INTERCOSTAL AND ABDOMINAL RESPIRATORY MOTONEURONS IN THE NEONATAL RAT SPINAL CORD: SPATIOTEMPORAL ORGANIZATION AND RESPONSES TO LIMB AFFERENT STIMULATION

Aurore GIRAUDIN, Marie-Jeanne CABIROL-POL, John SIMMERS and Didier MORIN

Universités Bordeaux 1 & 2
Centre National de la Recherche Scientifique
Bordeaux, France

Running head: Spinal respiratory motoneurons and limb sensory inputs

Corresponding author:
Dr Didier MORIN
Université Victor Segalen Bordeaux 2, UMR CNRS 5227, Laboratoire Mouvement - Adaptation - Cognition bâtiment 2A, 146 rue Léo Saignat, 33076 Bordeaux, France

Phone: (33) 05 57 57 47 73
Fax: (33) 05 56 90 14 21
E-mail: didier.morin@u-bordeaux2.fr

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ABSTRACT

Respiration requires the coordinated rhythmic contractions of diverse muscles to produce ventilatory movements adapted to organismal requirements. During fast locomotion, locomotory and respiratory movements are coordinated to reduce mechanical conflict between these functions. Using semi-isolated and brainstem-spinal cord preparations from neonatal rats, we have characterized for the first time the respiratory patterns of all spinal intercostal and abdominal motoneurons, and explored their functional relationship with limb sensory inputs. Neuroanatomical and electrophysiological procedures were initially used to locate intercostal and abdominal motoneurons in the cord. Intercostal motoneuron somata are distributed rostro-caudally from C7-T13 segments. Abdominal motoneuron somata lie between T8 and L2. In accordance with their soma distributions, inspiratory intercostal motoneurons are recruited in a rostro-caudal sequence during each respiratory cycle. Abdominal motoneurons express expiratory-related discharge that alternates with inspiration. Lesioning experiments confirmed the pontine origin of this expiratory activity which was abolished by a brainstem transection at the rostral boundary of the VII nucleus, a critical area for respiratory rhythmogenesis. Entrainment of fictive respiratory rhythmicity in intercostal and abdominal motoneurons was elicited by periodic low-threshold dorsal root stimulation at lumbar (L2) or cervical (C7) levels. These effects are mediated by direct ascending fibers to the respiratory centers and a combination of long-projection and polysynaptic descending pathways. Therefore, the isolated brainstem-spinal cord in vitro generates a complex pattern of respiratory activity in which alternating inspiratory and expiratory discharge occurs in functionally identified spinal motoneuron pools that are in turn targeted by both forelimb and hindlimb somatic afferents to promote locomotor-respiratory coupling.

KEYWORDS: Respiration, neonatal rat brainstem-spinal cord, retrograde labeling, locomotory-respiratory coupling, sensori-motor integration.

INTRODUCTION
Respiration is a complex cyclic motor act that requires an alternation between a phase of inspiration necessary to inhale oxygen and an expiratory phase to exhale carbon dioxide. Similarly to diverse rhythmic movements such as those involved in locomotion, the basic alternating phases of respiratory muscle contractions are driven by pattern-generating neural circuitry within the central nervous system (for a recent review, see Feldman and Del Negro 2006). Although the cellular and synaptic mechanisms engaged in respiratory rhythm generation still remain to be completely elucidated, our understanding of respiratory circuit operation has benefited greatly from the study of various in vitro preparations of the neonatal rat and mouse (for review see Funk and Feldman 1995; Onimaru et al. 1997; Ballanyi et al. 1999; Feldman and Del Negro 2006). Since the pioneering studies of the respiratory system in isolated brainstem-spinal cord preparations (Suzue 1984; Smith and Feldman 1987), attention over the last two decades has focused mainly on the central neural mechanisms responsible for the inspiratory phase of respiration. In this perspective, accumulated findings have shown that inspiration results from a combination of emergent network and intrinsic membrane properties (reviewed by Ramirez and Viemari 2005), and that medullary neurons likely to be involved in respiratory rhythm generation are located within two distinct but interconnected brainstem populations, the preBötzinger complex (Smith et al. 1991) and the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG; Mellen et al. 2003; Onimaru and Homma 2003; Onimaru et al. 2006; Barnes et al. 2007). However, because expiration has often been considered as a passive component of the respiratory cycle at rest and because it occurs only occasionally in caudal thoracic ventral roots in brainstem-spinal cord preparations in vitro (Smith et al. 1990) or only in response to an acidosis of the extracellular environment (Iizuka 2004), the site(s) of generation, the spatio-
temporal organization of spinal motor activity (in particular at the thoraco-lumbar level) and the neural mechanisms underlying the expiratory phase of respiration are less well documented.

Although the central respiratory rhythm-generating networks continue to function alone in isolated brainstem-spinal cord or slice preparations, in intact animals including humans, the respiratory circuitry must interact with other major functions (for example cardiovascular, thermoregulatory, locomotory) to adapt rhythmic breathing movements to changing environmental and behavioral demands. During vertebrate locomotion, for example, a 1:1 coupling between locomotory and respiratory cycles has been observed in several species of bipeds and quadrupeds when running or galloping speeds have been attained (Bramble and Carrier 1983; Boggs 2002). Furthermore, we have previously reported that in isolated brainstem-spinal cord preparations from neonatal rats, the rhythmic activation of hindlimb sensory pathways by electrical stimulation of lumbar dorsal roots (DR) can reset and entrain respiratory rhythmicity (Morin and Viala 2002). On this basis, we proposed that, in quadrupeds at least, hindlimb somatic sensory inputs could provide timing information to the respiratory rhythm generators in order to couple the frequency of breathing movements to the locomotor cycle. Moreover, both short-latency excitatory and GABA-mediated inhibitory postsynaptic potentials are produced in inspiratory phrenic motoneurons (PMNs) in response to activation of lumbar afferent pathways (Morin and Viala, 2002). Although the exact role of these apparently direct lumbar synaptic inputs to PMNs remains to be established, we proposed that they could contribute to respiratory entrainment by modulating the sensitivity of PMNs to the descending excitatory drive from the medullary respiratory rhythm-generating centers. Whether functioning in synergy or in alternation with the PMNs, other spinal
neuronal populations, such as intercostal and abdominal motoneurons, participate actively in respiration to produce inspiratory movements of the rib cage or to facilitate expiration, respectively (reviewed by Monteau and Hilaire 1991; Iscoe 1998). However, to date the actions of limb somatic afferents on intercostal and abdominal respiratory motoneuron activity have not been investigated. Moreover, although stimulation of forelimb somatic afferent pathways can increase the cycle frequency of phrenic nerve discharge (Potts et al. 2000) and entrain the respiratory rhythm (Potts et al. 2005) in heart-brainstem preparations from 6-8 week old rats, the effects of forelimb sensory inputs on respiration during the perinatal period remain to be determined.

In the present study, in vivo neuroanatomical labeling and in vitro electrophysiological approaches with brainstem-spinal cord preparations were first used to determine the position of intercostal and abdominal motoneurons in the neonatal rat spinal cord. Next, we characterized the spatio-temporal organization of all inspiratory- (intercostal) and expiratory-related (intercostal and abdominal) motor patterns along the spinal cord. Finally, the influence of fore- and hindlimb sensory inputs on thoraco-lumbar respiratory motor activity was examined, as was the nature of the intraspinal pathways that mediate such interactions. Part of this work has been presented previously in abstract form (Giraudin et al. 2006).
MATERIALS AND METHODS

Experiments were conducted on 0-4 day old Wistar rats from different litters obtained from timed pregnant female rats raised in our laboratory breeding colony. All experiments were performed in accordance with the local ethics committee of the Bordeaux 2 University and the European Communities Council Directive.

