Reinnervation of the Rat Levator Ani Muscle after Neonatal Denervation

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ABSTRACT: After axonal injury on postnatal day 14 (P14), but not P21, motoneurons in the spinal nucleus of the bulbocavernosus (SNB) do not display their normal response to circulating testosterone levels. This could result from a permanent disruption of communication between motoneurons and their testosterone-sensitive target muscles. We assessed the extent of reinnervation of one of these target muscles, the levator ani (LA) muscle, 5 months after the pudendal nerve was cut either on P14 or P21. The number of motoneurons innervating the LA in control and nerve cut animals was determined using retrograde labeling procedures. Functional recovery of the LA muscle was determined via the testing of its in situ contractile properties. Compared to control muscles, reinnervated LA muscles were smaller, had fewer muscle fibers, generated a lower maximum tetanic tension, and were more fatigable. In spite of the fact that fewer motoneurons reinnervated the LA muscle after nerve cut on P14 than on P21, there were no differences in the weight or contractile properties of the LA muscle between these two groups. These data suggest that motoneurons that survived injury on P14 innervated more muscle fibers than normal and exhibited a similar ability to functionally reinnervate the target muscle as those motoneurons that survived injury on P21.

Keywords: axotomy; motoneuronal death; denervation; motor unit; fatigability; maximum tetanic tension; development

INTRODUCTION

Nerve injury during early postnatal life has severe effects on neuromuscular systems, including massive motoneuronal death and muscle atrophy (Romanes, 1946; Schmalbruch, 1984; Crews and Wigston, 1990; Mousavi et al., 2002; reviewed by Lowrie and Vrbová, 1992; Houenou et al., 1994). As neuromuscular systems mature, the effects of nerve injury become less severe. In the spinal nucleus of the bulbocavernosus (SNB) of rats, no motoneurons survive axotomy on postnatal day 1 (P1) or P7, fewer than half survive axotomy on P14, and all survive axotomy on P21 or later ages (Lubischer and Arnold, 1995a). Furthermore, neonatal motoneurons do not respond to partial denervation of their muscle in a mature manner, failing to expand to reinnervate denervated muscle fibers after the axons of neighboring motoneurons have been removed (Brown et al.,...
Because the SNB motoneurons (Lubischer and Arnold, 1995b), due in part to a failure to extend terminal sprouts (Lubischer and Thompson, 1999), Neonatal denervation (full or partial) leaves muscle fibers permanently denervated, resulting in their eventual loss and a corresponding decrease in muscle weight (Lowrie et al., 1987; Mousavi et al., 2002). Immature neuromuscular systems show a striking inability to compensate for denervation injury.

Motoneurons that do survive axonal injury in early postnatal development also can show lasting effects not seen after axonal injury at later ages (e.g., Navarrete et al., 1990). The SNB provides an interesting example of this. SNB motoneurons normally respond to changes in circulating testosterone levels in adulthood with changes in soma size, with higher levels of testosterone resulting in larger somata (Breedlove and Arnold, 1981). However, after nerve cut on P14, surviving SNB motoneurons no longer show this response to testosterone in adulthood (Lubischer and Arnold, 1995b). The SNB is composed of motoneurons innervating the levator ani (LA), bulbocavernosus (BC), and anal sphincter muscles (Breedlove and Arnold, 1980; McKenna and Nadelhaft, 1986), and the LA and BC muscles have been well documented to be highly responsive to circulating androgen levels (e.g., Rand and Breedlove, 1992; Wainman and Shipounoff, 1941). Although testosterone appears to increase SNB motoneuron soma size by acting through androgen receptors expressed in SNB motoneurons (Watson et al., 2001), it is possible that interactions with the target muscles modulate this effect. The LA and BC muscles are known to be important in regulating SNB dendritic length (Rand and Breedlove, 1995) and androgen receptor expression (Al-Shamma and Arnold, 1995; Lubischer and Arnold, 1995c) in adult rats. Therefore, one possible hypothesis for the lack of testosterone-sensitivity by SNB motoneurons axotomized on P14 is that after nerve cut at an early developmental stage, the motoneurons that survive are somehow deficient in reinnervating their hormone-sensitive target muscles, thereby disrupting the motoneuronal response to testosterone.

