An overview of phrenic nerve and diaphragm muscle development in the perinatal rat

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Greer, John J., Douglas W. Allan, Miguel Martin-Caraballo, and Robert P. Lemke. An overview of phrenic nerve and diaphragm muscle development in the perinatal rat. J. Appl. Physiol. 86(3): 779–786, 1999.—In this overview, we outline what is known regarding the key developmental stages of phrenic nerve and diaphragm formation in perinatal rats. These developmental events include the following. Cervical axons emerge from the spinal cord during embryonic (E) day 11. At ~E12.5, phrenic and brachial axons from the cervical segments merge at the brachial plexi. Subsequently, the two populations diverge as phrenic axons continue to grow ventrally toward the diaphragmatic primordium and brachial axons turn laterally to grow into the limb bud. A few pioneer axons extend ahead of the majority of the phrenic axonal population and migrate along a well-defined track toward the primordial diaphragm, which they reach by E13.5. The primordial diaphragmatic muscle arises from the pleuroperitoneal fold, a triangular projection of the body wall composed of the fusion of the primordial pleuroperitoneal and pleuropericardial tissues. The phrenic nerve initiates branching within the diaphragm at ~E14, when myoblasts in the region of contact with the phrenic nerve begin to fuse and form distinct primary myotubes. As the nerve migrates through the various sectors of the diaphragm, myoblasts along the nerve’s path begin to fuse and form additional myotubes. The phrenic nerve intramuscular branching and concomitant diaphragmatic myotube formation continue to progress up until E17, at which time the mature pattern of innervation and muscle architecture are approximated. E17 is also the time of the commencement of inspiratory drive transmission to phrenic motoneurons (PMNs) and the arrival of phrenic afferents to the motoneuron pool. During the period spanning from E17 to birth (gestation period of ~21 days), there is dramatic change in PMN morphology as the dendritic branching is rearranged into the rostrocaudal bundling characteristic of mature PMNs. This period is also a time of significant changes in PMN passive membrane properties, action-potential characteristics, and firing properties.

THE FUNDAMENTAL PROBLEM of how the phrenic nerve and diaphragm muscle develop in utero has been a long-standing issue in embryology. The standard drawings and descriptions found in medical textbooks reflect the traditional views of phrenic nerve and diaphragm embryogenesis, which have been based largely on interpretations of anatomic dissections (33). Some texts add the important caveat stating that these are simply interpretations and that there is actually very little data on which to base the assumptions (17, 29). Regardless, these descriptions have been propagated in the literature to the point where they are often cited as dogma. This point, as it pertains to the diaphragm, was succinctly put in a recent commentary by Professor J. C. McLachlan (26): “I would be happy to give a pound to everyone who really understands the development of the diaphragm, if in return I received a pound from everyone who has merely committed the official description (and those diagrams) to memory.” The lack of concrete data on which to base an understanding of...
phrenic nerve and diaphragm development could be attributed largely to the previous unavailability of appropriate experimental tools for studying mammalian nerve-muscle formation. However, with the advances in the field of developmental biology allowing for a clearer delineation of developing axons and musculature, this issue can now be rigorously examined. In this overview, we reassess phrenic nerve-diaphragm development based on data from studies that take advantage of the improved methodology. Specifically, we discuss the embryology and functional properties of the rat phrenic nerve and diaphragm during the perinatal period (key events are summarized in Fig. 1). Whereas much has been learned from other animal models regarding the ontogeny of fetal breathing, from the sheep in particular, the majority of data specifically concerning multiple aspects of phrenic nerve-diaphragm development in the embryonic period has arisen from studies in which rat models were used and thus, this is the focus of this review.