Retrograde labeling of intercostal and abdominal motoneurons

Motoneurons innervating intercostal and abdominal muscles were localized in the spinal cord by means of retrograde staining. Newborn animals were anaesthetized by hypothermia and small incisions in the skin were made to expose intercostal or abdominal muscles. Crystals of the cholera toxin subunit B coupled to Alexa Fluor 488 (CTX-AF 488; Molecular Probes; Eugene, Oregon) were placed on a selected muscle with a pin and then the wound was left to dry for 5 mins and disinfected with Betadine. Animals were allowed to recover from anesthesia and returned to their nest for 24 hrs. Spinal cords were then dissected (see below) and fixed in a 4% paraformaldehyde solution diluted in 0.1M phosphate buffer (PB; pH=7.4) for 2 hrs at room temperature or were stored overnight at 4°C. The cords were dehydrated through a graded ethanol series (50°, 70°, 95° and 100°) and cleared for 2 hrs in methylessalicylate at room temperature. Finally, spinal cords were mounted in Fluoromount (BDH), observed under a fluorescent microscope (DMRB; Leica) at 488nm and images were acquired using a CCD camera (Sony DXC-990P).

In vitro isolated brainstem-spinal cord preparations

Animals were deeply anaesthetized and decerebrated from the rostral end of the fifth cranial nerves. The skin and muscles were removed and preparations were then
placed in a 100 ml chamber filled with artificial cerebrospinal fluid containing (in mM): 100 NaCl, 4 KCl, 1.2 NaH2PO4, 2 CaCl2, 1.3 MgCl2, 25 NaHCO3 and 30 D-glucose. This standard saline was continuously equilibrated with 95% O2 / 5% CO2 (pH 7.4) and maintained at 10°C during the dissection. The brainstem and spinal cord with its dorsal and ventral roots still attached were isolated, then the ensemble was placed in a 10 ml recording chamber and fixed on a Sylgard resin block with the ventral surface upwards. The bath temperature was progressively raised and thereafter maintained at 26°C by means of a Peltier system. A post-dissection resting period of 30 min was systematically respected before recording procedures began.

**Recordings and stimulations**

Respiratory-related activity in both spinal ventral roots and cranial nerves was recorded using glass suction electrodes. Signals were amplified (x10000) by differential AC amplifiers (AM System; Phymep, Paris), bandpass-filtered (0.1-3 kHz), rectified, integrated ($\tau=20$ ms) (Neurolog System; Digitimer Ltd., England), digitized and stored on a computer hard disk (using Spike 2 software; Cambridge Electronic Design, Cambridge, UK) for off-line analysis. Single or train stimulus pulses (0.2-2V, 0.5 ms at 5-10Hz) were applied respectively to ventral or dorsal spinal roots via glass suction electrodes using an 8-channel digital stimulator (A.M.P.I.; Jerusalem, Israel). The stimulation of dorsal roots followed the procedure used by Morin and Viala (2002), which was based on their finding that the activation of large diameter, and therefore presumed proprioceptive, dorsal root axons in isolated *in vitro* preparations requires relatively low stimulus intensities, with thresholds consistently ranging from 0.6 to 1.1 V. Accordingly, in our experiments the threshold stimulus for so-called "low threshold" DR afferents was determined by increasing the train shock intensity from a
subthreshold value until either a maximum of 1.5 V or an intervening level at which an effect on the timing of the subsequent cycle of ongoing spontaneous respiratory rhythmicity had occurred. Once the latter was observed, and unless otherwise stated in the following, this threshold stimulus intensity was applied to a given root throughout the course of the experiment.

In a series of experiments, a stimulating tungsten electrode (Frederick Haer; Brunswick, USA) was manipulator-positioned in contact with the ventrolateral surface of the brainstem in order to stimulate (single shocks of 300 µA, 0.1 ms) or to electrolytically destroy (400 µA for 3-5 s) the region containing the pre-Bötzinger complex. The final position of the stimulating electrode was adjusted using the hypoglossal root exits as anatomical landmarks.

**Drug application**

In order to reversibly isolate the lumbar spinal cord from the cervical region, axonal conduction in the thoracic spinal segments was prevented by means of sucrose blockade. For this, the recording chamber was partitioned into three compartments with barriers of syringe-ejected Vaseline and the intermediate thoracic cord compartment (containing at least 4 contiguous spinal segments) was irrigated with an isotonic sucrose solution (10% in distilled water). The narrow petroleum jelly bridges allowed the cord to remain functionally connected between the different baths, and water tightness was checked at the end of each experiment by adding methylene blue to the bathing medium on either side of a given bridge.

In other experiments, a low calcium concentration (0.1 mM CaCl2, 5 mM MgCl2) solution, which has been found previously to block chemical synaptic transmission in the neonatal rat spinal cord (Morin and Viala 2002; Tresch and Kiehn...
2000; Cazalets 2005), was used to reversibly attenuate synaptic transmission in the mid-cord region using the same bath partitioning and verification procedures.

**EMG and semi-isolated preparations**

In a further set of experiments, a spinal cord-rib cage preparation was used to make electromyographic recordings from intact intercostal or abdominal muscles during respiratory activity. For this the spinal cord after brainstem removal was exposed as described above but with all ventral and dorsal roots left uncut. The preparation was then pinned down ventral side upwards and bipolar EMG electrodes (made from 50 µm silver wire) were inserted either into external intercostal muscles of the rib cage, or medial or lateral abdominal muscles.

**Brainstem transections and histological controls**

In several isolated brainstem-spinal cord preparations, expiratory activity was recorded from thoracic ventral roots while serial caudally-directed transverse sections of the brainstem were performed with a scalpel blade in order to locate pontine structures involved in the genesis of the expiratory phase of the respiratory cycle. Recordings were performed about 100 minutes after each section in order to allow recovery of the preparation. For histological verification of the lesion location, the brainstem region caudal to the section (see Fig. 5C) was fixed for 48H at 4°C in Lillie solution (10% formalin in PB; pH= 7.0). The tissue was then rinsed twice in PB and cryoprotected overnight with 25% sucrose in PB. After embedding in Tissue Tek and freezing by using cooled isopentane until -80°C, frozen 40µm thick serial parasagittal sections were cut on a cryostat. Sections were mounted on gelatin-coated slides and adjacent sections were alternately stained for acetylcholinesterase (AchE) or with
cresyl violet to visualize the approximate boundaries of the pontine nuclei. For AchE staining, sections were slide-mounted and left to dry at room temperature (RT). They were then rinsed in 0.2M acetate buffer (pH=5.9) for 5 minutes before being immersed in 0.2M acetate buffer containing 0.04M glycine and 0.01M copper sulfate pentahydrate for 18 hours under agitation at RT. The slides were incubated in the same solution containing 1% acetylthiocholine iodide for 2 minutes under agitation at RT and rinsed 3 times in acetate buffer. They were then dehydrated in an ascending ethanol series, cleared in two changes of xylene and mounted in Eukitt before observation under microscopy (DMRB; Leica).

Data analysis
Inspiratory and expiratory-related motor activities were characterized in terms of the onset delays and durations of spinal ventral root bursts. For this, 20 to 30 burst cycles were chosen randomly from sequences of respiratory activity recorded from each preparation. Burst onset delay was taken as the interval (in ms) between the beginning of an inspiratory burst in the C5 ventral root and the onsets of corresponding bursts recorded in more caudal roots. The absolute duration of each burst was also measured. Data analysis was performed with Spike 2 software (Cambridge Electronics Design, Cambridge, UK) and pooled burst onset delay and duration measurements for a given ventral root were expressed as means ± SEM. Differences between means were analyzed using a statistical software package (Sigma Stat for Windows; SPSS, Chicago, IL) and assessed by one-way ANOVA with a Student-Newman-Keuls post-test. In a further analysis, values of thoracic burst durations were plotted against the corresponding burst onset delay and a linear regression line was fitted to the scatter plot. The coefficient of linear regression (r)
was calculated and the statistical significance was established using the Pearson test (Prism 4 for Windows, GraphPad software).