We studied the LA muscle after nerve cut on P14 or P21 to assess the extent of functional reinnervation after neonatal denervation. These ages were chosen for two reasons: previous studies of the SNB motor pool showed differential survival of motoneurons after nerve cut on P14 and P21 (Lubischer and Arnold, 1995a), and nerve cut on P14, but not P21, results in a loss of testosterone responsiveness by SNB motoneurons (Lubischer and Arnold, 1995b). Because the in situ physiological properties of the LA muscle have not been determined previously, we fully characterized the contractile properties of age-matched control, as well as 5 month reinnervated, LA muscles. Our data show that despite the loss of 58% of LA motoneurons and 25% of LA muscle fibers after nerve cut on P14, the functional recovery of the LA muscle was the same as was seen after nerve cut on P21, which showed no loss of motoneurons and only a 12% loss of fibers in the LA muscle. LA motoneurons that survive axotomy on P14 are as capable of reinnervating LA muscle fibers as are those that survive axotomy on P21. These data argue against the hypothesis that communication between SNB motoneurons and their target muscles is permanently disrupted after nerve cut on P14.

**METHODS**

All experiments used Sprague-Dawley rats from litters culled to eight pups on the day after birth. Male rats were anesthetized with inhalation anesthetic on P14 or P21, and under aseptic conditions the motor branch of the right pudendal nerve was cut where it passes Cowper’s gland near the LA and BC muscles. This procedure severed all axons innervating the LA, BC, and anal sphincter muscles on one side. No portion of the nerve was removed, and the cut ends were left in place but not sutured together. This approach was used to facilitate comparison with previous studies of SNB motoneuronal axotomy (Lubischer and Arnold, 1995a,b). A third group of rats was not subjected to any surgery during development. Each group included animals from at least three different litters, and littermates were used in different experimental groups.

**Muscle Physiology**

At 5 to 6 months of age, animals (n = 5 per group) were prepared for in situ assessment of the contractile properties of control (i.e., no surgery) and reinnervated LA muscles. Terminal experiments were performed under sodium pentobarbital anesthesia (55 mg/kg, i.p.), supplemented as needed to suppress eye blink and withdrawal responses. Atropine sulfate (10 mg/kg) was administered to prevent respiratory failure. At the end of the experiment, animals were overdosed with sodium pentobarbital (100 mg/kg).

The right and left LA muscles join at a connective tissue, midline raphe located on the dorsal surface of the rectum. Connective tissue attaches both LA muscles to the rectum, and this served to anchor one end of the LA (the proximal end) during the experiment. The LA extends rostrally as it wraps laterally and ventrally around the rectum toward its attachment site on the penile bulb. The right LA muscle and the motor branch of the right pudendal nerve were carefully dissected free from surrounding tissues. Innervation of the two LA muscles is separate and
unilateral and is provided by the motor branch of the pudendal nerve (Cihak et al., 1967 as cited in Cihak et al., 1970; McKenna and Nadelhaft, 1986). The BC muscle was cut away to expose the distal end of the LA muscle, which attaches to the penile bulb. The penile bulb was then cut and 1.0 surgical silk (6.21 ± 0.35 cm in length) was tied to the penile bulb to attach the LA muscle in series to a force transducer (Statham model UC3:ULA-20) for isometric tension measurements. Care was taken not to impede blood flow to the muscle throughout the duration of the experiment, and every effort was made to maintain a reasonable approximation of the normal rostro-caudal and medio-lateral angle of pull of the muscle. The rat was securely anchored by clamps on the base of the tail and the foot. A bipolar silver hook electrode was placed on the muscle nerve for stimulation, and a bipolar ball electrode was positioned on the surface of the LA to record the electromyogram (EMG). The muscle and nerve were kept submerged in a bath of mineral oil maintained at 35 ± 1°C.

