Phrenic motoneuron morphology during rapid diaphragm muscle growth

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PREVIOUS STUDIES HAVE SUGGESTED a correspondence between the phenotypic properties of a motoneuron and the muscle fibers it innervates (motor unit; Refs. 7, 17, 46, 49, 50). For example, the “size principle” of Henne-man predicts that larger fast-twitch motor units (in terms of both muscle fiber size and innervation ratio, i.e., the number of muscle fibers innervated by a motoneuron) are innervated by larger motoneurons than smaller, slow-twitch motor units (26, 27). The characteristic heterogeneity of muscle fiber size in adult motor units is brought about by a differential growth of fibers during the postnatal development that occurs after weaning (32, 40). For example, type I and type II muscle fibers, which comprise slow- and fast-twitch muscle units, respectively, are approximately the same size until postnatal day 21 (D21) in the rat diaphragm muscle (Dia_m). Thereafter, type II muscle fiber growth is disproportionate to that of type I fibers (32).

During the preweaning period of postnatal development, processes such as synapse elimination and secondary myogenesis make it difficult to isolate the specific effect of muscle fiber growth on motoneuron growth. Whereas the process of synapse elimination continues during early postnatal development, motor unit innervation ratio changes (1, 2, 4) and may affect motoneuron size. Partial denervation studies in adults have suggested that an increase in innervation ratio results in an increase in motoneuron somal size (47). Thus synapse elimination, which proceeds until postnatal day 14 in the rat Dia_m (35, 40), would result in a progressive decrease in innervation ratio and, if anything, would tend to decrease phrenic motoneuron size. Conversely, secondary myogenesis, which is incomplete until postnatal day 21 in the rat Dia_m (25, 32), would tend to increase motor unit innervation ratio. The period of postnatal development just after weaning, when singular innervation is established and secondary myogenesis is completed, presents an excellent opportunity to examine the correspondence between Dia_m fiber and phrenic motoneuron growth. Accordingly, in the present study, changes in motoneuron morphology were compared with changes in Dia_m fiber cross-sectional area (CSA) in male rats between D21 and postnatal day 84 (adulthood). We hypothesized that there is corresponding growth between Dia_m fibers and phrenic motoneurons during this period of development.

METHODS

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were used for all experiments. Nineteen animals at D21 (body wt 45–55 g) and nineteen adult animals (~15 wk old; body wt 350–400 g) were studied. At each age,
seven animals were used to measure Dia \(_m\) fiber size, and twelve animals at each age were used to determine phrenic motoneuron morphology (six each for motoneuron somal morphometry and motoneuron dendritic arborization). Differences in the tissue extraction procedures necessitated separate analyses for muscle fiber and motoneuron analyses.

All procedures involving animals were in strict accordance with the guidelines established by the American Physiological Society and were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic.

**Muscle Fiber Morphometry**

Animals were anesthetized with pentobarbital sodium (120 mg/kg body wt). The right midcostal Dia \(_m\) was excised, and resting muscle length was measured. The samples were then stretched to \(\sim 1.5\) times the resting excised length [to approximate optimal fiber length for force generation (32)], pinned on pieces of cork, and quickly frozen in melting isopentane that was cooled by liquid nitrogen.

**Histology.** Serial cross sections of the muscles were cut at 10 \(\mu\)m using a Reichert-Jung cryostat kept at \(-20^\circ\)C. Alternate muscle sections were stained for myofibrillar ATPase (mATPase) as follows: 1) mATPase after alkaline (pH = 9.0) preincubation to classify fibers as type I or type II (41, 44), 2) mATPase after acid (pH = 4.55) preincubation to subclassify fibers as IIA or IIB/IIX (5, 42), and 3) mATPase after light (4%) paraformaldehyde fixation and alkaline (pH = 10.4) preincubation to distinguish type IIA/IIB and IIX fibers (20).

**Imaging and analysis.** The techniques for imaging and analyzing muscle cross sections have been described in detail previously (29, 39, 43). Briefly, muscle sections were digitized using a dedicated image processing system (MegaVision 1024XM) that was attached to a cooled charge-coupled device camera (Texas Instruments) and mounted on an Olympus BH-2 microscope. Fiber type was identified from staining intensities (light or dark) for mATPase at different preincubation pHs, as described above. Individual fibers were delineated using an interactive cursor. Fiber CSA was determined by measuring the number of pixels within encircled fibers. The proportions and CSA of each fiber type were determined from samples of 100–150 fibers from each muscle segment.

