Inhibitory Synaptic Transmission Governs Inspiratory Motoneuron Synchronization

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Sebe, Joy Y., Johannes F. van Brederode, and Albert J. Berger. Inhibitory synaptic transmission governs inspiratory motoneuron synchronization. J Neurophysiol 96: 391–403, 2006. First published March 1, 2006; doi:10.1152/jn.00086.2006. Neurons within the intact respiratory network produce bursts of action potentials that cause inspiration or expiration. Within inspiratory bursts, activity is synchronized on a shorter timescale to generate clusters of action potentials that occur in a set frequency range and are called synchronous oscillations. We investigated how GABA and glycine modulate synchronous oscillations and respiratory rhythm during postnatal development. We recorded inspiratory activity from hypoglossal nerves using in vitro rhythmically active mouse medullary slice preparations from P0–P11 mice. Average oscillation frequency increased with postnatal development, from 17 Hz in P0–P1 mice to 38 ± 7 Hz in P7–P11 mice (n = 37; P < 0.0001). Bath application of GABA_A and GlyR antagonists significantly reduced oscillation power in neonates (P0–P6) and juveniles (P7–P10) and increased peak integrated activity in both age groups. To test whether elevating slice excitability is sufficient to reduce oscillation power, Substance P was bath applied alone. Substance P, although increasing peak integrated activity, had no significant effect on oscillation power. Prolonging the time course of GABAergic synaptic currents with zolpidem decreased the median oscillation frequency in P9–P10 mouse slices. These data demonstrate that oscillation frequency increases with postnatal development and that both GABAergic and glycineergic transmission contribute to synchronization of activity. Further, the time course of synaptic GABAergic currents is a determinant of oscillation frequency.

INTRODUCTION

Neural oscillations are widespread in the nervous system and are commonly associated with specific behaviors. Among the various brain regions and behaviors, oscillations occur in human sensorimotor cortex during sustained muscle contraction (Baker and Baker 2003), in corticothalamic neurons during non-REM sleep (Steriade 1999), and in the insect brain in response to odor presentation (Laurent and Naraghi 1994). Oscillations are also exhibited by inspiratory motoneurons and motor nerves in phase with inspiration (Cohen et al. 1997; Funk and Parkis 2002).

Respiratory motoneurons in the brain stem and spinal cord, which innervate respiratory muscles such as the diaphragm and tongue (phrenic and hypoglossal motoneurons, respectively), are synchronized on long and short timescales. On a long timescale, respiratory motor nerves exhibit bilateral bursts of action potentials during inspiration or expiration. On a short timescale, respiratory motoneurons synchronously fire at a particular frequency during inspiration (Cohen et al. 1997; Funk and Parkis 2002), creating a pattern of activity that we will refer to as synchronous oscillations. Inspiratory motoneurons are synchronized on a short timescale within a given motor nucleus (Solomon et al. 2003), across bilateral phrenic motor nuclei (Cohen et al. 1987), and across different motor nuclei (Richardson and Mitchell 1982). Synchronous oscillations have been recorded from several inspiratory nerves including those that innervate the diaphragm (Cohen et al. 1974; O’Neal III et al. 2005; Richardson and Mitchell 1982), tongue (Bou-Flores and Berger 2001; Cohen et al. 1987), and laryngeal muscles (Richardson and Mitchell 1982).

In the 15- to 120-Hz range within which these oscillations occur, there are two categories of oscillations: medium- and high-frequency oscillations (MFOs and HFOs, respectively). Oscillations can be differentiated into one of these two categories according to their frequency range and, more reliably, by the region of the brain stem or spinal cord in which they are generated (Cohen et al. 1997; Funk and Parkis 2002). According to the latter criterion, HFOs are observed within the 15- to 50-Hz frequency range, whereas HFOs are usually recorded within the 50- to 120-Hz range. However, oscillation frequency is dependent on changes in temperature (Dittler and Garten 1912; Richardson and Mitchell 1982), hypercapnia, anesthetics, the preparation used (Funk and Parkis 2002), and the age of the animal studied (Suthers et al. 1977). With respect to the latter criterion, HFOs recorded bilaterally in the same inspiratory motoneuron population (i.e., left and right phrenic nerves) and across different inspiratory nerves (i.e., phrenic vs. recurrent laryngeal) show strong coherence, suggesting that they are generated by common inputs from medullary neurons (Richardson and Mitchell 1982). In contrast, MFOs show little or no coherence when recorded from bilateral nerves from the same motoneuron population and no coherence when recorded from different inspiratory nerves, indicating that they are generated at or immediately upstream of the motoneuron level (Cohen et al. 1987). In addition to MFOs and HFOs, hypoglossal motoneurons (HMs) that innervate the tongue exhibit low-frequency oscillations (4–8 Hz) in response to metabotropic glutamate receptor (mGluR) activation. However, these oscillations do not require respiratory activity and are likely generated by a separate mechanism (Sharifullina et al. 2005).

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Similar to oscillations observed in cortex (Buzsáki and Draguhn 2004; Farmer 1998; Steriade 1999), hippocampus (Mann et al. 2005), and olfactory bulb (Gelperin 2006), the functional significance of oscillations in the respiratory network has been of interest. For example, Parkis et al. (2003) recorded endogenous, inspiratory-phase depolarizing currents containing oscillations from rhythmically active phrenic motoneurons. When this current was injected into the same motoneuron, the oscillations increased the probability of action potential firing at the oscillation peaks and thereby increased the precision of spike timing. Although the functional significance of synchronous oscillations in the respiratory network remains unknown, they may be involved in enhancing inspiratory muscle activation possibly by tuning motoneuron firing frequency.