All contractile measurements were made at the muscle length that produced maximal twitch tension (L0). Maximum twitch tension (P0), time to peak tension (TPT), and half-relaxation time (HRT) were measured for single, unpotentiated twitches by stimulating the muscle nerve once every 40 s (pulse duration 0.02 ms) using a stimulus intensity three times the intensity required to generate P0. Values from five to twelve twitches were averaged for each muscle. The frequency-tension response was obtained using stimulation frequencies from 5 to 200 Hz (stimulus duration 800 ms). After determining maximum tetanic tension (P0) of the muscle at 200 Hz, fatigue resistance was assessed using 40 Hz trains of 330 ms duration delivered once per second and continuing for 2 min (Burke et al., 1973). The fatigue index (FI) was calculated as the ratio of tension at the end of the 2 min fatigue test to the maximum tension produced during the fatigue test. Data were displayed on a computer and an oscilloscope during the experiment and were recorded on tape for further analysis.

**Muscle Histochemistry**

Immediately after the fatigue test, the LA muscle was dissected and separated from the contralateral LA muscle by cutting along the midline raphe. Age-matched controls were used for comparison instead of the contralateral muscle because of our concern that changes in reinnervated LA muscles might affect the contralateral muscle due to their connection along the midline raphe. The experimental LA muscle was weighed, cut into two blocks through the midbelly, and frozen in isopentane cooled in liquid nitrogen. Cross sections (12 or 20 μm) through the midbelly of the muscle were cut on a cryostat. Qualitative staining of serial sections for glycogen using the periodic acid-Schiff (PAS) reaction (20 μm sections), for succinate dehydrogenase activity (SDH; an oxidative marker enzyme; 12 μm sections), or with haematoxylin and eosin (12 μm sections) was performed as reported elsewhere (Pearse, 1961; Martin et al., 1985).

Muscle fibers were counted in 12 μm SDH-stained sections taken from the midbelly of the muscle. Fiber counts at three different levels of the muscle (Breedlove and Arnold, 1983) suggest that LA muscle fibers extend from their distal attachment on the penile bulb to the midline raphe. Consistent with this is the existence of a single endplate band across the full width of the muscle (not shown). Furthermore, acid digestion of the LA muscle revealed bundles of fibers extending the full length of the muscle from the midline raphe to the penile bulb (not shown). Therefore, fiber counts of one section through the midbelly of the muscle should give a reasonably accurate measure of the total number of fibers in the LA muscle.

**Statistical Analyses**

Statistical analyses of data using StatView (SAS Institute, Inc.) involved analyses of variance (ANOVA) followed by planned comparisons, with p values of less than 0.05 accepted as statistically significant. Frequency-tension data and data from the fatigue test were assessed using repeated measures ANOVAs. Pearson’s correlation coefficient was used to test for significant relationships among the variables. Data are presented as mean ± standard error of the mean (SEM).
RESULTS

Despite differences in motoneuronal survival after axotomy on P14 or P21, the level of functional recovery of the LA muscle did not differ. Although less than half of the motoneurons injured on P14 survived, those that did survive appeared capable of compensating for the loss of neighboring motoneurons, resulting in comparable recovery of the functional properties of the LA muscle after injury on P14 or on P21.

More LA Motoneurons Survive and Reinnervate the LA Muscle after Axotomy on P21 Than on P14

