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Identification of the neural pathway underlying spontaneous crossed phrenic activity in neonatal rats

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Abstract

Cervical spinal cord hemisection at C2 leads to paralysis of the ipsilateral hemidiaphragm in rats. Respiratory function of the paralyzed hemidiaphragm can be restored by activating a latent respiratory motor pathway in adult rats. This pathway is called the crossed phrenic pathway and the restored activity in the paralyzed hemidiaphragm is referred to as crossed phrenic activity. The latent neural pathway is not latent in neonatal rats as shown by the spontaneous expression of crossed phrenic activity. However, the anatomy of the pathway in neonatal rats is still unknown. In the present study, we hypothesized that the crossed phrenic pathway may be different anatomically in neonatal and adult rats. To delineate this neural pathway in neonates, we injected Wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP), a retrograde transsynaptic tracer, into the phrenic nerve ipsilateral to hemisection. We also injected Cholera toxin subunit B-horseradish peroxidase (BHRP) into the ipsilateral hemidiaphragm following hemisection in other animals to determine if there are midline-crossing phrenic dendrites involved in the crossed phrenic pathway in neonatal rats. The WGA-HRP labeling was observed only in the ipsilateral phrenic nucleus and ipsilateral rVRG in the P2, P7, and P28 hemisected rats. Bilateral labeling of rVRG neurons was shown in P35 rats. The BHRP study showed that many phrenic dendrites cross the midline in P2 neonatal rats at both rostral and caudal parts of the phrenic nucleus. There was a marked reduction of crossing dendrites observed in P7 and P28 animals and no crossing dendrites observed in P35 rats. The present results suggest that the crossed phrenic pathway in neonatal rats involves the parent axons from ipsilateral rVRG premotor neurons that cross at the level of obex as well as decussating axon collaterals that cross over the spinal cord midline to innervate ipsilateral phrenic motoneurons following C2 hemisection. In addition, midline-crossing dendrites of the ipsilateral phrenic motoneurons may also contribute to the crossed phrenic pathway in neonates.

Keywords

phrenic motoneurons; spinal cord; hemisection; crossed phrenic pathway

Cervical spinal cord hemisection results in the paralysis of the ipsilateral hemidiaphragm, subsequently leading to respiratory insufficiency which is the major cause of morbidity and mortality in human spinal cord injury (Golder and Mitchell, 2005; Fuller et al., 2006;

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Goshgarian et al., 1986). The hemisection interrupts the descending respiratory drive from bilateral respiratory premotor neurons in the medulla to phrenic neurons in the spinal cord (Dobbins and Feldman 1994; Ellenberger et al., 1990). The respiratory premotoneurons are located mainly in the rostral ventral respiratory group (rVRG) and Bötzing complex in the medulla and innervate phrenic motoneurons (Monteau and Hilaire 1991, Dobbins and Feldman 1994). We have previously shown that after C2 hemisection, function of the paralyzed hemidiaphragm can be restored after the administration of such drugs as theophylline or rolipram in adult rats (Nantwi and Goshgarian, 1998; Kajana and Goshgarian, 2008). The restored function of the paralyzed hemidiaphragm is mediated by activating a latent, non-functional neural pathway which has been referred to as “the crossed phrenic pathway” (Goshgarian, 2003; Moreno, et al., 1992; Goshgarian, 1979). This neural pathway in adult rats has been delineated anatomically as the collaterals from both crossed and uncrossed descending bulbospinal axons that cross the midline of the spinal cord and monosynaptically innervate both left and right phrenic motoneurons (Boulenguez, et al., 2007; Moreno, et al., 1992). Recently, there has been a suggestion that propriospinal neurons may relay descending respiratory drive to the phrenic nucleus (Lane et al., 2008).