**Motoneuron Morphometry**

A fluorescent labeling technique combined with confocal imaging was used for examining somal volume and surface area, as well as primary dendrite numbers and dimensions. A choleratoxin-based technique was used for analysis of dendritic arborization. The advantage of using fluorescent confocal imaging is that optical slices could be used to accurately determine somal volume and surface areas. In addition to allowing three-dimensional (3D) reconstruction of phrenic motoneuron soma, the use of confocal imaging also provided better visualization of the emergence of primary dendrites, because contamination by overlying structures was minimized. On the other hand, the extent of dendritic arborization was better visualized using a choleratoxin-based technique with a light-stable reaction product.

**Fluorescent labeling.** Animals were anesthetized with a mixture of ketamine (60 mg/kg body wt) and xylazine (2 mg/kg body wt). The Dia \(_m\) was exposed by laparotomy, and 5–10 \(\mu\)l of 2% tetramethylrhodamine dextran (molecular weight = 3,000; Molecular Probes) in 0.15M NaCl were injected into the right Dia \(_m\) at multiple sites.

After a 48- to 72-h survival period, animals were re-anesthetized with pentobarbital sodium (120 mg/kg body wt) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH = 7.4). The cervical spinal cords were excised, postfixed in the same fixative for 3–4 h, and then stored in 25% sucrose in PB at 4°C.

Subsequently, 150-\(\mu\)m longitudinal sections of the spinal cords were cut using a Reichert-Jung cryostat. Sections were mounted, air-dried on chrome alum-subbed slides, and coveredslipped with a gelatin-based mounting medium (Gel/ Mount, Biomeda) containing 5% p-phenylenediamine, to reduce photobleaching. The refractive index of the mounting medium, measured using a series 510 liquid refractometer (Bausch and Lomb), was 1.37 in the fluid state.

**Confocal system validation.** A Bio-Rad laser-scanning confocal system (MRC 500), mounted on an Olympus BH-2 upright microscope and equipped with an Ar-Kr laser, was used to image labeled motoneurons. A manufacturer-supplied software package (CoMOS), running on a Northgate 386 personal computer, was used to control image acquisition. Optical sectioning was controlled via a stepper motor attached to the microscope-focussing knob.

An Olympus DPlan 40 X/1.3 numerical aperture oil-immersion objective was used for imaging. Low-fluorescence immersion oil (refractive index = 1.47; Cargill) was used for the objective lens. The XY plane, parallel to the microscope stage by convention, was used to make the confocal optical sections. Each optical section was digitized and stored into arrays of 384 \(\times\) 256 pixels. The XY plane was calibrated using a stage micrometer, and the dimension of each pixel was 0.6 \(\mu\)m, which matched the calculated thickness of optical sections using the \(\times\)40 objective. Optical sections were obtained by moving the stage in only one direction, thus eliminating backlash error in the stepper motor.

A number of factors may influence the determination of cell morphology from confocal optical sections. These factors include inaccuracy of the stepper motor; a mismatch among the refractive indexes of the tissue, mounting medium, and objective lens immersion oil; and tissue compression. Overall, these factors introduce distortion along the Z-axis. The techniques for estimating individual errors introduced by these factors have been described in detail elsewhere (33). Overall correction factors for volume and surface area measurements were obtained using these techniques.

**Confocal imaging and analysis.** Fluorescently labeled motoneurons were sampled from all labeled segments of the cervical spinal cord (C3–C6). Motoneurons that were damaged by the tissue sectioning process and those that were >100 \(\mu\)m below the surface of the tissue section (beyond the working distance of the \(\times\)40 objective lens) were not analyzed further.

Images of the optical sections were analyzed using a comprehensive image display and manipulation package [ANALYZE; Biomedical Imaging Resource, Mayo Foundation (36)] running on a Sun 4/330 workstation. The procedures for measurements of motoneuron somal volume have also been described in detail in a previous study (33). Briefly, with the use of a fuzzy-gradients-hashing algorithm, each set of optical sections was rendered in 3D (minimum threshold = 25 gray levels, maximum threshold = 255 gray levels). From these 3D reconstructions, motoneuron somal volumes were directly measured using a voxel-counting program in ANALYZE, based on a 0.216 \(\mu\)m/\(\mu\)m calibration. Motoneurons with overlapping somal boundaries (in both the XY plane and along the Z axis) were ignored, because accurate delineation of the somal boundaries was not possible.