Among the mechanisms underlying synchronous oscillations recorded throughout the brain, y-aminobutyric acid type A receptor (GABA$_A$R)–mediated synaptic transmission plays a prominent role (Cobb et al. 1995; Fisahn et al. 1998; Legier et al. 2004; Schoppa 2006; Steriade 1999; Whittington and Traub 2003). In the hippocampus, where network oscillations coincide with exploratory behavior, unitary inhibitory postsynaptic potentials (IPSPs), produced by GABAergic interneuron stimulation, initiate subthreshold oscillations in CA1 pyramidal cells (Cobb et al. 1995). Rhythmic unitary GABAergic IPSPs are able to evoke and synchronize spike firing in simultaneously recorded pyramidal cells by postinhibitory rebound firing. In the olfactory bulb, recovery from GABAergic IPSPs also results in oscillations and spike synchrony in simultaneously recorded mitral cells (Schoppa 2006). In both cases, recovery from IPSPs mediated by GABAergic interneurons underlies principal cell oscillations and spike synchronization. Previous work from our laboratory has shown that GABAergic and glycinenergic synaptic inhibition are also important in generating synchronous oscillations recorded from hypoglossal nerves of the neonatal respiratory network (Bou-Flores and Berger 2001). As the brain stem develops, inhibitory synaptic transmission undergoes multiple changes that may affect synchronous oscillations.

In the present study, we extended work by Bou-Flores and Berger (2001) to examine inspiratory-phase synchronous oscillations during a later stage in postnatal development. To record inspiratory-phase activity from hypoglossal rootlets, we used the rhythmically active medullary slice preparation (Smith et al. 1991). This rhythmically active slice preparation contains pacemaker neurons of the pre-Bötzinger complex (PBC), which generate inspiratory rhythm (Johnson et al. 2001; Koshiya and Smith 1999; Smith et al. 1991), as well as the hypoglossal nerve rootlets from which inspiratory-phase motor outflow can be recorded. We recorded inspiratory-phase activity from hypoglossal nerves of neonatal and juvenile mice to measure oscillation frequency and examine the role of GABA and glycine in generating oscillations. To further probe the mechanisms by which oscillations are produced, we investigated how oscillation frequency is modulated by prolonging the time course of GABAergic inhibition.

**METHODS**

**Preparations**

In vitro experiments were performed on either the rhythmically active (Fig. 1) or the nonrhythmically active medullary slice preparations from Swiss–Webster mice (P0–P11). For experiments in which we measured oscillation frequency over postnatal development (Fig. 2), we used P0–P11 mice. However, in all other figures, we divided the mice into two age groups: neonates (P0–P6) and juveniles (P7–P10) to divide the sample population into the first and second weeks of postnatal development, respectively. Mice were anesthetized with halothane and killed by decapitation in accordance with the regulations of the University of Washington Institutional Animal Care and Use Committee (IACUC).

Methods used in dissecting the rhythmically active medullary slice preparation were previously described (Funk et al. 1993). In brief, the medulla and cervical spinal cord were isolated and removed from the mouse. The brain stem and spinal cord were pinned onto a Sylgard block and the block was mounted into a vibratome platform (Pelco 101 Series 1000, PELCO, Redding, CA). Brain stem slices were then cut from rostral to caudal. After the facial nucleus was no longer visible, another 200-μm slice was cut before cutting the rhythmic slice. The thickness of the rhythmic slice was increased from 500 to 700 μm according to the age of mouse. Slices from younger mice were thinner than those obtained from older mice. This slice was placed into the recording chamber and superfused for ≥20 min with 8 mM K+ artificial cerebrospinal fluid (ACSF) before recording began.

For studies using the nonrhythmically active medullary slice preparation, the brain stem was removed and 300-μm slices containing the hypoglossal motor nucleus were cut. Slices were incubated for 1 h at 37°C before recording.

**Recording**

For the rhythmically active slice preparation, the temperature of the custom-made recording chamber was maintained between 27 and 28°C. Glass suction electrodes were pulled from borosilicate glass and filled with ACSF to record from the cut ends of hypoglossal rootlets. Raw nerve signals were amplified and AC filtered at 0.1 Hz using CyberAmp 320 and pClamp8 (Axon Instruments, Union City, CA) and the signal was sampled at 5 kHz. To measure integrated nerve activity, the filtered signal was rectified and integrated using a custom-built “leaky” integrator with a time constant of 100 ms.

Whole cell voltage-clamp recordings were made from hypoglossal motoneurons (HMs) in nonrhythmically active medullary slices visualized using IR-DIC microscopy (Zeiss) and camera (Hamamatsu). Cells were voltage clamped at a holding potential of −70 mV (after correction for the measured liquid junction potential of 10 mV) using an Axopatch 200B amplifier (Axon Instruments). Recording electrodes were pulled from borosilicate glass and had a resistance of 3–3.5 MΩ. Access resistance ($R_a$) was monitored periodically during the recording and recordings were rejected if $R_a$ was >30 MΩ or changed by >25% during the recording. Whole cell current was converted from AC to DC at 20 kHz using pClamp8 (Axon Instruments) after low-pass filtering at 5 kHz with an eight-pole Bessel filter.

**FIG. 1.** Mouse rhythmically active medullary slice preparation. A: this slice preparation contains inspiratory rhythm–generating neurons in the pre-Bötzinger complex (PBC) that transmit inspiratory-phase activity, possibly through a premotor area, to motoneurons of the hypoglossal nucleus (XII). Hypoglossal nerve roots exit the ventral surface of the brain stem slice and exhibit inspiratory-phase activity (B, top trace). Raw hypoglossal nerve root recordings are rectified and integrated (B, bottom trace).
Solutions and drug application

The normal ACSF used for rhythmically active slice preparations contained (in mM): 118 NaCl, 3 KCl, 1 MgCl$_2$, 1 NaH$_2$PO$_4$, 25 NaHCO$_3$, 30 d-glucose, and 1.5 CaCl$_2$. The osmolarity of the ACSF was 300 mOsm and the ACSF was pH adjusted to 7.4 with NaOH. For recording spontaneous rhythmic activity, the same ACSF was used except that KCl concentration was elevated to 8 mM KCl. ACSF was superfused over the preparation at 2–3 ml/min and recycled using a peristaltic pump (Rainin). The ACSF used for nonrhythmically active medullary slice preparations contained (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO$_4$, 1 NaH$_2$PO$_4$, 26.2 NaHCO$_3$, 11 d-glucose, and 2.5 CaCl$_2$. During dissection, slicing, and recording, both ACSFs were gassed with 95% O$_2$-5% CO$_2$. The internal solution for whole cell voltage-clamp recordings contained (in mM): 140 CsCl, 1 CaCl$_2$, 3.45 Cs-BAPTA, 5 Mg$_2$-ATP, 10 HEPES, and 10 QX-314 (pH = 7.3 with CsOH; osmolarity = 290 mOsm).