Although the crossed phrenic pathway is latent and non-functional in adult rats, we have recently shown that the neural pathway is active and functional during early postnatal development in both an *in vitro* study as well as an *in vivo* study (Zimmer and Goshgarian, 2005; Huang and Goshgarian, 2009). Both studies indicated that there was a gradual reduction in spontaneous crossed phrenic activity during postnatal development. During postnatal development, the crossed phrenic pathway converted from a functional to a latent state by postnatal day P35 (Huang and Goshgarian, 2009). Despite this important discovery, the bulbospinal respiratory neural pathway which underlies spontaneous crossed phrenic activity has not been delineated in neonatal rats.

Many studies suggest that the anatomical organization of the central respiratory network during early development is different than it is in adult rats (Allan and Greer, 1997; Ellenberger, 1999; Prakash, et al., 2000; Li and Duffin, 2004; Li, et al., 2003). For example, the dendrites of phrenic motoneurons cross the midline of the spinal cord in neonatal rats and the percentage of crossing phrenic dendrites is significantly higher in very young rats as compared to adult rats (Song, et al., 2000; Prakash, et al., 2000). Furthermore, other aspects of respiratory control and breathing patterns in mammals undergo change during development (Thoby-Brisson and Greer, 2008; Cameron and Nunez-Abades, 2000). Some developmental studies have shown that left and right phrenic nerve outputs are synchronized by using cross-correlation analyses in neonatal and adult rats (Li and Duffin, 2004; Li, et al., 2003). In adult rats, respiratory premotor neurons project bifurcating axons to excite phrenic motoneurons located on both sides of the spinal cord. These bifurcating axons are primarily responsible for the synchronized output of both the right and left phrenic discharge (Li and Duffin, 2004). However, the respiratory transmission and synchronization are different between neonatal rats and adult rats (Li and Duffin, 2004; Li, et al., 2003). Therefore, it has been indicated that the organization of central respiratory pathway changes during postnatal development. These changes may contribute to the conversion of spontaneous crossed phrenic activity from an active to a latent state. Accordingly, the purpose of the present study was to delineate the crossed phrenic pathway in neonatal rats.

Materials and methods

Animal surgery protocol

Timed pregnant female Sprague-Dawley rats were purchased from Harlan Rodent Laboratories and allowed to give birth in the animal care facilities at Wayne State University, School of Medicine. Litters of rat pups were housed with mothers together and individual postnatal rats

were brought to the laboratory before each experiment. P2 was selected as the initial postnatal time to be analyzed because rat pups younger than P2 are too small to undergo the following surgery.

Postnatal rats were prepared for aseptic hemisection surgery of the left C2 spinal cord following anesthetization with ketamine (30-40mg/kg, ip) and xylazine (10mg/kg, ip). A dorsal midline incision (1cm) was made through the cervical skin and the paravertebral muscles above the first three cervical vertebrae to expose the second vertebra. A laminectomy of the C2 vertebral bone and durotomy were performed to expose the cervical spinal cord. A left C2 spinal cord hemisection was performed with microscissors just caudal to the C2 dorsal rootlets under magnification. Then, the paravertebral muscles were sutured at the midline with 5.0 absorbable sutures and the skin was closed with tissue glue.

