Published in final edited form as:

Respir Physiol Neurobiol. 2008 December 31; 164(3): 338–349. doi:10.1016/j.resp.2008.08.004.

Respiratory pattern in midline-lesioned brainstems and hemibrainstems from adult turtles

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Abstract

Discrete midline lesions uncouple left and right respiratory motor output in mammals, but not in frogs and lampreys. To address this question in reptiles, isolated adult turtle brainstems were cut along the midline while recording respiratory motor output (bursts of action potentials) on left and right hypoglossal (XII) nerves. XII motor bursts were synchronized as long as a small portion of the midline was still intact. When turtle brainstems were completely cut along the midline and separated into hemibrainstems, XII motor bursts were produced that could be abolished by mu-opioid receptor (MOR) activation or exposure to high pH (7.80) solution. Also, 13/57 hemibrainstems expressed episodic discharge (>1.75 bursts/episode). To test whether crossed connections were necessary to express a long-lasting increase in burst frequency (*i.e.*, frequency plasticity), phenylbiguanide (PBG, 5-HT₃ receptor agonist, 20 μ M) was bath-applied to hemibrainstems. Although PBG significantly increased burst frequency by 0.43 ± 0.10 bursts/min after 60 min, no frequency plasticity was observed because burst frequency returned to near baseline levels after a 2-h washout. Thus, crossed connections in turtle brainstems synchronize respiratory motor output and are not required for normal respiratory pattern formation, but are required for PBG-dependent frequency plasticity.

1. Introduction

During vertebrate embryonic development, several oscillatory motor neural networks are distributed rostrocaudally in the brainstem. These highly coupled oscillatory neural networks spontaneously generate synchronized motor output (Jacquin et al., 1996; Chatonnet et al., 2002; Borday et al., 2003). During maturation, the oscillatory networks uncouple from each other to varying degrees and eventually become coordinated rhythmic motor networks that contribute to breathing, chewing, or swallowing (Champagnat and Fortin, 1997). In mammals, the respiratory control system retains these basic characteristics and is hypothesized to be composed of several oscillatory neural networks located bilaterally in the brainstem. For example, oscillatory neural networks in the pre-Bötzinger Complex (preBötC) and para-facial respiratory group (pFRG) are located on the left and right side of the brainstem, and are coupled to each other via ipsilateral and crossed propriobulbar connections (Onimaru et al., 2006). These crossed connections are necessary to generate coordinated and synchronized neural activity that controls respiratory muscles involved in ventilation and maintaining airway patency.

Cutting the midline of the brainstem at different rostrocaudal levels provides insights as to where crossed connections are necessary for normal breathing. For example, small (5–10 mm)

midsagittal cuts at the level of the obex in cats and rabbits uncouple left and right XII and phrenic motor output (St. John, 1983; Janczewski and Karczewski, 1984; Kubin et al., 1987; Eldridge and Paydarfar, 1989). In contrast, left and right respiratory motor output on cranial nerve roots of frog and lamprey brainstems are synchronized as long as one-third of crossed connections are intact anywhere along the midline (Mc Lean et al., 1995a; Rovainen, 1985). This suggests that there may be fundamental differences in the organization of the respiratory control systems in different classes of vertebrates. As for reptiles, the extent to which crossed connections are necessary for synchronized respiratory motor output is not well understood.

When isolated vertebrate brainstems are completely hemisected along the midline to produce two “hemibrainstems”, the hemibrainstems can spontaneously produce respiratory-related motor output as defined by the presence of expiratory and inspiratory activity, and appropriate responsiveness to pH/CO₂ changes (Mc Lean et al., 1995a). However, it is not known whether hemibrainstems are capable of expressing more complex forms of behavior, such as neuroplasticity. Neuroplasticity is defined as a long-lasting change in neural output based on past experience (Mitchell and Johnson, 2003). One example is respiratory frequency plasticity, which is a long-lasting increase in breathing frequency following an experimental perturbation, such as repetitive episodes of exercise with increased dead space in goats (Martin and Mitchell, 1993) or intermittent hypoxia in unanesthetized rats and cats (Morris et al., 2000; Olson et al., 2001). Frequency plasticity is also expressed under *in vitro* conditions following intermittent hypoxic exposures in rhythmically active neonatal rat medullary slices (Blitz and Ramirez, 2002) or following bath-application of phenylbiguanide (PBG, serotonin 5HT₃ receptor agonist) to isolated adult turtle brainstems (Johnson et al., 2001). To our knowledge, it is not known whether vertebrate hemibrainstems express frequency plasticity.