Fluorogold injections into the LA muscle contralateral to the nerve cut filled an average of 36 motoneurons in the SNB (37.4 ± 2.5 and 34.6 ± 3.4 in animals subjected to surgery on P14 and P21, respectively; \( p = 0.61 \)). This number is expected to reflect the number of SNB motoneurons in control animals, because axotomy has no contralateral effect on motoneuron number (Lubischer and Arnold, 1995a). Furthermore, this estimate of LA motoneuron number is consistent with that of Jordan and coworkers (1992), who counted 29 motor units using graded stimulation of nerve filaments, a technique likely to somewhat underestimate the number of motoneurons in fully innervated muscles. Fluorogold injections into LA muscles that had been denervated on P21 filled the same number of motoneurons (35.4 ± 2.1) as in contralateral LA muscles (\( p = 0.88 \); Fig. 1). In contrast, after nerve cut on P14, an average of only 16.6 ± 6.0 motoneurons were labeled (Fig. 1), indicating regrowth to the muscle of 41.7 ± 12.1% of the LA motoneuronal population. These results for LA motoneurons are consistent with the percentages of SNB motoneurons found to survive after axotomy on P14 (42.4 ± 4.6%; Lubischer and Arnold, 1995a), indicating that there was no preferential loss or sparing of LA motoneurons relative to other SNB motoneurons.

Average Motor Unit Size Is Increased in P14-Reinnervated LA Muscles

Five months after cutting the pudendal nerve on P14 or P21, there was a 25 and 12% decrease, respectively, in the total number of fibers in the LA muscle compared to control values (Table 1). There was no indication of degenerating fibers at 5 months after axotomy in either group, that is, there was a normal distribution of muscle fiber sizes and no centrally located nuclei in sections stained with haemotoxylin and eosin (data not shown), suggesting that all fibers counted had been reinnervated. There were a lower number of fibers and a greater range in fiber number after nerve cut on P14 (5428 to 6994 fibers) than after nerve cut on P21 (7036 to 7651) or in age-matched controls (7886 to 8547). LA muscles also weighed less in P14- and P21-reinnervated muscles than in control rats, whereas body weight did not differ among the three groups (Table 1). The decrease in muscle fiber number after nerve cut on P14 was modest (25%) compared to the decrease in motoneurons (58%). This suggests that at least some motoneurons that survived P14 nerve cut were capable of innervating more muscle fibers than they normally would in adulthood.

LA Muscles from Control Rats Display Expected In Situ Contractile Properties

LA muscles from control rats displayed properties characteristic of a fast, fatigable muscle. LA muscles produced an average \( P_t \) of 20.8 g, with a mean TPT and HRT of 15.7 and 14.3 ms, respectively (Table 2). During assessment of the frequency-tension relationship, LA muscles consistently showed a maximal increase in force between 50 and 75 Hz and between 75 and 200 Hz (Fig. 3). During the 2 min fatigue test, tension decreased dramatically within 60 s (29% of the maximum tension during the test), and the mean FI for control LA muscles was 0.13 ± 0.03. The EMG amplitude usually decreased during the fatigue test.
test, often starting to drop after about 30 s of stimulation, parallel to the loss of force. This observation indicates the possibility of synaptic fatigue in addition to muscle fatigue. A majority of the fibers in LA muscles of control rats stained lightly for PAS at the end of the in situ contractile tests, indicating that most of the fibers were depleted of glycogen. Some fibers, however, were dark or intermediate in PAS-stained sections, consistent with possible synaptic fatigue. In contrast, fibers in muscles of control rats not subjected to physiological studies stained uniformly darkly for PAS.

P14- and P21-Reinnervated LA Muscles Have Similar Contractile Properties

There were no differences in the contractile properties of LA muscles between the two reinnervated groups (Table 2; Fig. 3). Mean $P_0$ of reinnervated LA muscles was about one-half that in control rats (main effect $p < 0.0001$; P14- and P21-reinnervated both lower than control, $p < 0.0001$). Mean $P_r$ tended to decrease (Table 2; main effect $p = 0.064$) and showed a large variability among the reinnervated muscles in both groups [Fig. 2(A)]. The twitch-tetanus ratio also tended to be higher (Table 2; main effect $p = 0.36$) in reinnervated than in control LA muscles, mainly reflecting the significant decrease in $P_0$ [Fig. 2(B)].