Surface areas of motoneuron soma were estimated by applying two techniques: 1) measuring the major and minor diameters of the soma and applying the equation for the
surface area of a prolate spheroid and 2) from the 3D reconstructions, using a surface-tracking algorithm (36). When using the prolate spheroid model, a correction factor was applied to compensate for the inequalities of the Z axis diameter and the minor axis diameter measured in the XY plane. This correction factor was previously calculated to be 0.94 for phrenic motoneurons (33). The results from this stereological estimation were compared with 3D surface area measurements.

The 3D reconstructions were also used to count the number of primary dendrites of each motoneuron. If necessary, the reconstructions were interactively rotated to view dendrites hidden behind the cell soma. For each primary dendrite, an orthogonal, two-dimensional section was taken using an interactive tool in ANALYZE. The section was taken at the end of the dendritic hillock, in which dendritic diameter was fairly uniform. Dendritic diameter was then measured from this two-dimensional image using tools in ANALYZE.

**Dendritic analysis.** The total dendritic surface area was estimated using a simple model described by Burke et al. (9). In this analysis, the primary dendrite was identified from the stack of optical sections, and the initial diameter of each primary dendrite was measured at a point 15 μm from the soma. On the basis of this measurement, dendritic tree surface area was estimated using the following formula

\[ A_d = 986d_o^{1.88} \]

where \( A_d \) is the total surface area of the family of dendrites that arise from a primary dendrite of diameter \( d_o \). The total motoneuron surface area was then calculated from the measured somal surface area and from the sum of the surface areas of each dendrite family of that motoneuron.

**Motoneuron Dendritic Arborization**

To better evaluate dendritic arborization patterns, phrenic motoneurons were retrogradely labeled with cholera toxin B-fragment (CTB), using a dense reaction product that provided greater contrast. The intent of these analyses was to provide a qualitative description of the arborization pattern of phrenic motoneuron dendrites. Quantification was not attempted because of the possibility that retrograde transport of CTB might yield incomplete or nonhomogeneous filling of motoneurons, especially the most distal dendrites.

**CTB labeling.** Animals were anesthetized with a mixture of ketamine (60 mg/kg body wt) and xylazine (2 mg/kg body wt). The right hemidiaphragm was exposed after laparotomy and injected at multiple sites with 1 μl of 1% CTB using a glass micropipette attached to a Hamilton syringe. The CTB solution contained 2% Evans blue dye for clear identification of the injection sites.

After a 48- to 72-h survival period, animals were re-anesthetized with pentobarbital sodium (120 mg/kg) and transected peritoneally with 4% paraformaldehyde in PB. Cervical spinal cords were excised and immersion-fixed for 3–4 h, and the spinal cords were then stored in PB at 4°C. Longitudinal tissue sections were cut at 10 μm using a vibratome. The CTB-labeled motoneurons were then visualized with an indirect immunocytochemical method similar to that previously described (31). Briefly, all reagents were diluted in 0.05 M Tris = 0.15 M NaCl containing 0.2–2% Triton X-100 (TBS-Tx, pH = 7.6). Incubations were done at room temperature with gentle agitation on a platform shaker. The sections were incubated successively in the following reagents: 10% normal donkey serum (30 min), goat anti-CTB (1:83,000, 12–16 h; List Biological Labs), biotinylated donkey-anti-goat (1:1,000 with 10% normal donkey serum, 12–16 h; Jackson Immunoresearch), and HRP-conjugated streptavidin (1:1,000, 4–6 h; Jackson Immunoresearch). The tissue was rinsed for 45 min in TBS-Tx after each incubation. After the final incubation, HRP was visualized using a glucose oxidase-imidazole-diaminobenzidine solution (45), which provided a brown deposition in labeled neurons that could be viewed under brightfield illumination. The labeled samples were mounted and air-dried on chrome alum-subbed slides, dehydrated in graded ethanol (50, 75, 95 and 100%), cleared with xylene, and coverslipped with DPX (Fluka).

A comprehensive, computer-controlled neuron tracing and morphometric system (NeuroLucida, Microbrightfield) attached to an Olympus BH2 microscope was used to render representations of labeled motoneurons. An Olympus DP27 ×40 objective lens was used to visualize the motoneurons. The morphometric system was calibrated using a stage micrometer. Depth (Z axis) information was obtained using a stepper motor (0.1 μm resolution) attached to the fine focus knob of the microscope. Dendrites were assumed to be cylinders of constant diameter between sampling points.