In experiments conducted to determine the effect of brainstem stimulation on the ongoing respiratory rhythm, the resultant changes in burst timing were expressed in phase-response plots. For this, the reference period (Pref) was taken as the mean cycle period over 3 spontaneous respiratory cycles prior to stimulation. The ratio of the stimulus latency (time elapsed from the stimulus to the onset of the ensuing evoked inspiratory burst) and Pref then determined the stimulus phase. The shift in phase of the inspiratory burst, expressed as the difference between Pref and the period in which the stimulation occurred and again divided by Pref, was plotted on the ordinate. Differences in mean values for all parameters were taken to be significant at p<0.05.
RESULTS

Spinal cord distribution of intercostal and abdominal motoneurons

To determine the location of intercostal motoneurons in the neonatal rat spinal cord, Alexa Fluor 488-conjugated cholera toxin (CTX-AF 488; Fig. 1) was inserted unilaterally into external muscles of the second (n=4), fifth (n=4), eighth (n=4) and eleventh (n=4) intercostal spaces of which there are twelve in the rat rib cage. An example of motoneurons stained after CTX-AF 488 insertion into the fifth intercostal space is shown in Figure 1A. Retrograde-labeled motoneuron somata were distributed ipsilaterally along the cord in a single column that extended rostro-caudally from the cervical C6 to the thoracic T12 segments. The rib space position of an injected external intercostal muscle within the rib cage was reflected in the cell body location of its innervating motoneurons within the spinal cord. Thus, more posterior intercostal muscles had more caudally-distributed motoneuron somata (see Fig. 2C, filled bars in left panel). For a given CTX-AF 488 injected intercostal muscle, the majority of stained somata was located within one to two spinal segments (as in Fig. 1A), although in most preparations (12 of 16), some labeling extended rostrally and/or caudally into one or two immediately adjacent segments.

In 8 other animals, injections of the dye-labeled toxin into the lateral (external and/or internal oblique; n=4) or medial (rectus abdominis; n=4) abdominal muscles also stained motoneuron somata that were confined to the ipsilateral (Fig. 1B) or bilateral (Fig. 1C) ventral spinal columns, respectively. The bilateral distribution of stained medial rectus motoneurons was presumably due to the entry of CTX-AF 488 via motor axons on both sides of the cord as a result of our labeling rectus abdominis at the abdominal midline. As illustrated in Figure 2C (right panel, filled bars), motoneurons innervating the abdominal muscles were distributed exclusively within
the lower thoracic cord region. Labeled cell bodies of lateral abdominal muscle motoneurons were found to lie between the T10 and T13 spinal segments, while those innervating the medial abdominal muscles were distributed from T8 to T13.

Although CTX-AF 488 crystals were applied directly to selected muscles, the possibility of nonspecific staining due to tracer leakage could not be totally excluded. In a series of electrophysiological experiments (n=4), therefore, a brainstem-spinal cord preparation that included the rib cage and still-attached abdominal muscles (Fig. 2A) was used to identify individual cervico-thoracic ventral roots according to their specific intercostal and abdominal muscle targets. This was achieved by applying single pulse stimuli to successive motor roots while monitoring activation of a specific muscle by EMG recording (Fig. 2B). In agreement with findings from the above anatomical experiments, this electrophysiological approach confirmed that motoneurons innervating external intercostal and abdominal muscles project their axons in C7 to T13 (Fig. 2C, left panel, unfilled bars) and in T9 to L1 (Fig. 2C, right panel, unfilled bars) ventral roots, respectively. Furthermore, on the basis of EMG signal amplitude, one and sometimes two ventral roots were found to be principally involved in the activation of a given muscle (for example, see the maximal response to T6 stimulation in the 7th intercostal muscle recordings of Fig. 2B). In all preparations studied, however, from three to seven adjacent ventral roots were also found to contribute to the motor command to a given intercostal (Fig. 2C, left) or abdominal (Fig. 2C, right) muscle, as seen in Fig. 2B where attenuated responses in the recorded intercostal muscle occurred with T5, T7 and T8 ventral root stimulation. The close coherence between results obtained with the two anatomical and electrophysiological approaches (compare data in Fig. 2C) therefore confirmed the
somatotopic rostro-caudal organization of intercostal and abdominal motoneurons in the spinal cord.

Spatio-temporal organization of spinal inspiratory and expiratory motor activity

Following the pioneering studies of Suzue (1984) and Smith and Feldman (1987) on the respiratory motor system of the neonatal rat brainstem-spinal cord in vitro, a rostro-caudal gradient in spinal respiratory motor outputs has been described in which rhythmic bursting in both inspiratory and expiratory phases can occur at various levels along the cord (Smith et al. 1990; Iizuka 2004). Here we wished to complete these earlier descriptions and to determine, when present, the function and the supra-spinal origin of spontaneous respiratory-related output at all cervical to lumbar cord segments.

Respiratory activity in isolated medullary-spinal cord preparations (with the brainstem sectioned at the level of the X cranial nerves; n=9) consists of cyclic motor bursts occurring conjointly in cranial (hypoglossal) nerves (see Fig. 4A) and in spinal ventral roots throughout the cervico-thoracic cord (Figs. 3A, 4A). As illustrated in Fig. 3A, single shock stimulation (300 µA, 0.1 ms) applied to the surface of the ventrolateral medulla, a region that includes the pre-Bötzinger complex which is thought to be critically involved in the inspiratory phase of respiratory rhythmogenesis (Smith et al. 1991), caused a resetting of ongoing bursts recorded simultaneously from phrenic (C4) and intercostal (T4) ventral motor roots (Fig. 3A1, A2). Consistent with typical features of endogenous biological oscillators (Pinsker 1977), premature (Fig. 3A1) or retarded (Fig. 3A2) spinal root bursts were triggered by the stimulation depending on the phase at which the stimulus occurred in the ongoing rhythm cycle (Fig. 3B). Thus, when a stimulus was applied relatively late in the interburst interval
(at >50% of the elapsed cycle), the onset of the next burst was advanced causing the ensuing rhythm to be phase-advanced (Fig. 3A1; 3B, left). By contrast, a relatively early stimulus (at <50% of the cycle) retarded the next spontaneous burst and consequently caused a phase-delay in the timing of subsequent cycles (Fig. 3A2; 3B, right). Finally, an electrolytic lesion (400 µA for 3-5 s) performed at the site of stimulation abolished all burst discharge in the C4 and T4 roots (Fig. 3C), thereby further confirming the inspiratory nature of the recorded cervical and thoracic motor activities.

