XII motor nucleus and at each level of Gly. Data are shown before (open bars), during (solid bar), and after (shaded bar) steady-state stimulation with 7% inspired CO₂. B: percent increase in GG activity with CO₂ stimulation was similar across all doses of Gly. Values are means ± SE. See text for further details.

Fig. 5. Group data (n = 7) showing Dia amplitude (A), respiratory rate (B), Dia minute activity (C), and blood pressure (D) during control microdialysis of aCSF into the XII motor nucleus and at each level of Gly. Data are shown before (open bars), during (solid bars), and after (shaded bars) steady-state stimulation with 7% inspired CO₂. Values are means ± SE. See text for further details.
ual motoneurons also containing receptors for both transmitters (24). Such corelease of GABA and glycine also occurs onto neurons in the spinal cord (2, 15, 21). Overall, the results of this study have direct relevance to the neural mechanisms and respiratory control of hypoglossal motor outflow to GG muscle. These results may also be relevant to the periods of major suppression of GG muscle activity that occur in REM sleep (22, 27, 36, 45), even in the presence of strong respiratory stimulation by CO2 (11).

Experimental Preparation and Implications for Sleep

The extrapolation of these results to a behaving preparation is, however, necessarily limited because studies were performed in anesthetized rather than sleeping rats. The experiments were performed in anesthetized rats to allow for delivery of the varying levels of glycine to the hypoglossal motor nucleus in a controlled and systematic fashion, with and without steady-state CO2 stimuli. Microdialysis was used to allow repeated switches between different drugs and doses without the need for removing and replacing a microinjection cannula. Although it is unlikely that the use of anesthetization would have altered the direction of change in GG activity to the applied glycine, it is possible that anesthesia may have altered the magnitude of responses (13, 26). For example, halothane has been shown to potentiate the effects of both GABA_A and glycine-receptor stimulation (42), although it does not alter extracellular glycine concentration as measured in the cerebral cortex (34). Nevertheless, this initial characterization of the GG muscle responses to glycine, and its modulation of reflex respiratory responses to CO2, was performed in this reduced and controlled preparation because of the added complexity in a behaving rat of varying sleep-wake states with their own different effects on GG muscle activities (14) and responses to CO2 (11).

Extrapolation to the intact behaving preparation may also be somewhat limited because the rats were vagotomized in these studies. Vagotomy was performed to prevent reflex inhibition of GG muscle originating in vagal afferents, particularly strong in the rat (1, 37), and to eliminate the confounding effect of vagal reflex responses to the changes in breathing rate and depth elicited by hypercapnic stimuli. Nevertheless, because the conditions of the experiments favored the
presence of strong inspiratory GG activation and the absence of tonic activity, the balance of tonic and phasic components within the respiratory control network may have been altered to some degree by vagotomy because more tonic activity and less phasic inspiratory GG activity are typically present in intact rats (11). It remains to be tested whether application of glycine and muscimol to the hypoglossal motor nucleus produces similar suppression of hypoglossal motor output to the GG muscle in intact rats.

We believe, however, that it is important to initially characterize the potential effects of glycine at the hypoglossal motor nucleus on GG muscle activity in a reduced and controlled preparation because controversy exists as to the potential role (if any) of such inhibitory neurotransmitters in the suppression of hypoglossal motor outflow. For example, although glycine has been implicated in the suppression of lumbar (39), trigeminal (38), and masseter (16) motoneuron activity, both in natural REM sleep or in the REM-like state produced by pontine carbachol, such mechanisms have not been demonstrated at the hypoglossal motor nucleus (17). Although, pontine carbachol does not produce the whole range of electrocortical and respiratory events characteristic of natural REM sleep (10), transient inhibitory postsynaptic potentials have been recorded at hypoglossal motoneurons in such a preparation, but the neurotransmitters responsible are currently unidentified (9, 46) and may be mediated by transmitters other than glycine or GABA.

Nevertheless, before adequate interpretation of whether or not postsynaptic inhibitory mechanisms play a role (or not) in the suppression of motor outflow to GG muscle, either in behaviors such as REM sleep or

**Fig. 7.** Raw traces from 2 rats (A) and group data from 7 rats (B) showing major suppression of GG muscle activity with combined administration of Gly and muscimol (Mus) into the XII motor nucleus compared with when either agent was administered alone. B: values are means ± SE.