Motoneurons were systematically sampled from all parts of the labeled phrenic nucleus. The phrenic nucleus typically spanned the cervical cord from C3 to C6. The first clearly labeled motoneuron in the C3 segment was as a reference point and also as the first sample. Thereafter, every discernible motoneuron along the rostrocaudal axis, at a distance of 100 μm from the previous sample, was sampled. Sample size ranged from 20 to 25 motoneurons per animal, spanning C3 to C6.

**Data Analysis**

Mean CSA of each fiber type in adults was compared with that at D21 using a two-way (age and fiber type) ANOVA with Student’s t-test and selected pairwise comparisons. A Bonferroni correction was applied for repeated comparisons.

Distributions of individual motoneuron somal volumes in the two age groups were compared using \( \chi^2 \) analysis. Furthermore, somal volume distributions at the two ages were summarized and compared, using \( t \)-tests to evaluate average changes in somal volume with growth. The average change in somal volume between D21 and adult groups was calculated as a percentage of D21 somal volume.

At each age, changes in fiber CSA were compared with changes in motoneuron somal volume by taking the ratio of the mean values at adulthood and D21 to estimate the proportional growth of muscle fibers and motoneurons. In addition, the distributions of fiber CSA and motoneuron somal volumes at each age were analyzed to determine whether the distributions were uni- and/or bimodal.

Dendritic data were summarized for each motoneuron, and then an overall summary across all sampled motoneurons within an animal was obtained. These summaries were compared across animals in each age group and across age groups using two-way ANOVA. Statistical distributions of total motoneuron surface areas were also compared, using \( \chi^2 \) analysis. Statistical significance was tested to a level of 0.05. Mean and standard errors were calculated for each parameter of interest.

**RESULTS**

**Muscle Fiber Morphometry**

Overall, the proportions of type I and II fibers in the Dia_m were not significantly different between D21 and adulthood (Table 1). The proportion of type IIx fibers in
the Diám decreased from ~40% at D21 to ~30% in adults. There were no type IIb fibers in the D21 Diám, whereas ~8% of all fibers in the adult Diám were type IIb.

The overall CSA of type II fibers was calculated by taking a proportion-weighted average of different subtypes. In the D21 Diám, the CSA of type I and II fibers were comparable, whereas, in the adult Diám, the CSA of type II fibers was significantly larger than that of type I fibers. Type I, IIa, and IIx fibers all showed significant increases in CSA from D21 to adulthood. Type IIa fibers doubled in size, whereas type IIx fibers more than tripled in size between the two ages. In adults, type IIb fibers were the largest among fiber types.

Motoneuron Somal Morphometry

The results of the validation procedures for confocal microscopy have been previously published (33). With the use of these procedures, a 16.8% overestimation in volume measurements was detected. Therefore, to compensate for the overall distortion introduced by the imaging procedures, a correction factor of 1.168 was applied to each confocally measured motoneuron somal volume.

Typical examples of phrenic motoneurons at D21 and adulthood are shown in Fig. 1. The morphometric measurements obtained from phrenic motoneurons are shown in Table 2. Both major and minor diameters of phrenic motoneuron cell soma were significantly larger in the adult than at D21. As a result, surface areas were also significantly larger in the adult compared with D21. The somal volumes of phrenic motoneurons increased significantly between D21 and adulthood, showing a 141% increase in volume (Table 2). The proportionate increase in phrenic motoneuron somal volume generally matched the overall increase (122%) in Diám fiber CSA.

The distributions of motoneuron somal volumes are shown in Fig. 2. Phrenic motoneuron somal volumes showed unimodal distribution both in D21 and adult animals. There was considerable overlap between the distributions for D21 and adult animals, suggesting either proportional growth of most motoneurons or growth of only a subpopulation of the entire phrenic pool.

Both primary and secondary dendrites of motoneurons could be labeled by rhodamine dextran; however, morphometric information could only be reliably obtained from the primary dendrites. In the confocal optical sections, the number of primary dendrites was found to be 5.4 ± 0.5 and 9.3 ± 1.1 in D21 and adult animals.