All drugs were bath applied in the ACSF and washed in or out for 10–15 min before recording inspiratory activity in drug condition or washout, respectively. In experiments to block GABA$_A$ and glycine receptors (GlyRs), SR95531 (0.5 μM, Sigma) and strychnine hydrochloride (1 μM, Sigma), respectively, were applied for 10 min before recording. Zolpidem (0.1–0.5 μM, Sigma) prepared from a 50 mM stock solution in ethanol was diluted to the desired concentration in bath solution just before the start of the experiment. Substance P (0.05 μM, Sigma) was bath applied to increase the excitability of the slice (Gray et al. 1999; Yasuda et al. 2001). In whole cell recordings of GABAergic miniature inhibitory postsynaptic currents (mIPSCs), the bath solution contained DNQX (10 μM, RBI), AP5 (25 μM, Tocris), tetrodotoxin (1 μM, Alomone Labs), and strychnine hydrochloride (1 μM). Bicuculline methiodide (5 μM, Sigma) was bath applied at the end of some whole cell experiments to confirm the GABAergic nature of mIPSCs.

Data analysis

Inspiratory bursts were selected in Clampfit (Axon Instruments) and analyzed in Igor Pro (WaveMetrics) using a routine developed by Dr. Randy K. Powers. For each recording condition, 15–20 inspiratory bursts were selected and used to create 15–20 absolute power spectra in which data points were at 1-Hz intervals. Average absolute power spectra were computed by averaging the 15–20 absolute power spectra for each condition. Average relative power spectra were calculated from the average absolute power spectra by dividing the absolute power at each data point (0–99 Hz) by the absolute power for all data points (0–99 Hz). For Fig. 2, oscillation frequency was measured by finding the frequency of the dominant peak in the average relative power spectrum. The dominant peak was defined as the largest peak in the average relative power spectrum, not including the power at low frequencies (0–5 Hz) that arises from the overall envelope of the inspiratory burst. Only average relative power spectra of control recordings were used to find oscillation frequency. A least-squares regression line was used to fit and determine the statistical significance of the change in oscillation frequency of the dominant peak over postnatal age. Unpaired t-tests were used to compare oscillation frequencies recorded from neonate and juvenile slices.

To measure relative power of synchronous oscillations, as opposed to measuring synchronous oscillation frequency, each average relative power spectrum was binned (10-Hz bin width). To bin the relative power within each average relative power spectra, we located the dominant peak in the average relative power spectra. The location of this peak and its concomitant 10-Hz bin defined the starting point of the binning process whereby all other bins were in 10-Hz increments above and below this starting bin. Then, we were able to compute the fraction of relative power within the 10-Hz bin that contained the dominant peak. To do this, we added all points within each 10-Hz bin and divided that number by the total relative power from 0 to 99 Hz. By comparing these fractions of relative power, we measured changes in the relative power of synchronous oscillations after various pharmacological manipulations. Paired t-tests were used to compare changes in relative power between control and drug conditions.

Peak integrated activity and burst duration were measured in Igor Pro using the rectified and integrated traces of the inspiratory bursts. Peak integrated activity was the highest point of the rectified and integrated traces. Burst duration was defined as the time at 95% of the integrated area subtracted by time at 5% of the integrated area. Peak integrated activity and burst duration values were computed for the same 15–20 inspiratory bursts used for power spectral analysis. The average peak integrated activity and burst duration were compared across recording conditions. Paired t-tests were used to determine statistical significance of changes in peak integrated activity, inspiratory burst duration, and inspiratory burst frequency, separately, between control and drug conditions.

Event detection and off-line analysis of the recorded spontaneous miniature GABAergic mIPSCs (mIPSCs) was performed using the MiniAnalysis 5.6 software program (Synaptosoft, Decatur, GA). We
determined the peak amplitude and decay time (measured as the time for the mIPSC to decay to 37% of its peak value) of all individual events whose amplitude exceeded the detection threshold (set manually by visual inspection of the data traces). Event detection was started after a minimum preequilibration period of 5 min in each bath solution and 1- to 4-min-long recorded segments during perfusion with control solution and after adding zolpidem were selected for automated event detection. Overlapping events or events with slow rise times (>10 ms) were excluded from analysis by visual inspection of the detected mIPSCs. Miniature event frequency varied considerably from cell to cell, although a minimum of 35 events was used per cell to calculate average values for mIPSC amplitude and decay time in individual cells. Only one cell per slice was tested and only one concentration of zolpidem (0.1, 0.2, or 0.5 μM) was tested per cell. Statistical significance was determined with paired and unpaired t-tests within and between cell groups and with the Kolmogorov–Smirnov test for comparisons within individual cells. Significance was set at \( P < 0.05 \).

To investigate the effect of zolpidem on oscillation frequency, we computed the average relative power spectrum, as described above, for the control condition, after bath application of strychnine, and subsequent bath application of strychnine and zolpidem. For each average relative power spectrum, we determined the oscillation frequency by computing the median oscillation frequency within the 9- to 59-Hz frequency range (Igor Pro). To calculate median oscillation frequency, we added all the relative power values between 9 and 59 Hz and computed the oscillation frequency that divided the relative power into two equal sums. Statistical significance was determined using paired t-test.

**RESULTS**

**Oscillation frequency increases over postnatal development**

We found that oscillation frequency gradually increases over postnatal development and spans a range of frequencies, from 6 to 57 Hz, in P0–P11 mouse slices. Figure 2A shows inspiratory bursts from P0 and P10 mouse slices acquired from hypoglossal nerve recordings. The clusters of action potentials are evident in both neonatal and juvenile slices. Using power spectrum is similar to that after bath application of strychnine and SR95531. The power spectra of 20 different inspiratory bursts were averaged for each of the four conditions and the average power spectra are shown in Fig. 2B.

