GABA<sub>A</sub>ergic and Glycinergic Inhibition in the Phrenic Nucleus Organizes and Couples Fast Oscillations in Motor Output

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Marchenko V, Rogers RF. GABA<sub>A</sub>ergic and glycinergic inhibition in the phrenic nucleus organizes and couples fast oscillations in motor output. J Neurophysiol 101: 2134–2145, 2009. First published February 18, 2009; doi:10.1152/jn.91030.2008. One of the characteristics of respiratory motor output is the presence of fast synchronous oscillations, at rates far exceeding the basic breathing rhythm, within a given functional population. However, the mechanisms responsible for organizing phrenic output into two dominant bands in vivo, medium (MFO) and high (HFO)-frequency oscillations, have yet to be elucidated. We hypothesize that GABA<sub>A</sub>ergic and glycinergic inhibition within the phrenic motor nucleus underlies the specific organization of these oscillations. To test this, the phrenic nuclei (C<sub>1</sub>) of 14 unanesthetized, decerebrate adult male Sprague-Dawley rats were microinjected unilaterally with either 4 mM strychnine (n = 7) or GABA<sub>Z</sub>ine (n = 7) to block glycine or GABA<sub>A</sub> receptors, respectively. Application of GABA<sub>Z</sub>ine caused an increase in overall phrenic amplitude during all three phases of respiration (inspiration, postinspiration, and expiration), while the increases caused by strychnine were most pronounced during postinspiration. Neither antagonist produced changes in inspiratory duration or respiratory rate. Power spectral analysis of inspiratory phrenic bursts showed that blockade of inhibition caused significant reduction in the relative power of MFO (GABA<sub>A</sub> and glycine receptors) and HFO (GABA<sub>Z</sub> receptors only). In addition, analysis of the coherence between the firing of the ipsi- and contralateral phrenic nerves revealed that HFO coupling was significantly reduced by both antagonists and that of MFO was significantly reduced only by strychnine. We conclude that both GABA<sub>A</sub> and glycine receptors play critical roles in the organization of fast oscillations into MFO and HFO bands in the phrenic nerve, as well as in their bilateral coupling.

INTRODUCTION

The generation of synchronous activity in motor outputs has been a subject of interest for many years. In addition to the normal three-phase rhythmic output during eupnea [inspiration, postinspiration, and stage 2 expiration (E2)] (Richter and Ballantyne 1983; Richter et al. 1986), the respiratory rhythm generator/controller produces oscillations within the inspiratory bursts of phrenic, hypoglossal (XII), and other respiratory motor nerves that are well above the frequency of the primary rhythm (Cohen et al. 1997). In the phrenic nerve, these fast oscillations are typically organized into two dominant bands known as high-frequency (HFO) and medium-frequency oscillations (MFO) (Cohen et al. 1987, 1997; Davies et al. 1985; Dittler and Garten 1912; Huang et al. 1996; Marchenko and Rogers 2006a; Marchenko et al. 2002; Richardson and Mitchell 1982; Wyss 1939) and presumably result from near-synchronous discharge of phrenic motoneurons.

Two major mechanisms have been investigated in the production of fast oscillations in phrenic and/or hypoglossal outputs: coupling via gap junctions and synaptic inhibition. In neonatal mouse in vitro brain stem slice and brain stem-spinal cord en bloc preparations, gap junctions appear to play a desynchronizing role as their systemic blockade significantly increases the amplitude of fast oscillations within the XII burst without significantly changing peak integrated activity (Bou-Flores and Berger 2001), presumably via the dampening effect of higher electrical load (associated with excessively coupled pools of neurons) on a network that is generating synchronized activity (Traub and Wong 1983). By contrast, blockade of gap junctions in juvenile/young adult arterially perfused rat preparations significantly reduced the amplitude of fast phrenic oscillations, altered the burst amplitude/shape, and increased respiratory rate when applied to the perfusate (Solomon et al. 2003). Simulation studies suggest either diminution or enhancement of synchrony may result from this mechanism (Marder 1998; Traub and Wong 1983). Thus the role of direct electronic coupling in the generation of fast oscillations in motor population activity may depend on many experimental (e.g., maturity, species, and type of preparation) and cellular/network properties (e.g., relative proportion of coupled neurons, voltage-dependent conductances).