To address these questions, adult turtle brainstems were isolated *in vitro* and used to determine: (1) the extent to which midline sagittal lesions of the brainstem uncouple respiratory motor output; (2) whether turtle hemibrainstems produce respiratory-related motor output; (3) whether turtle hemibrainstems express PBG-dependent frequency plasticity. *In vitro* turtle brainstems are advantageous because they produce inspiratory- and expiratory-related motor output that is qualitatively similar to that produced by intact turtles (Douse and Mitchell, 1990; Johnson et al., 1998). Also, since turtle brainstems are isolated from adult ectothermic reptiles that have a low metabolic rate and are extremely resistant to hypoxia (Jackson, 2000; Johnson et al., 1998), respiratory-related motor output can be produced by turtle brainstems for several days at physiologically relevant temperatures (Wilkerson et al., 2003). A preliminary report of the work was published in abstract form (Majewski, et al., 2006).

2. Methods

All procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison School of Veterinary Medicine. Adult red-eared slider turtles (*Trachemys*, n = 53, 851 ± 30 g) were obtained from commercial suppliers and kept in a large open tank where they had access to water for swimming and heat lamps and dry areas for basking. Room temperature was set to 27–28°C with light provided 14 h/day. Turtles were fed ReptoMin® floating food sticks (Tetra, Blackburg, VA) 3–4 times per week.

2.1. Turtle brainstem preparations

Turtles were intubated and anesthetized with 5% isoflurane (balance oxygen) until the head and limb withdrawal reflexes were eliminated, upon which the turtles were decapitated. The brainstem was removed and pinned onto Sylgard® in a recording chamber. The tissue was superfused with artificial cerebrospinal fluid (aCSF) containing HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethane-sulfonic acid]) buffer as follows (mM): 100 NaCl, 23 NaCHO₃, 10 glucose, 5 HEPES (sodium salt), 5 HEPES (free acid), 2.5 CaCl₂, 2.5 MgCl₂, 1.0 K₂PO₄, 1.0

KCl. The HEPES solution was bubbled with 5% CO₂-95 % O₂ to maintain pH 7.28 ± 0.01 , as measured periodically with a calomel glass pH electrode (Cole-Parmer Inst. Co., Vernon Hills, IL, USA). All experiments were performed at room temperature (22-23°C).

2.2. Electrophysiological recordings

Glass suction electrodes were attached to hypoglossal nerve rootlets. Signals were amplified (10,000x) and band-pass filtered (10-10,000 Hz) using a differential AC amplifier (model 1700, A-M Systems, Everett, WA, USA) before being rectified and integrated (time constant = 200 ms) using a moving averager (MA-281/RSP, CWE, Inc., Ardmore, PA, USA). The signals were digitized (50 Hz sampling rate) and analyzed using Axoscope software (Axon Instruments, Foster City, CA, USA) and MiniAnalysis software (Synaptosoft, Inc., Decatur, GA, USA).

2.3. Experimental protocols

Brainstem preparations were allowed to equilibrate for 3-4 h before initiating a protocol. For sequential cut experiments, baseline data were recorded for 30 min before making a cut along the midline. Brainstems were allowed to recover for 30 min before recording data for 30 min. The next cut was made and the cycle repeated until the brainstems were completely separated. Rostral-to-caudal sequential midline cuts were made from rostral edge of the brainstem to the levels of abducens (VI), vagus (X), and XII cranial nerves. Caudal-to-rostral sequential midline cuts were made from caudal edge of the tissue (at spinal segment C1) to the levels of XII, glossopharyngeal (IX), and VI cranial nerves. In some experiments, a caudal-to-rostral midline lesion was made directly to the level of the VI cranial nerve, or midline cuts were made such that only the region between the VI and rostral XII roots was left intact. In separate experiments, brainstems were completely separated along the midline to generate hemibrainstems, which were allowed to equilibrate for 150 min, the last 30 min of which was recorded as baseline data. Afterward, either high pH solution (pH=7.8, ~1.3% CO₂), DAMGO solution (1.0, 0.1 or 0.01 μ M; [D-Ala²,N-Me-Phe⁴, Gly⁵-ol] enkephalin; MOR agonist) or PBG solution (20 μ M; serotonin 5-HT₃ agonist) were applied for 60 min followed by washout with aCSF. All drugs and chemicals were obtained from Sigma/RBI Aldrich (St Louis, MO, USA).