To more completely establish the spatio-temporal properties of spinal inspiratory activity, simultaneous recordings were made from all cervical and thoracic ventral roots, while using motor output at C5, which contains phrenic motoneuron axons, as the reference for the inspiratory phase of each respiratory cycle. In close agreement with our previous anatomical and electrophysiological data (see Fig. 2C, left panel), these experiments (n=14) showed clearly that burst discharge, which would normally be responsible for inspiratory-phase activation of the intercostal muscles, extended from the C6 to T12 cord segments. On the basis of the differing delays to burst onset (Fig. 4A, right traces; Fig. 4B, left histograms), and in general agreement with the timing of corresponding burst endings and the resultant burst durations (Fig. 4B, right histograms), three groups of intercostal motoneurons were distinguishable in the inspiratory phase of each cycle. The first pool included cervical (C6-C8) and rostral thoracic (T1-T3) intercostal motoneurons that were activated shortly after C5 phrenic burst onset, with delays ranging from 11 to 23 ms (Fig. 4B). No significant differences were observed between the end times and durations of bursts (1.1-1.4 s) in this group and those of reference C5. The mid-thoracic intercostal motoneurons, whose axons exit the cord in the T4 to T6 ventral roots,
constituted the second group. The burst durations of these motoneurons, which ranged from 0.9 to 1.3 s, were significantly shorter than C5 bursts (0.001<p<0.05), due to their significantly longer delay to onset (29-34 ms after C5) and their relatively earlier termination (142-245 ms prior to C5 bursts). Intercostal motoneurons with axons in the T7 to T12 ventral roots made up the third most caudal inspiratory motoneuron pool. The still longer delays to burst onset in this group relative to phrenic bursts ranged from 38-64 ms, bursts ended from 199-434 ms earlier, and they lasted from 0.8 to 1.2 s, which was even shorter again than reference C5 (p<0.001). Finally, the strong negative correlation between thoracic burst durations and the corresponding delays to burst onset seen in the scatter plot of Fig. 4C further supports the conclusion that inspiratory intercostal motoneurons are recruited in a strict rostrocaudal gradient in which bursts occur progressively later, terminate earlier, and thereby become shorter with distance down the cord (Fig. 4D).

In ponto-medullary-spinal cord preparations (n=17), simultaneous recordings from lower thoracic (T12 and T13) ventral roots that carry abdominal motor axons (see Fig. 2C, right panel) also displayed spontaneous rhythmic bursts that now occurred in alternation with C5 inspiratory discharge (Fig. 5A). Because of their out-of-phase relationship with phrenic discharge and the positions of the spinal ventral roots where they occurred, these thoracic motor bursts were considered to be expiratory in function. Transections of the brainstem at different levels were then conducted to determine the location of the supra-spinal structures involved in the generation of this expiratory-related activity. In a first step, a single transection was performed just rostral to the X cranial nerves in order to accomplish a global removal of the brainstem pontine structures (n=10 preparations). In all cases, an increase in respiratory cycle rate that is classically attributed to the removal of the A5
noradrenergic nucleus (Hilaire et al. 1989) was observed. Significantly, moreover, in such reduced medullary preparations, expiratory-related activity was no longer expressed (Figs. 5B, D, lesion c). Therefore, in a second series of experiments on initially intact ponto-medullary-spinal cord preparations, consecutive transverse sections of the brainstem were made in the rostro-caudal direction to more precisely define the location the pontine structure(s) involved in expiratory-phase rhythm generation. In all seven preparations examined, expiratory-like activity persisted until a section was made just caudally to the VII cranial nerves (Figs. 5C and D, lesion b). The exact rostro-caudal positioning of this lesion was also verified histologically following physiological experimentation. For this, we made parasagittal sections of the caudal brainstem and stained the tissue for acetylcholinesterase (AchE) or with cresyl violet to visualize the approximate boundaries of remaining pontine motor nuclei (see Methods; Fernandes et al. 1998). As seen in Fig. 5C which shows two AchE-stained parasagittal sections taken at different planes (1 and 2 in right panel) from the same brainstem, the labeled facial nucleus provided a clear landmark for the slightly more rostral position of the original in vitro brainstem transection. The above findings strongly suggest therefore that the brainstem area in the vicinity of the VII motor nucleus in the neonatal rat participates either directly (i.e. in actual rhythmogenesis) or indirectly (i.e. as a necessary relay) in the production of spinal expiratory-phase activity.

To assess the spatio-temporal organization of expiratory activity along the cord, therefore, simultaneous ventral root recordings were made from cervical to lumbar cord levels in brainstem-spinal cord preparations that now included the pontine structures (Fig. 6A, B). Here again, the onsets and durations of individual root discharges were compared to C5 motor bursts as the reference for the inspiratory
activity phase of each cycle. Such experiments (n=10) revealed that expiratory bursting occurred from T8 to L2 ventral roots (Fig. 6B, traces at left; 6C, filled histogram bars in left panel) in agreement with our previous conclusions (see Fig. 2C, right panel) that these motor roots innervate the abdominal muscles. Typically, the spinal expiratory discharge occurred in a double bursting pattern that consisted of a short pre-inspiratory burst (mean duration: 0.26±0.02 s) and a prolonged post-inspiratory discharge (mean duration: 1.8±0.1 s). However, when a double inspiratory burst in a cycle occasionally occurred spontaneously (Fig. 6B, traces at right), expiratory-related activity was recorded in otherwise solely inspiratory (T5 to T7) or previously quiescent (L3) ventral motor roots (Fig. 6C, filled histogram bars in right panel). Therefore, the isolated pons-medulla-spinal cord preparation is capable of generating complex and spatio-temporally variable patterns of fictive respiration in the ensemble of functionally-identified (inspiratory, mixed inspiratory-expiratory, and expiratory) motor roots throughout the cervical-to-lumbar cord region.

Influence of limb sensory inputs on intercostal and abdominal motoneuron activity

We have previously reported that in the neonatal rat, phrenic respiratory entrainment-induced polypnea can be achieved by phasic low-threshold lumbar afferent stimulation in the isolated brainstem-spinal cord preparation (Morin and Viala 2002). On this basis, it was proposed that in quadrupeds, hindlimb somatic afferent inputs could serve normally to couple breathing frequency to the locomotor rhythm. Two unaddressed questions that arose from these earlier findings were firstly, whether such a respiratory entrainment could also be driven by the cyclic activation of cervical low-threshold sensory afferents originating from forelimb muscles, and secondly, how
intercostal and abdominal respiratory motor outputs might respond to both cervical and lumbar somatic inputs.

To explore these issues, hindlimb and forelimb afferent pathways were rhythmically activated by applying electrical stimuli (0.5-1 s pulse trains over a range of 0.2-1.5V at 10Hz; see Methods) to lumbar (L2-L5) or cervical (C7-C8) DRs, respectively, in isolated pontomedullary-spinal cord preparations in which respiratory activity was monitored simultaneously from the C4 (phrenic), T8 (intercostal) and T13 (abdominal) ventral roots (Fig. 7, see schematics). In such preparations (n=13), the rhythmic activation of either low-threshold lumbar (Fig. 7A) or cervical (Fig. 7B) afferents was able to fully entrain (with 1:1 coupling) ongoing respiratory bursting at all three cord levels. As already described (Morin and Viala 2002), this entrainment derived from a resetting action of the DR stimulation, which was characterized by a near constant interval between each stimulus train and the respiratory burst it evoked (mean C4 burst latency after lumbar DR stimulation: 1.56±0.05 s; Fig. 7A right; after cervical DR stimulation: 1.34±0.06 s; Fig. 7B right). Importantly, no differences were observed between spontaneous and DR stimulation-induced burst patterns (compare traces in Fig. 7A,B with those in Fig. 6B), with both beginning by a brief pre-inspiratory discharge in thoracic ventral roots. Together these results suggest that entrainment of respiratory activity in cervical (phrenic) and thoraco-lumbar (intercostal and abdominal) motoneurons by lumbar or cervical DR afferent stimulation is mediated by long-loop neuronal pathways via the respiratory rhythm-generating centers in the brainstem (see Discussion).

