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Brief Communications

Synaptic Depression Influences Inspiratory–Expiratory Phase Transition in Dbx1 Interneurons of the preBötzing Complex in Neonatal Mice

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The brainstem preBötzing complex (preBötC) generates the rhythm underlying inspiratory breathing movements and its core interneurons are derived from Dbx1-expressing precursors. Recurrent synaptic excitation is required to initiate inspiratory bursts, but whether excitatory synaptic mechanisms also contribute to inspiratory–expiratory phase transition is unknown. Here, we examined the role of short-term synaptic depression using a rhythmically active neonatal mouse brainstem slice preparation. We show that afferent axonal projections to Dbx1 preBötC neurons undergo activity-dependent depression and we identify a refractory period (~2 s) after endogenous inspiratory bursts that precludes light-evoked bursts in channelrhodopsin-expressing Dbx1 preBötC neurons. We demonstrate that the duration of the refractory period—but neither the cycle period nor the magnitude of endogenous inspiratory bursts—is sensitive to changes in extracellular Ca²⁺. Further, we show that postsynaptic factors are unlikely to explain the refractory period or its modulation by Ca²⁺. Our findings are consistent with the hypothesis that short-term synaptic depression in Dbx1 preBötC neurons influences the inspiratory–expiratory phase transition during respiratory rhythmogenesis.

Key words: breathing; central pattern generator; oscillation; respiration

Introduction

Breathing movements emanate from neural rhythms in the preBötzing complex (preBötC) of the ventrolateral medulla (Smith et al., 1991; Feldman et al., 2013; Moore et al., 2013). The breathing cycle consists of an inspiratory phase in which preBötC neurons discharge bursts of spikes synchronously followed by a two-part expiratory phase in which preBötC neurons remain quiescent, at least during the initial postinspiratory stage (but may recover spontaneous activity during the second expiratory stage). Core rhythm-generating preBötC interneurons are derived from progenitors that express the homeobox gene Dbx1 (henceforth referred to as Dbx1 neurons), which are glutamatergic and interconnected bilaterally in the preBötC (Bouvier et al., 2010; Gray et al., 2010). AMPAR-mediated excitatory interactions are required to initiate inspiratory bursts (Funk et al., 1993; Wallen-Mackenzie et al., 2006), but there is no consensus regarding the cellular and synaptic mechanisms that terminate inspiratory bursts and lead to the quiescent postinspiratory phase of respiratory cycle (i.e., inspiratory–expiratory phase transition).

A longstanding view posits that the inspiratory–expiratory phase transition depends on respiratory circuits throughout the pons and medulla operating via postsynaptic inhibition (Richter, 1982; Bianchi et al., 1995). However, the obligatory role of inhibi-
bition is falsified by experiments that block chloride-mediated
synaptic transmission in respiratory networks but do not signif-
icantly perturb respiratory rhythm in vitro (Brockhaus and Bal-
lanyi, 1998; Ren and Greer, 2006; Feldman et al., 2013) or in vivo
(Janczewski et al., 2013; Sherman et al., 2015). One alternative
mechanism that does not depend on distributed inhibitory cir-
cuits is the “group pacemaker,” which instead focuses on collec-
tive activity among preBo¨tC neurons. According to this model,
recurrent excitatory synaptic activity initiates inspiratory bursts
(for which there is strong evidence: Rekling and Feldman, 1998;
Pace et al., 2007; Carroll and Ramirez, 2013; Carroll et al., 2013)
and short-term synaptic depression promotes burst termination
and inspiratory–expiratory phase transition (but this latter part
remains an untested model prediction; Rubin et al., 2009).

Using the group-pacemaker model as our conceptual frame-
work, we investigated the role of short-term synaptic depression
in respiratory rhythm generation. Our results in a reduced slice
context support the group-pacemaker model and are consistent
with the notion that presynaptic depression in Dbx1 preBo¨tC
neurons facilitates inspiratory burst termination and influences
postinspiratory network activity.

Materials and Methods
The Institutional Animal Care and Use Committee at the College of
William & Mary approved these protocols. We used female mice that express Cre recombinase fused to a tamoxifen-sensitive mutant form of
the human estrogen receptor (CreERT2) in cells that express the
Dbx1 gene (Fig. 1). For optical stimulation experiments, female Cre-
driver mice were mated with male reporter mice with a Rosa26 locus
that was modified by targeted insertion of a loxp-flanked STOP
cassette followed by a channelrhodopsin-tdTomato fusion gene
(Rosa26<CreERT2;ChR2(H134R)-tdTomato, stock no. 12567; The Jackson Laboratory).
To record Dbx1 neurons, female Cre-driver mice were mated with male
reporter mice that express Cre-dependent tdTomato (Rosa26<CreERT2;tdTomato,
stock no. 007905; Jackson Laboratory). Tamoxifen (22.5 mg/kg body
mass) was administered to pregnant females at embryonic day 9.5. Cre-
mediated recombination resulted in the expression of the hChR2-
tdTomato or cytosolic tdTomato in neurons with progenitors that
express Dbx1.