2.4. Data analysis and statistics

Respiratory burst frequency was defined as the number of bursts/min while delta frequency was the difference between measured burst frequency and baseline burst frequency. Burst interval was the time between bursts while the burst interval coefficient of variation was the burst interval standard deviation divided by the burst interval mean. Two or more bursts separated by less than the average duration of a burst within that recording was defined as an episode. Respiratory motor output with bursts/episode greater than 1.75 was considered episodic, whereas output with bursts/episode less than 1.40 was considered non-episodic (all other preparations were not considered episodic or non-episodic). Amplitude was defined as the maximum height of the burst relative to baseline burst amplitude. Burst rise time was the time from the start of the burst to the burst maximum height, and duration was the time from burst start to burst termination. All measurements were averaged into 30-min bins and reported as the mean $\pm$ SEM. One-way and two-way ANOVA with repeated measures design (Sigma Stat, Jandel Scientific Software, San Rafael, CA, USA) were used to determine if data were significantly different ($p < 0.05$) from baseline and time controls. If normality or equal variance assumptions were violated, data were ranked, and the ANOVAs recalculated. Multiple comparisons were made using Student-Newman-Keul's test.

3. Results

3.1. Synchronization and pattern changes following sequential midline sagittal lesions

To determine the levels of crossed connections that are necessary for XII burst synchronization, caudal-to-rostral cuts along the brainstem midline were made in a sequential manner. After cutting from the caudal edge of spinal segment C1 to the XII root level, bilateral XII motor output was synchronized in 8/8 preparations (Fig. 1A). Burst frequency in lesioned brainstems (0.63 ± 0.05 bursts/min) was unchanged from the frequency in intact brainstems (0.60 ± 0.08 bursts/min, $p=0.66$, Fig. 2A). Burst duration was 18.0 ± 1.0 s in intact brainstems and was unchanged at 15.5 ± 1.4 s in lesioned brainstems ($p=0.36$, Fig. 2B). Burst rise time decreased from 7.0 ± 0.6 s to 5.7 ± 0.6 s ($p=0.04$, Fig. 2C). After cutting to the IX root level, bilateral XII motor output was synchronized in 8/8 brainstems, but 3/8 exhibited a pattern in which XII burst amplitude on one side was larger compared to the synchronized XII burst on the other side (Fig. 1B). The XII root with the larger burst amplitude was not always restricted to one side of the brainstem (see arrows in Fig. 1B). Burst frequency, duration, and rise time in brainstems lesioned to the IX root level were unaltered compared intact brainstems ($p>0.05$ for all three variables; Fig. 2). After cutting to the VI root level, 3/8 brainstems stopped bursting on one or both sides while the remaining 5/8 were completely uncoupled (Fig. 1C). Compared to values measured in intact brainstems, burst frequency decreased to 0.16 ± 0.04 bursts/min ($p=0.001$, Fig. 2A), burst duration remained unchanged at 18.1 ± 2.4 s ($p=0.36$; Fig. 2B) and burst rise time decreased to 5.6 ± 0.6 s ($p=0.01$, Fig. 2C). In separate experiments ($n=6$), turtle brainstems were lesioned with a single cut along the midline from the spinal cord segment C1 to the level of the VI cranial nerve. In 5/6 lesioned brainstems, left and right XII respiratory bursts were synchronized even though burst amplitude was decreased (Fig. 3A). In two lesioned brainstems, coupling between left and right XII bursts was enhanced with bath-applied PBG ($20 \mu\text{M}$; Fig. 3B). Thus, brainstems lesioned to the VI nerve can produce synchronized XII respiratory bursts, and the degree of coupling may depend on overall network excitability.

For rostral-to-caudal sequential cuts, 9/9 brainstems had synchronized XII motor bursts after the cut from the rostral edge of the brainstem to the VI root level (Fig. 4A). Burst frequency (0.52 ± 0.05 s), burst duration (17.8 ± 0.6 s) and burst rise time (7.9 ± 0.7 s) in lesioned brainstems were all unchanged compared to intact brainstems (0.58 ± 0.08 bursts/min, 17.5 ± 0.8 s and 8.2 ± 1.0 s; $p>0.05$ for all three variables; Fig. 5). After midline cuts to the X root level, XII respiratory motor output was coupled in 9/9 brainstems (Fig. 4B). Burst frequency in lesioned brainstems (0.33 ± 0.05 bursts/min) was lower compared to the intact brainstem frequency ($p<0.001$, Fig. 5A), but burst duration (17.9 ± 2.9 s, Fig. 5B) and burst rise time (9.5 ± 2.1 s, Fig. 5C) in lesioned brainstems were unaltered ($p>0.05$ for both variables). After cutting to the XII root level, no respiratory motor bursts were observed on both XII roots in 4/9 brainstems, but synchronized XII motor bursts continued on 5/9 brainstems (Fig. 4C). Burst frequency in lesioned brainstems (0.24 ± 0.06 bursts/min) was lower compared to the frequency in intact brainstems ($p<0.001$, Fig. 5A). Burst duration (18.4 ± 0.8 s, $p=0.82$; Fig. 5B) and burst rise time (8.7 ± 0.6 s, $p=0.83$; Fig. 5C) remained unchanged compared to intact brainstems ($p>0.05$ for both variables).