To further establish the indirect nature of these sensori-motor influences, isotonic sucrose-blockade of axonal conduction in thoracic segments was employed in experiments conducted on 6 preparations. Under control conditions, cyclic
stimulation of C7 (Fig. 8A, left) or L5 (Fig. 8B, left) DRs was able to entrain respiratory bursting monitored from C4 (phrenic) and T12 (intercostal and abdominal) ventral roots. As expected, during a sucrose block on the thoracic T1-T9 cord region, all respiratory bursting at T12 was abolished, consistent with an interruption of the descending respiratory drive to this low thoracic level. Under these conditions, moreover, although a phasic activation of low-threshold C7 DR afferents could still entrain C4 respiratory bursting (Fig. 8A, middle), DR stimulation at L5 was now unable to affect the ongoing respiratory rhythm (Fig. 8B, middle), and despite an increase in the stimulus intensity (to twice the threshold for afferent fiber activation) in order to verify the efficacy of the stimulating electrode. The occurrence of unidentified, short-latency T12 motor discharge in response to this stronger L5 DR stimulation, which were absent at the lower stimulus intensities in control conditions (Fig. 8B, left), was probably due to the activation of local sensori-motor circuitry in the low thoraco-lumbar region of the cord. Finally, both T12 respiratory activity and the capacity for rhythm entrainment by cervical and lumbar DR stimulation were rapidly restored by a return to normal saline superfusion of the thoracic cord region (Fig. 8A-B, right panels).

In a final series of in vitro experiments (n=9 ponto-medullary spinal cord preparations), we wished to determine the nature of the propriospinal pathway(s) responsible for driving spinal motoneurons during spontaneous or DR (cervical or lumbar) stimulation-entrained respiratory activity. In a first step, the thoracic cord from T1 to T10 was selectively bathed with low calcium/high magnesium saline to reversibly block synaptic transmission (see Methods) and thereby suppress local intersegmental connections within the mid-cord region. Under these conditions, the ability of C8 (Fig. 9A) or L2 (Fig. 9B) DR stimulation to entrain respiratory rhythmicity
persisted in all cases, as is evident in both the phrenic (C4) and low thoracic (T11) recordings of Fig. 9A,B (middle panels). In a second step, respiratory bursts occurring spontaneously at the cervical and low thoracic levels were integrated and their areas compared before and during blockade of synaptic transmission in the mid-thoracic cord region (Fig. 9C). Consistent with the maintained effectiveness of both cervical and lumbar afferent stimulation to influence the timing of ongoing respiratory rhythmicity, individual patterns of spontaneous discharge at C4 remained unaffected by a mid-thoracic synaptic blockade (Fig. 9C1, C2, upper panels). In contrast, in 8 of the 9 preparations, the intensity of low-thoracic T11 respiratory bursts was significantly decreased (Fig. 9C1, C2, lower panels), indicating a substantial decline in the strength of the descending respiratory drive as it crossed the synaptically-blocked mid-cord region. In 4 of the 8 preparations, inspiratory phase discharge at T11 was diminished, as illustrated by the experiment of Fig. 9C1 (lower panel), while in the 4 remaining experiments inspiratory activity was abolished. Similarly, in 3 preparations in which the generation of more labile expiratory activity persisted throughout the entire experiment, T11 expiratory discharge was also significantly and reversibly decreased and even abolished (as in Fig. 9C1, lower) by a mid-thoracic synaptic blockade (Fig. 9C2, lower right panel).

These results therefore support the conclusion that in the newborn rat, both forelimb and hindlimb afferent, presumed proprioceptive, inputs responsible for locomotor-respiratory coupling are carried by direct, long-projecting ascending pathways to the brainstem, whereas a combination of long-fiber tracts and local segmental circuitry appears to convey the descending drive from the respiratory centers to low thoracic (intercostal and abdominal) motoneurons.
DISCUSSION

In this study we have shown for the first time in the neonatal rat that the somata of motoneurons innervating intercostal and abdominal muscles are distributed in an ipsilateral rostro-caudal column extending from lower cervical segments to the upper lumbar region of the spinal cord. Consistent with this spatial organization, during spontaneously generated respiratory activity in vitro, spinal inspiratory (intercostal) motoneurons are recruited sequentially in a rostro-caudal gradient and are active in strict alternation with expiratory (intercostal and abdominal) motoneurons in the lower thoracic and upper lumbar segments of the cord. Moreover, the generation of this complex spinal respiratory pattern in isolated preparations depends on the integrity of the pons-medulla-spinal cord since, in agreement with recently reported lesion experiments conducted on juvenile (Janczewski and Feldman 2006) and newborn rats (Ruangkittisakul et al. 2007), thoraco-lumbar expiratory-like activity is abolished by a brainstem transection performed at the rostral boundary of the parafacial nucleus. This implies, therefore, that the rostral pFRG and more anterior pontine structures are necessary for the production of the expiratory phase of respiration. Finally, our study provides new evidence that somatic sensory pathways from both the forelimbs and hindlimbs have direct access to the medullary respiratory centers, thereby providing the substrate for coupling the ensemble of spinal respiratory outputs (including phrenic, intercostal and abdominal motoneurons) to ongoing locomotory movements.

Intercostal motoneurons and inspiratory-phase discharge

Intercostal motoneurons are located in discrete rostrocaudally-aligned populations in the cervico-thoracic (C6-T13) region of the cord (Fig. 1). Although individual external intercostal muscles appear to receive their motor innervation principally from one or
two cord segments, in most cases, a small number of motoneurons located in immediately adjacent rostral and/or caudal segments also contribute to a given intercostal muscle's motor command (Fig. 2). This anatomical organization corresponds closely to that reported in adult cat and rat (reviewed by Monteau and Hilaire 1991), although it differs from the segmental specificity of intercostal motoneuron distributions in the developing chicken embryo (Stirling et al. 1995).

In further agreement with previous studies (Smith et al. 1990; Iizuka 2004), our neonatal rat experiments have shown that all spinal ventral roots containing external intercostal motor axons (i.e. from C6 to T12) continue to express respiratory-related bursting even after the brainstem-spinal cord has been isolated in vitro (Figs. 3, 4). Multi-site nerve recordings and resetting experiments involving direct medullary stimulation confirmed the inspiratory nature of this rhythmic cervico-thoracic activity. Firstly, the intercostal motoneurons located in this cord region fire in phase with C5 ventral roots which normally convey motor axons to the main inspiratory muscle, the diaphragm. Secondly, as reported in fetal (Di Pasquale et al. 1994) and neonatal rats (Onimaru et al. 1988), electrical stimulation of the rostral ventro-lateral medulla causes a resetting of rhythmicity at both cervical and thoracic levels (Fig. 3), presumably via a direct perturbation of Pre-I neurons (Onimaru et al. 1988) in the pre-Bötzinger complex, the inspiratory rhythm generator (Smith et al. 1991).

A detailed temporal analysis of these cervico-thoracic inspiratory outputs also showed that the intercostal motoneurons are activated differentially in the respiratory cycle and, on the basis of their burst onsets, endings and durations, can be divided into three populations (Fig. 4). Inspiratory intercostal motoneurons that fire longer bursts are generally recruited earlier in the inspiratory phase and are therefore located in more rostral spinal segments, whereas intercostal burst onsets and
durations become respectively later and shorter with distance down the cord. The functional significance of the discontinuity in rostro-caudal activation of the intercostal motoneurons remains unclear. Presumably, this reflects regional differences in the organisation of descending respiratory pathways in the spinal cord and/or is related to a functional requirement for intercostal muscles to produce discrete regional synergisms in their control of rib cage movements.

Thus, the rostrocaudally-graded activation of intercostal muscles that was previously described in cat (Greer and Martin 1990), dog (De Troyer and Legrand 1995) and humans (De Troyer et al. 2003) also appears to occur in the neonatal rat. In a functional context, moreover, such a decrementing recruitment pattern would ensure that the inspiratory intercostal muscles with the greater breathing mechanical advantage \((i.e.\) those located in the rostral intercostal spaces of the rib cage) are the most rapidly and extensively activated during each respiratory cycle (for review, see De Troyer et al. 2005).