We anesthetized neonatal mice via hypothermia and dissected their
nerve roots. The XII discharge was amplified (2000

0.3 Na3-GTP, and 50

martic pressure ejection system (5 pulses at 5 psi for 5 ms each; Toohey). Midline
electrical stimulations and AMPA application experiments were per-
formed in 3 mm extracellular K+ to decrease baseline membrane potential
fluctuations and to minimize contributions from spontaneous EPSPs
(sEPSPs).

We measured the peak amplitude and area of inspiratory bursts in
Dbx1 preBo¨tC neurons by digitally smoothing the membrane potential
trajectory to eliminate spikes but preserve the underlying envelope of
depolarization. Ionotropic receptor antagonists were applied at these
concentrations (in µM): 10 CNQX, 20 -APV, 5 picrotoxin, and 5
strychnine (Sigma–Aldrich).

We report all measurements as mean ± SD. SPSS software (IBM)
compared group means and probability distributions for statistical hy-
thesis testing. Paired-sample t tests evaluated mean differences be-
tween two groups and the Kolmogorov–Smirnov test compared
cumulative probability distributions. Nonparametric Friedman tests
were used in repeated-measures experiments when data
did not meet the assumptions of normality and homogeneity of variance
required for parametric analysis.

Results
Dbx1 Cre-driver mice (Dbx1<CreERT2) were coupled with two dif-
ferent flox-stop reporter strains to selectively record and optically
manipulate Dbx1 neurons in transverse brainstem slices that pro-
vide optimal experimental access to the preBo¨tC. We identified
Dbx1 neurons by native fluorescence in the region of the slice
ventral to the semicompact division of the nucleus ambiguous
and orthogonal to the dorsal boundary of the principal loop of the
inferior olive, which corresponds to the rostral face of the
preBo¨tC (Fig. 1A; Ruangkittisakul et al., 2014). Dbx1 preBo¨tC
neurons, dyaled with Alexa Fluor 488 through the patch pipette

Figure 1. Rhythmically active slices expose Dbx1 preBo¨tC neurons. A, Rostal slice surface from aDbx1<CreERT2;Rosa26<tdTomato mouse pup showing hypoglossal motor nucleus (XII), semi-
compact division of the nucleus ambiguus (scNA), and the principal loop of the inferior olive (IOloop) sites colocalized with the preBo¨tC (left). Dotted box marks the preBo¨tC. B, Whole-cell
recordings in Dbx1<CreERT2;Rosa26<tdTomato (top) and Dbx1<CreERT2;Rosa26<ChR2R2-tdTomato (bottom) mouse slices. Shown are tdTomato (left), Doct contrast microscopy (middle), and Alexa Fluor
488 introduced via patch pipette (right). C, Inspiratory bursts in the Dbx1 preBo¨tC neuron from B (top) with XII motor output.

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neurons, dyaled with Alexa Fluor 488 through the patch pipette
(Fig. 1B), discharged inspiratory bursts in phase with inspiratory-related XII nerve output (Fig. 1C).

Dbx1 preBo¨tC interneurons project commissural axons to form synaptic connections with the contralateral preBo¨tC (Bouver et al., 2010). To determine whether excitatory synapses onto Dbx1 preBo¨tC neurons undergo activity-dependent depression, we repetitively stimulated commissural axons during whole-cell recordings in Dbx1 CreERT2; Rosa26 tdTomato mouse slices. We observed a progressive amplitude reduction of evoked EPSPs (Fig. 2A, n = 8 Dbx1 neurons, 3 trials/neuron). Furthermore, the number of failures increased during each trial such that the last five pulses experienced a 40–50% failure rate (Fig. 2B). These data indicate that excitatory synapses among Dbx1 preBo¨tC neurons undergo activity-dependent depression even when activated at a rate of 5 Hz, which is lower than typical intraburst spike frequency of 15–50 Hz.