In Fig. 2B, the 8- to 17-Hz frequency bins contains the dominant peaks for the average power spectra. The fraction of relative power within the 8- to 17-Hz frequency bins is reduced after strychnine bath application compared with control. After combined strychnine and SR95531 bath application, the fraction of relative power within the same 8- to 17-Hz frequency bin is further reduced. This reduction in relative power is reflected in the average power spectra (Fig. 2B, inset) as the lack of a dominant peak when both GABAAR and GlyRs are blocked. The return of the dominant peak during wash demonstrates the partial reversibility of this reduction in oscillation power. During wash, oscillation power in the average power spectrum is similar to that after bath application of strychnine (Fig. 3B, inset). This suggests that strychnine does not wash out. In Fig. 3C, the average power spectra for a similar experiment in a P9 mouse are shown. Relative power in the frequency bins containing the dominant peaks (32–41 Hz) is reduced after GlyR blockade and relative power is further reduced after GABAAR and GlyR blockade. The lack of a dominant peak in the average power spectrum during GABAAR and GlyR blockade (Fig. 3D) reflects this reduction in relative power. The dominant peak partially returns after washout of GABAAR and GlyR antagonists.

Summary data demonstrating the role of GABAergic and glycineric transmission in synchronizing HM activity over postnatal development are shown in Fig. 4. In neonatal mouse slices, bath application of strychnine alone decreased oscillation power by \( -21 \pm 7\% (n = 7, P < 0.05) \) and bath application of SR95531 alone decreased oscillation power by \( -39 \pm 11\% (n = 6, P < 0.05) \) compared with control. Bath application of strychnine and SR95531 together in neonates decreased oscillation power by \( -49 \pm 7\% (n = 10, P < 0.0001) \) compared with control and this effect was partially reversed after wash (\( -23 \pm 12\%, n = 13, \text{n.s.} \)). In juveniles (P7–P10), GlyR blockade alone reduced oscillation power by \( -20 \pm 7\% (n = 14, P < 0.01) \) and bath application of GABAergic and glycineric transmission contribute to motoneuron synchronization

In each experiment, we recorded inspiratory-phase activity from a hypoglossal rootlet in control ACSF. Next, strychnine, a glycine receptor (GlyR) antagonist, or SR95531, a GABAAR antagonist, was bath applied separately, and then both antagonists were bath applied together. We used SR95531 because it is a more specific GABAAR antagonist than bicuculline methiodide, which also blocks small-conductance Ca2+-activated potassium channels (Khawaled et al. 1999). The antagonists were washed out for \( \geq 20 \) min before recording hypoglossal nerve rootlet activity in the wash condition. In Fig. 3A, representative inspiratory bursts recorded from a P0 mouse slice in control, 1 μM strychnine, 1 μM strychnine + 0.5 μM SR95531, and wash conditions are shown. Using the P0 slice recording shown in Fig. 3A, the power spectra of 20 different inspiratory bursts were averaged for each of the four conditions and the average power spectra are shown in Fig. 3B.

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SR95531 alone decreased oscillation power by $33 \pm 6\%$ ($n = 5$, $P < 0.05$). Bath application of GABA$_A$ and GlyR antagonists together decreased oscillation power by $61 \pm 6\%$ ($n = 12$, $P < 0.0001$), and this effect was partially reversible ($-41 \pm 9\%$, $n = 12$, $P < 0.001$).

The effect of GABA$_A$ and GlyR blockade on synchronous oscillations was also apparent in the recording of the rectified and integrated hypoglossal nerve traces (representative examples of this are shown in Fig. 5, A and B). In a P0 slice (Fig. 5A), the control trace (black) shows a 10-Hz oscillation within the inspiratory burst that is abolished after GABA$_A$ and GlyR blockade (red), and returns in the wash condition (gray). In a P10 slice (Fig. 5B), oscillation frequency in control (black) is higher, at 34 Hz, and this oscillation is less prominent in the rectified and integrated trace after GABA$_A$ and GlyR blockade (red).

Blocking GABAergic and glycinerergic transmission increase slice excitability

Blocking GABA$_A$ and GlyRs increased slice excitability for both neonate and juvenile mice. We measured changes in peak integrated activity, inspiratory burst duration, and inspiratory burst frequency relative to control for neonates and juveniles. In neonates (Fig. 5, A and C1), bath application of SR95531 or strychnine, separately, increased mean peak integrated activity (SR95531: $36 \pm 9\%$, $n = 8$, $P < 0.01$; strychnine: $36 \pm 5\%$, $n = 10$, $P < 0.01$). Bath application of SR95531 and strychnine together further increased mean peak integrated activity ($103 \pm 17\%$, $n = 18$, $P < 0.0001$) and this effect was partially reversed after wash ($65 \pm 10\%$, $n = 18$, $P < 0.01$). In Fig. 5A, the rectified and integrated traces of inspiratory bursts for a single neonatal mouse show the increase in peak integrated activity after antagonist application. After strychnine bath
application (blue), peak integrated activity increased compared with control (black) and increased further when GABA\textsubscript{A} and GlyRs were blocked together (red). In juveniles, bath application of strychnine alone did not affect peak integrated activity (Fig. 5C1). In the same age group, bath application of SR95531, separately, and both antagonists together increased peak integrated activity (SR95531: 38 ± 5\%, n = 7, P < 0.001; SR95531 + strychnine: 55 ± 8\%, n = 13, P < 0.0001) and this effect was partially reversible (10 ± 5\%, n = 13, P < 0.01) (Fig. 5C1). In Fig. 5B, the rectified and integrated traces show the increase in peak integrated activity in a juvenile mouse after combined GABA\textsubscript{A} and GlyR blockade (red) compared with control (black).