### Table 1. Age-related morphological changes in muscle fiber types

<table>
<thead>
<tr>
<th>Fiber Type Proportion, %</th>
<th>Fiber Type CSA, μm²</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>D21</td>
<td>33 ± 4</td>
</tr>
<tr>
<td></td>
<td>404 ± 29(230)</td>
</tr>
<tr>
<td>Adult</td>
<td>37 ± 2</td>
</tr>
<tr>
<td></td>
<td>697 ± 40(309)*</td>
</tr>
<tr>
<td>%Change</td>
<td>73%</td>
</tr>
</tbody>
</table>

Values are means ± SE. Diaphragm muscles from postnatal day 21 (D21) and adult rats (n = 7 for each group) were analyzed. At least 100 fibers were sampled from each diaphragm muscle. Cross-sectional area (CSA) measurements (in parentheses) represent total numbers of fibers from all animals within an age group. Percentage changes are relative to D21 values. *Significant difference between D21 and adult; †significant difference between type I and II muscle fibers; ‡type II data is proportion-weighted average from IIa, IIb, and IIx data.

Fig. 1. Three-dimensional reconstructions of phrenic motoneurons at postnatal day 21 (D21; A) and adulthood (B). Note the age-related difference in motoneuron sizes.
Table 2. Age-related morphological changes in motoneuron soma

<table>
<thead>
<tr>
<th></th>
<th>Somal Volume, μm³</th>
<th>Major</th>
<th>Minor</th>
<th>Somal Surface Area, μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>D21</td>
<td>4,882 ± 692 (150)</td>
<td>41.3 ± 3.2</td>
<td>28.5 ± 3.8</td>
<td>1,431 ± 412</td>
</tr>
<tr>
<td>Adult</td>
<td>11,771 ± 1,541 (166)*</td>
<td>65.5 ± 4.3*</td>
<td>34.8 ± 4.7*</td>
<td>3,321 ± 594*</td>
</tr>
<tr>
<td>% Change</td>
<td>141%</td>
<td>59%</td>
<td>22%</td>
<td>135%</td>
</tr>
</tbody>
</table>

Values are means ± SE. At each age, motoneurons from 6 animals were analyzed. For each animal, at least 20 motoneurons were sampled. Numbers in parentheses represent total numbers of motoneurons. Percentage changes are relative to D21 values. *Significant difference between D21 and adult.

animals, respectively. Mean diameter of primary dendrites was 1.2 ± 0.1 μm in D21 animals and 2.7 ± 0.1 μm in adults.

Motoneuron Dendritic Morphometry

CTB labeling. The intramuscular injections of CTB into the Diam extensively labeled the phrenic nuclei of both the D21 and the adult animals (Fig. 3). The phrenic somata formed a column in the medial aspect of the ventral horn in the cervical spinal cord (C3–C6), with an axis approximately parallel to the long axis of the spinal cord, as has been previously observed (21, 30). However, in contrast to previous studies with HRP labeling via a nerve dip (21, 30), cell somata could be easily outlined, in spite of the extensive dendritic labeling, because the confined injections into the diaphragm limited the total number of labeled cell somata. Conglomerates of phrenic cell bodies (cell clusters) were observed in both age groups, with dendrites traversing these clusters (Fig. 3). There was no age-related difference in this unique feature of the phrenic nucleus, which has been previously described in both neonates and adults (21, 30).

Extensive dendritic labeling was seen in both age groups, with extremely fine processes being easily discernible over a distance of 500 μm from their cell bodies. Distal dendrites could be consistently visualized, up to the third order of branching in both age groups, and, occasionally, up to the fifth order in the adults. The dendritic fields of phrenic motoneurons overlapped extensively and were primarily oriented in the horizontal plane, which corresponded to the plane of tissue sectioning. Dendrites from different motoneurons traversed in close proximity, suggesting dendritic “bundles” that extended along either the rostrocaudal or mediolateral axes. These bundles have also been seen in previous studies (21, 30). In both age groups, dendrites extended to either the medial or lateral limits of the ipsilateral spinal cord, and some dendrites also projected dorsolaterally, especially in the D21 group.

In the adult rats, the medial and lateral dendritic projections frequently terminated within the ipsilateral ventral and lateral funiculi, respectively. Additionally, in D21 animals, dendrites were frequently seen crossing the anterior commissure into the contralateral ventral funiculus (Fig. 4); however, none of these dendrites extended to locations that contained the cell somata of the contralateral phrenic nucleus. In contrast to those in D21 animals, dendrites in adult motoneurons were rarely found to cross over to the contralateral spinal cord.

In both age groups, the rostrocaudal projections were confined to the phrenic nucleus and frequently traversed one or two cell clusters from the parent cluster. These dendrites also did not appear to be as tightly bundled as the mediolateral projections. The dorsolateral projections of the phrenic motoneurons did not extend more than 50 μm in the dorsal direction and were mainly oriented toward the lateral funiculus.