The study of phrenic nerve-diaphragm ontogeny is of general interest to those examining the development of the mammalian respiratory system. From a clinical perspective, an understanding of the normal developmental processes will provide the necessary foundation for examining the pathogenesis of the often lethal developmental anomaly congenital diaphragmatic hernia (1). In the broader context of developmental biology, a comprehensive examination of the embryogenesis and of the multitude of controlling factors operating to bring about the formation of a single, well-identified mammalian nerve-muscle functional unit is lacking. The phrenic nerve-diaphragm system in perinatal rats has a number of characteristics that make it particularly amenable and valuable for addressing these issues. First, the complete migratory path of the growing axons and developing musculature can be clearly visualized via immunolabeling, because they are not co-localized anatomically with other motor nerves and muscles and the diaphragm is a thin, essentially two-dimensional structure (e.g., see Figs. 2–4). Second, these anatomic features facilitate the elucidation of the spatiotemporal expression patterns of developmentally regulated molecules via immuno- and in situ hybridization labeling at various stages of nerve-muscle formation and interaction. Third, the rat phrenic nerve-diaphragm is a relatively simple system consisting of ~220 motoneurons and one muscle (11). In contrast, when examining the development of the hindlimb before individual muscles in the limb separate from the undifferentiated primordial muscle mass. Fourth, considering that axonal outgrowth and myogenesis are in part controlled by changes in synaptic inputs and electrophysiological properties, a system in which the multiple facets can be studied in parallel is desirable. As described below, this is being achieved in the rat phrenic nerve-diaphragm model where we have utilized in vitro spinal cord models to determine when phrenic motoneurons (PMNs) first receive [embryonic day 17 (E17)] descending inspiratory drive and undergo changes in motoneuron firing properties, ionic currents, and cell morphology, in parallel with our studies of phrenic nerve-diaphragm embryogenesis.

INITIAL STAGES OF PHRENIC AXON OUTGROWTH AND CONTACT WITH THE PRIMORDIAL DIAPHRAGM

There are two very basic questions that require clarification pertaining to the early outgrowth of phrenic axons. First, what is the migratory pathway that phrenic axons traverse to innervate the primordial diaphragm? Second, what is the primordial diaphragmatic target? The classic view of phrenic nerve-diaphragm embryology states that phrenic axons migrate from the cervical spinal cord to innervate the dorsal portion of the septum transversum, located at approximately the same rostrocaudal level as the emerging phrenic axons. Subsequently, the septum transversum and the attached phrenic nerve descend to a lower
level in the thorax as the heart and lung enlarge within the thoracic cavity (22). However, these ideas were challenged by Noakes et al. (27), who proposed that the primordial diaphragm has descended to the lower thoracic cavity before innervation by phrenic axons. Furthermore, they proposed that phrenic axons migrate caudally en masse adjacent to the cardinal vein, without a clear indication of leading axons, which could act as pioneers toward the target.

Recently, we systematically readdressed the issues of phrenic axon outgrowth and target primordial musculature (2, 4) by using immunohistochemical markers that delineate developing nerve and muscle tissue in fetal rats. These studies demonstrated the following details regarding phrenic nerve-diaphragm embryogenesis. 1) Cervical axons emerge from the spinal cord during E11 (gestational period is ~21 days). 2) At ~E12.5, phrenic and brachial axons from the cervical segments merge at the brachial plexi. 3) Subsequently, the two populations diverge as phrenic axons continue to grow ventrally toward the diaphragmatic primordium and brachial axons turn laterally to grow into the limb bud (Fig. 2). A few pioneer axons extend ahead of the majority of phrenic axonal population and migrate along a well-defined track toward the primordial diaphragm, which they reach by E13.5. 4) In agreement with the classic view, the primordial target for phrenic nerve outgrowth is initially positioned rostrocaudally at the approximate level of emerging phrenic axons from the cervical spinal cord (Fig. 3). However, the primordial target appears to be the pleuroperitoneal fold (PPF), rather than the septum transversum (33) or posthepatic mesenchymal plate (14), as often suggested. The PPF is a triangular protrusion of the body wall composed of the fusion of the primordial pleuroperitoneal and pleuropericardial tissues. This fold of tissue extends medially, tapering as it does so, to fuse with the ventral aspect of the primary esophageal mesentery. The ventral portion of this fold appears fused with the dorsal aspects of the liver and septum transversum.

Our present focus is directed toward examining and understanding the embryogenesis of the PPF. This will necessitate delineating the somitic source of the muscle progenitors in conjunction with studies of muscle-precursor migration, proliferation, and differentiation. Such data will provide key information toward clarifying the early stages of diaphragm embryogenesis and are of clinical interest in light of recent studies demonstrating that the diaphragmatic defect associated with an animal model of congenital diaphragmatic hernia can be traced back to the formation of the PPF (1).