In both modeling and experimental studies, synaptic inhibition via GABAergic and glycinergic transmission has also been implicated as playing a major role in short timescale (ms) synchronization of motor neuron firing. Studying the behavior of simulated motor neuron pools, Turker and Powers (2001) demonstrated that either excitatory or inhibitory inputs may induce synchronous discharge. Also working in simulation, Taylor and Enoka (2004) found that although active membrane conductances may decrease motor- unit synchronization due to saturation in firing rates, the addition of just 10% background inhibition rescues synchronicity. In experimental studies, Bou-Flores and Berger (2001) observed a significant reduction in phrenic and XII synchrony when GABA<sub>A</sub> and glycine receptor antagonists were applied to the superfusate of neonatal mouse brain stem/spinal cord en bloc and in vitro slice preparations. In brain stem slices derived from neonatal and juvenile mice, Sebe et al. (2006) demonstrated that blockade of GABA<sub>A</sub> and glycine receptors significantly reduced the power of fast XII oscillations even while increasing the amplitude of integrated activity.
Although there appears to be general accord in the finding that inhibition promotes synchrony, exactly how and where these effects are manifest in the generation of fast oscillations in respiratory motor output remains unclear for several reasons. When applying antagonists to an entire preparation systemically, one cannot ascribe specific sites for their action. Brainstem slice preparations contain both rhythm-generation circuitry (i.e., in the ventrolateral medulla) as well as the premotor/motor circuitry (e.g., XII) and thus suffer from lack of specificity of drug action, as does intravenous application of (ant)agonists in vivo (e.g., Lalley 1983). In addition, the degree to which the mechanisms producing rhythmic output in XII rootlets of in vitro neonatal slice or en bloc preparations (in the presence of high concentration of extracellular potassium) are related to those producing the eupneic rhythm in adults in vivo is open to debate (e.g., Rybak et al. 2002).

To avoid these complications in the present study, we utilize an unanesthetized, decerebrate, vagotomized adult in vivo rat model that generates classical HFO and MFO, and bilateral coupling detected by left-right coherence, in the phrenic output (Marchenko and Rogers 2006a,b). Using this model, we seek to test the hypothesis that local inhibition in the phrenic motor nucleus in vivo is required for the functional organization of fast synchronous oscillations as well as for the functional coupling between left and right phrenic motor neurons. In addition to the use of time-frequency and power spectral estimates to detect the dynamics of fast oscillations, coherence (a measure of phase constancy) is used to evaluate the degree to which the left and right phrenic outputs are functionally coupled. That is, high degrees of coherence may indicate a shared input (to both phrenic nuclei) at a given frequency, or a direct coupling between the nuclei, or both. Because left-right phrenic nerve coherence is typically much greater in the HFO than MFO band (e.g., Marchenko and Rogers 2006b), it has been assumed that HFO is generated supraspinally and transmitted to both nuclei from a common source. To evaluate the role of synaptic inhibition in generation and bilateral coupling of fast oscillations, our experimental approach is to block GABAergic and glycinegic transmission within one phrenic nucleus and quantify the effects on both relative power within, and coherence between, the phrenic nerve outputs. This approach has an advantage over other preparations because the pontine and brainstem networks responsible for respiratory rhythmogenesis are physically remote from the phrenic nucleus and therefore unaffected by the microinjection of antagonists therein, thereby increasing specificity of the site of action of the receptor classes involved.

METHODS

Experiments were conducted on 14 adult male Sprague-Dawley rats (356–428 g). All procedures were approved by the University of Delaware Institutional Animal Care and Use Committee.

General surgical preparation

Spontaneously breathing rats were anesthetized with isoflurane vaporized in O₂ (Matrix; 4–5% induction, 2.0–2.5% maintenance) via a snout mask. Anesthetic depth was maintained at a level such that withdrawal reflexes and changes in heart rate and blood pressure in response to pinches of the distal hind limbs were absent. Following intubation with an atraumatic glass tube, animals breathed the same gas mixture spontaneously until paralysis after decerebration (see following text). One femoral artery and vein were cannulated for measurement of arterial pressure and infusion of drugs, respectively. Electrocardiography (EKG) was measured via three small subcutaneous electrodes using conventional amplification and filtering (Neurolog; Digitimer, Hertfordshire, UK) and monitored using an audio amplifier (model AM10; Grass Instruments). During all surgical procedures, rectal temperature was maintained at 37.0 ± 0.3°C via a servocontrolled heating blanket coupled to a rectal thermometer (Harvard Apparatus). Using a ventral approach, the medial branches of the hypoglossal nerves (XII), and the phrenic nerves (PhNs), were dissected free from the surrounding tissue, transected, and desheathed. Both internal carotid arteries were tied off just below the pterygopalatine artery to prevent bleeding after decerebration.

Decerebration and laminectomy

After the initial surgical preparation, rats were placed prone in a stereotaxic device. Arterial and tracheal cannulae were connected to pressure transducers (CDXII; Argon Medical) for monitoring blood pressure and inflation pressure using conventional amplifiers (Gould Statham). Using a variable-speed surgical drill (Foredom Electric), the parietal bones were removed, the superior sagittal and straight sinuses were ligated using suture, and the neuraxis was carefully transected at the rostral border of the superior colliculus using a microspatula. Brain tissue rostral to the transection was removed by suction, and residual bleeding was arrested by applying small pieces of gelfoam (USP; Pharmacia) soaked with cold thrombin solution (50 U/ml USP) dissolved in 0.9% saline.

Following decerebration, animals were repositioned in a supine orientation. Branches of the external carotid artery (e.g., superior thyroid, ascending pharyngeal, lingual) were ligated as needed, and the larynx, thyroid, and portions of the esophagus removed. The C2–C3 vertebrae were exposed using a ventral approach by removal/retraction of the rectus capiti, superior oblique and ventral portions of the longus colli muscles. The ventral surface of the C1–C2 spinal cord was exposed by gradual grinding of the vertebral bodies using a variable-speed drill and sealing them with bone wax, followed by opening of the dura using iridectomy scissors. In some rats, continuous C2–C4 exposure was not possible due to large superficial blood vessels and/or significant bleeding from the vertebrae, and vertebral disks were removed and the intervertebral space carefully expanded rostrocaudally. In all cases, access to the entire ventral surface of the C₄ cord was achieved.