Although the mechanisms underlying this sequential activation of inspiratory intercostal motoneurons remain unknown, differences in excitability related to cell size or intrinsic membrane properties, and/or a differential modulation of the descending respiratory drive to intercostal motoneurons could be involved (Gandevia et al. 2006). Clearly, however, the possible contribution of peripheral afferent inputs in this recruitment pattern can be eliminated, at least in the case of our isolated medullary-spinal cord preparations. Our experiments have also provided evidence that the descending respiratory drive to intercostal motoneurons in the lower thoracic spinal region is mediated at least in part by indirect segmentally-relayed pathways (Fig. 9; see below), as was recently reported in the upper thoracic segments of the neonatal rat (Juvin and Morin 2005). It is likely therefore, that the propriospinal
interneurons engaged in the polysynaptic respiratory drive to upper cord segments may also participate in the rostrocaudal activation of the entire intercostal motoneuron population.

**Abdominal motoneurons and expiratory-phase discharge**

Expiratory-phase activity occurring in alternation with inspiration is also expressed spontaneously in caudal thoracic (T8) to lumbar (L2) segments, with expiratory bursts occurring alone from T13 to L2 (Fig. 6). Combined anatomical and electrophysiological evidence established that these spinal roots contain motor axons that innervate abdominal muscles (Figs. 1, 2). This conclusion is also supported by previous neonatal rat experiments showing that L1 ventral root activity recorded *in vitro* corresponded to abdominal EMG activity recorded *in vivo* (Janczewski et al. 2002). Moreover, with a sudden reduction in inspiratory cycle period during fictive respiration (i.e. when two inspiratory bursts per cycle are produced spontaneously), expiratory discharge can appear in more rostral ventral roots (T5-T7) that otherwise appeared to be uniquely inspiratory in function. Presumably this auxiliary recruitment reflects an adaptive ability of the central respiratory system to reinforce expiratory-phase activity in caudal intercostal motoneurons when breathing frequency is suddenly increased. Furthermore, in adult rats, firing patterns in the T6-T8 spinal segments have been shown to be mostly expiratory in function (Tian and Duffin 1996) with the more caudal interspaces of the rib cage having basically an expiratory mechanical advantage (De Troyer et al. 2005). Our present results are therefore consistent with, and add to, the first description of the spatiotemporal patterns of motoneuronal activity during fictive respiration (Smith et al. 1990): respiratory-generating networks *in vitro* are able to produce a complex pattern of respiration,
consisting of alternating inspiratory- and/or expiratory-phase outputs from the cervical to lumbar regions of the spinal cord.

According to these and other authors, expiratory-phase discharge occurs either occasionally in caudal thoracic roots in vitro (Smith et al. 1990) or only in response to central chemoreceptor stimulation (Iizuka 1999, 2004). In contrast to our investigation, however, in these earlier studies the pons was sometimes conserved (Smith et al. 1990) or was consistently removed (Iizuka 1999, 2004). In juvenile vagotomized rats, a transection performed at the caudal end of the facial nucleus which thereby removes the pons, also eliminates abdominal EMG expiratory activity while inspiratory bursts are preserved (Janczewski and Feldman 2006; also see lesion c in Fig. 5D). In agreement with these latter findings and recently reported lesion experiments on the isolated brainstem of newborn rats (Ruangkittisakul et al. 2007), our study has shown that all thoraco-lumbar expiratory-related activity disappears without affecting inspiratory discharge when a brainstem transection is performed near the rostral margin of the parafacial nucleus (Fig. 5), an area that probably includes elements of the RTN/pFRG respiratory networks. Although the structural boundaries of the pFRG have not been clearly established, this brainstem region is known to contain pre-inspiratory neurons (Onimaru et al. 1987, 1988) which fire in a characteristic pattern consisting of a short pre-inspiratory burst and longer post-inspiratory discharge. This pattern is strikingly similar to the double (pre-inspiratory and expiratory) bursting activity we observed in T13-L2 motoneurons. Although pFRG pre-inspiratory neurons do not contact spinal motoneurons directly, they send projections to the caudal ventro-lateral medulla (Janczewski et al. 2002) wherein lies the nucleus retroambiguus, a group of bulbospinal neurons known to monosynaptically excite abdominal expiratory motoneurons (Boers et al. 2006). This
therefore accounts for the elimination of spinal expiratory-phase discharge, but not inspiratory bursts, that occurred after RTN/pFRG removal (Fig. 5), and is further consistent with current doctrine (Feldman and Del Negro 2006) that mammalian respiratory rhythmogenesis resides with two interconnected oscillatory networks in the RTN/pFRG and the preBötzinger complex, which are responsible respectively for the expiratory- and inspiratory phases of respiration.

**Respiratory rhythm entrainment by cervical and lumbar somatic afferents**

As mentioned above, the respiratory system must interact continuously with other CNS regions in order to produce respiratory patterns adapted to changing behavioral circumstances. During fast locomotion, for example, a variety of mammals coordinate respiratory rhythmicity with ongoing limb movements (Bramble and Carrier 1983; Viala 1997; Boggs 2002). It is now well-established that diverse combinations of mechanical (Bramble and Carrier 1983) and neurogenic (Eldridge et al. 1981; Viala et al. 1987; Romaniuk et al. 1994) interactions between the respiratory and locomotor systems are likely to underlie the coupling of these two primary functions. Limb movement-activated sensory inputs have access to the respiratory system (Palisses et al. 1988; Funk et al. 1992) and cyclic somatic afferent stimulation can fully entrain inspiratory phrenic nerve activity in juvenile (Potts et al. 2005) and adult animals (Iscoe and Polosa 1976). Similarly, in the isolated brainstem-spinal cord from neonatal rats, periodic activation of low-threshold lumbar afferents in time with bursts of fictive locomotion leads immediately to locomotor-respiratory rhythm coupling (Morin and Viala 2002). However at birth, freely moving rats use mainly their forelimbs for overground locomotion due to a lack of sufficient hindlimb postural tonus to support their body weight (Altman and Sudarshan 1975; Clarac et al. 1998;
Brocard et al. 1999). Our present results now show that the phasic activation of low-threshold sensory inputs to the cervical (C7-C8) spinal cord region, which contains the forelimb locomotor networks (Ballion et al. 2001; Juvin et al. 2005, 2007), is also capable of entraining fictive respiration (Fig. 7). Furthermore, we find that periodic electrical stimulation of either cervical or lumbar afferent pathways fully activates the different populations of inspiratory (phrenic and external intercostal) and expiratory (internal intercostal and abdominal) spinal motoneurons, in a manner attributable to a resetting action on the brainstem respiratory generators themselves (see also Morin and Viala 2002; Potts et al. 2005). Interestingly, the two-burst patterning of abdominal expiratory motoneurons, which is also a particular feature of medullary pre-inspiratory neuron activity (Onimaru et al. 1987, 1988), is expressed during both spontaneous (Fig. 6B) and DR stimulation-entrained (Fig. 7B) fictive respiration. It is likely, therefore, that the pre-inspiratory neurons, which are thought to be responsible for the rhythmic activation of abdominal expiratory motoneurons (Onimaru et al. 1987, 1988; Janczewski et al. 2002; Ruangkittisakul et al. 2007), serve as preferential targets for limb afferent circuitry involved in locomotor-respiratory coupling.