We also measured the effect of GABA\textsubscript{A} and GlyR antagonists on mean inspiratory burst duration in neonates and juveniles (Fig. 5C2). In neonates, bath application of strychnine (−13 ± 2\%, n = 10, P < 0.001), but not SR95531 (−11 ± 4\%, n = 8, P = 0.12), decreased mean burst duration. When SR95531 and strychnine were bath applied together, mean burst duration decreased (−17 ± 4\%, n = 18, P < 0.001) and this effect was not reversed (−13 ± 2\%, n = 18, P > 0.001). In juveniles, bath application of SR95531 or strychnine, separately, decreased mean burst duration (SR95531: −9 ± 2\%, n = 8, P < 0.01; strychnine: −10 ± 2\%, n = 6, P < 0.001). Bath application of SR95531 and strychnine together decreased mean burst duration (−13 ± 3\%, n = 13, P < 0.001) and this effect was not reversible by wash (18 ± 2\%, n = 13, P < 0.0001). The decrease in burst duration, in neonates and juveniles, is reflected in the rectified and integrated traces shown in Fig. 5, A and B. GlyR blockade (blue) and combined GABA\textsubscript{A} and GlyR blockade (red) reduced mean burst duration in the P0 and P9 slices compared with control (black).

Bath application of GABA\textsubscript{A} and GlyR antagonists changed the pattern of respiratory rhythm in neonates and juveniles. After GABA\textsubscript{A} and GlyR blockade, the hypoglossal nerve output occasionally exhibited seizurelike activity. It was difficult to consistently measure the inspiratory burst frequency during these seizurelike events because the bursts often merged into one long burst as the antagonists washed into the slice. For this reason, mean inspiratory burst frequency was measured in blocks of activity that did not include seizurelike activity.

Neonate and juvenile slices in control conditions exhibited a primary rhythm composed only of large-amplitude bursts that occurred at a relatively low frequency (Fig. 6, A and B, respectively). This primary rhythm persisted, in both age groups, after separate blockade of GABA\textsubscript{A} or GlyRs (Fig. 6, A and B). Subsequent combined GABA\textsubscript{A} and GlyR blockade gave rise to a secondary rhythm, in neonates (Fig. 6A) but not in juveniles (Fig. 6B). The secondary rhythm consisted of low-amplitude inspiratory bursts occurring at a higher burst frequency than the inspiratory bursts of the primary rhythm (Fig. 6A).

For all recordings, we used only the high-amplitude inspiratory bursts for our power spectral analysis. As a result of the two inspiratory rhythms exhibited by neonatal slices after GABA\textsubscript{A} and GlyR blockade, we approached mean inspiratory burst frequency analysis, for all conditions and both age groups, in two ways. First, we included all inspiratory bursts, those in the primary and secondary rhythm, in measuring inspiratory burst frequency; these data are summarized in Fig. 6C1. Second, we counted only the high-amplitude bursts; these data are summarized in Fig. 6C2.

First, we report the effects of GABA\textsubscript{A} and GlyR blockade on inspiratory burst frequency measured from neonatal slices. When all inspiratory bursts were counted (Fig. 6C1), GlyR blockade did not affect mean inspiratory burst frequency (0 ± 7\%, n = 9, n.s.). GABA\textsubscript{A}R blockade alone substantially increased inspiratory burst frequency in a single P2 slice out of eight neonatal slices studied by giving rise to a secondary inspiratory rhythm. This result increased the variability of the data points and when all inspiratory bursts were counted, GABA\textsubscript{A}R blockade did not significantly affect mean inspiratory burst frequency in neonates (204 ± 190\%, n = 8, n.s.). When counting all inspiratory bursts, we found that combined GABA\textsubscript{A} and GlyR blockade increased mean inspiratory burst frequency in neonates (415 ± 114\%, n = 17, P < 0.001). This effect was reversible during wash (−13 ± 6\%, n = 17, P < 0.001).

**FIG. 5.** GABA\textsubscript{A} and GlyR blockade increased peak integrated hypoglossal nerve activity and decreased inspiratory burst duration in neonates and juveniles. A: representative rectified and integrated traces from a P0 slice show that with Stry bath application peak integrated activity increased and with subsequent combined SR and Stry bath application it increased further. Dotted lines in A and B are aligned with synchronous oscillation peaks in the control traces. B: representative rectified and integrated hypoglossal nerve traces from a P9 slice show that peak integrated activity did not change with Stry bath application but increased with subsequent SR and Stry bath application. Washing reversed this effect. C1: peak integrated hypoglossal nerve activity increased with Stry application alone in neonates but not in juveniles. Bath application of SR increased peak integrated activity in both age groups as did bath application of both SR and Stry. This effect was partially reversed after wash. C2: blockade of GlyRs, separately, or GABA\textsubscript{A} and GlyRs, together, decreased inspiratory burst duration in neonates. In juveniles blockade of GABA\textsubscript{A} and GlyRs, separately and together, decreased inspiratory burst duration. Mean ± SE. ***P < 0.01; ****P < 0.001; paired t-test vs. control.
In neonatal slices, when only high-amplitude bursts were counted (Fig. 6C2), GlyR blockade alone did not affect inspiratory burst frequency. Because GlyR blockade alone did not induce a secondary rhythm, both forms of analysis yielded the same result as reported above. GABA_A-R blockade alone (17 ± 8%, n = 8, n.s.) and combined GABA_A and GlyR blockade (22 ± 7%, n = 17, n.s.) did not affect inspiratory burst frequency in neonates. Inspiratory burst frequency decreased during wash (−13 ± 6%, n = 17, P < 0.001).

In juveniles, GABA_A and GlyR blockade did not yield a secondary rhythm and thus both forms of analysis yielded the same result (Fig. 6, C1 and C2). GlyR or GABA_A-R blockade, separately, did not affect mean inspiratory burst frequency (−7 ± 14%, n = 6, n.s. and 14 ± 13%, n = 7, n.s., respectively). Bath application of the same concentrations of strychnine and SR95531 as used on neonatal slices decreased inspiratory burst frequency (−31 ± 10%, n = 13, P < 0.05) in juveniles and inspiratory burst frequency did not recover after wash (48 ± 8%, n = 13, P < 0.001).

Overall, GABA_A and GlyR blockade increases slice excitability in neonates and juveniles by increasing mean peak integrated activity. However, mean inspiratory burst frequency showed an age-dependent sensitivity to GABA_A and GlyR blockade in that bath application of GABA_A and GlyR antagonists together induced a secondary rhythm in neonates, but not in juveniles.