To verify that crossed connections exist between the VI and the rostral XII cranial nerve roots, midline cuts were made from the rostral edge of the brainstem to the VI root level and from the caudal edge of the brainstem to the rostral XII root level. Following these cuts, right and left XII motor output was synchronized in 6/6 brainstems (Figs. 6A, 6B). Burst frequency, duration, and rise time in lesioned brainstems were all unchanged compared to intact brainstems ($p>0.05$; Figs. 6C-E).

3.2. Hemibrainstems produce respiratory motor output similar to intact brainstems

To test whether hemibrainstems produced motor output similar to intact brainstems, turtle brainstems ($n=16$) were completely cut sagittally along the midline and separated into two hemibrainstems (Fig. 7A). Burst frequency in intact brainstems started at 0.52 ± 0.05 bursts/min, decreased to 0.12 ± 0.07 bursts/min following hemisection ($p<0.001$), and steadily increased over time to 0.44 ± 0.07 bursts/min at 120 min post-hemisection, and remained near the level for intact brainstems over the next 150 min (Fig. 7B). For example, burst frequency at 270 min post-hemisection was 0.47 ± 0.05 ($p=0.46$ compared to intact brainstem frequency). Because hemibrainstem burst frequency reached levels similar to intact brainstems at 150 min post-hemisection and remained constant, the 150-min time point was chosen as the time to start subsequent experiments on hemibrainstems. Burst duration (12.9 ± 1.6 s) and rise time (6.5 ± 1.0 s) in intact brainstems were unaltered in hemibrainstems (15.6 ± 0.9 s and 6.4 ± 0.6 s, respectively; $p>0.05$) at 30-min post-hemisection, and remained constant up to 270-min post-hemisection (Fig. 7C, 7D).

To test whether XII motor output in hemibrainstems responded to perturbations known to alter respiratory motor output in intact turtle brainstems, hemibrainstems were exposed to either DAMGO (MOR agonist; Fig. 8A) or high pH conditions (Fig. 8B). After establishing a baseline burst frequency of 0.45 ± 0.09 bursts/min, bath application of $1.0 \mu\text{M}$ DAMGO abolished motor output within 30 min in 5/5 hemibrainstems ($p<0.001$; Fig. 8A). Motor output resumed 2 h after drug washout, but at a very low frequency of 0.07 ± 0.04 bursts/min ($p<0.001$). Likewise, bath-application of $0.1 \mu\text{M}$ DAMGO abolished motor output in 2/2 hemibrainstems within 60 min ($p<0.001$) and burst frequency recovered to 0.39 ± 0.16 bursts/min 2 hr after drug washout, which was similar to the pre-drug baseline frequency of 0.35 ± 0.09 bursts/min (Fig. 8A). Exposure to $0.01 \mu\text{M}$ DAMGO decreased burst frequency from 0.38 ± 0.07 bursts/min (baseline) to 0.12 ± 0.06 bursts/min ($p=0.001$; motor output was abolished in 3/11 hemibrainstems; Fig. 8A). After 90 min of washout, burst frequency increased to well above baseline and reached a maximum of 0.65 ± 0.16 bursts/min ($p<0.001$). In separate experiments ($n=5$), hemibrainstems were switched from a low pH solution ($\text{pH}=7.31 \pm 0.04$; equilibrated with 5% CO_2) to a high pH solution ($\text{pH}=7.80 \pm 0.01$; equilibrated with 1.3% CO_2) (Fig. 8B). Burst frequency in the low pH solution was 0.59 ± 0.12 bursts/min, but motor output was reversibly abolished in 4/5 brainstems within 60 min following the switch to the high pH solution ($p<0.001$, Fig. 8B). Thus, turtle hemibrainstems responded to MOR activation and high pH conditions similarly to intact turtle brainstems (Johnson et al., 2002).