We reasoned that, if short-term synaptic depression influences inspiratory–expiratory phase transition, then its effects would be measurable at the network level immediately after an endogenous inspiratory burst. First, we tested whether inspiratory-like bursts could be triggered by light in Dbx1 CreERT2; Rosa26 tdTomato mouse slices. We evoked bursts using 100 ms pulses of blue light delivered to the preBo¨tC contralateral to the whole-cell recording site. Evoked bursts were reversibly blocked by the AMPAR antagonist CNQX, but not by coapplication of the NMDA receptor antagonist AP5 with the GABAA and glycine receptor antagonists picrotoxin and strychnine (Fig. 3A). These data demonstrate that evoked bursts depend on AMPAR-mediated synaptic interactions, akin to endogenous inspiratory bursts (Funk et al., 1993; Wallen-Mackenzie et al., 2006), whereas NMDA receptors and chloride-mediated inhibition are dispensable (Brockhaus and Ballanyi, 1998; Ren and Greer, 2006; Feldman et al., 2013; Janczewski et al., 2013; Sherman et al., 2015).

We applied transient light pulses at varying intervals after endogenous inspiratory bursts, which reliably evoked subsequent bursts for intervals >2 s. However, intervals <2 s failed to evoke a burst (Fig. 3B). In this context, we defined the refractory period as the minimum duration after an endogenous inspiratory burst necessary to evoke a subsequent inspiratory-like burst of amplitude ≥75% of the average endogenous inspiratory burst (Fig. 3C). The refractory period duration was significantly shorter than the endogenous burst period (1.94 ± 0.74 s vs 5.80 ± 1.49 s, n = 10 preparations, Student’s t(8) = 7.62, p = 5.6E-6). These data suggest that an ~2 s refractory period is a previously unrecognized component of the respiratory cycle in Dbx1 preBo¨tC neurons. This refractory period is relevant during the postinspiratory phase (~2 s in duration), but not the remaining 3–4 s of the expiratory phase in vitro.

Presynaptic and postsynaptic factors cause short-term synaptic depression and both could potentially contribute to the refractory period (Fig. 3). First, we examined postsynaptic AMPAR desensitization as a potential contributing factor to the refractory period by comparing the cumulative probability sEPSPs that were measurable before and after endogenous inspiratory bursts in Dbx1 preBo¨tC neurons (Fig. 4A, left). We observed no difference in the cumulative probability sEPSP amplitude histogram (Fig. 4A, right, n = 8 Dbx1 neurons, 10 cycles/neuron, Kolmogorov–Smirnov test statistic D = 0.081, p = 0.89), suggesting that AMPARs do not remain desensitized for seconds after endogenous inspiratory bursts and therefore do not contribute to the refractory period. Nevertheless, it is conceivable that the sEPSPs we

**Figure 2.** Activity-dependent synaptic depression in Dbx1 preBo¨tC neurons. A. Evoked EPSPs in response to 5 Hz electrical stimulation of midline-crossing axons (top); group data from eight Dbx1 preBo¨tC neurons (time synced) are also shown (bottom). Red circles show EPSP amplitudes; black bars show mean ± SD. B. Relative frequency of failures to evoke an EPSP as a function of pulse number.

**Figure 3.** Light-evoked inspiratory-like bursts in Dbx1 CreERT2; Rosa26 tdTomato mouse slices. A. Pharmacology of evoked bursts. Time calibration applies to A. B. Laser pulses delivered at increasing intervals after endogenous inspiratory bursts. Voltage calibration applies to A and B. C. Burst amplitude and area plotted versus the time interval between the endogenous inspiratory burst and stimulus onset. Endogenous control bursts are plotted at the 0 s tick. Time calibration for B and C is the abscissa.
measured might originate from tonic (noninspiratory) neurons upstream of the preBoTc, the excitatory synaptic properties of which may differ from excitatory synapses among Dbx1 neurons in the preBoTc. Therefore, to further assess the contribution of AMPAR desensitization to the refractory period, we pressure-ejected AMPA onto Dbx1 neuron dendrites in five-pulse trains and then measured transient postsynaptic depolarizations. In 0.75 mM Ca$^{2+}$, the refractory period measured $2.88 \pm 1.18 \text{s}$ in 1.50 mM Ca$^{2+}$, it measured $2.22 \pm 0.94 \text{s}$; and in 2.25 mM Ca$^{2+}$, it measured $1.66 \pm 0.81 \text{s}$ (Fig. 4Ca). The duration of the refractory period is inversely proportional to the level of extracellular Ca$^{2+}$ (Fig. 4Cb, $n = 5$, Friedman $\chi^2 = 6.4, p = 0.04$). In contrast, these same changes in extracellular Ca$^{2+}$ caused no significant change in the respiratory cycle period (Fig. 4Cc, $n = 5$, Friedman $\chi^2 = 0.7, p = 0.7$) and did not affect the amplitude ($n = 5$ Dbx1 neurons, Friedman $\chi^2 = 2.8, p = 0.25$) or area ($n = 5$ Dbx1 neurons, Friedman $\chi^2 = 1.6, p = 0.45$) of endogenous inspiratory bursts (Fig. 4Cd). Ca$^{2+}$ manipulations did not affect the input resistance of Dbx1 preBoTc neurons (Fig. 4Ce, $n = 4$, Friedman $\chi^2 = 0.5, p = 0.78$). These data indicate that extracellular Ca$^{2+}$ manipulations influence the duration of the refractory period, but do not affect excitability or basic intrinsic properties of Dbx1 preBoTc neurons, suggesting that the refractory period and its modulation by extracellular Ca$^{2+}$ cannot be attributed to postsynaptic factors.