**Fig. 8.** Group data from 7 rats showing phasic respiratory GG activity during control microdialysis of aCSF into the XII motor nucleus and combined perfusion of Gly and Mus. Data are shown before (open bars), during (solid bars), and after (shaded bars) steady-state stimulation with 7% inspired CO₂. See text for further details.
otherwise, it is first necessary to characterize the presence of such inhibitory mechanisms at the hypoglossal motor nucleus and the effects of the applied neurotransmitters. Accordingly, this study provides such information in vivo for glycine and its interactions with GABA<sub>α</sub>-receptor-mediated effects at the hypoglossal motor nucleus and the ability of these mechanisms to suppress GG muscle activity in normocapnia and during reflex hypercapnic stimulation. After this initial characterization, future studies using in vivo microdialysis of the hypoglossal motor nucleus in freely behaving rats (14) can be used to determine the potential role of such inhibitory mechanisms in the major suppression of GG activity and responses to CO<sub>2</sub> in natural REM sleep (11). Such mechanisms may indeed be operative, because there is an interesting case report of increased pharyngeal muscle activity and improvements of obstructive sleep apnea after blockade of putative glycnergic inhibition of pharyngeal motoneurons with systemically applied strychnine (32). However, the effects on GG muscle activity in REM sleep were not specifically distinguished in that study, and responses were observed predominantly in the tensor palatini muscle innervated by trigeminal motoneurons (32).

**Specificity of Responses**

The tongue electrodes were placed under direct vision into GG muscle, but there is the possibility that other muscles were being recorded as well as GG. For example, tongue retractors are coactivated with GG during respiratory stimulation (7, 8). In addition, it is possible that retractor motoneurons such as those innervating the styloglossus and hyoglossus muscles would be influenced by the interventions at the hypoglossal motor nucleus, although this may be to a lesser degree than GG motoneurons because the probe sites were located in rostral regions of the hypoglossal motor nucleus (Fig. 2 and Ref. 28). Protruder motoneurons, such as those innervating GG, are predominantly located in rostral regions of the hypoglossal motor nucleus, whereas retractor motoneurons are clustered in more caudal regions (4).

It is also a concern that neural structures close to the hypoglossal motor nucleus may have been influenced by diffusion of drug from the microdialysis probe and that this would complicate data interpretation. Significant suppression of GG muscle activity with glycine at the hypoglossal motor nucleus occurred at a threshold dose of 0.001 mM (Fig. 3). Although glycine also caused a significant decrease in respiratory rate, possibly indicating diffusion to nearby respiratory neurons (e.g., see Ref. 3), the glycine dose that caused this change (1 mM) was much higher than that which produced a change in GG activity. Moreover, there was no effect on phasic diaphragm activity or overall diaphragm minute activity with any dose of glycine. Similarly, although there was a statistically significant decrease in blood pressure with glycine at the hypoglossal motor nucleus, this decrease of 5–10 mmHg was observed at a dose that was also higher (0.1 mM) than that which produced a change in GG activity. Moreover, a decrease in blood pressure would be expected to cause a reflex increase in GG activity (35, 44), i.e., the opposite of the GG suppression that was observed with glycine (Fig. 3). Although there was no effect of glycine at the hypoglossal motor nucleus on the distribution of frequencies in the EEG signal, total EEG power declined with glycine at a dose similar to that which produced a change in GG activity. However, a decrease in total EEG power is indicative of an arousal response, an effect that would be expected to produce an increase in GG activity (13, 26) rather than the decrease that was observed with glycine.

Overall, these observations suggest that the effects of glycine on GG muscle activity were likely primary responses to modulation of hypoglossal motor outflow via local inhibitory mechanisms similar to those observed in vitro (5, 6, 24). However, the changes in other physiological variables (e.g., blood pressure and respiratory rate), albeit at higher doses, suggest the likelihood of spread of the perfusate because of prolonged exposure. This suggestion is supported by the additional results with shorter exposures that showed that when the three higher doses of glycine were applied to the hypoglossal motor nucleus without the preceding lower doses, the changes in GG activity were applied to the hypoglossal motor nucleus without the preceding lower doses, the changes in GG activity occurred without any associated changes in phasic diaphragm activity, respiratory rate, diaphragm minute activity, blood pressure, or total EEG power.

**Summary**

This study shows that glycine receptor stimulation at the hypoglossal motor nucleus in vivo suppresses GG muscle tone and activity during hypercapnia. Moreover, the combined effects of glycine and the GABA<sub>α</sub>-receptor agonist muscimol at the hypoglossal motor nucleus was additive in the suppression of GG muscle activity. It remains to be determined whether these inhibitory neural mechanisms are recruited in natural REM sleep to explain the major decrease in GG muscle activity and reflex GG responses to systemic hypercapnia observed in that sleep state (11).

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