3.3. Frequency plasticity is not expressed in hemibrainstems

PBG-dependent frequency plasticity in isolated turtle brainstems is characterized by a long-lasting (>2 h) increase in XII burst frequency (Johnson et al., 2001), and long-lasting decreases in burst interval, burst interval coefficient of variation (*i.e.*, increased burst regularity), and episodic discharge (S.M. Johnson, unpublished observations). To test whether hemibrainstems express PBG-dependent frequency plasticity, PBG ($20 \mu\text{M}$) was applied to hemibrainstems ($n=14$) for 60 min followed by a 2-h washout period (Fig. 9A). Burst frequency started at 0.36 ± 0.05 bursts/min (baseline), increased to 0.79 ± 0.09 bursts/min at 60 min following drug application ($p<0.001$), but decreased back to 0.44 ± 0.07 bursts/min after 2 h of washout with control solution ($p=0.74$, Fig. 9B). When graphed as delta frequency (change in frequency/min compared to baseline), burst frequency increased by 0.43 ± 0.1 bursts/min ($p<0.001$) in response to PBG after 60 min and decreased back to near baseline levels during washout. There was a significant drug effect ($p<0.001$), but no PBG data points differed from controls at 60-120 min post-drug exposure (Fig. 9C). Likewise, PBG decreased burst interval from 4.1 ± 0.7 min to 1.5 ± 0.2 min ($p<0.001$) after 60 min, but the burst interval returned to 3.5 ± 1.0 after 2 h washout ($p=0.25$, Fig. 9D). PBG decreased burst interval coefficient of variation from 1.01 ± 0.16 (baseline) to 0.51 ± 0.11 after 60 min of PBG ($p<0.001$), but increased to a range of 0.71

to 0.81 during the 2-h washout period ($p < 0.001$ for drug effect; Fig. 9E). PBG also did not alter other XII burst properties in hemibrainstems. For example, burst duration was 11.0 ± 1.5 s, 9.8 ± 1.0 s, and 9.7 ± 0.9 s at baseline, 60 min after PBG, and after 2 h washout, respectively ($p > 0.05$, data not shown). In control hemibrainstems ($n=15$) exposed only to aCSF, there were no time-dependent changes in frequency, burst interval, or burst interval coefficient of variation (Figs. 9B-E).

3.4. Episodic discharge expressed in hemibrainstems

In 13/57 hemibrainstems with episodic discharge (bursts/episode > 1.75), the average bursts/episode was 2.28 ± 0.10 (range = 1.89 – 3.00; Figs. 10A-B). There was no correlation between episodic discharge in intact brainstems prior to hemisection and hemibrainstems after hemisection. Some intact brainstems ($n=10$) discharged episodically while the resulting hemibrainstems discharged non-episodically. Other intact brainstems ($n=9$) discharged non-episodically and the resulting hemibrainstems discharged episodically, while some intact brainstems ($n=6$) discharged episodically before and after hemisection. One feature of PBG-dependent frequency is a long-lasting decrease in episodic discharge. In turtle hemibrainstems producing episodic discharge that were exposed only to aCSF ($n=2$), bursts/episode were constant for a 3-h period (Fig. 10D). In turtle hemibrainstems producing episodic discharge ($n=7$), PBG (20 μ M) decreased bursts/episode from 2.59 ± 0.17 to 1.76 ± 0.39 after 60 min, but bursts/episode returned to 2.41 ± 0.64 after the 2-h washout ($p=0.52$, Figs. 10C-D).

4. Discussion

The main finding was that the turtle respiratory control system more closely resembles the amphibian, rather than the mammalian, respiratory control system because only small section of connectivity across the midline is necessary for synchronized bursting of left and right respiratory motor output. Thus, synaptic pathways throughout the rostrocaudal extent of the brainstem connect the two rhythm generating networks on either side of the brainstem. After cutting the turtle brainstem into two hemibrainstems, the pattern of respiratory motor output produced by turtle hemibrainstems was similar to that produced by intact turtle brainstems, including episodic discharge. Turtle hemibrainstem respiratory motor output was abolished by well-known respiratory depressants, such as exposure to high pH/low CO_2 solution or MOR receptor activation. Finally, hemibrainstems did not express PBG-dependent frequency plasticity since there were no long-lasting changes in burst frequency, regularity, and episodic discharge. These findings suggest that an intact pontomedullary respiratory network contained within a turtle hemibrainstem is capable of producing eupnea, but not one form of neuroplasticity.