WGA-HRP and BHRP labeling

The hemisected postnatal rats were divided into 2 groups. Group 1 was subjected to Wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP, Sigma, St. Louis, MI) injection and group 2 was subjected to Cholera toxin subunit B-horseradish peroxidase (BHRP, Sigma, St. Louis, MI) injection. Furthermore, each group of postnatal rats from different litters was separated into 4 subgroups with different ages: P2, P7, P28, and P35 (N=10 per age group, female/male). Other rat pups served as non-hemisected controls (N=4 per age group, female/male). Immediately following the hemisection surgery, the rats were placed in a supine position and the left phrenic nerve was exposed after a small incision was made on the ventral side of the neck. WGA-HRP (2% 10 μ l diluted in 0.9% saline) was injected into the left phrenic nerve (i.e., ipsilateral to C2 hemisection) slowly by using a 10 μ l Hamilton syringe fitted with a special needle (Hamilton 27-33 gauge RN needle). The neck muscles were sutured and skin closed with tissue glue. Following injection with warm 0.9% saline to prevent dehydration (10 μ l/g, subcutaneous), the pups were placed on a heating pad until awake (approximately 1 hr) and then returned to their mother. Rat pups were monitored afterward to ensure the mother would accept and feed them. Mother rats were desensitized to odors related to the surgical procedures and human handling by daily contact with lab personnel and by placing gauze soaked with antiseptics and surgical glue into the cage for several days before the birth of the rat pups. The desensitization procedure helped to ensure that rat pups were accepted by the mother when they were returned to her after surgery. Since WGA-HRP is a transsynaptic tracer which retrogradely crosses active and functional synapses (Moreno et al., 1992; Jankowska, 1985), the P35 day animals were subjected to a contralateral phrenicotomy to activate the latent crossed phrenic pathway immediately before WGA-HRP injection. This was not necessary in the younger animals since an earlier study indicated that the crossed phrenic pathway is spontaneously active after hemisection in these animals (Huang and Goshgarian, 2009).

One day (N=6) or two day (N=4) following WGA-HRP injection and retrograde labeling, EMG recordings were taken from both sides of the diaphragm under anesthetization (details shown in Huang and Goshgarian, 2009). After this, the pups were transcardially perfused with 10-20 ml 0.9% saline followed by 20-50 ml 2.5% glutaraldehyde in 0.1 M phosphate buffer. The brain stem and spinal cord were dissected, postfixed with 2.5% glutaraldehyde for 2 hours and cryoprotected with 30% sucrose for 3 days. A small steel pin was inserted into the dorsolateral part of the right spinal cord and medulla to make a pinhole to identify the right side from the left side in free-floating transverse sections. The medulla and spinal cord (C1-C6) were transversely sectioned (50 μ m) with a cryostat. The WGA-HRP was visualized by using the TMB technique (described in Moreno et al., 1992) and sections were mounted on chrome alum-gelatin coated slides. The slides were counterstained with 1% neutral red, dehydrated in a graded series of acetone, and then coverslipped for microscopic viewing.

To test for the presence of midline-crossing dendrites of phrenic motoneurons in postnatal rats, animals at P2, P7, P28 and P35 were injected with BHRP (four injections of 5 μ l 0.1% BHRP) into the left hemidiaphragm immediately after hemisection. BHRP has been shown to be more sensitive than the other HRP conjugates in labeling the distal dendrites of motoneurons (Wan et al., 1982). Injections were distributed evenly in all regions of the hemidiaphragm. After 48 hours of retrograde labeling, the animals were perfused as described above and the spinal cord (C1 to C6) was dissected, horizontally sectioned, stained by the TMB technique (Moreno et al., 1992) and also stained by neutral red. Histological verification of a complete C2 hemisection was carried out in each animal.

Statistic analysis

Quantification of the labeled rVRG neurons and midline-crossing phrenic dendrites in different age groups was performed in the present study. All the transverse sections of the rostral medulla and horizontal sections of the spinal cord were examined and counted for rVRG neurons and midline-crossing phrenic dendrites individually under 20 $\times$ magnification. There is variability between animals including tracer diffusion and differences in uptake, transport and histochemical staining. Thus, the quantitative data presented is meant to provide only a general idea of the changes of premotor neurons and midline-crossing dendrites involved in the crossed phrenic pathway during postnatal development. Statistical significance was analyzed by using SPSS 11 software for the Western blot experiment. A one-way multiple analysis of variance (ANOVA) was used to compare different age groups and the successive two group difference was tested by a Tukey test of Post-Hoc analysis (SPSS 11). Results were expressed as means $\pm$ SE. Significance was set at $P < 0.05$.