Morphometric analysis. There was extensive labeling of the phrenic nucleus, as illustrated in Fig. 3. In D21 animals, an estimated 188 ± 6 motoneurons per animal were labeled vs. 191 ± 8 in adults. Because not all sections of the Diam were injected with CTB, it was not expected that the entire phrenic nucleus would be labeled. In agreement with our findings, previous studies have estimated the total number of phrenic motoneurons to be more than 200 per side (21, 30).

The total number of dendrites with mediolateral orientation was greater at D21 than in adulthood (67 ± 5 vs. 38 ± 3%, respectively; P < 0.01). Conversely, the proportion of dendrites oriented along other directions was greater in the adult. The percentage of dendrites crossing over to the contralateral spinal cord was markedly higher in D21 motoneurons than in adult
motoneurons (26.3 ± 2.0 vs. 3.1 ± 1.5%, respectively; P < 0.01).

The average number of primary dendrites was significantly higher in adults compared with D21 (Table 3). However, in D21 animals, the number of primary dendrites ranged from 4 to 7, whereas, in adults, the number ranged from 4 to 14. The number of primary dendrites in CTB-labeled motoneurons did not differ significantly from the number of primary dendrites in rhodamine-labeled motoneurons. Both primary and secondary dendritic diameters were significantly greater in the adult animals. There was no significant difference in primary dendritic diameters of fluorescently-labeled motoneurons and CTB-labeled motoneurons. Primary dendritic length was also significantly greater in the adult. Total dendritic surface area, as estimated using the mathematical model described in Dendritic analysis, was ~375% greater in the adult. This large increase in surface area was a result of both greater dendritic length and increase in dendritic diameter.

Dendritic surface area comprised 89% of total motoneuronal surface area in D21 and 95% in adult animals (Table 3). Total dendritic surface area was almost fourfold smaller in the D21 rats (Table 3, P < 0.05). Accordingly, total motoneuron surface area was also significantly smaller in D21 animals. Figure 5 illustrates the distributions of total motoneuron surface areas across all animals from each age group. The surface areas displayed a relatively narrow, unimodal distribution at D21 but a considerably wider, and bimodal, distribution in the adult (P < 0.05 for bimodality). There was considerable overlap between the distributions for areas <10,000 μm².

DISCUSSION

The present study compared the age-related growth of Δ2m fibers to the age-related growth of phrenic motoneurons. Both type I and II muscle fibers showed significant growth between D21 and adulthood, with the growth of type II fibers being disproportionately larger than type I fiber growth. Phrenic motoneurons also displayed significant growth between D21 and adulthood, at both the somal and, especially, the dendritic levels. The proportionate growth of motoneuron soma, in terms of volume and surface area, matched the growth of Δ2m fibers; however, the maturational increase in dendritic surface area was far in excess of both muscle fiber and motoneuron somal growth. These correlative data suggest that establishment of motoneuron recruitment order in the adult involves differential morphological adaptations of the motoneuron vs. muscle fiber that will regulate intrinsic excitability.

Qualitative Features of Motoneuron Growth

The qualitative features of adult phrenic motoneurons observed using CTB labeling confirm observations from previous studies (18, 21). Characteristic features, such as cell clustering, preferred orientations of dendritic projections, and dendritic “bundling,” were observed at both ages. These features have been previously reported, even in the neonate (30). However, the present study also found that, between D21 and adulthood, there is a significant decrease in the proportion of dendrites with mediolateral orientations, suggesting that the preferential axes for dendritic projections are shifted during maturation, with a tendency to favor the rostrocaudal direction. This reorganization may serve to integrate activity among phrenic motoneurons and may also facilitate interactions between the phrenic nucleus and nearby motoneurons that control accessory respiratory muscles (37, 38). These changes may also be related to changes in the afferent input from premotor neurons (in the brainstem) to phrenic motoneurons, which have been shown to be predominantly along the axis of the phrenic motor column (15, 16, 18).
Previous studies have suggested that the dendritic arborization of phrenic motoneurons is primarily limited to the ipsilateral spinal cord, except in neonates, in which dendrites occasionally cross over to the contralateral spinal cord (30). The present study also found a higher incidence of contralateral dendritic projections in the D21 animals compared with adults. In contrast to previous studies, contralateral dendritic projections were also found in adult rat phrenic motoneurons. It is possible that the greater sensitivity of CTB labeling, compared with HRP labeling, highlighted this feature in the present study. However, it must be emphasized that accurate quantitative measurements of the extent of contralateral projections and age-related changes in the extent of these projections cannot be made with CTB labeling and do not necessarily result in a complete filling of the entire dendritic arborization (compared to intracellular fills). Accordingly, our data only underestimate the extent of contralateral projections in D21 animals, and it is likely that there is indeed an age-related reduction in the extent of contralateral projections. The reduced incidence of contralateral dendritic projections with maturation may be due to either a retraction of dendrites, as is the case in the postnatal development of certain sensory and motor nuclei (14, 19, 22), or a higher growth rate of the spinal cord compared with dendritic extension. It has been suggested that the higher incidence of contralateral dendritic projections in the neonate may be due to a higher growth rate of motoneurons compared with the spinal cord (30).