Frequency-tension profiles did not differ between the two reinnervated groups. As was seen in the control group, LA muscles in the reinnervated groups dramatically increased their force production between 50 and 75 Hz and again between 75 and 200 Hz, with $P_0$ occurring at the highest frequency of stimulation (200 Hz) in all groups. The force produced at most frequencies, however, was significantly lower in the reinnervated than in control groups, including $P_0$ (Fig. 3, Table 2). This decrease in force potential of the reinnervated muscles is consistent with the observed decrease in muscle fiber number and muscle weight (Table 1). Both fiber number ($r = 0.65; p < 0.01$) and muscle weight ($r = 0.76; p < 0.001$) correlated positively with $P_0$ across all muscles in all groups.

Mean TPT and HRT were similar across all groups, although the values were more variable in the reinnervated than in control groups (Table 2). These measures were positively correlated across all muscles in all groups ($r = 0.54; p < 0.05$). Reinnervated LA muscles were more fatigable than control muscles (Table 2, $p < 0.01$), with tension decreasing to 17% (P14-reinnervated) and 19% (P21-reinnervated) of maximum tension by 60 s. The shape of the force-time curve during the fatigue test was similar for all three groups, with maximum tension reached between 15 and 30 s, a dramatic decrease in tension between 30 and 60 s, and a more gradual decrease after 60 s (Fig. 4). Approximately one-half of the reinnervated muscles produced no detectable force by the end of the 2 min fatigue test (two of five in the P14 group and three of five in the P21 group). There was also a decrease in EMG during the fatigue test, similar to that seen in control muscles. Nevertheless,

### Table 1 Body Weight and Morphological Properties of Control and Reinnervated LA Muscles

<table>
<thead>
<tr>
<th></th>
<th>Body Weight (g)</th>
<th>Muscle Weight (mg)</th>
<th>Total Fiber Number</th>
<th>Number of Dark SDH Fibers [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4–5)</td>
<td>608 ± 37</td>
<td>282 ± 15</td>
<td>8296 ± 143</td>
<td>0</td>
</tr>
<tr>
<td>P14 nerve cut (n = 5)</td>
<td>637 ± 35</td>
<td>176 ± 14</td>
<td>6216 ± 295</td>
<td>248 ± 39 [4.1 ± 0.9]</td>
</tr>
<tr>
<td>P21 nerve cut (n = 5)</td>
<td>638 ± 31</td>
<td>201 ± 12</td>
<td>7286 ± 120</td>
<td>53 ± 17 [0.7 ± 0.3]</td>
</tr>
<tr>
<td>$p$ value for main effect</td>
<td>0.80</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.

*Significantly different from control at $p < 0.01$ and $p < 0.001$, respectively.

### Table 2 In Situ Contractile Properties of Control and Reinnervated LA Muscles

<table>
<thead>
<tr>
<th></th>
<th>$P_0$ (g)</th>
<th>$P_r$ (g)</th>
<th>TPT (ms)</th>
<th>HRT (ms)</th>
<th>$P_r : P_0$</th>
<th>FI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 5)</td>
<td>305.6 ± 8.5</td>
<td>20.8 ± 0.8</td>
<td>15.7 ± 0.5</td>
<td>14.3 ± 1.6</td>
<td>0.068 ± 0.002</td>
<td>0.132 ± 0.029</td>
</tr>
<tr>
<td>P14 nerve cut (n = 5)</td>
<td>149.4 ± 15.4</td>
<td>14.8 ± 2.6</td>
<td>16.6 ± 1.6</td>
<td>13.5 ± 2.6</td>
<td>0.101 ± 0.017</td>
<td>0.014 ± 0.013</td>
</tr>
<tr>
<td>P21 nerve cut (n = 5)</td>
<td>143.9 ± 22.1</td>
<td>13.2 ± 2.6</td>
<td>14.2 ± 0.7</td>
<td>13.3 ± 2.5</td>
<td>0.106 ± 0.029</td>
<td>0.023 ± 0.014</td>
</tr>
<tr>
<td>$p$ value for main effect</td>
<td>&lt;0.0001</td>
<td>0.06</td>
<td>0.29</td>
<td>0.94</td>
<td>0.36</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.