Results

Spontaneous crossed phrenic activity in postnatal rats

Crossed phrenic activity was recorded in P2, P7 and P35 animals as shown by EMG recordings immediately following complete C2 hemisection (Fig. 1). This activity was spontaneously expressed in the ventral, lateral and dorsal parts of the left hemidiaphragm in P2 neonatal rats although the activity ipsilateral to hemisection was markedly lower in amplitude qualitatively compared to the activity of the contralateral hemidiaphragm, (Fig. 1A). The respiratory activity of the left hemidiaphragm was coincident with activity on the right non-hemisectioned side. In P7 rats, crossed phrenic activity was still observed spontaneously, but only in the ventral part of the hemidiaphragm ipsilateral to hemisection and qualitatively reduced as compared to the activity in the P2 animals (Fig. 1B). However, during further postnatal development, P35 animals did not display any crossed phrenic activity as shown by EMG recording of the left hemidiaphragm following acute left C2 hemisection (Fig. 1C). All the cervical spinal cord lesions were analyzed and confirmed anatomically as complete hemisections in the present study (data not shown).

WGA-HRP labeling of phrenic motoneurons and rVRG premotor neurons

After WGA-HRP injection into the left phrenic nerve of P2, P7, P28 and P35 postnatal rats, labeling was observed in left phrenic motoneurons from C3 to C6 ipsilateral to the site of injection (Fig. 2). No contralateral phrenic motoneurons were labeled in the right cervical spinal cord. The retrograde labeling of spinal cord neurons was restricted to the left phrenic nucleus. In particular, there were no transynaptically labeled propriospinal neurons labeled in the spinal cord in spite of careful analysis of all the spinal cord sections from C1 to C6. Phrenic motoneurons in the P2 animal are small and are loosely separated (Fig 2A, B, C). During postnatal development, the phrenic motoneurons become larger (Fig 2D-I) and more tightly organized within the phrenic nucleus at P35 (Fig 2J, K, L).

Retrograde transynaptic transport of WGA-HRP resulted in labeling of respiratory pre-motor neurons (rVRG, rostral ventral respiratory group) in the rostral ventrolateral medulla (Fig. 3). Unlike adult rats, the WGA-HRP labeling was observed only in the left rVRG neurons, ipsilateral to the C2 hemisection in the P2, P7 and P28 rats by either one day or two days after WGA-HRP injection (Fig. 3A-I). There was no labeling in the right rVRG neurons contralateral to the C2 hemisection in these neonatal animals. Furthermore, no labeling in any other medullary nucleus was observed after careful analysis of every section of the rostral medulla in the present study. The WGA-HRP medullary labeling in P35 rats, on the other hand, was observed bilaterally in rVRG neurons (Fig. 3J, K, L) and thus was significantly different from the younger groups. For the non-hemisectioned control animals, the WGA-HRP was retrogradely and transynaptically transported to rVRG neurons bilaterally (data not shown). In addition, the number of the WGA-HRP labeled rVRG neurons was quantified in different age groups and analyzed by ANOVA (Fig. 4). The number of rVRG neurons in P2 was significantly higher than P7 and P28 ($P<0.05$, $P<0.01$ respectively). The labeled rVRG neurons in P28 was also much less compared to P7 animals ($P<0.01$).