Possible sensory-motor pathways that mediate locomotor-respiratory coupling

Figure 10 summarizes our overall findings and proposes neural substrates for locomotor-respiratory coupling in the neonatal rat, and possibly quadrupedal mammals in general. During normal resting levels of respiration, a combination of direct-line and segmentally-relayed inspiratory and expiratory drives are conveyed down the cord to the various cervical and thoraco-lumbar motoneuron pools responsible for rhythmic inspiratory (diaphragm and external intercostal) and expiratory (internal intercostal and abdominal) muscle contractions (Fig. 10A). During
rapid locomotion, the cyclic contractions of fore- and hindlimb muscles activate somatic proprioceptors which in turn, via direct ascending spinal pathways, provide feedback signals that entrain the respiratory rhythm-generating networks through a phasic excitation of the expiratory generator (Fig. 10B). Consequently, a coordination of locomotor and respiratory functions occurs in which stride and breathing cycle periods are coupled in a strict 1:1 phase relationship. Earlier evidence from intracellular recordings indicated that the ascending lumbar sensory pathways also provide complex collateral synaptic inputs to phrenic motoneurons en route to the brainstem (Morin and Viala 2002). However, whether (1) the fore- and hindlimb sensory pathways also directly influence other more caudal spinal motor populations and (2) afferent inputs from the four limbs operate independently or in combination and act on the same or different supra-spinal target(s), remain unanswered questions.
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REFERENCES


FIGURE LEGENDS

FIG. 1. Location of intercostal and abdominal motoneurons in the neonatal rat spinal cord determined by the application of cholera toxin coupled to Alexa Fluor 488 (CTX-AF 488) to selected external intercostal muscles in anaesthetized animals. A: Schematic of the spinal cord (left) and corresponding distribution of retrogradely-labeled intercostal motoneuron somata in the rostral thoracic (T) cord after ipsilateral CTX-AF 488 application into the left fifth intercostal space of the rib cage (right). An example of stained intercostal motoneurons at higher magnification is shown in inset (top right). B-C: Ipsilateral (B) and bilateral (C) ventral columns of stained abdominal motoneurons in the lower thoracic cord region after dye insertion into the lateral and medial abdominal muscles, respectively. Note that CTX-AF 488 was applied to the medial rectus muscle at the abdominal mid-line, which explains the staining of motoneuron somata on both sides of the spinal cord. In all photographs, the spinal cord is ventral side upwards. CC, central canal.

FIG. 2. Distribution of ventral spinal motor roots innervating intercostal and abdominal muscles. A: Schematic of the in vitro brainstem spinal cord rib cage preparation. Ventral roots were successively stimulated by single shocks during simultaneous EMG recordings from intercostal or abdominal muscles. B: Example of EMG recordings from the seventh intercostal muscle in response to successive electrical stimulation of the thoracic T4-T8 ventral roots. Maximal (max) and minimal (min) EMG response amplitudes were used to construct histograms in C. C: Distribution of ventral motor roots (or motoneurons) and their corresponding intercostal (left) and abdominal (right) muscles as determined by retrograde labeling (filled bars) and individual root stimulations (unfilled bars). For the latter, open boxes show positions...
of roots responsible for maximal muscle responses, while open bars indicate minimal response distributions (see B). Data were pooled from 24 neuroanatomical and 4 electrophysiological experiments. C, cervical; T, thoracic; L, lumbar.

FIG. 3. Rhythm resetting of spontaneous fictive respiration by electrical stimulation of the ventro-lateral medulla. Left: Schematic of the experimental protocol. The hypoglossal (XII) nerves were used as a landmark for stimulating electrode positioning. PBC, pre-Bötzinger Complex. A: Resetting of cervical (C4) and thoracic (T4) inspiratory bursting. A single stimulation (300µA, 0.1ms; at arrows) applied to the ventral surface of the medulla elicited phase-advanced (A1) or -delayed (A2) inspiratory bursts in the ongoing rhythm cycle. Arrowheads above traces show the expected time of occurrence of spontaneous C4 and T4 bursts in the absence of resetting. B: Phase-response histograms showing phase-advance (<0%) or delayed (>0%) rhythmicity as a function of stimulus timing. Numbers of stimuli in each phase (expressed as a percentage of the cycle) are indicated in parentheses. Data were collected from 9 experiments. * p<0.05; ** p<0.01. C: Suppression of respiratory-related bursting in spinal roots after an electrolytic lesion (400µA for 5s) at the site of medullary stimulation in A and B.

FIG. 4. Temporal organization of inspiratory bursting in cervico-thoracic intercostal motoneurons. A: Simultaneous multi-site recordings of spontaneous inspiratory bursts in the cranial XII (hypoglossal) nerves and spinal (C, cervical; T, thoracic; L, lumbar) ventral roots. Traces are expanded at right to show rostro-caudal delay in motoneuron burst onsets. B: Bar diagrams showing pooled measurements of the timing of rhythmic bursts in the indicated cervical and thoracic ventral roots in relation
to C5 discharge. Horizontal bars at left represent mean delays (± SEM) to burst onset relative to C5 (at 0 on lower left scale), while bars at right indicate the mean durations (± SEM) of bursts (upper scale) and the mean timing of their termination in relation to C5 (at 0 on lower right scale). NS, not significantly different. C: Scatter plot showing relationship between burst duration and burst onset delay for each thoracic ventral root. The coefficient (r) of the regression line (solid line) is indicated. Data were collected from 14 experiments. D: Schematic representation of the graded rostro-caudal activation (onset and duration) of 3 intercostal motoneuron (mn) pools along the spinal cord.

FIG. 5. Brainstem structures necessary for the genesis of expiratory phase discharge. **A-B**: Ventral root recordings of spontaneous inspiratory (C5), mixed inspiratory-expiratory (T12) and expiratory (T13) bursting in pontomedullary- (A) and medullary- (B) spinal cord preparations (also see D). Traces are expanded at right to clearly show absence of expiratory phase discharge after pons removal (in B). C: Histological control. Schematic (left) indicates positions of brainstem transection (plane b, see also D) and parasagittal sections (1, 2). Ipsilateral parasagittal sections at right (1 and 2 were made at 1360 µm and 1000 µm from midline, respectively) stained for acetylcholinesterase and showing remaining brainstem structures in a medullary preparation. D: Proportions of preparations (ratios indicated in parentheses) producing expiratory-like activity after brainstem transection. Positions of transections (at a, b, c) corresponding to each point are indicated below in a parasagittal schematic of the medullary ventral respiratory column. A5/VLP, A5 noradrenergic neurons/ventrolateral pons; BC, Bötzinger complex; LRN, lateral reticular nucleus; VII, facial nucleus; NA, nucleus ambiguous; PBC, pre-Bötzinger
complex; RTN/pFRG, retrotrapezoid nucleus/parafacial respiratory group; SO, superior olive; VII nerve, facial nerve; X nerve, vagal nerve; XII nerve, hypoglossal nerve.

FIG. 6. Spatiotemporal organization of inspiratory/expiratory activity in spinal intercostal and abdominal motoneurons. A: Raw inspiratory- and expiratory-phase activity recorded from cervical (C5) and thoracic (T11, T13) ventral roots. B: Expanded cervical and thoracic ventral root traces showing alternation between intercostal inspiratory and abdominal expiratory discharge (left panel). Shading indicates expiratory activity. In the same preparation, when two inspiratory bursts occurred spontaneously in a cycle, expiratory-timed discharge also occurred in addition to inspiratory bursts at the T7 level (right panel). C: Compilation of data from 10 experiments. Bars indicate the timing of intercostal inspiratory (unfilled bars) and abdominal expiratory activity (filled bars) in the indicated ventral roots during the production of single (left) or double inspiratory bursts per cycle (right).

FIG. 7. Respiratory rhythm entrainment by cyclic activation of hindlimb (A) or forelimb (B) somatic afferent axons. Schematic representations of the experimental protocol (at left) indicating that hind- or forelimb sensory input pathways were activated by low-threshold lumbar (L2 in A) or cervical (C8 in B) dorsal root (DR) stimulation, respectively. Corresponding recordings (right panels), showing that DR stimulation (indicated by vertical dotted lines) at either lumbar (A) or cervical (B) cord levels caused respiratory rhythm entrainment. Arrowheads above traces indicate the expected time of occurrence of spontaneous C4, T4 and T13 respiratory bursts in the
absence of entrainment. Expanded traces of single DR stimulation-evoked cycles are shown at right in A and B.