**Discussion**

Breathing movements emanate from neural activity in a central pattern generator circuit for which core interneurons are known, Dbx1-derived preBoTc neurons are bilaterally interconnected glutamatergic interneurons that express membrane properties consistent with rhythmogenic function. Dbx1 knock-out mice form no preBoTc and suffer fatal asphyxia at birth (Bouvier et al., 2010; Gray et al., 2010). Therefore, elucidating the cellular and synaptic basis for rhythmogenesis in Dbx1 preBoTc neurons is crucial for understanding the neural origins of the full breathing motor pattern.

We hypothesized that synaptic depression influences the phase transition from inspiratory to postinspiratory (i.e., early expiratory) activity in Dbx1 preBoTc interneurons. To quantify short-term depression, we stimulated midline-crossing axons and observed a progressive diminution of evoked EPSP amplitude and increasing probability of synaptic failure (reaching 50% by the end of the burst). Because the decay time constant of evoked EPSPs was $\sim 100 \text{ms}$, stimulation rates could not exceed 5 Hz without causing temporal summation and obscuring the analysis. Nonetheless, these data establish that excitatory synapses among Dbx1 preBoTc neurons experience short-term depression when activated repetitively. We can infer that, in the context of rhythmogenesis, when Dbx1 preBoTc neurons discharge 5–20 intraburst spikes at 15–50 Hz and thus more intensely activate excitatory transmission among the interconnected Dbx1 population, synaptic depression could decrease the magnitude of synaptic potentials by 50–70% and increase the probability of synaptic failure to 10–50%. Therefore, we surmise that synaptic depression could curtail recurrent excitation and thus contribute to inspiratory burst termination. However, this remains to be demonstrated.

Activity-dependent synaptic depression could contribute to terminating, not only bursts, but also “burstlets” in the preBoTc, which are periodic inspiratory events of lower intensity that do not result in motor output, but have been proposed as a basic substrate of rhythmogenesis (Kam et al., 2013). preBoTc neurons generate $\sim 6$ spikes per burstlet at an average rate of 15 Hz (range

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**Figure 4.** sEPSPs, dendritic AMPA pulses, and extracellular Ca$^{2+}$ modulation of the refractory period. A, sEPSPs before (magenta) and after (cyan) an endogenous inspiratory burst and the cumulative probability histogram for sEPSP amplitude. Calibrations apply to A, B. Postsynaptic responses to repetitive dendritic AMPA pulses separated by 4, 3, 2, 1, or 0.5 s (left). The amplitude of the second (black) or fifth (gray) postsynaptic response, normalized to the first response, is plotted for each time interval (right). Bars show means. Voltage calibration applies to B; time calibration can be inferred by AMPA pulse interval timing. C, Minimum refractory period after an endogenous inspiratory burst (e.g., Fig. 3B) plotted for different [Ca$^{2+}$]o. Symbols show individual experiments; solid lines represent sample means. D, Exponential fit of cumulative probability of synaptic failure to $10^{-50}$. Therefore, we surmise that AMPAR desensitization to the refractory period, which are periodic inspiratory events of lower intensity that do not result in motor output, but have been proposed as a basic substrate of rhythmogenesis (Kam et al., 2013). preBoTc neurons generate $\sim 6$ spikes per burstlet at an average rate of 15 Hz (range
5–28 Hz, see Fig. 6 in Kam et al., 2013) and our results (Fig. 2) show that, at a minimum, synaptic potentials could decline by 40% and failure probability could rise to 10% within the constraints of these intraburstlet spiking parameters.