4.1. Crossed connections between respiratory rhythm generators in the brainstem

Breathing requires precise spatiotemporal control of motoneuron firing to produce expiration and inspiration, and to maintain airway patency. Symmetrical pools of cranial and spinal motoneurons, which are located on each side of the CNS, need to be activated simultaneously for ventilation to be effective and efficient. Synchronization of motoneuron firing can be achieved by synaptic coupling at the level of the rhythm generator or at the level of premotoneurons. Coupling at the level of the respiratory rhythm generator requires that excitatory synaptic connections cross the midline. In vertebrates, the extent to which crossed connections exist along the rostrocaudal length of the brainstem is organized differently. Crossed connections required for synchronization in mammals appear to be localized within a discrete section of the medulla near the obex, whereas crossed connections in amphibians and jawless fish appear to be localized throughout nearly the entire length of the brainstem.

For example, midline sagittal cuts at the level of the obex desynchronized left and right phrenic motor output in rats (Peever et al., 1998), cats (St. John, 1983; Eldridge and Paydarfar, 1989), rabbits (Janczewski and Karczewski, 1984), and monkeys (Gromysz and Karczewski, 1982). These midline sagittal cuts typically started at 1-3 mm caudal to obex and extended to 5-7 mm rostral to obex. In most of these studies, frequency and amplitude changes in respiratory motor output were reported or shown following the midline cuts, but lesion-induced changes in respiratory burst frequency, pattern, and shape were not systematically analyzed. However, in rats, midline cuts that desynchronized phrenic motor output resulted in a 65-81% amplitude decrease with no change in burst frequency or duration (Peever et al., 1998).

In contrast, midline lesions extending for up to two-thirds of the length of adult frog brainstems did not desynchronize respiratory motor output produced by left and right trigeminal nerves (McLean et al., 1995a). Following complete midline hemisection, there was no change in respiratory burst frequency and duration, although evidence for a two-peak respiratory burst on trigeminal nerves was less apparent. In isolated lamprey brainstems, synchronized left and right cranial nerve respiratory motor output was observed when 5-45% of the midline was intact (Rovainen, 1985). Interestingly, in two lamprey preparations, desynchronized cranial nerve respiratory output was obtained when the midline was cut throughout the brainstem except for the region between the caudal trigeminal and rostral XII nuclei (Rovainen, 1985). In lamprey brainstems with extensive rostral midline lesions, respiratory motor bursts were synchronized, but one side was delayed relative to the other, double bursts were frequently observed, and burst frequency and duration were altered (Rovainen, 1985). When lamprey brainstems were completely lesioned along the midline, cranial respiratory motor output was desynchronized with little change in burst frequency or pattern (Kawaski, 1979, 1984). The lamprey results suggest that crossed connections contribute to determining respiratory burst frequency and pattern, but it's important to note that these were *in situ* preparations with the spinal cord and some cranial nerves left intact. Thus, muscle sensory afferent inputs and spinobulbar inputs may have influenced the responses of the respiratory control system to various midline lesions.

In the present study on isolated turtle brainstems, synchronized left and right XII respiratory motor output was observed as long as ~25% or less of the midline was intact. There was no evidence that a midline lesion at a particular location could completely desynchronize respiratory motor output on left and right XII nerves. In this respect, the response of isolated turtle brainstems to midline lesions is similar to frogs and lampreys. Likewise, similar to the isolated frog brainstems, there was little change in respiratory burst frequency, duration, and rise time following a series of rostral-to-caudal or caudal-to-rostral midline cuts. When turtle brainstems were completely hemisected, only minor changes were observed in respiratory burst frequency duration, and rise time, and hemibrainstem respiratory motor output was abolished following MOR activation or exposure to high pH solutions.

These findings are consistent with the hypothesis that each side of the turtle brainstem contains a respiratory rhythm generator that is sufficient to produce respiratory motor output similar to intact brainstems. Thus, crossed connections in the turtle brainstem are likely only necessary for synchronization and not for regulating burst frequency or pattern. In contrast, crossed connections play a more critical role in rhythm generation in some motor neural networks. For example, for the swimming rhythm generator in the lamprey spinal cord, crossed reciprocal inhibitory connections are necessary to regulate left/right alternation and regulation of burst frequency (Cangiano and Grillner, 2003).

The putative respiratory rhythm generator appears to be located in the brainstem between the facial (VII) and glossopharyngeal (IX) cranial nerves in frogs (McLean et al., 1995b) and lampreys (Kawaski, 1979, 1984). Since extensive midline lesions in this area do not

desynchronize respiratory motor output, there must be extensive polysynaptic pathways that travel rostrocaudally in the ipsilateral hemibrainstem before crossing over at locations along the midline that are far removed from the region between the VII and IX nerves. In mammals, there is evidence for diffuse pathways from the rostral pons to respiratory rhythm generating centers in the medulla. For example, electrical stimulation in the rostral pons of cats produces premature termination of respiratory activity on both phrenic nerves, even if the pontomedullary junction is hemisected either ipsilateral or contralateral to the pontile electrical stimulation (St. John, 1986). It's possible that these connections exist in mammals and provide a sufficient polysynaptic pathway for synchronizing left and right respiratory motor output, but these pathways may be silent *in situ*. In contrast, if the brainstems were isolated under *in vitro* conditions (similar to isolated frog and turtle brainstems), then these previously silent pathways may become active and allow for synchronization following extensive midline lesions.