BHRP labeling of the dendrites of phrenic motoneurons

After BHRP injection into the left hemidiaphragm, phrenic motoneurons and labeled phrenic dendrites were identified in the horizontal sections of the cervical spinal cord in P2, P7, P28 and P35 postnatal rats (Fig. 5, 6). The cell somata of phrenic motoneurons became larger qualitatively with increasing postnatal development. Many phrenic dendrites crossed the midline of the cervical spinal cord and reached the opposite side in P2 rats (Fig. 5A). High magnification showed that these midline crossing dendrites were distributed from the rostral to caudal parts of the phrenic nucleus (Fig. 5B, C). In the P7 age group, the phrenic dendrites also crossed over the midline of the cervical spinal cord (Fig. 5D). However, the midline-crossing dendrites were observed primarily in the rostral half of the phrenic nucleus (Fig. 5E). Phrenic dendrites projecting medially were much less conspicuous at the caudal pole of the nucleus in P7 rats (Fig. 5F). During postnatal development, the clustering of somata of the phrenic motoneurons was clearly apparent (Fig. 5). As compared to the younger age groups, the extent of the midline-crossing dendrites at P28 was much reduced not only in the rostral part, but also in the caudal part of the phrenic nucleus (Fig. 6A, B, C). In P35 rats, very few medially projecting phrenic dendrites were observed (Fig. 6D).

The number of the midline-crossing dendrites in different age animals were quantified and statistically analyzed by ANOVA (Fig. 7). In P2 rats, the number of the rostral crossing phrenic dendrites was significantly higher compared to P7 as well as P28 ($P<0.01$, Fig. 7A). At the caudal level of the phrenic nucleus, the P2 crossing phrenic dendrites was also much more than P7 ($P<0.01$, Fig. 7B). There were no caudal crossing phrenic dendrites in P28 and P35 animals. In addition, there was no significant difference in the number of the P2 crossing phrenic dendrites between rostral and caudal parts (Fig. 7C). However, a significant difference between rostral and caudal phrenic nucleus was found in the P7 crossing phrenic dendrites ($P<0.01$, Fig. 7D). There was no significant difference in the crossing dendrites between the hemisection and control animals within the same age group (Fig. 7).

Discussion

The present study has identified the bulbospinal respiratory pathway which may underlie the spontaneous expression of crossed phrenic activity in postnatal rats and has reported its major changes during postnatal development. At P2, P7 and P28, midline crossing collaterals from ipsilateral rVRG premotor neurons are involved in the crossed phrenic pathway after C2 hemisection (Fig. 8A). This conclusion is based on the observation that only ipsilateral rVRG neurons are transynaptically labeled in the spinal-hemisectioned neonatal rats when the phrenic

nucleus is labeled with WGA-HRP. Figure 8A shows how crossed rVRG axons cross back in the spinal cord to project to the ipsilateral phrenic motoneurons following C2 hemisection. After four postnatal weeks, the axon collaterals from the contralateral rVRG premotor neurons also cross the midline of the cervical spinal cord below the C2 hemisection site and join the crossed phrenic pathway (Fig. 8B). Therefore, the crossed phrenic pathway at the fifth postnatal week is composed of the midline-crossing collaterals from bilateral rVRG premotor neurons and is similar to the pathway of adult rats (Fig. 8B). Furthermore, no transynaptically labeled propriospinal neurons were identified in the cervical spinal cord sections from C1 to C6 in the present study. This suggests that the rVRG premotoneurons monosynaptically transmit respiratory drive to phrenic motoneurons in the postnatal rats when the WGA-HRP technique is used. In addition, our results also indicated that bilateral rVRG premotoneurons were involved in the bulbospinal respiratory pathways in non-hemisectioned postnatal animals of all ages. This finding is consistent with previous reports which have shown ipsilateral and contralateral rVRG premotoneurons monosynaptically projecting to phrenic motoneurons in adult rats by using pseudorabies virus labeling (Dobbins and Feldman, 1994; Ellenberger et al., 1990). An important distinction must be made, however. Although there are both crossed and uncrossed descending bulbospinal respiratory pathways in young postnatal rats, only the crossed descending bulbospinal pathway contains collaterals that cross back over the midline of the spinal cord to innervate phrenic motoneurons ipsilateral to hemisection. The uncrossed descending pathway projects only to ipsilateral phrenic motoneurons (Fig. 8A). By P35, the uncrossed pathway develops collaterals that project to contralateral phrenic motoneurons and thus the adult circuitry is established (Fig. 8B).