All animals were vagotomized via bilateral transection of the vagi (just below the nodose ganglion) so that synchronous oscillations produced by the central pattern generator’s interaction with the phrenic nucleus could be studied without confounding effects of pulmonary mechanoreceptor feedback. A bilateral pneumothorax was performed before recording to eliminate lung inflation-related movement artifacts and to eliminate chest wall mechanoreceptor feedback, with a positive end-expiratory load of 1.0 cm H₂O. Fifteen to 20 min after ventral laminectomy, anesthesia was slowly withdrawn, and the animals were paralyzed by an intravenous bolus injection of 2 mg/kg followed by continuous infusion (3 mg·kg⁻¹·h⁻¹) of vecuronium bromide (Abbott Labs), during which they were artificially ventilated (~60 cycles/min, 2.3–3.0 ml tidal volume; Columbus Apparatus) with O₂-enriched air. End tidal CO₂ was maintained between 4.0 and 4.5% (Capstar CWE) by adjusting minute volume. PhN recordings were not initiated until ≥1 h after decerebration. If necessary, animals were continuously infused with 5% dextrose in 0.9% saline (1.0–1.5% body wt or 1.5–2.0 ml/h) to maintain a minimum mean blood pressure of at 90 mmHg. The central ends of the PhN and XII were placed on bipolar silver electrodes and immersed in a mineral oil pool formed by skin flaps.
Data collection and injection protocol

Monophasic recordings (10–5,000 Hz; Neurolog, Digitimer) of efferent activity were obtained after crushing the peripheral end of each PhN and XII between the electrodes. The electrical activity of the four nerves, expiratory CO_2_ level, arterial blood pressure, lung inflation pressure, and injection trigger signals were recorded onto the hard disk of a personal computer at 10,000 sample/s for each signal using a 16-bit A/D converter with visualization software (AD Instruments). Prior to analysis, nerve recordings were digitally low-pass filtered with a −3-dB roll off between 250 and 400 Hz.

GABA_\text{A} and glycine receptor antagonists were dissolved in physiological saline (0.9% NaCl). Following a 15-min control period of PhN and XII recording, single-, double-, or triple-barrel micropipettes (20–25 μm tip) filled with 10 mM L-glutamate, 4 mM strychnine (Sigma), or 4 mM GABA\text{Zinc} (Tocris) and/or physiological saline were lowered into the ventral horn using a piezoelectric microstepper motor (Nano stepper, Scientific Precision Instruments). Small volumes (20–25 nl) of glutamate were pressure-injected (Picospitzer III, Parker Hannifin) into C_4 ventral horn to determine the optimal injection site, identified by the rapid increase in tonic discharge of the ipsilateral PhN (see Fig. 1A). Once the optimal location was established, 25 nl saline was injected as a vehicle control. More than 10 min after this, two 50-nl volumes of either 4 mM GABA\text{Zinc} or 4 mM strychnine were injected unilaterally into the phrenic nucleus, separated by ~4 mm rostrocaudally, centered on C_4. As described in a preliminary report of this work (Marchenko and Rogers 2007b), this concentration-volume combination was chosen for maximum duration effect without significant spread to intermediate or dorsal spinal levels. Lower concentrations (1 or 2 mM) and/or smaller volumes (25 nl) produced either very transient (e.g., 20 s) or negligible effects on phrenic nerve activity, whereas two 50-nl injections of 4 mM antagonist produced lasting (>30 min) and marked effects (Fig. 2). Chicago sky blue (1%) was dissolved in the antagonist solution and used to mark injection sites (Fig. 1B). All injection volumes were verified by noting the change in level of the meniscus within the micropipette using a ×40 graduated ocular lens mounted to the stereotaxic frame. Only one antagonist, either GABA\text{Zinc} (n = 7 rats) or strychnine (n = 7 rats), was injected in the left or right phrenic nucleus per experiment. After enough breaths were collected for analysis in the “receptor blocked” condition, the animals were killed by transcardial perfusion with heparinized saline, followed by 10% formalin in saline and refrigerated overnight in the same solution. 100-μm sections were cut the following day to determine injection sites, and these were mapped onto a standardized stereotaxic atlas (Paxinos and Watson 2005).

Data analysis

General PhN output variables including respiratory rate, inspiratory (T_i) and expiratory (T_e) duration, T_i/T_e ratio, and inspiratory burst amplitude were determined as ≥300 respiratory cycles (each) were analyzed under control and treatment conditions. Respiratory rate was slower and T_i correspondingly longer than in our previous studies because expiratory CO_2 was kept lower (4.0–4.5 vs. 4.5–5.0) (cf. Marchenko and Rogers 2006a) to diminish brain/spinal cord edema in the comparatively longer experiments conducted in the present study. To compare PhN amplitudes, digitally integrated signals generated from the raw neurograms (t = 50 ms) were used. The statistical significance of any differences was assessed using a t-test.