Episodic breathing is observed in many reptiles and amphibians, as well as a few mammals under certain conditions (Milsom, 1991). In frogs, episodic breathing appears to be regulated or modulated by chemosensory and mechanosensory inputs (Kinkead and Milsom, 1997), structures rostral to the brainstem (Kinkead et al., 1997; Milsom et al., 1997; Reid et al., 2000; Gargaglioni et al., 2007), and structures within the brainstem during GABA_B receptor blockade (Straus et al., 2000). In contrast, very little is known about mechanisms underlying episodic discharge in turtles other than the fact that some isolated turtle brainstems produce episodic cranial nerve motor bursts (Douse and Mitchell, 1990; Johnson et al., 1998). In this study, we found that crossed connections were not necessary for episodic respiratory motor output since some turtle hemibrainstems produced XII motor bursts in episodes of doublets and triplets. To our knowledge, this is the first report of vertebrate hemibrainstems producing episodic discharge. At the least, this suggests that some of the mechanisms controlling episodic breathing in turtles are located within the hemibrainstem pontomedullary network.

4.2. Turtle hemibrainstems do not express neuroplasticity

The ability of the central nervous system to adapt to changing physiological conditions is vital to the survival of an organism. Neuroplasticity can occur when neural circuits undergo alterations to maintain homeostasis in response to injury, disease, or varying environmental conditions. The respiratory control system expresses many forms of neuroplasticity during both development and adulthood (Mitchell and Johnson, 2003; Morris et al, 2000). Since breathing is vital to life, the respiratory rhythm generator itself, or the neurons modulating the rhythm generator, have the capacity to adapt to changing circumstances. For example, long-lasting changes in respiratory frequency following a perturbation (defined as “frequency plasticity”) occurs during the expression of long-term facilitation *in vivo* following exposures to intermittent hypoxia in goats and rats (Turner and Mitchell, 1997; Olson et al, 2001) and repeated bouts of exercise with increased dead space in goats (Martin and Mitchell, 1993). Likewise, exposing neonatal murine rhythmically active medullary slices to repeated bouts of anoxic solution produced frequency plasticity (Blitz and Ramirez, 2002).

In isolated turtle brainstems, a long-lasting augmentation of respiratory burst frequency occurs following a 60-min exposure to serotonin or PBG (Johnson et al., 2001). PBG-dependent frequency plasticity is also characterized by long-lasting decreases in burst interval, burst interval coefficient of variation, and episodic discharge (unpublished observations). Turtle hemibrainstems exposed to PBG transiently increased burst frequency and decreased burst interval and episodic discharge, but most of these changes were nearly abolished during the washout period. This suggests that crossed connections are required for the full manifestation of PBG-dependent frequency plasticity. It also suggests that frequency plasticity in turtle

brainstems is not solely due to changes within rhythm-generating neurons, but somehow requires the respiratory network contained within intact brainstems.

Acknowledgments

This work was supported by National Science Foundation Grant (IOB 0517302). David Majewski was supported by a National Heart Lung Blood Institute training grant (T32 HL07654).

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Fig. 1.

Caudal-to-rostral sequential cuts alter respiratory motor output in turtle brainstems. Drawings of turtle brainstems (left) are shown with traces of left ($\int$ LXII) and right ($\int$ RXII) integrated XII respiratory motor output (right). Brainstems were cut along the midline starting at the caudal surface up to the level of XII (A), IX (B) and VI (C) cranial nerve roots. The arrows in the integrated XII traces in part (B) show examples where left and right bursts were coupled, but the burst amplitude was larger on one side versus the other side.

Fig. 2.

Caudal-to-rostral sequential cuts decrease frequency but not burst shape. Frequency (A) of respiratory-related bursts remains constant until the cut to VI. Burst duration (B) and time to peak (C) remain constant throughout the experiment. Symbols: * = $p < 0.05$ compared to intact brainstems.

Fig. 3.