The mechanisms responsible for inspiratory burst termination and postinspiratory activity are vigorously debated. The group-pacemaker hypothesis posits that convoluted synaptic and intrinsic properties of preBo¨tC neurons mediate inspiratory–expiratory phase transition (Rekling et al., 1996; Rekling and Feldman, 1998). In a mathematical model of the group pacemaker, presynaptic depression can intervene during the inspiratory burst to curb recurrent excitation, which facilitates inspiratory burst termination and promotes the inspiratory–expiratory phase transition (Rubin et al., 2009). Simultaneously, activity-dependent outward currents, namely Na/K ATPase electrogenic pump current (I_{pump}), Na^+-dependent K^+ current (I_{Na,K}), and ATP-dependent K^+ current (I_{K,ATP}) play a complementary role in burst termination and phase transition. These outward currents in preBo¨tC neurons have been identified experimentally (Del Negro et al., 2009; Krey et al., 2010), but the potential role for short-term synaptic depression remained an unsettled prediction of the group-pacemaker model. I_{pump}, I_{Na,K}, and I_{K,ATP} relax within 15–300 ms after burst termination, which is too short a time to explain the refractory period in vitro that measured 2 s in response to hChR2 activation but measured ~1 s when a bolus of AMPA was administered unilaterally to the preBo¨tC after inspiratory burst termination (Del Negro et al., 2009). This shorter refractory period is most likely attributable to the stronger stimulus. AMPA application depolarized preBo¨tC neurons by 40 mV and then slowly decayed for 1 min, whereas hChR2 depolarized Dbx1 preBo¨tC neurons <10 mV for exactly 100 ms. In both cases, the refractory period outlasts postsynaptic contributions of I_{pump}, I_{Na,K}, and I_{K,ATP}. Therefore, we propose that activity-dependent outward currents and synaptic depression act in concert to influence inspiratory–expiratory phase transition, terminating the inspiratory burst and then causing a transient phase of postinspiratory quiescence in the network.

Could this mechanism identified in models and in vitro apply in vivo? We observed an ~2 s refractory period after inspiratory bursts, which comprises 25–33% of the respiratory cycle period in vitro. By optogenetically stimulating the preBo¨tC in adult rats in vivo, Alsaafari et al. (2015) recently documented a refractory period of 200–400 ms after inspiration, which comprises ~25–60% of the breathing cycle period. The refractory period measured in vitro constitutes the same proportion of the cycle period as the refractory period measured in vivo, suggesting that the refractory period is a real phenomenon and not an artifact of the in vitro preparation. Therefore, the biophysical mechanisms that we identify in vitro could govern the inspiratory–expiratory phase transition in vivo as well.

The most common form of short-term synaptic depression involves presynaptic vesicle depletion during high-frequency activity. Manipulations of Ca^{2+}, which affect release and recovery processes (Neher and Sakaba, 2008), modulated the refractory period during the postsynaptic phase but did not affect respiratory cycle period, inspiratory burst magnitude, or input resistance of Dbx1 preBo¨tC neurons (Fig. 4). The readily releasable vesicle pool recovery time varies from hundreds of milliseconds to seconds depending on the experimental model (Stevens and Tsujimoto, 1995; Hosoi et al., 2007; Cohen and Segal, 2011). The refractory period in vitro and the refractory period Alsaafari et al. (2015) measured in vivo are within this range, suggesting that vesicle depletion is a viable explanation for the lack of synaptic efficacy after inspiratory bursts in vitro or inspiration in vivo.

AMPA desensitization is unlikely to contribute on the time scale associated with the refractory period based on measurements of sEPSPs before and immediately after inspiratory bursts, as well as the lack of modulation of postsynaptic responses to repetitive AMPA pulses. However, it remains possible that we measured sEPSPs from a source extrinsic to the preBo¨tC; for example, from an upstream tonic source of excitation to the preBo¨tC with postsynaptic AMPARs that do not exhibit the same biophysical properties as AMPARs involved in recurrent excitation. Further, receptors may desensitize during bursts (and thus assist in burst termination) but then recover faster than could be measured using pressure-ejection techniques in which the decay kinetics of postsynaptic response precluded pulse rates in excess of 2 Hz. It is also possible that some portion of AMPARs targeted by local AMPA application are not inspiratory modulated, so we cannot rule out postsynaptic effects entirely.

Conceptual models of respiratory rhythm often feature obligatory roles for postsynaptic inhibition in inspiratory–expiratory phase transition. The present results indicate that excitatory synaptic dynamics of core rhythmogenic Dbx1 interneurons could influence inspiratory burst termination and the quiescent postinspiratory phase of the respiratory cycle, which should be added to existing frameworks for analysis and models of breathing’s neural bases.

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