Quantitative analysis showed more rVRG neurons were labeled in P2 rats as compared to older age animals. Since retrograde transynaptic transport of WGA-HRP is directed across physiologically active connections (Moreno et al., 1992; Jankowska, 1985), this suggests that the crossed phrenic pathway is more active in younger neonatal rats than older neonatal rats. We have reported previously that crossed phrenic activity is expressed spontaneously and extensively in P2 neonatal rats following C2 hemisection (Huang and Goshgarian, 2009). Furthermore, the extent of the crossed respiratory activity gradually decreases and eventually disappears by postnatal day 35. The reduced activation of the crossed phrenic pathway results in decreased crossed phrenic activity. Thus, the gradual decrease of the spontaneous crossed phrenic activity during development is coincident with the reduced labeling of rVRG neurons in older postnatal rats. The quantification did not involve P35 animals because the crossed phrenic pathway is latent at this age. In the present study, right phrenicotomy was used to activate the latent crossed phrenic pathway in the P35 hemisectioned rats and label the rVRG neurons. Furthermore, the anatomy of the pathway at this time point is not the same as the early neonatal crossed phrenic pathway, but is closer to the adult neural pathway (Moreno et al., 1992; Boulenguez, et al., 2007).

The phrenic motor neuron pool establishes a rostrocaudal somatotopic organization on the diaphragm muscle (Laskowski and Owens, 1994; Laskowski and Sanes, 1987). Specifically, the rostral phrenic motor neurons tend to innervate the ventral part of the diaphragm while caudal phrenic motor neurons tend to innervate the dorsal part of the diaphragm. Based on this, the changes in the anatomy of the phrenic motoneurons during postnatal development as noted in the current study could represent interesting anatomical /functional correlations. It is interesting that the phrenic dendrites of P2 rats crossing the midline in both rostral and caudal parts of the phrenic nucleus corresponded to our previous observation that spontaneous crossed phrenic activity can be recorded in the ventral, lateral, and dorsal parts of the hemidiaphragm at the same age (Huang and Goshgarian, 2009). Thus, it is possible that the phrenic crossing dendrites in P2 rats contribute to the neonatal crossed phrenic activity (Fig. 8A). Moreover, the midline-crossing dendrites of phrenic motoneurons in P7 rats were markedly reduced in the caudal part of the phrenic nucleus and this developmental change may be related to the fact

that spontaneous crossed phrenic activity disappears in the lateral and dorsal parts of the ipsilateral hemidiaphragm in P7 neonates following hemisection (Huang and Goshgarian, 2009). During postnatal development at P35, no midline crossing dendrites were observed either in the rostral or caudal part of the phrenic nucleus (Fig 6D-F, 8B). This change is parallel to the timing when the crossed phrenic pathway becomes latent in the fifth postnatal week (Huang and Goshgarian, 2009). In the present study, midline crossing dendrites were confirmed only if we could follow in one section, the same dendrite from one side across the midline to the other side. This often involved changing the focal plane of the rather thick (50 μ m) sections used. Since phrenic dendrites are much less than 50 μ m in diameter, there is no possibility that we were counting the same dendrites more than once. Admittedly, however, this technique may not reveal the true length of each dendrite, because they could move out of the plane once they got to the other side. The quantification may also be an underestimate of total crossing dendrites because of this issue.

Previous anatomical studies on the perinatal development of the respiratory network have shown that phrenic motoneuron dendrites of the newborn rats extend for long distances into the white matter of the lateral funiculus and medially cross the midline (Allan and Greer, 1997; Song et al., 2000; Prakash, et al., 2000). Before the present study, however, no one has associated crossing phrenic dendrites with any functional capability of phrenic motoneurons. The medially projecting crossing phrenic dendrites may receive descending respiratory afferents from the contralateral side of the cervical spinal cord in neonates (Fig. 8A). It is presently not known if the retraction of the crossed phrenic dendrites results in an anatomical loss of contralateral descending respiratory afferents or if many of the afferent connections persist and are drawn across the spinal midline by the retracting dendrites. We do know that when crossed phrenic dendrites disappear in neonatal rat so does the spontaneous expression of the crossed phrenic pathway in the somatotopically appropriate region of the diaphragm. The fact that the latent crossed phrenic pathway can be activated in adult rats suggests that at least some of the contralateral descending respiratory axon connections persist on phrenic motoneurons ipsilateral to a complete hemisection even though many of these connections may be lost during postnatal development.