Zero-interval subtraction (ZIS) time-frequency methods for producing dynamic power and coherence representations have been previously described (Marchenko and Rogers 2006a,b, 2007a) and are therefore described only in brief here. Representative epochs were selected during control and treatment periods, and tags marking the beginning and end of PhN-defined inspiration were created automatically and subsequently verified visually. Only inspiratory PhN bursts were analyzed, with a 2.44-Hz resolution (band-pass: 10–400 Hz), to create power spectra and coherence estimates produced by averaging ZIS time-frequency representations in the time domain (Marchenko and Rogers 2006a,b). A minimum of 300 inspiratory bursts (~7 min data) in control and treated epochs were used to create dynamic and time-averaged estimates of power and coherence. To avoid temporal smearing in time-frequency representations, only bursts with consistent durations were selected within a given condition with a maximum variation of 5%. Postinjection epochs beginning 10 min after the second injection were analyzed to determine the nature of the spectral changes associated with local GABA_\text{A} and glycine receptor blockade.

Antagonist-evoked changes in power and coherence in MFOs and HFOs were analyzed. Individual power spectral estimates were normalized (total power = 100% within each condition) and smoothed using Savitsky-Golay filter (5 points wide, 3rd-order polynomial), and major MFO and HFO peaks were identified as previously described (Marchenko and Rogers 2006a). Peak locations were chosen based on preinjection ipsilateral PhN spectra. In cases where previous postinjection peak locations did not match in frequency, the closest postinjection peak within 20 Hz of the preinjection peak was considered equivalent. In most cases, functionally equivalent peaks were present at slightly higher frequencies in treated than control conditions. In cases where no clear postinjection peak was found within 20 Hz of the preinjection peak, the postinjection power or coherence value at the preinjection peak frequency was used. Group MFO and HFO peak data were compared using paired Student’s t-test.

In addition to examining changes in internally normalized power, bin-by-bin frequency analysis of group data were also performed on absolute power between control and blockade conditions as previously described (Marchenko and Rogers 2006a,b). In these cases, control conditions were set to a total power equal to 100% and blockade conditions reported relative to that. Thus the effects on two aspects of power and coherence were assessed: the change in organizational structure of phrenic power during GABA_\text{A} or glycine receptor antagonism (via relative power measurement; Fig. 3) and the changes in absolute power relative to control (Figs. 5 and 7).

RESULTS

Injection sites

Figure 1A provides an example of physiological determination of a site at which local injection (arrow) of 25 nl of 10 mM glutamate evokes an immediate increase in tonic activity of the ipsilateral PhN that persists for ≤1 min (not shown). Figure 1B shows the location and extent of one injection site, centered in and restricted to the ventral horn at the C_4 level. The location of the centers of one injection location from each animal is shown in Fig. 1C.

Changes in PhN activity

Neither respiratory rate (36 ± 0.6 min^-1), T_i (0.32 ± 0.02 s), T_e (1.34 ± 0.04 s), or T_i/T_e (0.24 ± 0.009) were significantly altered by local injection of GABA\text{Zinc} or strychnine. However, GABA\text{Zinc} injection consistently caused an increase (43.8 ± 4.2%; mean ± SE; P < 0.05) in peak ipsilateral PhN burst amplitude in all seven rats, whereas strychnine injection had no effect on peak PhN amplitude in five rats and caused modest (17.3 ± 7.6%; P < 0.05) increases in the remaining two animals. Ipsilateral PhN burst amplitude was unaffected by saline injection (P > 0.87). As shown in Fig. 2, strychnine injection caused an increase in asynchronous activity during both inspiration and postinspiration, whereas GABA\text{Zinc} injection resulted...
in increased activity during all phases of respiration, including stage 2 expiration (E2). The examples of individual bursts under control versus treated conditions (Fig. 2C) illustrate that major oscillations still could be readily observed in bursts following GABA\textsubscript{A} injection but were less conspicuous following strychnine injection. Figure 2D compares the oscillatory behavior of smoothed epochs of these bursts under control and treated conditions. No significant changes in burst pattern or amplitude were evident in the activity of the PhN contralateral to the injection of antagonist (Fig. 2, A and B). The ipsilateral effect persisted for the duration of the recording session in all 14 animals.

Effects on normalized power and coherence

To evaluate the effects of blockade of local GABA\textsubscript{A} and glycine receptors, time-averaged ZIS spectra and coherence estimates were produced. Figure 3, A and B, illustrates the typical effects on PhN power and coherence from two different rats using GABA\textsubscript{A}zine and strychnine, respectively. Figure 3, left, demonstrates the typical effect observed, that of power spectral “whitening” (a decrease in peak height relative to broad-band power levels, an increase in peak width, and/or an increase in number of peaks compared with control) caused by local injection of GABA\textsubscript{A}zine (top) and strychnine (bottom) in individual rats. This effect is manifest as a reduction in relative power within the dominant spectral peaks (MFO, HFO; all spectra independently normalized). Although both animals generated significant fast oscillations in these two classical bands under control conditions (Marchenko and Rogers 2006a), the relative amplitudes of the distinct peaks correlating to MFO were diminished ipsilateral to blockade of either GABA\textsubscript{A} or glycine receptors. Across all animals, the diminution in relative power at MFO was significantly reduced (Fig. 4, top left) by GABA\textsubscript{A}zine (G, 33.2%) and strychnine (S, 21.6%) injection into the phrenic nucleus. While GABA\textsubscript{A}zine application significantly reduced HFO peak amplitude by 26.9%, strychnine had no significant effect on HFO over all animals (Fig. 4, top right). The relative MFO and HFO power in the contralateral PhN (Fig. 3, right) for the two animals was unchanged by antagonist application, and this was true for all animals (data not shown).