Table 3. Age-related morphological changes in motoneuron dendritic architecture

<table>
<thead>
<tr>
<th>Parameter</th>
<th>D21</th>
<th>Adult</th>
<th>%Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of primary dendrites</td>
<td>4.8 ± 0.3</td>
<td>7.4 ± 0.8*</td>
<td></td>
</tr>
<tr>
<td>Mean primary dendritic length, μm</td>
<td>75 ± 8</td>
<td>161 ± 7*</td>
<td>114%</td>
</tr>
<tr>
<td>Primary dendritic diameter, μm</td>
<td>1.36 ± 0.01</td>
<td>2.32 ± 0.04*</td>
<td>70%</td>
</tr>
<tr>
<td>Secondary dendritic diameter, μm</td>
<td>0.71 ± 0.02</td>
<td>1.16 ± 0.04*</td>
<td>63%</td>
</tr>
<tr>
<td>Calculated dendritic surface area, μm²</td>
<td>8,719 ± 219</td>
<td>41,726 ± 2,347*</td>
<td>378%</td>
</tr>
<tr>
<td>Total motoneuron surface area, μm²</td>
<td>9,767 ± 226</td>
<td>44,716 ± 2,395*</td>
<td>357%</td>
</tr>
</tbody>
</table>

Values are means ± SE. At each age, motoneurons from 6 animals were analyzed. For each animal, at least 20 motoneurons were sampled. Percentage changes are relative to D21 values. *Significant difference between D21 and adult.
Correlations Between Motoneuron and Muscle Fiber Growth

The results of the present study suggest a relationship between the growth of muscle fibers and the growth of motoneurons, although causality could not be established. The intrinsic capacity of motoneurons to maintain target size (size of the motor unit) may be one factor in modulating these morphological adaptations. Previous studies have addressed the issue of increased motor unit size using partial denervation, in which innervation ratio increases due to axonal sprouting (3, 6, 34). These changes in innervation ratio lead to a significant increase in somal diameter (47), suggesting that, when the intrinsic capacity for target size is exceeded, motoneuron size increases. However, it is unclear from these previous studies whether the increase in motor unit size after partial denervation was accompanied by any changes in muscle fiber size. It is possible that the increased innervation ratio observed in these studies may be accompanied by either a decrease or an increase in muscle fiber size. Because ~90% of the motoneuron area is composed of dendrites, it is also possible that, in addition to changes at the somal level, dendrites may also adapt with partial denervation. In this regard, increased target size in the present study was represented purely by an increase in muscle fiber size, because motor unit innervation ratio is set by D21 in the rat Dia_m. Therefore, the shift towards larger somal volumes in the distribution of adult phrenic motoneurons, albeit with considerable overlap with the distribution of somal volumes in D21 animals, suggests that the intrinsic capacity for target maintenance is exceeded for some motoneurons during age-related muscle fiber growth and results in an increase in motoneuron size. The overlap of distributions also suggests that there may be some motoneurons in which increased muscle fiber size may not exceed this intrinsic capacity of the motoneuron.