Increasing excitability by Substance P does not result in a decrease in oscillation power

We have shown that blocking inhibitory synaptic transmission decreases the power of synchronous oscillations whereas it increases the excitability of the slice, that is, there is an increase in peak integrated hypoglossal nerve activity. Although GABAergic and glycineergic transmissions are likely involved in synchronizing motoneuron activity, it is possible that any intervention that increases slice excitability and therefore increases nerve activity will reduce the power of the oscillations. The oscillations are characterized by clusters of action potentials within a burst that are separated by short periods of little or no activity. Increasing excitability may reduce oscillation power by filling in the gaps between the clusters of action potentials. If this interpretation of the data is true, increasing the excitability using another method should also decrease oscillation power.

To test this hypothesis, we increased slice excitability by bath applying 0.05–0.1 μM Substance P to rhythmically active slices from juvenile mice (P8–P10). Substance P increases HM excitability by inhibiting the TASK-1 K⁺ channel (Talley et al. 2000). Extensive studies that investigated the effects of Substance P on respiratory rhythm have shown that local application of Substance P in the PBC (Gray et al. 1999) or hypoglossal nucleus (Yasuda et al. 2001) increase inspiratory burst frequency and inspiratory burst amplitude. We bath applied Substance P to examine whether increasing slice excitability also reduces oscillation power.

As expected, Fig. 7A shows that bath application of 0.05–0.1 μM Substance P elevated the activity recorded from hypoglossal nerve roots by increasing mean peak integrated activity (Fig. 7B: 123 ± 55%, n = 12, P < 0.05) and this effect was reversed by wash (Fig. 7, A and B: 5 ± 33%, n = 10, n.s.). Substance P bath application also increased mean inspiratory burst frequency (Fig. 7C: 170 ± 24%, n = 12, P < 0.0001) from 0.07 ± 0.03 Hz in control to 0.18 ± 0.05 Hz in Substance
P. Mean inspiratory burst frequency returned to 0.07 ± 0.05 Hz after wash (Fig. 7C: 3 ± 19%, n = 10, n.s.). Unlike the GABA<sub>A</sub> and GlyR antagonists, Substance P increased mean burst duration (7 ± 3%, n = 12, P < 0.05) and this effect was reversible (5 ± 3%, n = 10, n.s.).

In contrast to the results seen with GABA<sub>A</sub> and GlyR blockade, the increase in slice excitability was not accompanied by a decrease in oscillation power. Examples of inspiratory bursts recorded from two different slices in control solution, after 0.05 μM Substance P application, and in wash are shown in Fig. 8, A and C. In the slice shown on the left, the relative power of the oscillation in the 35- to 44-Hz bin increases after bath application of 0.05 μM substance P and oscillation power is diminished during wash (Fig. 8B). A similar increase in oscillation power was observed in six of eight cells tested (100 ± 41%, P < 0.05). In the slice shown on the right, relative power in the 33- to 42-Hz bins does not change after Substance P application (Fig. 8D). A summary of the effect of Substance P on relative power of the oscillations is shown in Fig. 8E. When data from all eight slices were pooled, bath application of Substance P does not significantly affect relative power (72 ± 33%, n = 8, n.s.). After washout, oscillation power was not different from control (−45 ± 13%, n = 6, n.s.).

These data reconfirm that Substance P increases slice excitability by increasing peak integrated activity and inspiratory burst frequency. GABA<sub>A</sub> and GlyR blockade results in a similar increase in peak integrated activity. However, increasing slice excitability by Substance P bath application does not reduce oscillation power. In fact, bath application of Substance P increases oscillation power in the majority of slices tested. These data demonstrate that the reduction in oscillation power after GABA<sub>A</sub> and GlyR blockade is not a result of increased slice activity, providing further evidence that GABAergic and glycinergic transmissions have a unique role in synchronizing inspiratory neurons on a short timescale.

Prolonging GABAergic current decreases oscillation frequency

Our data demonstrate that GABAergic transmission is important in modulating respiratory rhythm and in synchronizing inspiratory motoneurons because blocking GABAergic current increased peak integrated activity and decreased oscillation power, respectively. Therefore we next investigated the effect of prolonging the time course of GABAergic transmission on inspiratory motoneuron synchronization and inspiratory rhythm by bath-applying zolpidem to the slice. Previous studies have shown that zolpidem prolongs the time course of GABAergic IPSCs recorded from cerebellar and hippocampal neurons (Goldstein et al. 2002; Vicini et al. 2001).

We confirmed these results in our system by recording GABA<sub>A</sub>-receptor–mediated mIPSCs from juvenile HMs (P8–P10) in the nonrhythmically active brain stem slice preparation in control and after bath application of 0.1, 0.2, and 0.5 μM zolpidem. The representative average GABAergic mIPSCs shown in Fig. 9A, left are normalized to baseline and show the increase in mIPSC peak amplitude and decay time after bath application of 0.5 μM zolpidem. Bath application of 0.1, 0.2, and 0.5 μM zolpidem increased GABAergic mIPSC decay time by 23 ± 8, 27 ± 8, and 45 ± 10%, respectively (Fig. 9B). In addition to the increase in decay time, 0.5 μM zolpidem increased mean mIPSC peak amplitude by 31 ± 16% (Fig. 8C).

Next, we investigated the effects of prolonging GABAergic currents on inspiratory activity and oscillation frequency recorded from hypoglossal nerve rootlets in the rhythmic slice preparation. By pharmacologically prolonging the time course of inhibition with zolpidem, we expected to decrease oscillation frequency. Synchronous oscillations within an inspiratory burst are characterized by clusters of action potentials that are separated by gaps of little or no activity. We hypothesized that inhibitory synaptic transmission contributes to the gaps of no activity and that by increasing the decay time of GABAergic currents, the duration of these gaps would also increase. This
effect would be reflected in the hypoglossal nerve recording as a decrease in oscillation frequency.