The coherence estimates (Fig. 3, center) demonstrate the effects of local blockade of GABA\textsubscript{A} (top) or glycine (bottom) receptors in the phrenic nucleus on coherence, respectively, in the same two animals. Coherence between ipsi- and contralateral PhN activity is reduced by blockade of synaptic inhibition in one phrenic nucleus. As illustrated in Fig. 4 (bottom right), HFO coherence is dramatically diminished by both GABA\textsubscript{A}zine (47.3%) and strychnine (32.6%). On the other hand, MFO coherence (bottom left) was significantly reduced only by strychnine application (17.7%).

Effects on absolute time-frequency power and coherence

To determine if changes in power distribution exhibited consistent time-dependent features across animals, we estimated time-frequency representations for the two populations of animals. Under control conditions, both sets of animals displayed consistent early-inspiratory HFO and late-inspiratory MFO peaks as previously described (March-
enko and Rogers 2006a), such that these features were retained in time-frequency representations averaged over all animals (Fig. 5, top, bands within dashed lines). Under conditions of unilateral GABA\textsubscript{A} blockade of GABA\textsubscript{A} receptors, oscillations were dispersed to other frequency bands (Fig. 5, A, bottom). The additional bands (100–150 and >180 Hz) present under GABA\textsubscript{A} influence reached maximum values in the last 2/3 of inspiration and began ≲20\% earlier than the major bands under control conditions. The population-wide effects illustrated in Fig. 5A were caused by the presence of multiple additional power bands in all animals (n = 7; cf. Fig. 3A, left) as opposed to large peaks present in non-MFO or HFO frequencies in a minority of animals. In general, strychnine treatment did not

FIG. 2. Effects of local blockade of inhibitory synaptic transmission in phrenic nucleus. A: contralateral and ipsilateral phrenic nerve records taken before (control) and after (GABA\textsubscript{A}zine) 2 50-nl injections of 4 mM GABA\textsubscript{A}zine. B: same as in A except that 2 50-nl injections of 4 mM strychnine were applied. In both cases, treated records were taken 10 min following the 2nd injection of antagonist. A and B recorded in different animals. Dashed vertical lines in A and B indicate borders of respiratory phases. I, inspiration; p-I, postinspiration; E2, expiration. C: phrenic inspiratory bursts under control (top) and after strychnine (bottom left) and GABA\textsubscript{A}zine (bottom right). D: smoothed (15.1 ms, 5th-order polynomial) neurograms of boxed epochs of C, demonstrating fast oscillations. Traces are in the same relative positions as in C. Voltage and time bars in A–D apply to all traces within those subplots.
cause power in additional bands to begin earlier in inspiration (Fig. 5B). Glycine receptor blockade caused HFO power to spread over much wider frequencies without diminishing its overall power significantly across the population (Fig. 4, top right), while at the same time inducing a shift and spread of MFO power to frequencies above that band under control conditions (Fig. 5B). These effects were found in all animals ($n = 7$; cf. Fig. 3B, left). Both strychnine and GABA<sub>z</sub>ine treatment resulted in significantly more ($P < 0.05$) absolute power over control at most frequencies (Fig. 7, left).

In both sets of animals, the dominant coherence band was in HFO during the first 2/3 of inspiration, and this feature produced population time-frequency coherence averages shown in

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FIG. 3. Effects of blockade of inhibitory synaptic transmission in the phrenic nucleus on power and coherence. A: GABA<sub>z</sub>ine effects on ipsilateral (left) and contralateral (right) phrenic power and ipsilateral-contralateral phrenic coherence (center). B: same as A except showing the effects of strychnine (in a different rat than A). MFO, HFO: medium- and high-frequency oscillation peaks under control (black traces) conditions. Note whitening of power in the ipsilateral phrenic nerve during blockade of inhibition (gray traces). Spectra are all independently normalized.

FIG. 4. Summary statistics for peak values of fast oscillations. Top: MFO (left) and HFO (right) peak amplitudes under control (C), GABA<sub>z</sub>ine (G), and strychnine (S) conditions. Data are mean amplitudes ± SE, with $n = 7$ for each condition. Bottom: coherence values at MFO and HFO peaks under same conditions. Significance values for pairwise comparisons: *$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; *****$P < 0.00001$; NS, not significant ($P > 0.05$).
Fig. 6 (top), although 5 of 14 individual animals demonstrated significant MFO coherence compared with E2 (E2 coherence not shown). Antagonism by either GABAzine or strychnine (Fig. 6, bottom) resulted in significant disruption of left-right phrenic HFO coherence. Within the each treatment group, these effects were statistically significant ($P < 0.05$) either within the HFO band (156–222 Hz; strychnine) or at all frequencies $>119$ Hz (GABAzine) as summarized in Fig. 7, right.