Synchronized XII bursts following a single caudal-to-rostral midline lesion to the level of the VI nerve. (A) Drawing of a turtle brainstem is shown with traces of left and right integrated XII respiratory motor output before and after a single caudal-to-rostral midline lesion. After the lesion was made, XII bursts were synchronized although burst amplitude was decreased (note increase in size of baseline noise). (B) Left and right XII respiratory motor output prior to (left) and following a single caudal-to-rostral midline lesion (middle). Following the lesion, left and right XII bursts are coupled, but the degree of coupling is highly variable. Following bath-application of PBG (20 μ M), the degree of coupling appears to be increased with robust XII bursts on both sides of the brainstem (right).

Fig. 4. Rostral-to-caudal sequential cuts alter respiratory motor output in turtle brainstems. Similar to Fig. 1, drawings of turtle brainstems (left) are shown with traces of left and right integrated XII respiratory motor output (right). Brainstems were cut along the midline starting at the rostral surface down to the level of VI (A), X (B) and XII (C).

Fig. 5.

Rostral-to-caudal sequential cuts decrease frequency but not burst shape. Frequency (A) of respiratory-related bursts remains constant until cut to X and remains below baseline in the cut to XII. Burst duration (B) and time to peak (C) remain constant throughout the experiment. Symbols: * = $p < 0.05$ compared to intact brainstems.

Fig. 6.

Crossed connections exist between VII and XII cranial nerve roots. (A) Drawing of an intact turtle brainstem is shown with traces of left and right integrated XII respiratory motor output. (B) Left and right XII respiratory motor output remains synchronized following a caudal-to-rostral midline lesion to XII and a rostral-to-caudal midline lesion to VI. The lesions did alter XII burst frequency (C), time to peak (D) or burst duration (E).

Fig. 7.

Hemisected turtle brainstems produce respiratory-related motor output similar to intact brainstems. (A) Drawing of hemisected turtle brainstem preparation with left and right XII integrated respiratory motor output. (B) Burst frequency decreases after hemisection but returns to baseline in 2.5 h. (C, D) Burst duration and time to peak are not affected by hemisection. The solid line above the graphs shows when the hemisection occurred while the dotted shows burst frequency, duration, and rise time for intact brainstems at 0 min. Symbols: * = $p < 0.05$ compared to intact brainstems; filled-in circles indicate time during which subsequent hemisection experiments were measured.

Fig. 8.

Respiratory motor output from turtle hemibrainstems is decreased following MOR receptor activation and high pH solution. (A) DAMGO applied at 0.01, 0.1, and 1.0 μM decreases respiratory burst frequency. (B) Respiratory burst frequency was reversibly decreased in response to high pH solution (pH=7.80). The solid lines above the graphs show the time of DAMGO and high pH solution application. Symbols: * = $p < 0.05$ compared to baseline; # = $p < 0.05$ compared to time controls; † = $p < 0.05$ for drug effect ‡ = $p < 0.05$ for time-dependent effect.

Fig. 9.

PBG increases burst frequency and regularity in turtle hemibrainstems, but does not induce frequency plasticity. (A) Traces of integrated XII respiratory motor output are shown during baseline (upper trace), during a 60 min bath application of PBG (20 μM; middle trace), and during a 2-h washout (lower trace). (B, C) PBG increased burst frequency, but only during PBG application. There was no long-lasting increase in burst frequency during the 2-h washout. (D) Burst interval decreased during PBG application, but returned to baseline levels during the 2-h washout. (E) Burst interval coefficient of variation decreased during the last 30-min of the PBG application and rebounded to levels close to baseline. The solid line above the graphs shows the time of PBG application. Symbols: * = p<0.05 compared to baseline; # = p<0.05 compared to time controls; † = p<0.05 for drug effect.

Fig. 10.

Episodic discharge is produced by turtle hemibrainstems and altered by PBG. (A) Turtle brainstems produce episodic XII motor bursts (episodic discharge occurs when bursts/episode > 1.75). (B) Episodic discharge is produced in turtle hemibrainstem. (C) PBG (20 μM) reduces the number of bursts per episode in turtle hemibrainstems. (D) Graph of group data for turtle hemibrainstems producing episodic discharge during exposure to aCSF (n=2; open circles) or PBG (20 μM; n=7; filled squares). PBG decreased bursts/episode during the PBG exposure, but returned to near baseline levels during washout. Thus, there were no long-lasting PBG-dependent changes in episodic discharge in turtle hemibrainstems. The solid line above the graphs shows the time of PBG application. Symbols: * = p < 0.05 compared to baseline; # = p < 0.05 compared to time controls; † = p < 0.05 for drug effect.