There may also be phenotypic differences in the intrinsic capacity of motoneurons for target expansion. A number of studies have suggested a correspondence between the morphological properties of muscle fibers within a motor unit, such as muscle fiber size and muscle fiber type, and the morphological properties of the motoneuron (7, 17, 46, 49, 50). There is currently some controversy whether there is any motoneuron size-related difference in motor unit innervation ratio (8, 13, 40). Nonetheless, because muscle unit size is determined by both innervation ratio and muscle fiber size, increases in muscle fiber size may influence motoneuron size. In this regard, the differential growth of muscle fibers types in the Dia_m from D21 to adulthood may result in differential growth of motoneurons. For example, assuming that there are no phenotypic differences in the intrinsic capacity of motoneurons for target expansion, the greater extent of type II fiber growth may result in a disproportionate growth of the motoneurons innervating these fibers. The larger phrenic motoneurons observed in the adult distribution may be these motoneurons. However, if motoneurons innervating type II fibers have greater intrinsic capacity for target expansion compared with those innervating type I fibers, the larger motoneurons observed in the adult may be motoneurons innervating type I fibers, because there is also considerable growth of type I fibers. This possibility seems unlikely, however, given the probable relationship between motoneuron size and earlier recruitment of slow-twitch motor units (7, 26, 27). Conversely, motoneurons innervating type I fibers may have greater capacity for target expansion and may not require significant growth to compensate for the maturational increase in type I fiber size. It was not possible, in the present study, to classify motoneurons on the basis of their motor unit phenotype, but a comparison of the adult-to-D21 ratio of motoneuron somal volumes (2.41) to the same ratio for muscle fiber CSA (2.27) suggests that motoneurons innervating both type I and II fibers may have grown to compensate for muscle fiber growth (because the ratio of somal volumes is intermediate to the ratio of type I fiber CSA and type II fiber CSA).

A comparison of the changes in somal vs. dendritic volumes clearly indicates that dendrites grow to a much greater extent between D21 and adulthood than do motoneuron soma. The contribution of dendrites to total motoneuron surface area was found to be between 85 and 90%, which consistent with recent data from Torikai et al. (48). An important difference between the present study and the study by Torikai et al. (48) is the number of motoneurons analyzed. The present study used a retrograde-labeling technique to analyze a larger number of motoneurons within an animal vs. the intracellular-filling technique used by Torikai et al.
physiological implications than changes in somal dimensions, in terms of motoneuron recruitment.

Physiological Implications

Motoneuron excitability is determined both by intrinsic electrophysiological properties and by extrinsic factors such as synaptic input. However, for a given synaptic input, a smaller motoneuron soma, and a smaller dendritic surface area, would imply greater excitability due to higher input resistance and lower rheobase. A number of other studies have also shown that motoneurons are smaller in size during early development compared with adult motoneurons (10–12, 30). Therefore, it is possible that D21 phrenic motoneurons are generally more excitable. It has been suggested that, during the early periods of postnatal maturation, the lower compliance of the chest wall may necessitate greater recruitment of \( \text{Dia}_{m} \) motor units to maintain ventilation (40). Greater excitability of D21 motoneurons may therefore facilitate recruitment. In the adult, recruitment of slow- and fast-twitch muscle motor units (innervated by smaller motoneurons) may be sufficient to maintain ventilation (40). Therefore, larger motoneuron somal volume and increased dendritic surface area may be directed toward reducing excitability of motoneurons and toward establishing an orderly pattern of recruitment, as suggested by the Henneman size principle (26, 27). These hypotheses, along with those presented in this study, are consistent with a recent report by Torikai et al. (48), who found that adult rat phrenic motoneurons recruited early during inspiration displayed smaller dendritic surface areas than quiescent motoneurons that were never recruited and also displayed the largest dendritic arborization. Although no electrophysiological measurements were made in our study, it is tempting to speculate that the quiescent motoneurons reported by Torikai et al. (48) innervate the fast fatigable type IIb muscle fibers in the \( \text{Dia}_{m} \) that are rarely recruited, except in forceful maneuvers (40).

The relationships between fiber and motoneuron sizes may be particularly important under pathological conditions in which the normal interactions between motoneurons and muscle fibers are disturbed. For example, denervation results in significant changes in motoneuron morphology and electrophysiological properties (23, 24). It has also been suggested that only the motoneurons that innervate fast motor units (type II fibers) exhibit adaptive changes after denervation and that fast and slow motor units in a mixed muscle respond differentially to denervation (28). Extending this hypothesis, it is possible that motoneurons innervating slow and fast motor units also respond differentially to altered activation other than denervation, such as in partial denervation and spinal transection.

In conclusion, the present study found both qualitative and quantitative differences between the growth of motoneurons and muscle fibers during a period of rapid muscle growth. The differential growth of muscle fibers is accompanied by changes in motoneuron soma and dendritic architecture. Changes at the motoneuron may be geared toward establishing a motor unit recruitment order.

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