ONSET OF PHRENIC NERVE INTRAMUSCULAR BRANCHING WITHIN THE DEVELOPING DIAPHRAGM

Initial contact and innervation of the PPF by the phrenic nerve occur on E13 (2). However, the actual initiation of phrenic nerve intramuscular branching

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Fig. 2. Phrenic axon migration toward the primordial diaphragm. A: schematic of early phrenic nerve-diaphragm development in an embryonic rat. Phrenic nerve exits cervical roots and follows a track of cells along the medial aspect of the body wall to innervate PPF. Large arrows point through pleuroperitoneal canals. B: growth-associated protein (GAP-43) immunolabeling of a transverse section of an E13 fetal rat illustrates the migratory path of phrenic axons (Ph) beyond brachial plexus toward PPF. CV, cardinal vein; Br, brachial axons; DRG, dorsal root ganglion; FL, forelimb. C: transverse section of E12.75 fetal rat immunolabeled for the low-affinity nerve growth (p75) receptor. Phrenic and brachial nerves also express the p75 receptor. Immunolabeling for the p75 receptor has been demonstrated to be a convenient means of detecting developing neural and muscle tissue, although the functional significance of p75 receptor expression on these tissues is presently unknown. Leading phrenic axons have just separated from the brachial plexus and are located at ventral end of a well-defined track (arrow) of p75 receptor-expressing tissue (~50 µm in diameter), situated along the migratory path toward the PPF (+). This track of cells could be providing a guidance or permissive substratum on which the subpopulation of leading phrenic axons grows. Axonal populations and the cellular track also express neural cell adhesion molecule (NCAM; data not shown). Scale bars = 50 µm. (Adapted from Allan and Greer (1, 2).)
commences ~24 h after initial contact, when the nerve and developing PPF have descended caudally toward the level of the middle to lower thoracic spinal cord (Fig. 3). It is not clear whether the waiting period results from limitations with the nerve and/or target, which prevent initial synapse and myotube formation. Previous electrophysiological studies have demonstrated that, by E14, the earliest age studied, cervical motoneurons are electrically excitable and their axons are capable of transmitting action potentials (10). Thus the potential for the induction and localization of acetylcholine-receptor expression in the diaphragm by presynaptic electrical activity in the phrenic nerve exists on E14 [see Hall and Sanes (12) for review]. Once the intramuscular branching commences within the diaphragm, there are precise, but as-yet-unidentified, guidance mechanisms for establishing the characteristic bilateral entry points of the phrenic nerves and the tertiary intramuscular branching pattern consisting of crural, sternal, and dorsolateral primary branches (20, 21).

RELATIONSHIP BETWEEN PHRENIC INTRAMUSCULAR BRANCHING AND DIAPHRAGM MYOTUBE FORMATION

There has been controversy in the literature regarding the nature of the interaction between intramuscular branches of phrenic axons and diaphragmatic myotubes. Bennett and Pettigrew (5) initially reported that primary myotubes formed in the diaphragm in a sequential fashion, which paralleled the extent of outgrowth of primary phrenic intramuscular branches. However, later reports by Harris (13) suggested that primary myotube formation within the diaphragm occurred throughout the muscle mass, with no apparent relationship between the arrival of growing phrenic intramuscular branching and myotube formation. Results from our recent studies (2) clearly support the earlier interpretation of Bennett and Pettigrew (5). At the earliest time of phrenic nerve branching within the diaphragm, the myoblasts in the region of contact with the nerve begin to fuse and form distinct primary myotubes. As the nerve migrates through the various sectors of the diaphragm, myoblasts along the nerve's...
path begin to fuse and form additional myotubes (Fig. 4). These data are in agreement with the general idea that, whereas some primary myotubes will eventually form in an aneural muscle, axons typically impose regulatory influences from the time of initial contact, which modulate and facilitate myotube formation via electrically mediated effects and/or diffusible substances (12, 16, 32). The data illustrated in Fig. 4 also demonstrate that there is a radiation of myotube elongation from the point of nerve innervation at the center of the fibers medially toward the central tendon and laterally toward the lateral edge of the diaphragm, as new myoblasts are likely absorbed at the ends of myotubes (34). Whereas further studies need to be performed, there is no clear indication to date that myoblasts originate from sources other than the PPF, as suggested in past interpretations of diaphragm embryogenesis (33). Phrenic nerve intramuscular branching and concomitant diaphragmatic myotube formation continue to progress through to age E17.5, at which time the mature pattern of innervation and muscle architecture are approximated. Thus close to the time of the inception of respiratory drive transmission in utero (E17; Ref. 10), the phrenic nerve-diaphragm system has developed to the point where it is operational. Further development of the phrenic nerve occurs during the first 1–2 wk postnatally, with the retraction of polysynaptic neuromuscular terminals (5) and the myelination of large-caliber afferent and efferent axons (28).