*Significantly different from normal at $p < 0.01$ and $p < 0.0001$, respectively. $P_0$, maximum tetanic tension; $P_r$, maximum twitch tension; TPT, time to peak tension; HRT, half-relaxation time; FI, fatigue index.
the EMG signal did not decrease to zero when force production had decreased to zero. Similar to what was observed in the control group, a majority of the fibers stained lightly for PAS at the end of the in situ contractile tests, indicating that most of the fibers were depleted of glycogen (data not shown).

**LA Muscles from the Reinnervated, but Not Control, Groups Show a Subpopulation of Fibers That Stain Dark for SDH**

The LA muscle in control rats was found to be composed of a homogenous population of fibers based on staining for PAS (dark) and SDH (light), consistent with previous work (see Discussion). Reinnervated muscles contained a small population of fibers that stained darkly for SDH activity (Fig. 5, top) that was not present in control muscles. The percentage of fibers that stained darkly for SDH was higher in the P14 than in P21 groups (4.1 ± 0.9 and 0.7 ± 0.3, respectively; \( p < 0.001 \)). Adjacent sections stained with haematoxylin and eosin showed that these fibers had peripherally located nuclei (Fig. 5, bottom), suggesting that they were not degenerating or regenerating. In addition, as noted above, many of these fibers were glycogen depleted at the end of the physiological testing, suggesting that they were functionally innervated.

**DISCUSSION**

**In Situ Contractile Properties of the LA Muscle**

To our knowledge, this is the first report of the physiological properties of the LA muscle deter-
mined in situ. Our data suggest that the force generating capability of the rat LA muscle is greater than reported in earlier, in vitro studies (Bass et al., 1969; Gutmann and Hanzlikova, 1975; Souccar et al., 1982; Vyskocil and Gutmann, 1977). In the LA muscle fibers of control rats, SDH staining was uniformly light and PAS staining was uniformly dark, consistent with previous reports of the LA muscle being composed of a homogeneous population of fast twitch, glycolytic fibers with low SDH activities (Blanco et al., 1995; Ishihara et al., 1997).

Reinnervation Capabilities of LA Motoneurons after Injury on P14 or P21

Nerve cut on P14 or P21 resulted in a loss of muscle fibers, muscle weight, and in situ maximum force production in response to tetanic stimulation. Nevertheless, recovery of these muscle properties was remarkably similar after nerve cut on P14 and on P21, despite the difference in motoneuron survival (42 vs. 100%, respectively). These data suggest that a subpopulation of LA motoneurons is capable of exhibiting a mature response to axonal injury on P14, and that the recovery capabilities of the LA muscle after denervation are, to some extent, independent of the number of motoneurons that reinnervate it. The most consistent effect of neonatal axotomy was an increase in variability on a number of measures, including muscle fiber number, \( P_v \), TPT, and HRT. This may reflect variability within the pool of surviving motoneurons in their relative state of maturation. Interestingly, the response of SNB motoneuron soma size to testosterone in

\[ \text{Figure 4} \] Neonatally reinnervated LA muscles were more fatigable than control muscles. The force produced by LA muscles during the fatigue test dropped dramatically for all groups, but more so for the P14- and P21-reinnervated groups. The force produced at each time point was normalized to maximum force produced by each muscle during the fatigue test. Values are mean ± SEM. Statistically significant group differences were seen at 90, 105, and 120 s (\( p < 0.01 \)). The force produced by P14- and P21-reinnervated muscles was similar at each time point.

\[ \text{Figure 5} \] Reinnervated LA muscles contained a subpopulation of fibers that stained darkly for SDH. Serial sections of a P14-reinnervated LA muscle stained for succinate dehydrogenase (SDH, top) and glycogen (PAS, bottom). Arrows point to a cluster of muscle fibers that stained darkly for SDH. Such fibers were not seen in control muscles. Some (arrows), but not all (arrowheads), of these dark SDH fibers were also dark in PAS sections. Scale bar = 0.2 mm.
adulthood also is more variable after nerve cut on P14 (Lubischer and Arnold, 1995b).