To test this hypothesis, we first bath-applied strychnine then strychnine and zolpidem (0.1–0.5 μM) to rhythmically active slice preparations from P9–P10 mice. Bath application of strychnine alone did not affect median oscillation frequency (−5 ± 2%, n = 7, n.s., paired t-test). An example of the unchanging oscillation frequency, after strychnine bath application, is shown in the average power spectra of Fig. 3B. For seven rhythmic slices where it was tested, subsequent bath application of strychnine and zolpidem reduced average median oscillation frequency from 35 ± 3 Hz, in the presence of strychnine, to 33 ± 3 Hz after combined strychnine and zolpidem bath application (−9 ± 2%, n = 7, P < 0.01, paired t-test). The average power spectra in Fig. 10, A and B show the decrease in median oscillation frequency after strychnine and zolpidem (Stry + Zolp) bath application relative to strychnine application alone.

For this sample of seven rhythmic slices, we observed that bath application of strychnine alone did not decrease the relative power of oscillations (−19 ± 11%, n = 7, P = 0.06). Relative to strychnine, which acts as the control condition in this set of experiments, combined bath application of strychnine and zolpidem reduced oscillation power (Fig. 10D: −25 ± 7%, n = 7, P < 0.05). Relative to the effect of strychnine alone, combined bath application of strychnine and zolpidem decreased peak integrated activity (−24 ± 12%, n = 7, P < 0.05) and burst duration (−12 ± 2%, n = 7, P < 0.01).

DISCUSSION

In the present work, we have investigated inspiratory-phase short timescale synchronization of hypoglossal motoneurons during postnatal development and the role of GABAergic and glycinergic transmissions in generating inspiratory-phase oscillations. Our principal findings are threefold. First, the fre-
frequency of synchronous oscillations increases with postnatal development in P0–P11 mice. Second, both GABAergic and glycinergic transmission are required to generate robust oscillations in P0–P10 mice. Third, prolonging the decay time of GABAergic currents with zolpidem reduces median oscillation frequency.

Changes in GABAergic and glycinergic transmissions during postnatal development

In the rodent brain stem, GABAergic and glycinergic transmissions undergo many changes during the first 2 wk of postnatal development. The changes most relevant to this study are the decrease in GABAergic and glycinergic current decay time (Sebe et al. 2003; Singer et al. 1998) and the shift in the Cl⁻ reversal potential (Ritter and Zhang 2000; Singer et al. 1998). As the postnatal rodent develops, the decay time of GABAergic and glycinergic mIPSCs recorded from HMs decreases (Sebe et al. 2003; Singer et al. 1998). The shortening of inhibitory currents may be partially responsible for the increase in oscillation frequency we observed with postnatal development. Synchronized oscillations during an inspiratory burst are characterized by clusters of action potentials separated by periods of little or no activity. The time course of inhibitory

FIG. 9. Effects of zolpidem on the amplitude and decay time of GABA_A-receptor–mediated miniature inhibitory postsynaptic currents (mIPSCs) recorded from hypoglossal motoneurons. A: superimposed average GABAergic mIPSCs recorded in a cell (P8 mouse) in control and after 0.5 μM zolpidem. In this cell, the decay time increased from 21.3 ms in control to 27.4 ms after zolpidem (Zolp). B: change in GABAergic mIPSC decay time as a percentage of control in response to bath application of 0.1, 0.2, or 0.5 μM Zolp. Asterisk indicates significantly different change from control (P < 0.05). Zolp increased decay time at all concentrations tested. C: change in GABAergic mIPSC amplitude as a percentage of control. GABAergic mIPSC amplitude increased only at the highest concentration of Zolp tested. Data are expressed as means ± SE with the number of cells indicated in parentheses. Amplitude and decay times were calculated from a total of 1,089 (0.1 μM zolpidem), 555 (0.2 μM), and 703 (0.5 μM) miniature events.

FIG. 10. Zolpidem (Zolp) bath application decreased median oscillation frequency. A: average power spectra in Stry and after bath application of Stry + 0.1 μM Zolp in a P10 mouse slice. Median oscillation frequency decreased from 41 Hz in Stry to 35 Hz after bath application of Stry + Zolp. In A and B, median oscillation frequencies in the presence of Stry and Stry + Zolp are represented by the black and gray lines, respectively. Leftward arrows (A and B) indicate direction of frequency shift. B: average power spectra in Stry and after bath application of Stry + 0.2 μM Zolp in a P9 mouse slice. Median oscillation frequency decreased from 40 Hz in Stry to 37 Hz after bath application of Stry + Zolp. C: median oscillation frequency decreased in the presence of Stry + Zolp (0.1–0.5 μM) relative to Stry alone. D: summary data showing the decrease in relative power of oscillations after bath application of Stry + Zolp (0.1–0.5 μM) relative to Stry alone. Mean ± SE. *P < 0.05.
currents may determine how many milliseconds the inspiratory motoneurons are active (Palva et al. 2000), during an inspiratory burst, until the next cluster of action potentials is triggered.

Given the depolarized Cl\(^-\) reversal potential during postnatal development, it is at first puzzling that GABAergic and glycinergic transmission is inhibitory. However, many studies, including the present work, have repeatedly shown that blocking GABA\(_A\) and GlyRs in the neonatal mouse respiratory network elevates inspiratory activity (Bou-Flores and Berger 2001; Paton and Richter 1995; Ramirez et al. 1996). These results demonstrate that Cl\(^-\)-mediated transmission suppresses excitability in the respiratory network even in newborn mice. In the brain stem, Cl\(^-\) reversal potential shifts from approximately \(-40\) to \(-70\) mV at P2 in mouse PBC neurons (Ritter and Zhang 2000) and between P3 and P10 in rat HM (Singer et al. 1998). Despite the depolarized Cl\(^-\) reversal potential in neonatal inspiratory neurons, GABA\(_A\)- and GlyR-mediated currents induce a shunting inhibition that suppresses firing in P0–P4 HM (Marchetti et al. 2002).

It is likely that shunting inhibition, in part, explains the inhibitory nature of Cl\(^-\)-mediated transmission in the newborn respiratory network. It is also possible that the Cl\(^-\) reversal potential of inspiratory neurons yet to be examined is already hyperpolarized at birth.