MATURATION OF PMN MORPHOLOGICAL AND ELECTROPHYSIOLOGICAL PROPERTIES

During the period of phrenic axon outgrowth and intramuscular branching, PMN somata and dendritic morphology are very immature (3). It is not until the inception of respiratory drive (~E17) that previously apposed PMN somata begin to separate and the characteristic rostrocaudally projecting dendrites begin to form. The first central afferent processes from the cervical dorsal root ganglion also reach the vicinity of PMN dendrites at age E17 (3). We have recently begun to perform functional studies to test the hypothesis that there will be a concomitant maturation of PMN electrophysiological properties prior and subsequent to the
critical developmental events spanning the E16-birth period, which include major morphological reorganization, the completion of target musculature innervation, the onset of afferent and descending respiratory synaptic drive in utero, and continuous rhythmic recruitment at birth. These studies utilize whole cell patch recordings of antidromically identified PMNs within a cervical slice phrenic nerve preparation, isolated from perinatal rats at ages E16, E18, and postnatal (P) day 0–1.

Age-dependent changes in passive membrane properties, action-potential characteristics, repetitive firing properties, and neuronal coupling have been examined (24, 25).

Measurements of PMN passive membrane properties during this period have demonstrated that between E16 and P0 the resting membrane potential becomes significantly more hyperpolarized (~10 mV) without a significant change in threshold, whereas there are significant decreases in the input resistance (~3 times).

Fig. 5. Perinatal phrenic motoneuron (PMN) firing properties. A: left top, examples of repetitive firing patterns generated in E16, E18, and P0 PMNs after injection of a depolarizing square pulse (1-s duration) from a membrane holding potential of approximately ~60 mV. Firing frequencies (means ± SE) in response to lowest stimulation current able to evoke repetitive firing (threshold (I_{th})) and at twice threshold are listed in right for PMNs at ages E16 (top; n = 18), E18 (middle; n = 16), and P0 (bottom; n = 21). B: representative current frequency plot for PMNs at various ages studied. With increasing age, there are increases the amount of current necessary to initiate firing, in maximal attainable firing frequencies, and in slope of current-frequency curves.

Fig. 6. Evidence for electrical and dye coupling between subpopulations of PMNs. A: multiple criteria were used as evidence for electrical coupling between motoneurons. Low-amplitude, short-latency depolarizations (SLDs) were evoked by subthreshold antidromic stimulation of phrenic nerve in a P0 slice (dorsal roots cut). In this particular case, subthreshold stimulations generated a graded response consisting of at least 2 SLDs (arrows). SLDs are depolarizations caused by spread of current from adjoining PMNs, which have reached threshold and fired action potentials in response to antidromic stimulation of phrenic nerve. Increments in stimulation intensity to the nerve eventually induced an antidromic action potential in the PMN being recorded from. Neither the antidromic spike nor the SLDs were eliminated when synaptically mediated events were depressed by bathing the sample in a calcium-free buffer (not shown). B: in a different P0 motoneuron, low-amplitude SLDs could be clearly seen when action-potential production within PMN being recorded from was prevented by addition of the intracellular blocker of sodium channels QX 314 (1.5 mM) to the recording pipette. Holding of membrane potential at various levels did not affect the amplitude of electrotonic potentials, providing further evidence for the lack of a synaptic source for the SLDs. C: photomicrograph of a 30-μm-thick transverse section from a 16-day-old embryo, showing presence of Lucifer Yellow labeling in at least 3 PMNs. Only 1 neuron per cervical slice preparation was recorded from when Lucifer-Yellow-containing electrodes were used. Dye coupling appears to be between dendrodendritic couplings, as the soma are not apposed. Scale bar = 10 μm.
lower) and time constant (~1.4 times shorter). Thus PMNs require significantly less depolarizing current to reach threshold at the inception of inspiratory drive (E17) compared with more mature states (Fig. 5; rheobase current at E16 is ~2.5 times less than at P0). Functionally, the increased propensity for reaching firing threshold will compensate for what appears to be a relatively weak descending inspiratory drive at this age (9, 10) and thus will facilitate the production of fetal breathing movements.