**Increased Fatigability of the Reinnervated LA Muscle**

We found an increase in fatigability of the LA muscle after injury on P14 or P21. In contrast, a decrease in fatigability has been reported for the extensor digitorum longus muscle after full (Lowrie et al., 1990) or partial (Tyc and Vrbova, 1995) denervation in early postnatal development (P3–P18). These authors also report decreased TPT, increased HRT, and higher numbers of slow fibers after neonatal denervation and reinnervation (Tyc and Vrbova, 1995), changes that were more pronounced in muscles denervated on P3 than P18. The decreased fatigability in these studies, therefore, may be due in part to either a conversion of fibers from fast to slow or a selective sparing of slow twitch fibers. The latter would not occur in LA muscles, because they contain no slow twitch fibers (Blanco et al., 1995; Ishihara et al., 1997). Interestingly, we did see some evidence for an increase in the oxidative capacity of a small subpopulation of fibers in the reinnervated muscles, that is, a small percentage of fibers stained darkly for SDH. Many of these fibers, however, also stained darkly in PAS sections, indicating that they were not glycogen-depleted during the fatigue test. Because we also saw concomitant decreases in EMG during the fatigue test, it is tempting to speculate that the fatigability measured reflects synaptic failure in addition to muscle fatigability, and perhaps this is more common in muscles denervated neonatally. If synapses on dark SDH fibers failed during the fatigue test, the muscle fiber properties would not contribute to the FI. A more quantitative assessment of EMG activity during the fatigue test and measures of synaptic strength in control and reinnervated LA muscles are needed to address this possibility.

**Expansion of Motor Unit Size after Nerve Cut on P14**

The present findings suggest that P14 motor units also have the ability to increase in size after injury. We estimated average LA motor unit size after nerve cut on P14 or P21 based on motoneuron counts (Fig. 1) and muscle fiber counts (Table 1). This estimate has some obvious limitations (discussed below), but strongly suggests that at least some LA motor units expanded in size after P14 axotomy. If there was no polynuclear innervation of fibers after neonatal reinnervation, then the LA motoneurons that survived P14 and P21 axotomy reinnervated an average of 374 and 206 LA muscle fibers, respectively (Fig. 6). These calculations assume that there is no overlap among motor units, but the normal LA muscle is unusual among mammalian muscles in that some polynuclear innervation is maintained in adulthood, that is, about 18% of the fibers in adult LA muscles receive inputs from two motoneurons (Jordan et al., 1988). Taking this into consideration, control animals in our experiment are estimated to have an average LA motor unit size of 272 fibers (Fig 6). If there were a similar level of polynuclear innervation after reinnervation, average motor unit size in the reinnervated groups would be 442 fibers (P14-reinnervated) and 243 fibers (P21-reinnervated, Fig. 6). Thus, whether or not polynuclear innervation is maintained after neonatal denervation and reinnervation, LA motor units can innervate more fibers (between 374 and 442 fibers on average) than in controls (272 fibers on average) to account for our results. This indicates an enlargement of motor unit size by at least a subpopulation of motoneurons that survive P14 nerve cut, suggesting that some LA motoneurons at this early stage of postnatal development are capable of innervating more muscle fibers than they normally would in adulthood.

This estimate of LA motor unit size necessarily ignores the fact that motor unit size varies, providing an estimate only of average motor unit size. In fact, it is likely that the range of motor unit sizes in LA muscles reinnervated after neonatal denervation is larger than in controls (Albani et al., 1988). This esti-
mate is also complicated by the possibility of an unknown amount of polynucleonal innervation in reinnervated LA muscles. In normal LA muscles, 18% of the fibers are polynucleonal innervated (Jordan et al., 1988). After neonatal reinnervation, the extent of polynucleonal innervation is unknown. Nevertheless, even a conservative estimate of average motor unit size (assuming no polynucleonal innervation) reveals the fact that at least some LA motoneurons were capable of innervating more fibers than normal. Almost the same total number of muscle fibers was innervated by about one-half the number of motoneurons in P14-reinnervated compared to P21-reinnervated muscles. If some degree of polynucleonal innervation was re-established in these muscles, an even greater increase in average motor unit size would be indicated.