DISCUSSION

In this study, we tested the hypothesis that inhibition within the phrenic nucleus is critical for the generation of synchronous oscillations in phrenic motor neuron activity and that functional coupling of these oscillations likewise requires local inhibitory synaptic transmission. Both hypotheses are supported by the data presented here.

Inhibitory receptors in the phrenic nucleus

Our results demonstrating increased PhN amplitude in response to local injection of a GABA_A receptor antagonist is consistent with previous studies. In an in vivo microinjection study by Chitravanshi and Sapru (1999) in urethane-anesthetized rats, single 50-nl injections of GABA_A and GABA_B agonists caused transient decreases in PhN amplitude, while application of the same receptor antagonists blocked or reversed these effects. The authors did not report increases in postinspiratory or expiratory PhN activity, but this may have been due to their use of less concentrated solution (typically 1 mM). In support of this general finding, the authors also reported that individually recorded inspiratory neurons (presumed to be phrenic motoneurons) responded similarly to whole nerve activity. Although we only used a GABAzine antagonist in the present study, our present results support their findings, as do our preliminary studies of phrenic motoneuronal behavior (Marchenko and Rogers 2007b). Our results are also in general accord with Lalley’s (1983) findings in anesthetized cats that bicuculline (a GABA_A antagonist) reverses the depression of PhN firing (and individual motoneuronal firing) caused by large intravenous doses of baclofen, although the potential for multiple sites of action of these compounds limits the interpretation of these results.
Our results suggest that the local GABA_A and glycine receptors play crucial roles in the organization of PhN synchronous oscillations into MFO and HFO bands. Thus our results are in accord with simulation studies that suggest that inhibition may serve important functions in synchronization of motor neuron activity (e.g., Taylor and Enoka 2004; Turker and Powers 2001). In studies using neonatal rat in vitro spinal cord preparations, Tresch and Kiehn (2002) found that synaptic activity was important for millisecond-timescale synchrony in motor neuronal firing during “locomotor” behavior. Our results are also in general accord with those derived from superfused neonatal rodent in vitro and en bloc preparations in which widespread blockade of inhibition caused decreases in observed fast oscillations (Bou-Flores and Berger 2001; Sebe et al. 2006). However, it seems that a fundamentally different oscillatory output may be produced in these preparations, and how those mechanisms relate to the adult in vivo is not clear.

Reduced preparations typically produce fast phrenic or hypoglossal oscillations that are dominated by a single spectral peak (Bou-Flores and Berger 2001; Sebe et al. 2006), akin to those produced in the arterially perfused juvenile rat preparation at low temperatures (e.g., 21–23°C) (Marchenko and Rogers 2007a). At higher temperatures, the in situ preparation produces oscillations with spectral features similar to those produced in unanesthetized decerebrate adult rats in vivo (Marchenko and Rogers 2006a), while it is not known if the in vitro preparations can do so.

The differences in phrenic burst shape between en bloc brain stem/spinal cord preparations (decrementing; e.g., Bou-Flores and Berger 2001) and in situ juvenile or adult in vivo (incrementing) (e.g., Marchenko and Rogers 2006a, 2007a) may reflect important differences in the mechanisms of rhythm generation and synchrony in these preparations in their baseline or “control” conditions. In addition, the use of preparation-wide application in vitro brain stem slices or in brain stem-spinal cord en bloc preparations differs from our in vivo microinjection approach in that the latter does not affect inhibitory mechanisms associated with respiratory rhythm generation or supraspinal motor neuron coordination. The constancy of respiratory variables (rate, \( T_i, T_{i/e} \), etc.) during antagonism of GABA_A and glycine receptors suggests that the rhythm-generating circuit (and presumably the structure of the descending drive) is relatively unaffected by the local pharmacological manipulation in the phrenic nucleus and that the disorganization of synchronous oscillations in phrenic motor neurons are a product of alterations in local processing, unlike complete brain stem exposure to these antagonists during which basic respiratory variables are altered (Bou-Flores and Berger 2001; Paton and Richter 1995; Sebe et al. 2006).

FIG. 6. GABAzine- and strychnine-evoked changes in coherence dynamics. A: average of time-frequency estimates of bilateral phrenic coherence over all rats under control conditions (top) and following unilateral GABAzine injection into the phrenic nucleus (bottom). B: as in A taken from the group of rats receiving strychnine injection. - - -, HFO band. Axis labels in B, bottom, apply to all plots. Time axes as in Fig. 5. Color bars specify absolute coherence values.
Interestingly, we observe marked qualitative similarities between power spectra during hypoxia-induced hyperpnea in situ (Marchenko and Rogers 2007a) and asphyxia-induced hyperpnea in vivo (Marchenko and Rogers 2006a) with those produced under blockade of GABAA receptor-mediated inhibition in vivo (present study). In both cases, an increase in spectral entropy (increase in number of peaks and reduction in peak/broadband power ratio compared with eupnea or control) is evident, suggesting that the hyperpneic state may be characterized by a reduction in inhibition or augmentation of excitation, which are not necessarily functionally equivalent. The potential relevance of two mechanisms is supported by the observation that GABAmimetic anesthesia reduces or eliminates fast oscillations in vivo (Cohen et al. 1976) and by observations that inspiratory ventral respiratory group neurons increase their firing rates during hyperpneic breathing in unanesthetized decerebrate preparations (e.g., St. John 1981; St. John and Bianchi 1985).