Along with changes in passive membrane properties, the characteristics of PMN action potentials develop during the period spanning E16 to P0. The amplitude increases by ~12 mV, and the duration decreases by ~50%. The action potentials of PMNs at all ages studied are sodium dependent; however, calcium currents contribute significantly to the broadening of action potentials at age E16. The action-potential spike is followed by afterpotentials of various shapes depending on age. At E16, the main spike is followed by a slowly decrementing, calcium-dependent afterdepolarization, with no clear indication of an afterhyperpolarization. Through ages E18 to P0, a prominent humplike afterdepolarization and a medium-duration afterhyperpolarizing potential, which is mediated by calcium-dependent potassium currents, develop. Concomitant with changes in the duration and shape of action potentials, there is a marked change in the repetitive firing properties of PMNs during the period spanning E16-P0 (Fig. 5). At P0, PMNs fire at approximately two times the maximum discharge frequency achieved at E16. The net results of the changes in the passive and action-potential properties are that, by birth, while requiring a stronger synaptic drive to initiate firing, PMNs are capable of driving the diaphragm musculature to produce greater contractile forces, in comparison with those generated in utero.

Both dye and electrical coupling have been detected among subpopulations of PMNs between ages E16 and P0 (Fig. 6). This is in contrast to the mature state, where it is clear that neuronal coupling among PMNs does not exist (23). We propose that the presence of neuronal coupling early in development and its absence in the adult would be functionally appropriate. The central nervous system utilizes two fundamental strategies for increasing the force produced by a muscle. First, the firing frequency of a given motor unit can be increased. Second, additional motor units can be recruited. At the inception of fetal respiratory movements, the maximum attainable firing frequency of PMN discharge is limited. However, the presence of neuronal coupling facilitates the second strategy of increasing the number of motor units recruited for a given descending synaptic drive. Thus, although the descending drive may be relatively weak and the maximum discharge frequency of PMNs low, the presence of coupling among the neuronal population will facilitate adequate synchronous drive to the diaphragm for the purposes of generating perinatal breathing movements. As the animal matures postnatally, the situation changes to one where <30% of the motoneuron pool is recruited during an inspiratory effort at rest (8, 31). Therefore, it would seem inappropriate and disadvantageous for neuronal coupling to persist among the PMN pool at a time when precise, graded recruitment is desired.

There are a number of important issues arising from the electrophysiological studies that deserve further study. First, it would be of interest to examine the continued differentiation of PMN electrophysiological properties throughout the postnatal period when polygonal neuronal innervation is withdrawn and neuromuscular synapses are stabilized [see Cameron et al. (6–8) for extensive analyses of PMN postnatal development in kittens]. Second, voltage-clamp analyses are being performed to determine the ontogenic profile of voltage-sensitive channel expression within PMNs to better understand the ionic mechanisms underlying the profound changes in action potential and repetitive firing properties observed. Third, ongoing examinations of diaphragm muscle contractile and histochemical properties during the stages E16-P0 will provide information regarding the concomitant development of motoneurons and muscle. Fourth, the correlation between changes in PMN electrophysiological and morphological properties with the onset of synaptic drive transmission and innervation of the target musculature requires further study to determine the degree of interdependence between these developmental processes. Of particular interest would be an assessment of the potential retardation of PMN and diaphragm maturation due to the suppression of inspiratory drive transmission in utero. There is an increasing impetus for understanding the neuromuscular mechanisms controlling fetal breathing movements and the potential link between the suppression of these movements (e.g., as a result of fetal exposure to hypoxia, alcohol, opiates, cigarette smoke) and neonatal respiratory disorders (15). Specifically, there has been speculation that abnormalities in the maturation of diaphragmatic control and/or function may be related to a subset of infant mortalities categorized as sudden infant death syndrome (18, 30).

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