FIG. 8. Effects of mid-thoracic cord axonal conduction blockade on cervical (A) and lumbar (B) DR stimulation-induced respiratory entrainment. Cyclic DR stimulation at C7 (A) or L5 (B) was used to activate fore- or hindlimb sensory pathways, respectively. Drawings of isolated preparations (at left) show position of isotonic sucrose application to the thoracic cord from T1 to T7. Conduction blockade did not affect respiratory rhythmicity recorded at C4 or its entrainment by C7 DR stimulation, although all spontaneous activity was abolished at T12 (A, middle panel). In contrast, L5 DR stimulation during mid-thoracic blockade did not affect respiratory activity recorded at more caudal C4 (B, middle panel). Arrowheads above each recording panel indicate the expected time of occurrence of C4 (and T12) respiratory bursts in the absence of entrainment. The lower panels in B show recording segments indicated in upper B on a faster time base. Note that during the mid-thoracic blockade, the T12 ventral root activity elicited by L5 DR stimulation (at an intensity two-fold stronger than in control) occurred at short latency and was unrelated to ongoing fictive respiration.

FIG. 9. Effects of mid-thoracic cord synaptic blockade on DR stimulation-induced entrainment (A, B) and spontaneous cervical (C4) and low thoracic (T11) respiratory bursting (C). A: Cervical C8 DR stimulation under control conditions (left), during exposure of the thoracic cord (T1-T10) to low calcium saline to block synaptic transmission (middle), and after washout with normal saline (right). B: Lumbar L2 DR stimulation under the same conditions. C1: Integrated C4 (upper) and T11 (lower)
discharge before and during low calcium saline application to the mid-thoracic cord. Traces are averages of 15 spontaneous bursts taken from the experiment shown in B. C2: Group analysis. Mean areas (± SEM) of integrated C4 (upper) and T11 (lower) spontaneous inspiratory (at C4 and T11) and expiratory (at T11) phase discharge before and during low calcium saline superfusion of the mid-thoracic cord. Whereas inspiratory activity at C4 was unaffected, both inspiratory and expiratory discharge at T11 was decreased. Data were pooled from preparations similar to that in B, with the total numbers of preparations indicated in parentheses. Analyses were only performed on preparations in which inspiratory or expiratory phase activity persisted until and following low calcium saline washout. a.u.: arbitrary unit; *** p<0.001; * p<0.05; NS, not significant.

FIG. 10. Summary diagram of spinal circuitry involved in the activation of phrenic, intercostal and abdominal motoneurons during normal respiration (A), and during fast locomotion when phasic sensory inputs from forelimbs and hindlimbs entrain locomotor-respiratory coupling (B). Corresponding patterns of inspiratory (insp) and expiratory (exp) burst activity at C4 and in three different thoracic ventral roots (VR) are schematized in lower panels. The intracellular activity of phrenic motoneurons (see C4 Mn trace) under the two conditions has been previously described (Morin and Viala 2002). See text for further explanation.
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FIG. 6. Spatiotemporal organization of inspiratory/expiratory activity in spinal intercostal and abdominal motoneurons. A: Raw inspiratory- and expiratory-phase activity recorded from cervical (C5) and thoracic (T11, T13) ventral roots. B: Expanded cervical and thoracic ventral root traces showing alternation between intercostal inspiratory and abdominal expiratory discharge (left panel). Shading indicates expiratory activity. In the same preparation, when two inspiratory bursts occurred spontaneously in a cycle, expiratory-timed discharge also occurred in addition to inspiratory bursts at the T7 level (right panel). C: Compilation of data from 10 experiments. Bars indicate the timing of intercostal inspiratory (unfilled bars) and abdominal expiratory activity (filled bars) in the indicated ventral roots during the production of single (left) or double inspiratory bursts per cycle (right).
FIG. 7. Respiratory rhythm entrainment by cyclic activation of hindlimb (A) or forelimb (B) somatic afferent axons. Schematic representations of the experimental protocol (at left) indicating that hind- or forelimb sensory input pathways were activated by low-threshold lumbar (L2 in A) or cervical (C8 in B) dorsal root (DR) stimulation, respectively.

Corresponding recordings (right panels), showing that DR stimulation (indicated by vertical dotted lines) at either lumbar (A) or cervical (B) cord levels caused respiratory rhythm entrainment. Arrowheads above traces indicate the expected time of occurrence of spontaneous C4, T4 and T13 respiratory bursts in the absence of entrainment.

Expanded traces of single DR stimulation-evoked cycles are shown at right in A and B.
FIG. 8. Effects of mid-thoracic cord axonal conduction blockade on cervical (A) and lumbar (B) DR stimulation-induced respiratory entrainment. Cyclic DR stimulation at C7 (A) or L5 (B) was used to activate fore- or hindlimb sensory pathways, respectively. Drawings of isolated preparations (at left) show position of isotonic sucrose application to the thoracic cord from T1 to T7. Conduction blockade did not affect respiratory rhythmicity recorded at C4 or its entrainment by C7 DR stimulation, although all spontaneous activity was abolished at T12 (A, middle panel). In contrast, L5 DR stimulation during mid-thoracic blockade did not affect respiratory activity recorded at more caudal C4 (B, middle panel). Arrowheads above each recording panel indicate the expected time of occurrence of C4 (and T12) respiratory bursts in the absence of entrainment. The lower panels in B show recording segments indicated in upper B on a faster time base. Note that during the mid-thoracic blockade, the T12 ventral root activity elicited by L5 DR stimulation (at an intensity two-fold stronger than in control) occurred at short latency and was unrelated to ongoing fictive respiration.
FIG. 9. Effects of mid-thoracic cord synaptic blockade on DR stimulation-induced entrainment (A, B) and spontaneous cervical (C4) and low thoracic (T11) respiratory bursting (C). A: Cervical C8 DR stimulation under control conditions (left), during exposure of the thoracic cord (T1-T10) to low calcium saline to block synaptic transmission (middle), and after washout with normal saline (right). B: Lumbar L2 DR stimulation under the same conditions. C1: Integrated C4 (upper) and T11 (lower) discharge before and during low calcium saline application to the mid-thoracic cord. Traces are averages of 15 spontaneous bursts taken from the experiment shown in B. C2: Group analysis. Mean areas (± SEM) of integrated C4 (upper) and T11 (lower) spontaneous inspiratory (at C4 and T11) and expiratory (at T11) phase discharge before and during low calcium saline superfusion of the mid-thoracic cord. Whereas inspiratory activity at C4 was unaffected, both inspiratory and expiratory discharge at T11 was decreased. Data were pooled from preparations similar to that in B, with the total numbers of preparations indicated in parentheses. Analyses were only performed on preparations in which inspiratory or expiratory phase activity persisted until and following low calcium saline washout. a.u.: arbitrary unit; *** p<0.001; * p<0.05; NS, not significant.
FIG. 10. Summary diagram of spinal circuitry involved in the activation of phrenic, intercostal and abdominal motoneurons during normal respiration (A), and during fast locomotion when phasic sensory inputs from forelimbs and hindlimbs entrain locomotor-respiratory coupling (B). Corresponding patterns of inspiratory (insp) and expiratory (exp) burst activity at C4 and in three different thoracic ventral roots (VR) are schematized in lower panels. The intracellular activity of phrenic motoneurons (see C4 Mn trace) under the two conditions has been previously described (Morin and Viala 2002). See text for further explanation.