**Potential sources of phrenic inhibition during inspiration**

Inhibition of phrenic motor neurons or nerve activity, including in rats, has been described. Parkis et al. (1999), working in neonatal rat brain stem-spinal cord en bloc preparations, described GABAergic inhibition during inspiration, which caused diminished response to intracellally injected currents compared with those elicited during expiration. These results are in accord with preliminary results from our laboratory (Marchenko and Rogers 2007b) in which extracellular recordings of phrenic motor neurons during nearby picoliter injections of GABA_A and glycine receptor antagonists (in the same unanesthetized, decerebrate in vivo adult rat preparation used in the present study) revealed that some phrenic motor neurons are facilitated/disinhibited by antagonism of GABA_A receptors during all three phases of respiration, whereas others react similarly following glycine receptor antagonism only during postinspiration. However, comparing results derived from neonatal versus adult preparations should be done with caution because GABA_A activation has been reported to cause inward/depolarizing currents (reversal potential = −29 mV) (Su and Chai 1998) in phrenic motor neurons in the same neonate rat en bloc preparation used by Parkis et al. (1999). In the present study, we note that antagonism of glycine receptors did not, in most animals, produce consistent increases in phrenic burst amplitude but rather produced increases in postinspiratory activity. Blockade of GABA_A receptors, on the other hand, produced significant increases in inspiration (Chitravanshi and Sapru 1999) and postinspiration. More studies will be required to determine if these differential effects are mediated directly (i.e., different receptor types expressed by different motoneurons), indirectly (e.g., via disinhibition of local interneurons), or both.

In the present studies, it is not known if the neurotransmitters that normally bind the antagonized receptors are released from neurons the somata of which are within the local spinal circuit, or in distant locations (e.g., descending bulbospinal inhibitory neurons), or both. Because inspiratory bulbospinal neurons described to date have exclusively excitatory connections with phrenic motor neurons, we assume that the inspiratory inhibitory effects reported here are mediated by local spinal interneurons. Working in the en bloc neonatal brain stem-spinal cord preparation, Parkis et al. (1999) observed that
bulbospinal GABAergic inputs, possibly originating at or caudal to the pre-Botzinger complex, inhibited phrenic motor neurons during inspiration. Because this effect was unaltered by treatment with nicotinic acetylcholine receptor antagonists injected into the C1 ventral horn, the authors concluded that the source was not from phrenic motor neuron axon collaterals. Although their firing patterns are not known (i.e., relative to respiratory phase), GABA-synthesizing bulbospinal neurons, projecting to the phrenic nuclear region, have been identified (Ellenberger 1999). In anesthetized cats in vivo, Hilaire et al. (1986) observed inhibitory postsynaptic potentials (IPSPs) in phrenic motor neurons via subthreshold electrical activation of the C3 phrenic root, which also activated Renshaw cells. Antidromic inhibition of phrenic motor neurons, axon collaterals in filled phrenic motor neurons, and Renshaw cell activation, and C6 inhibition by C5 antidromic stimulation were also observed by Lipski et al. (1985) in deafferented cats. Thus there is evidence for both local and descending inhibition. The present study is the first of which we are aware that describes the role of inspiratory phase inhibition in the production of fast phrenic oscillations.

Our results are also the first to suggest different roles for local phrenic GABA_A and glycine receptors in the organization and bilateral coupling of fast oscillations. For example, GABAzone injection did not significantly reduce MFO coherence, suggesting that coupling of MFOs does not depend significantly on local GABA_A transmission even though relative power is substantially reduced by blockade of these receptors (Fig. 4). Also, (relative) HFO power was not affected by strychnine injection even though right-left coupling of HFOs is reduced by the same treatment (Fig. 4). Our preliminary observations are that “HFO” phrenic motoneurons (see following text) are disinhibited by local picoliter injection of GABAzone (and not strychnine), and only during postinspiration (Marchenko and Rogers 2007b). If their activity underlies HFO, then the reasons why local inhibition should play an important role in the generation of synchronous oscillations in the phrenic population. Given this descending input structure, a simple model using monosynaptic excitatory connections to two phrenic motor neuron types, a larger size with low input resistance (and short dendritic space constants) and a smaller size with higher input resistance (and longer dendritic space constants), could (theoretically) account for MFO and HFO firing responses to high-frequency inputs, respectively. Indeed, support for (at least) two different types of phrenic motor neurons based on input resistance is supported by studies in the adult cat (Berger 1979) and rat (Hayashi and Fukuda 1995; Marchenko and Rogers 2007b). In this simplified scheme driven by feed-forward excitation, HFO and MFO could result from the two different sets of intrinsic properties (e.g., membrane time constants, afterhyperpolarizing potassium currents) would serve to produce two I/O functions with their characteristic frequency and with each phrenic motoneuron firing once per oscillatory cycle in near-synchrony with others in its class.