**Prolonging the decay time of GABAergic currents decreases median oscillation frequency**

By using a network model, Pauluis et al. (1999) demonstrated that prolonging the time course of inhibitory currents decreased oscillation frequency. In the present experiment, we pharmacologically prolonged the decay time of GABAergic currents and found that median oscillation frequency decreased. Together, these findings support the hypothesis that time courses of inhibitory currents constitute a determinant of oscillation frequency.

**Blocking GABA and glycinergic transmissions disrupts inspiratory burst frequency**

When all inspiratory bursts were counted, we found that GABA\(_A\)R blockade alone did not significantly affect inspiratory burst frequency in neonates or juveniles. This result is inconsistent with previous in vitro studies, using rodents and turtles, in which blocking GABA\(_A\)Rs was sufficient to increase inspiratory burst frequency (Bou-Flores and Berger 2001; Hayashi and Lipski 1992; Johnson et al. 2002; Ritter and Zhang 2000). We suspect that the reason for this discrepancy is the nonsaturating concentration of the GABA\(_A\)R antagonist, SR95531 (0.5 \(\mu\)M) we used. When we recorded GABAergic mIPSCs from HM in the nonrhythmically active brain stem slices, 0.5 \(\mu\)M SR95531 blocked most but not all GABAergic mIPSCs (data not shown). However, we selected this nonsaturating concentration of SR95531 based on data from preliminary experiments in which we recorded inspiratory phase activity from the rhythmically active brain stem slice. In these experiments, we found that bath application of strychnine (1 \(\mu\)M) and a saturating concentration of SR95531 (1 \(\mu\)M), together, induced continuous seizurelike activity (data not shown). We were unable to perform power spectral analysis on this continuous seizurelike activity because we could not distinguish individual inspiratory bursts. To circumvent this problem, we used a nonsaturating concentration of SR95531 that increased peak integrated activity, when applied alone, without inducing continuous seizurelike activity, when applied with a saturating concentration of strychnine. This approach was used to fulfill the primary purpose of the present work: to examine the role of GABA and glycine in generating inspiratory motoneuron synchronization.

As seen with the nonsaturating concentration of the GABA\(_A\)R antagonist, GlyR blockade increased peak integrated activity without affecting inspiratory burst frequency. In the developmental time period we examined (P0–P10), glycinergic transmission is still immature in the mouse brain stem (Kandler and Gillespie 2005; Singer et al. 1998). Therefore, measures of respiratory activity, such as inspiratory burst frequency, may be less sensitive to glycinergic modulation in an immature animal than in an adult. Consistent with this conclusion, in vitro and in vivo studies have shown that strychnine does not affect inspiratory burst frequency recorded from mice ranging from P0 to P14 (Lieske et al. 2000; Paton and Richter 1995). However, the effects of GlyR blockade are still variable in this age range because strychnine increased inspiratory burst frequency recorded from hypoglossal and phrenic nerves from P0–P5 mouse slices (Bou-Flores and Berger 2001). In mice P15 or older, a developmental stage at which the respiratory network is considered mature, GlyR blockade decreases inspiratory cycle length (Paton and Richter 1995). Since that study by Paton et al., Pierrefiche et al. (1998) demonstrated that strychnine increases in inspiratory burst frequency in adult animals. Although blocking GABA\(_A\)Rs or GlyRs, separately, did not increase inspiratory burst frequency blocking both forms of inhibition significantly elevated peak integrated activity.

In neonatal slices, when all inspiratory bursts were counted we found that GABA\(_A\) and GlyR blockade induced a secondary rhythm, characterized by low-amplitude and high-frequency inspiratory bursts. Using the rhythmically active medullary slice preparation, Ritter and Zhang (2000) also recorded inspiratory activity from hypoglossal rootlets and reported a secondary rhythm after GABA\(_A\)R blockade with bicuculline. Using the mouse rhythmically active medullary slice preparation, Ramirez et al. (1996) recorded simultaneously from PBC neurons and a hypoglossal rootlet. They showed that PBC neurons fired inspiratory bursts at a higher frequency than hypoglossal rootlets. The secondary rhythm we observed may be generated by the PBC and not normally transmitted to the hypoglossal nucleus. When inhibitory synaptic transmission is blocked, the secondary rhythm generated in the PBC is revealed in hypoglossal motor output.

**MFOs versus HFOs**

As stated earlier, oscillations in the respiratory network are classified into MFOs and HFOs based on the frequency range in which they occur and the presence or absence, respectively, of oscillation coherence across different motoneuron populations. Frequency range of the oscillations changes with many factors, including temperature (Richardson and Mitchell 1982), developmental age (Kocsis et al. 1999), and animal preparation used (Cohen et al. 1987; Liu et al. 1990; O’Neal III et al. 2005). Although synchronous oscillations observed in the present
work fall within the MFO range, oscillation frequency per se is not a reliable criterion in determining the MFO or HFO nature of synchronous oscillations because of the factors previously mentioned.

Without coherence analysis of synchronous oscillations, it is unknown whether the oscillations reported here are MFOs, which are generated at the motoneuron level, or HFOs, which are generated by a common medullary input. In the present work and previously (Bou-Flores and Berger 2001), we have shown that synaptic inhibition is required for the generation of robust inspiratory-phase oscillations. It is possible that the oscillations are MFOs that are generated within the hypoglossal motor nucleus itself because this nucleus contains both motoneurons and a small population of inhibitory interneurons (Peever et al. 2002). During inspiration, hypoglossal motoneurons receive concurrent excitatory and inhibitory inputs (Saywell and Feldman 2004) that could be involved in producing such oscillations. Alternatively, the oscillations may be HFOs or outside of the PBC. Immunohistochemical studies have shown that the medullary reticular formation, in which a rrons receive concurrent excitatory and inhibitory inputs (Saywell and Feldman 2004) that could be involved in producing such oscillations. Alternatively, the oscillations may be HFOs or outside of the PBC. Immunohistochemical studies have shown that the medullary reticular formation, in which a
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INHIBITION GOVERNS MOTONEURON SYNCHRONY