In conclusion, the pathway underlying spontaneously expressed crossed phrenic activity in spinal hemisected neonates is comprised of not only spinal cord midline-crossing collaterals from the ipsilateral rVRG premotor axons but also the midline-crossing dendrites of phrenic motoneurons. The developmental changes of the crossed phrenic pathway occur within the first four postnatal weeks. After that, the neural pathway is similar to adult rats. These new findings on the developmental change of the crossed phrenic pathway in the respiratory control system may be important in developing new strategies for promoting respiratory recovery after spinal cord injury in humans.

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Abbreviation

WGA-HRP

Wheat germ agglutinin conjugated to horseradish peroxidase

BHRP

Cholera toxin subunit B-horseradish peroxidase

rVRG

rostral ventral respiratory group

postnatal day 2

P2

Figure 1.

Electromyographic (EMG) recordings from the left and right ventral hemidiaphragm of postnatal rats before and after a complete left C2 spinal cord hemisection showing activity on both sides of the diaphragm. A, B) The spontaneous crossed phrenic activity which drives the left ventral hemidiaphragm after left C2 hemisection was expressed in a P2 and P7 neonatal rat. C) No spontaneous crossed activity was observed in a P35 hemisected rat. This reflects the adult rat pattern in which no crossed phrenic activity is expressed after ipsilateral spinal cord hemisection.

Figure 2.

Phrenic motoneurons are labeled ipsilateral to the C2 spinal hemisection. Low power (A) and high power photomicrographs (B, C) of a transverse section of the C4 spinal cord in a P2 rat showing that the left phrenic nucleus, B (dark circled area in A) was retrogradely labeled with WGA-HRP after left phrenic nerve injection. C). No labeling in the phrenic nucleus contralateral to hemisection (light circled area in A). Low power (D) and higher power photomicrographs (E, F) of a transverse section of the C4 spinal cord at P7. Low power (G) and higher power photomicrographs (H, I) of a transverse section of the C4 spinal cord at P28. Low power (J) and higher power photomicrographs (K, L) of a transverse section of the C4 spinal cord at P35. The rest of the sections examined also show the same pattern of labeling

shown by A-L. P, pinhole on right side in A, D, G and J. Magnification in A is the same as in D, G and J. Magnification in B is the same as in C, E, F, H, I, K and L.

Figure 3. rVRG neurons in the medulla labeled with WGA-HRP in the hemisected rats at different ages. Low-power (A) and higher power photomicrographs (B, C) of a transverse section through the medulla in a P2 rat showing that the ipsilateral rVRG neurons (dark circled area in A) were transynaptically labeled with WGA-HRP. This area is enlarged in B. (C). No labeled rVRG neurons noted on the right side (light circled area in A). P, pinhole marking right side in A, D, G, J. Low power (D) and higher power photomicrographs (E, F) of a transverse section through the medulla in a P7 rat showing that the labeled rVRG neurons are ipsilateral to hemisection (dark circled area in D magnified in E). (F). Contralateral rVRG neurons are not labeled (light circled area in D). Low power (G) and higher power photomicrographs (H, I) of a transverse

section through the medulla in a P28 rat showing that the labeled rVRG neurons are ipsilateral to hemisection (dark circled area in G magnified in H). (I). No Contralateral rVRG labeling is observed (light circled area in G). Low power (J) and higher power photomicrographs (K, L) of a transverse section through the medulla in a P35 rat. Note, for the first time that bilateral rVRG premotor neurons are labeled by WGA-HRP. These neurons are delineated by the dark and light circled areas and magnified in K and J respectively. IV, fourth ventricle. Magnification in A is the same as in D, G and J. Magnification in B is the same as in C, E, F, H and I. Magnification in K is the same as in L.