One major problem with this hypothetical model is the failure of any previous studies to observe individual phrenic motoneuronal firing at frequencies correlating to HFO (see Marchenko and Rogers 2006a for review). A possible solution to this problem has been suggested by the results of van Brederode and Berger (2008), who observed “cycle skipping” by XII motoneurons during high-frequency sinusoidal current injections in vitro. Although not demonstrated directly in that study, it is possible that subsets of motoneurons fire during every cycle at high frequencies even though no individual motoneuron does. The investigators showed that at least for some epochs during which XII motoneurons fire every other or every third current injection cycle, action potential timing may be relatively phase-locked. However, in other epochs it clearly is not. Thus it is unclear whether this mechanism can account for major oscillatory behavior at high frequencies. A different potential mechanism for producing HFO is suggested in our preliminary observations of a new type of phrenic motoneuron (HFO motoneurons) that fire at appropriately high frequencies (Marchenko and Rogers 2007b). But this finding requires more verification to assess its plausibility of generating enough power at HFO-related frequencies. These two (potential) mechanisms for HFO generation are not mutually exclusive, and this question remains unsettled even though HFO has been observed in the firing rates of bulbospinal inputs to the phrenic nucleus (Davies et al. 1985; Huang et al. 1996; Tian and Duffin 1997).

However, our present results suggest that a feed-forward excitatory mechanism is wholly inadequate in producing the population effects of synchronous oscillations (Figs. 2 and 3). When inhibitory transmission is blocked, the output of the population becomes disorganized, resulting in power dispersion from the two major frequency peaks (Fig. 3), an effect that is consistently observed across animals (Fig. 7). Simulation studies of motor neuron population firing (Taylor and Enoka 2004; Turker and Powers 2001) suggest that inhibition has two main enhancing effects on synchrony. First, tonic inhibition causes sustained membrane hyperpolarization that requires significant correlated input to reach threshold, thereby increasing the likelihood of synchronous firing. When this hyperpolarization is removed, the motor neurons’ membrane potentials are near threshold far more often during which smaller, uncor-
related inputs may cause asynchronous spiking across the population. Second, if inhibitory neurons receive convergent common drive (from motor neurons themselves, from descending drive, or both) and in turn provide powerful divergent inputs to many motor neurons, then the latter may be synchronized by virtue of the near-simultaneous release from inhibition, i.e., via rebound excitation.

Bilateral coupling

It has been assumed that HFOs and MFOs are derived from separate sources because their bilateral coherences differ. That is, because HFO coherence is typically higher than MFO coherence, it is assumed that HFO is supraspinal in origin (i.e., providing a shared input to left and right motoneuron pools), whereas MFO is generated at the spinal level and weakly coupled across the midline. Davies et al. (1985) and Tian and Duffin (1997) found HFO-related firing frequencies in phrenic-projecting bulbospinal neurons, and Duffin and van Alphen (1995) were able to detect bilateral projections to phrenic motoneurons from individual bulbospinal inspiratory neurons using cross-correlation analysis. Using an in situ preparation, Duffin and Li (2006) failed to find bulbospinal axonal crossing at the C6–C8 level in neonatal through juvenile-aged rats, suggesting that functional coupling occurs at the brain stem level in contra- and ipsilateral projecting neurons. These observations lend support to the existence of a shared bulbospinal input (containing HFO) to the two phrenic nuclei. However, they do not exclude the possibility that both HFO and MFO in phrenic output result from the selective processing of a shared oscillatory input drive (see preceding text).

The present results suggest that the production of HFO, in particular, is not a purely supraspinal, feed-forward phenomenon. In addition to demonstrating the importance of local GABA
 and glycnergic mechanisms in the generation of fast phrenic oscillations, our findings highlight their role in coupling those oscillations between both phrenic motor pools. Because coherence is a measure of phase constancy, our results demonstrate that unilateral strychnine and GABA/azine injection cause reduction in HFO coherence, possibly due to recruitment of asynchronously (but rapidly) firing small motor neurons, a result supported by preliminary studies of individual motor neuronal responses to blockade of GABA and glycine receptors (Marchenko and Rogers 2007b). Moreover, at the individual motor neuron level, the same absolute variability in spike time accounts for a significantly larger phase displacement for fast-firing than slow-firing motoneurons (van Brederode and Berger 2008), which makes those operating at higher frequencies more susceptible to coherence reduction. Because HFO coherence is significantly reduced by strychnine and GABA/azine (Fig. 7), inhibitory mechanisms must play a critical role in bilateral HFO coupling, regardless of its ultimate origin. Alternatively (or in addition), our findings suggests that a single, common HFO input of strength large enough to produce high coherence is not shared by the left and right phrenic pools. Further investigation will be required to unravel which, if either, of these conceptual models is correct.

References


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