LA Motoneuron Soma Size Is Smaller after Nerve Cut on P14 Despite Larger Motor Unit Sizes

Although LA motoneurons injured on P14 innervated more muscle fibers on average, they have smaller soma sizes than control motoneurons (Lubischer and Arnold, 1995a,b). Previous reports have found decreases in soma size after neonatal axotomy, but at shorter survival times (Lowrie et al., 1987; Crews and Wigston, 1990) or if motoneurons were prevented from reinnervating any muscles (Snider and Thanedar, 1989). Conventionally, motor unit size is thought to correlate positively with motoneuron soma size (Burke, 1981; Krishnan et al., 1982). It may be possible that maintaining abnormally large terminal arbors reduces the metabolic ability of motoneurons to maintain a larger soma size. However, while some work suggests that extensive axonal branching might impair the ability of motoneurons to maintain synapses (Lubischer and Thompson, 1999; Tian and Thompson, 2000), we know of no evidence for a similar effect on soma size. In fact, chronic increases or decreases in neuromuscular activity levels have a minimal effect on the soma size of cat lumbar motoneurons (Chalmers et al., 1992). It is most likely that the smaller soma size of LA motoneurons injured on P14 is due to their failure to respond normally to circulating testosterone levels (Lubischer and Arnold, 1995b). After castration of adult male rats, SNB motoneurons, including those innervating the LA muscle, decrease in size, and this effect can be prevented or reversed by treating with exogenous testosterone (Breedlove and Arnold, 1981). The response to testosterone is lost after nerve cut on P14, resulting in soma sizes comparable to those in castrated animals (Fig. 3a in Lubischer and Arnold, 1995b). Soma size correlates with a number of features, such as dendritic arbor size, recurrent collateral synaptic terminal numbers, and input resistance (e.g., Burke, 1981), suggesting that these motoneurons may be impacted severely by the early injury, despite their ability to reinnervate the muscle. The failure of these motoneurons to respond to testosterone also has implications for their function in male sexual behavior, especially if it extends to features other than soma size (e.g., dendritic arbor, synaptic connectivity).

Reduced Testosterone-Sensitivity after Nerve Cut on P14 Is Not Explained by a Deficiency in Reinnervation of the Target Muscle

In the SNB system, nerve cut on P14 results in survival of less than half of the injured motoneurons (Lubischer and Arnold, 1995a), and they show a lasting effect of the injury. Specifically, they do not respond normally to testosterone in adulthood, in contrast to motoneurons that survive the same injury on P21 (Lubischer and Arnold, 1995b). The present studies argue against the hypothesis that this is due to a failure of these motoneurons to fully reinnervate their target muscles. With the exception of slightly less maximum force produced, consistent with a loss of muscle fibers, muscle properties after nerve cut on P14 matched those seen in control muscles and in muscles reinnervated after nerve cut on P21. LA muscles reinnervated by an average of 16 motoneurons generated forces similar to LA muscles reinnervated by an average of 35 motoneurons, indicating no impairment of reinnervation after nerve cut on P14 relative to the reinnervation seen after nerve cut on P21. Therefore, the failure to respond to testosterone does not appear to be due to any deficiency in reinnervation of the LA muscle. Instead, the most likely explanation for the lack of response to testosterone after P14 axotomy is that normal development of steroid sensitivity by these motoneurons requires contact with the target muscle during an early, critical period in development. During this window of time, the target muscle may be providing signals for the differentiation of motoneurons that innervate it. The fact that motoneuronal injury on P21 does not result in the same deficit in response to testosterone indicates that any such critical period for contact with the target muscle is over by this time.

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REFERENCES