Figure 4.

Quantification of the labeled rVRG neurons in P2, P7 and P28 rats. The WGA-HRP labeled rVRG premotor neurons in the medulla were ipsilateral to hemisection in neonatal rats. ** represents that significantly more labeled rVRG neurons in P2 than P7 and P28. * represents that significantly less labeled rVRG neurons in P28 compared to P7 animals.

Figure 5.

Horizontal sections of the cervical spinal cord in different aged rats showing the anatomy of phrenic dendrites during postnatal development. Lower power (A) and higher power photomicrographs (B, C) of phrenic motoneurons labeled with BHRP in a P2 rat. B shows the rostral half of the nucleus while C shows the caudal half. Note that phrenic dendrites (indicated by the solid arrows) cross the midline (dashed arrow in A) at both rostral and caudal levels of the cervical spinal cord. (D-F). Phrenic motoneurons labeled with BHRP in a P7 hemisected rat. (E), Note that phrenic dendrites cross the midline of the cervical spinal cord only at the rostral level. Arrows indicate the crossing dendrites. (F), Note that there are no midline-crossing dendrites at the caudal pole of the phrenic nucleus. P, pinhole marking the rostral part of the

cervical spinal cord in A and D. Magnification in A is the same as in D. Magnification in B is the same as in C, E and F.

Figure 6.

Horizontal sections of the cervical spinal cord in P28 (A-C) and P35 rats (D-F) showing the anatomy of phrenic dendrites during postnatal development. Lower power (A) and higher power photomicrographs (B, C) of phrenic motoneurons are labeled with BHRP in a P28 hemisected rat. Solid arrows (B) point to the dendrites approaching, but not crossing the midline. (D-F). Phrenic motoneurons labeled with BHRP in a P35 hemisected rat. None of the phrenic dendrites tend to cross the midline of the cervical spinal cord. Dashed arrows in A and D approximate the midline of the spinal cord. Magnification in A is the same as in D. Magnification in B is the same as in C, E and F.

Figure 7.

Quantitative summary of BHRP labeled midline-crossing phrenic dendrites in P2, P7 and P28 rats. A) At the rostral level of the phrenic nucleus, the number of P2 crossing phrenic dendrites were significantly more than P7 and P28 crossing dendrites. ** represents significance between P2 and P7 as well as P28 following hemisection. * represents the same change in the control animals. ## represents significance between P7 than P28 following hemisection. # represents the same change in the control animals. B) At the caudal level of the phrenic nucleus, crossing phrenic dendrites in P7 were significantly fewer than P2. Caudal crossing phrenic dendrites were not found in P28. C) The number of the crossing phrenic dendrites was similar at both rostral and caudal spinal cord in P2 rats. D) In P7 animals, the rostral crossing dendrites were significantly more than caudal crossing dendrites. ** represents a significance in P7 hemisection group. * represents that a significance in P7 control animals.

Figure 8.

Diagrams of the crossed phrenic pathway in neonatal rats and young rats at five postnatal weeks (P35). A). Diagram of the crossed phrenic pathway in neonatal rats. The pathway in neonatal rats is comprised of spinal cord midline-crossing collaterals from the rVRG descending axons that cross in the medulla as well as midline-crossing dendrites from the ipsilateral phrenic motoneurons. B). Diagram of the crossed phrenic pathway in P35 rats. The pathway involves bilateral rVRG respiratory premotoneuron axons with axon collaterals that cross the midline of the cervical spinal cord. Arrows indicate the direction of impulses along the crossed phrenic pathway.